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EFFECTS OF FLUCTUATING ENVIRONMENTS ON THE SELECTION OF HIGH YIELDING MICROALGAE

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NOTICE

ABSTRACT

Microalgae have the potential of producing biomass with a high content of lipids at high productivities using seawater or saline ground water resources. Microalgal lipids are similar to vegetable oils and suitable for processing to liquid fuels. Engineering /cost analysis studies have concluded that, at a favorable site, microalgae cultivation for fuel production could be economically viable. The major uncertainties involve the microalgae themselves: biomass and lipid productivity and culture stability.

The mass culture of microalgae for low cost lipids production requires a detailed understanding of both the physiology of lipid biosynthesis by the selected algal strains and of the factors making these strains competitive in outdoor ponds. Mass culture systems will, because of climatic variability, the day-night cycle, and inherent design factors, exhibit fluctuations in key parameters affecting algal growth, productivity and competitiveness: temperature, pH, pO_2, pCO_2, light, and nutrient availability. This project has for its overall objective to quantitate the effects of such fluctuations on culture productivity and species competitiveness. A second objective has been the development of basic information on algal physiology as it relates to lipid productivity.

Species competition experiments with several strains of algae isolated by the SERI/DOE Aquatic Species Program, some of which were previously used in outdoor mass cultures, were carried out. In these experiments certain parameters proved to be more important then others in determining species succession: fluctuating pO_2 was more important than fluctuating temperature, which was more important than fluctuating pH. These results must be extended to other species and conditions to determine how far these findings can be generalized. Future work will concentrate on comparing unialgal to mixed culture experiments and on elucidating the specific environmental factors that impact on species productivity and competitiveness.

To help guide this work, a computer model was developed capable of predicting the amplitude and frequency of the environmental parameters (pO_2, pH, pCO_2, temperature, and light intensity) over the diurnal cycle throughout the year at any location for which climatic data is available. This model must still be validated with data collected from operating ponds.

Using one algal strain, "Nanno Q" (Nannochloropsis sp.), as a test organism we also have investigated strategies for maximizing lipid productivity. Our data suggest a two stage process: the first stage is operated at the highest density permitting maximal productivity and the second stage at a higher light input (lower standing biomass or cell density) and under conditions of nitrogen starvation. Such a two stage system is more productive than a continuous nitrogen limited culture process. Future work in this area should utilize algal strains that have previously been selected for their competitiveness under outdoor conditions.

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SECTION 1.0 INTRODUCTION

1.1. MICROALGAE CULTIVATION

Microalgae have the ability to convert sunlight, carbon dioxide, water and inorganic fertilizers into useful products: proteins, lipids, carbohydrates, pigments, vitamins, etc. Their commercial potential has attracted the attention of biochemists, microbiologists, chemical engineers, and, most recently, entrepreneurs. The appeal of microalgae is that "cheap" sunlight energy and carbon sources can substitute for the expensive fermentable substrates required in industrial microbiology. Advantages can also be cited for microalgal cultures over agricultural systems: very high yields (photosynthetic efficiencies), use of saline waters and marginal lands, and potential for continuous automated production. The ability to manipulate the metabolism of microalgae through control of the environment, resulting in overproduction of specific metabolites, is also appealing. The possibility of future improvements in productivity of algal mass culture systems by genetic engineering techniques is frequently suggested.

However, significant disadvantages for microalgal systems are also apparent to even the casual observer: The use of sunlight requires reactors with extremely high ratios of surface area to volume, compared with conventional reactors. The lowest cost systems that achieve this requirement are simple open ponds which subject the cultures to contamination, a major technical difficulty. Microalgal cultures have very low volumetric productivities and low cell densities. Moreover, open ponds The harvesting of microalgae, from such dilute reactors, has proven to be a difficult problem to solve economically. CO_2 can be a major cost factor, commercial CO_2 can sometimes cost almost as much as fermentable substrates. Compared to agriculture the capital costs required to establish algal cultivation systems have been very high. Table 1.1 summarizes the advantages and disadvantages of microalgae production.

Up to the present the disadvantages have outweighed the advantages. The few commercial systems currently operating produce only high value products (> $10/kg of algal biomass, dry weight basis) primarily for the health food market. Almost forty years of applied research and development, involving many thousand scientist-years of effort and the investment of hundreds of millions of (current) dollars have not solved the fundamental problems which prevent the application of this technology to lower cost products - species control in outdoor mass cultures, harvesting of the biomass, CO_2 supply, low cost reactor (pond) designs, maximizing productivity, biomass processing, and development of marketable products.

TABLE 1.1 ADVANTAGES AND DISADVANTAGES OF MICROALGAE TECHNOLOGY

ADVANTAGES:

- HIGH YIELDS (PHOTOSYNTHETIC EFFICIENCIES)
- ENVIRONMENTALLY MANIPULATABLE
- USES LOW QUALITY SALINE WATER
- USES LOW QUALITY NON-ARABLE LAND
- WATER LOSS BY EVAPORATION ONLY
- NUTRIENT (FERTILIZER) RECYCLE POSSIBLE
- LARGE PORTION OF BIOMASS UTILIZED
- GENETIC IMPROVEMENTS NOT YET BEGUN

DISADVANTAGES:

- HARVESTING OF DILUTE CULTURES
- CO_2 REQUIREMENTS
- SPECIES CONTROL PROBLEMS IN OPEN PONDS
- HIGH CAPITAL COSTS

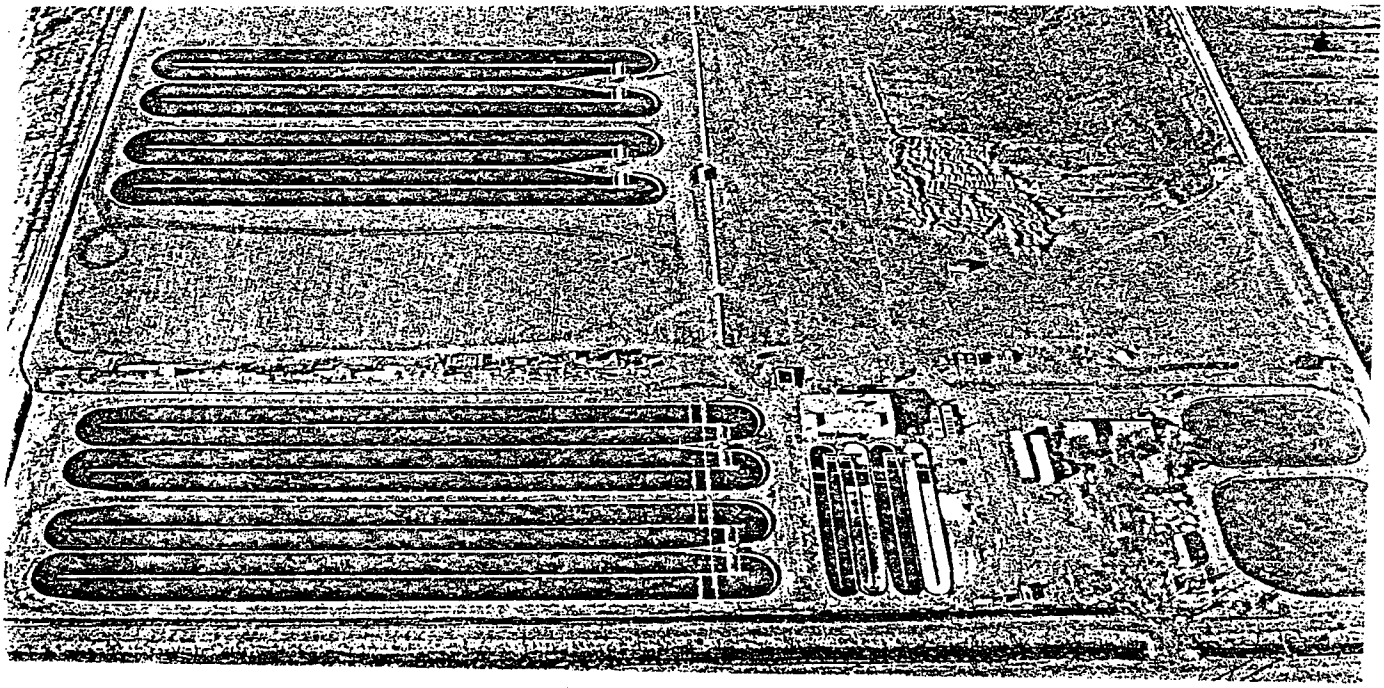
In 1953 the first comprehensive account of microalgal mass culture technology was published: "Algal Culture: from the Laboratory to the Pilot Plant" (1). That classic book, by its tile alone, already implied that the commercial development of microalgal systems was near at hand. The failure to realize these visions can be ascribed to a variety of factors: lack of sustained interdisciplinary research projects, emphasis on low cost commodities (foods, feeds, fuels, fertilizers) which were not realistically achievable in the near term, failure to develop low cost harvesting processes, the disappointingly low productivities observed with most outdoor pond systems, the difficulties inherent in processing the wet algal biomass into useful products, and the difficulties of reliably maintaining desirable algal species in outdoor ponds. In brief, development of microalgal mass culture proved more difficult than anticipated. For microalgae to become a source of low value, commodity type, products a large number of biological and engineering problems must be solved, and the solutions integrated into an overall process capable of producing algal biomass for less then $500/ton (ash-free dry weight basis).

As stated above, at present commercial microalgae production systems produce algal biomass, prior to processing and extraction, at a cost of above $10/kg. This has limited the worldwide commercial microalgal production to an aggregate of less then 50 hectares or 2,000 tons per year, the output of one mid-sized U.S. farm. Most of these (rather small, < 5 ha) plants are located in the Japan and Taiwan, where Chlorella and Spirulina are cultivated and sold as "health foods". Spirulina production is also taking place in Mexico, Israel and the U.S., where a 4 hectare plant (Figure 1) has operated since 1981 near the Salton Sea in Southern California. Smaller scale commercial Spirulina production is also taking place in Hawaii, and Israel.

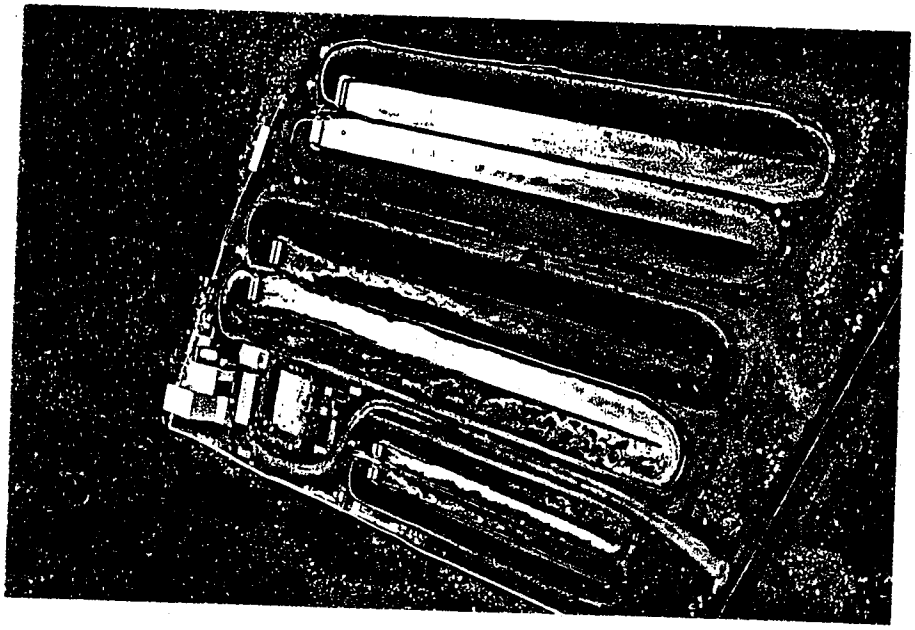
The discovery that some strains of Dunaliella salina have a high content (sometimes over 10%) of beta-carotene (2), a provitamin A and food color, with a value of over $300/kg (for the synthetic product) led to the expectation that this product would quickly become the first significant non-health food commercial product from microalgae. Although development was rather slow, several beta-carotene production plants are now operating, or are currently staring up, in Israel, the U.S. (California and Hawaii) (Figure 1) and Australia. However, even in this case production costs have turned out to be high, and the current plants can not yet compete with the synthetic product. The few other high value products (soil inoculum, aquaculture feeds, food colors, pigments) derived from microalgae are very small in scale (<$1 million total).

A recent engineering and cost analysis of the inherent economics of algal mass culture (3) concluded that no fundamental engineering or economic constraints prevent low cost (< $500/ metric ton) microalgae production. This requires, however, simple, unlined,

FIGURE 1.1 COMMERCIAL MICROALGAL POND SYSTEMS IN THE U.S.
(Photos courtesy or Earthrise Farms, Inc. and Cyanotech, Inc.)



1.1a Earthrise Farms, Inc. Spirulina Plant, Salton Sea, Calif.



1.1b Cyanotech, Inc. Spirulina and Dunaliella Ponds, Hawaii

large scale (>500 hectares) pond systems at sites with favorable climatic conditions, low cost water, and cheap carbon dioxide. In the U.S. such locations exist in Southern California and other Southern and Western States. A high biomass productivity and low cost harvesting process are the key technical assumptions underlying such a cost analysis. If a high productivity of "lipids" (hydrocarbons or triglycerides) can be achieved, and the overall site and cost assumptions (e.g. capital charges) are favorable, liquid fuels could be produced from microalgae (3).

Recent research sponsored by the SERI Aquatic Species Program has demonstrated sustained high productivities with outdoor algal cultures (4,5). Low cost harvesting, either by bioflocculation (6) or chemical flocculation (7) has also been demonstrated. The development of low cost processes for algal production of lipids, still requires fundamental and applied R&D in the areas of algal species control and metabolic control of product formation. These are the objectives of this project.

1.2. MICROALGAE SPECIES CONTROL

Maintaining a monoalgal culture of a specific inoculated strain in outdoor ponds is necessary since only specific, genetically selected, algal strains will exhibit the high lipid productivities required for a practical process. The difficulty of cultivating one desired microalgal strain in the presence of innumerable potential contaminants, predators, and parasites, is indeed considered formidable (8). It is, however, not unlike the agricultural problem of growing crops in competition with weeds and pests. The solution in agriculture has been a combination of plant breeding, chemical inhibition of undesirable organisms (weeds, pests, diseases), and mechanical tillage and (where possible) weed removal. Species control (or, technically more correct, strain maintenance) in algaeculture will be inherently more difficult than in agriculture: mechanical weed removal is usually not feasible and chemical approaches suffer from dilution in the pond medium and potential contamination of the product more severe then with agricultural crops.

Some limited advances in this area have been made. For example, it has been found that high ammonia or pH will inhibit most zooplankton infestations. However, in general, current methods for algal species control have relied on using extremes in environmental conditions which inhibit most other algae (and other organisms) and select for specific microalgae species: very high alkalinity selects for Spirulina and high salinity for Dunaliella, the two major species currently being cultivated. These techniques are expensive, as they require large quantities of salts. They also result in severe reductions in algal productivities.

Production of Chlorella, the other major species commercially mass cultured, suffered from severe contamination problems, solved only by the use of very large inoculum levels (produced under sterile conditions) and limited duration semi-batch operations, which reduce overall productivity and greatly increase costs. An even more extreme example are the diatoms and flagellates mass cultured for bivalve feed: Isochrysis, Tetraselmis, Chaetoceros, etc. These are grown under highly controlled, laboratory, conditions because of invasion by undesirable microalgae. Costs are very high (>$100/kg dry weight basis) and limit the application of algae feeds in aquaculture.

Even though the problems of contamination and culture "crashes" have been frequently referred to in the literature on algal mass cultures, to date there have been few specific publications dealing with species control in algal mass cultures. No systematic investigation has been carried out into the factors determining species dominance in mass culture systems. The obvious solution to this problem is to identify strains which easily dominate under specific outdoor pond environments. Indeed this applies to the mass culture of Spirulina and Dunaliella, where the ionic environment overwhelms most (but not all) other environmental factors.

Another example of this approach was the cultivation of a Scenedesmus strain in Germany and other locations (9). This strain was apparently an isolate from one of the early mass culture ponds that was operated in Germany, and was successfully maintained in outdoor systems operated with similar media in a variety of locations. Only when it was attempted to mass culture this strain in Thailand, did the contamination problem become intractable and other algae became dominant (10).

One of the objectives of this project is to identify factors which are important in determining selection and dominance of specific algal strains in outdoor systems. Although maximum growth rates under laboratory conditions (typically, 28^{o}C, low light) are often measured for algal species being screened for suitability for mass culture, these conditions have little, if any, relevance to outdoor pond situations, where high incident light intensities, dense cultures, light limited growth rates, and diurnally fluctuating environmental parameters present a very different situation from the conventional laboratory environment. However, not every environmental factor can be decisive, and the issue reduces to identifying which parameters affect algal strain competition to the greatest extent. The hypothesis being tested is that a few factors are critical in determining species dominance and maintenance, while others play a relatively minor role in the outcome of species (strain) competition.

If this hypothesis is correct, it may be possible to select algal strains suitable for mass culture based on specific physiological adaptations, which might be reproduced in the laboratory, rather

than depending on self-selecting strains in outdoor ponds. This would have significant advantages in the development of an algal technology, from the screening of strains for suitability for outdoor systems to their improvement for productivity and lipid formation through strain selection and genetic engineering.

The approach followed by this project has been to model the outdoor cultivation environment as a function of climatic and system design related inputs and to predict the time behavior and magnitude of specific environmental parameters and their fluctuations. Specifically the variables of temperature, pH, dissolved oxygen, and light intensity have been modelled. Based on results from the model, these conditions can be reproduced in a laboratory reactor and species productivity measured under fluctuating conditions as a function of controllable variables (dilution rate, nutrient supply) for several algae. The basic outcome of these experiments is productivity as a function of the imposed variables. The basic assumption that productivity is synonymous with competitiveness in such systems can be validated with mixed culture experiments. When compared with data obtained by other researchers operating outdoor systems with the same species, the results of this work will determine whether it is possible to use laboratory systems to select and screen microalgal species that are suited for cultivation in outdoor ponds. If this is successful it would greatly simplify species screening, strain selection and genetic engineering of microalgae useful in mass culture.

1.3 LIPID PRODUCTION BY MICROALGAE

The second objective of this project was to determine the effects of nitrogen deficiency on the kinetics of lipid biosynthesis and to develop an optimized process design for lipid production by microalgae. Nutrient supply, particularly nitrogen, is one pond operating parameters that can be relatively easily manipulated. Nitrogen starvation of algae causes a shift in their metabolism, from growth associated to storage pathways, carbohydrates and lipids, which can accumulate in large amounts. Although carbohydrate storage is more common than lipid storage, microalgae from several species that accumulate lipids in large amounts (up to and over 50% of dry weight) can be readily isolated.

However, the effect of nitrogen limitation on productivity, which is central to the question of whether lipid production by microalgal cultures is technically feasible, is not understood. Previous work by a preceding contract (10), involving a survey of eight strains of algae indicated that one, Nannochloropsis Q had a rapidly inducible pathway of lipid biosynthesis. This was also reported by other groups working with this organism (11). We have subjected this strain to a detailed study of the interactions of nitrogen limitations and light supply on biomass and lipid productivity as a model of lipid production by microalgae.

1.4 CONTENTS OF THIS REPORT

In this Report we first review the problem of species control in microalgal mass culture to present a theoretical framework for the present and future work in this area (Section 2.0). Next we develop a computer model for the behavior of a mass culture (pond) system in terms of key environmental parameters (temperature, light, pH, pO_2) (Section 3.0) which can guide the laboratory experiments by setting realistic boundaries to the parameters that need to be considered in these studies. The experiments with mixed cultures and species competition are presented in Section 4.0. A brief review of algal lipid content and productivity is presented in Section 5.0, followed by the experimental results(Section 6.0). The final Section (7.0) summarizes the results and the proposed future work for this project next year.

SECTION 2.0 GROWTH AND COMPETITION OF MICROALGAE IN MASS CULTURES

2.1 INTRODUCTION

The problem of species dominance and competition in outdoor mass cultures is considerably simpler than the problem of phytoplankton species dominance and succession in natural bodies of water. The variations of biota, light, temperature, pH, and oxygen in an algal growth pond are more predictable, and less random, than in ponds, lakes, estuaries, or oceans. This is principally because of the control over nutrient supplies and the uniform mixing in the mass culture growth pond.

Thus most of the problems that preoccupy phytoplankton ecologists and physiologists are not of direct concern in outdoor algal mass cultures. For example, the central problem for phytoplankton ecology is inorganic nutrient limitations. This can be avoided or controlled in algal cultivation. Environmental patchiness, a major focus of much of the ecological literature, is also not an issue in mass culture ponds. Light limitation, the key limiting factor in algal mass cultures, is less frequently addressed as a primary limiting factor by phytoplankton ecologists. Nevertheless, any theoretical framework of algal species control in algal mass culture must draw from the enormous body of research on phytoplankton ecology, physiology, and biochemistry, that has accumulated, almost in exponential fashion, over the past few decades. This is the objective of this Section. A brief review of the algal pond environment is presented first (see also Section 3.0).

2.2 THE HIGH RATE POND ENVIRONMENT

In essence the algal mass culture growth pond consists of a raceway type channel mixed by paddlewheels at between 15 and 30 cm/sec. The depth of the cultures is generally 10 to 30cm. The channels can be quite wide and long with individual ponds, mixed by a single paddle wheel, up to 10 hectares in size, theoretically. In current practice, individual growth ponds would be about 3 to 4 hectares in size, maximally. The pond is supplied with CO_2 through a sump spanning the channel, preferably near the paddlewheel. Flow deflectors are used around bends and in other strategic locations to prevent dead spots. The culture is harvested on a daily basis, with anywhere from 20 to 70% of the culture liquid withdrawn and replaced with make-up water and media recycled from the harvesting system. Nutrients are supplied with the make-up water or added directly into the ponds. Although a

number of variations are possible on this basic design, this is the essential algal production system envisioned in this project, as it is, in principle, of very low cost (3). It also has been adopted by most recent experimental projects and commercial ventures in algal mass culture, although the largest individual growth ponds currently existing are about 0.5 hectares in size.

The size of the pond has significant effects on the environment experienced by the algae. For example, if only one CO_2 supply station is provided (which is likely the case, for economic reasons) then the larger the pond the larger the amplitude and longer the periodicities in pH fluctuations to which the algae will be subjected between CO_2 resupply events. Keeping the fluctuations in pH (and the associated changes in pCO_2) within acceptable limits, as dictated by considerations of species control and productivity, are a major factor in the design of the pond system. Thus the commonly observed dominance of the diatom Phaeodactylum tricornutum in seawater algal cultures has been ascribed to the high pH found in most such culture systems (11). Since this organism is usually not among those that would be desirable, a seawater system design and operation should avoid extremes in pH to the extent feasible. A key question in this case, as well as that of the other factors addressed below, is the effect of periodic fluctuations as compared to continuous steady state conditions.

Besides pond size, factors affecting the pH range experienced by the algal cells in the culture are the mixing velocity, the outgassing coefficient, the alkalinity (and ionic composition) of the media, the productivity of the culture, and the efficiency of the carbonation system. Although there is some flexibility, the pH fluctuations in the ponds will to a large degree be predetermined by engineering and operating (that is economic) considerations. This suggests that the algal species to be cultivated must be either chosen or genetically selected to meet the pH/pCO_2 constraints imposed by the mass culture system. To a greater or lesser degree, similar considerations apply to the other environmental factors affecting species competition and productivity.

The key abiotic environmental parameters most likely to affect algal productivity and species competition are light, pH, pCO_2, temperature, pO_2, salts, dilution rate and nutrient levels. Some of these cannot be controlled to any significant extent (light, temperature), others to only a limited degree (pH, pCO_2, pO_2, salts), while some can be manipulated almost at will (nutrients, dilution rate). The abiotic pond environment can be modeled given detailed site specific climatic input parameters and assumptions about the design of the system (e.g. scale, length to width ratio, pond depths, etc.), water quality, and system operations (dilution rate and schedules, nutrient supplies, mixing velocities, etc.). Such a model is presented in Section 3.0. This Section reviews the effects of these key parameters, singly and in combination, on microalgal growth, productivity, and species competition. First some theoretical considerations are discussed.

2.1 THEORETICAL BASIS FOR SPECIES COMPETITION.

Darwin's basic observation about evolution was that a given environment will select for the most capable organism. Capable is defined as the ability to increase the number of decedents relative to other, competing, organisms. Competition refers to utilization of the resources which limit the overall population size and growth rate of the competing organisms. This is the fundamental concept underlying competition, selection, and evolution.

In algal mass culture ponds the fundamental limiting factor should be, at all times, light. The cost of the biomass is very sensitive to the overall productivity ($g/m^2/day$ or mt/ha/yr) of the system, which can only be maximized by absorbing essentially all the light and utilizing it efficiently. It must, however be recognized, that efficiency of light utilization by the individual cell (the competing organism) does not translate into high productivity by the overall culture. This fundamental point is addressed further below.

The environment of an algal mass culture pond is unlike that of any natural system in terms of the specific combination of factors that will affect growth rates and productivities by microalgae. Thus it is not surprising that the ecological literature has significant gaps that prevent a more detailed understanding of algal physiology and species dynamics in mass culture environments. Perhaps more importantly, algae isolated from natural environments are unlikely to be optimally adapted to the pond environment. This suggests that genetic selection may significantly improve future productivities by algal mass cultures and the ability to maintain monocultures. A major corollary of this hypothesis is that care must be taken to prevent the adaptation of unwanted organisms to the pond environment.

At its most basic, when a single limiting resource exists such as a chemical substance (nutrient), then those organisms most capable of capturing that substance (and, but not necessarily, utilizing it most efficiently) will be better able to compete and eventually become dominant in the environment. However, if two organisms differ, through a hereditable genetic difference, in their capability for capture of the same limiting substance, then the one that is, even if only slightly, better at capturing the limiting nutrient will prevail in that environment. Eventually such a competition for one limiting factor results in a single type of dominant organism. Since for any individual organism, according to Liebing's Law of the Minimum, as applied by Gause (13) (see also Hardin [14] and Levins [15])), only a single factor can be limiting at one time, the logical result is that for each limiting factor or nutrient in the environment only one organism can win the struggle for survival.

The obvious falsehood of this conclusion was pointed out for the specific case of microalgae in Hutchinson's famous paper on the "Paradox of the Plankton" (16). Since a single drop of water can

contain dozens or even hundreds of different algal species and since in such environments only a relatively few nutrients (N, P, Fe, etc.) can possibly limit algal growth, the coexistence of so many different species can not be explained by the above arguments. Either the arguments are wrong or other factors must play a role in allowing so many species to survive, or both.

One explanation for the Paradox of the Plankton was that resource - growth rate relationships were similar enough among different algae, and environmental conditions variable enough to prevent a final equilibrium result from occurring. The variation in conditions has been termed patchiness - with the implication being that each patch is its own ecological niche, a microcosm with different limiting nutrients from those of surrounding patches. As the patches decay and new ones form, the competition for resources never is carried out long enough to reach equilibrium, e.g. extinction of the majority of the species. Another, perhaps better, explanation was that different mechanisms for removal of organisms, particularly sinking and zooplankton grazing can reduce the yield of the most dominant species thus providing an increased resource base for the less capable. Inhibition of one organism by another through excreted substances (allelopathy) is another potential mechanism to allow coexistence of several species under conditions of a single nutrient limitation. These arguments were deemed sufficiently satisfactory explanations of the Paradox to concentrate most research into the questions of what the limiting factors were which caused the dominance of specific species and the frequent successions of species in nature.

Another explanation for the Paradox has more recently been proposed: in a fluctuating temporal environment resource utilization is quite different from that in a steady state environment (as has been assumed by most models and laboratory studies). For example, during a diurnal light cycle some algae are better able to utilize a limiting nutrient (e.g. N) in the dark period and others in the light period. Thus both can coexist. If the nutrient itself is added in a cyclic fashion, different species may be adapted to preferential utilization during periods of high and low supply (17). Thus fluctuating environments essentially act to increase the number of potentially co-exisiting organisms.

This may not be desirable in mass culture ponds, where a single species (strain) is to be maintained as a monoculture. However, the common observation is that upon nutrient enrichment of lakes or ponds, or in mass culture one organism usually dominates (90%+ of the total biomass present) at a time. Thus, although theoretically many "niches" exist, allowing two or more algae to coexist, one strain usually has a sufficient advantage in the capture of most of the limiting nutrient (light) to prevent any significant competition. This is the major paradigm on which our research on species competition and control in algal mass cultures is built: the pond environment will select for a single species as an essential monoculture and species succession can be prevented or minimized by adjustments in pond operations.

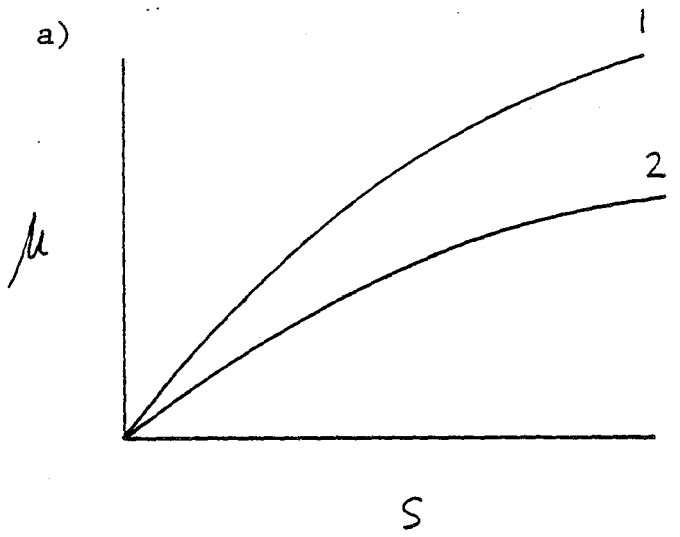
The mathematical treatment of competition is a simple matter of writing the material balances for each species coupled with an accurate rate expression for growth (see Ref. 18). If necessary one can include terms for inoculum make-up, allelopathic or antagonistic interactions, and losses (predation, sinking). In the simplest case the competition between two organisms whose growth follows Monod kinetics can be easily modeled (19). Four different steady state results are possible: the trivial result of each population equalling zero, and the non-trivial cases of each population by itself dominating, or both populations co-dominating. Co-dominance is only possible in this model if the growth rate-substrate curves intersect (e.g. the substrate concentration at which both had identical growth rates).(See Figure 2.1c). But co-dominance at this point, although theoretically possible is inherently unstable as any small deviation in substrate concentration (e.g. dilution rate) or even stochastic events would result in an irreversible decline of one or the other of the organisms. However, if two different potentially limiting substrates are present and the two competing organisms differ in their affinity for the two substrates, then a stable coexistence is possible under conditions where the two organisms are limited by different nutrients. Small deviations in substrate levels would not result in long term changes in species composition.

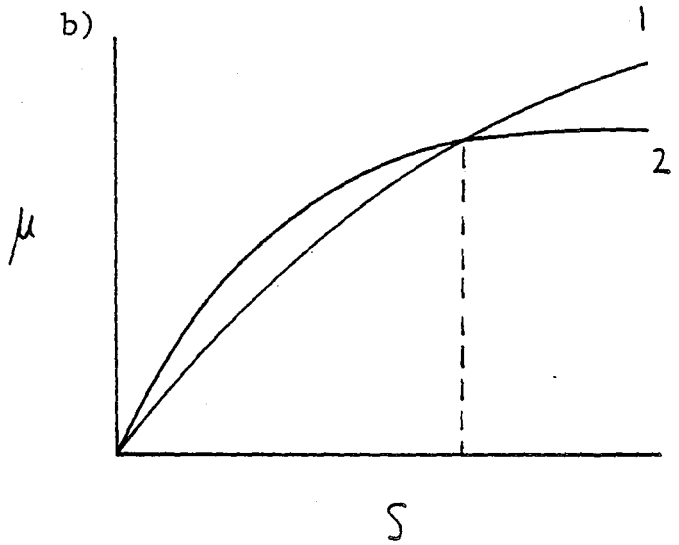
Tilman (20) tested the validity of this simple competition model by determining species dominance for two diatoms limited by different resources. The affinity constant for silicate for Asterionella formosa was higher than that for Cyclotella meneghiniana while the opposite was true for phosphorous. Maximum growth rates were essentially the same. By setting the two growth rate expressions equal he was able to predict under what concentration ratios of Si and P co-dominance would be observed. He then verified this experimentally and found that experimental results for the mixed cultures agreed with the theoretical predictions form the unialgal culture experiments. Although expected, these results demonstrated that mixed culture experiments can in practice be predicted from unialgal culture data (e.g. growth rate vs. substrate concentrations).

Of course, these results would not be obtained if there was interference between the organisms, specifically if allelopathic effects were involved. Allelopathic interactions between microalgae have been observed repeatedly (21) and would affect such studies. Allelopathic effects can be determined by growing the algae in the "spent" culture media (suitably fortified with the limiting nutrients) of each other. Any gross inhibition that could not be corrected by the addition of nutrients would reflect the presence of inhibitory compounds. It must be noted that for allelopathy to become a significant problem the inhibitory alga must have established a significant presence in the culture, as it acts through its metabolic products. Thus allelopathy and interference mechanisms generally could account for stable coexistence with only one limiting nutrient (22). In this case the starting point (e.g. the proportion of species initially present) determine the dominance pattern observed at steady state.

FIGURE 2.1 GROWTH RATE - SUBSTRATE RELATIONSHIPS

A) $K_1 < K_2$, $u_{1max} > u_{2max}$, $u = 0$ at $s = 0$
B) $K_1 > K_2$, $u_{1max} > u_{2max}$, $u = 0$ at $s = 0$





Returning to Fig. 2.1b, for the case where $u_{1max} > u_{2max}$ and $K_1 < K_2$, an argument can be made for stable coexistence if the resource supply profile fluctuates from a low to a high concentrations in some time dependent manner. Under these conditions, assuming instantaneous adaptation to the nutrient level and coupled nutrient uptake and growth, organism a will grow faster during periods of low and organism b during periods of high nutrient concentrations. Depending on the amplitude of the fluctuations (but not the periodicity) one or the other or both of the organisms can stably coexist in the system. However since none of the assumptions is true in real life, an accurate determination of competitiveness must include adaptation (time lags) to the nutrient levels as well as distinction between the instantaneous growth rate and nutrient uptake rate (see discussion below). This would result in both the amplitude and periodicity of the fluctuations in nutrient supply determining the outcome of the competition for the resource. It could also result in more then two organisms coexisting on one fluctuating limiting factor.

Another consideration is that for a given nutrient there may be more then one potential mechanism by which one organism can utilize it, resulting in a change in both V_{max} and K_m for the nutrient, depending on its concentration and the time allowed for the organism to metabolically adapt to it (as well as the organism's growth rate and other physiological characteristics at the time). For example if the resource was CO2 supply, and it was suddenly changed from a high to a low concentration, an active bicarbonate assimilation mechanism may be induced utilizing the enzyme carbonic anhydrase and other (as yet unidentified) cell components. Since it may take from 30 minutes to several hours for such a shift in metabolism to develop, it is clear that the situation in the case of a fluctuating limiting nutrient can be indeed complex. Although it should be possible to predict the behavior of the organisms under such conditions, the amount of data required would be very large indeed.

A further complication is that in many cases when a culture is exposed to a limiting level of a resource, a sudden increase of that resource will result in an overshoot in assimilation. These have time constants on the order of 30 minutes to several hours. Such an overshoot will then result in an undershoot, as the cell tries to adapt to the new environment. The result is an approach to the new equilibrium through a damped oscillation that may be detected for a considerable period of time.

In conclusion, growth (and thus prediction of the outcome of competition) during the transient or fluctuating supply of a single limiting substrate will not be a straightforward extrapolation of steady state uptake kinetics for that nutrient, as they are affected by the adaptation and overshoot (or undershoot) time constants. In fact these time constants may be the most important selective force in determining species dominance, depending upon the frequency, amplitude, and variance of the fluctuations.

Finally, another major complicating problem is that environmental factors tend to interact. Thus the results obtained with cultures subjected to variations in single factors under constant environmental conditions for other factors can not be extrapolated to what would be observed if under conditions where more then one factor was varied in a time dependent manner, as would be the case in mass culture ponds.

Indeed it is often not even possible to predict how two invariant factors will interact. Although models for the interactions of limiting nutrients with temperature of the temperature effects and light limitations have been proposed (24,25), the actual (very limited) data collected have shown that neither multiplicative nor additive models can explain the interactions of temperature or light with nutrient (N or P) limitation (26,27). Varying two factors simultaneously resulted in larger effects on growth rates then would be predicted by such models.

In conclusion, a logical approach to the study of algal mass culture is to carry out experiments under conditions that simulate, as much as possible, the dynamic environments observed or predicted in ponds and to determine the effects of specific variables, or combination of variables, that may be of interest in the operation, design, or siting of an algal mass culture facility. The results and predictions of single species experiments would then need to be confirmed, on a selected basis with mixed cultures. The key issue is whether it is indeed possible to simulate in a laboratory setting the conditions in outdoor ponds sufficiently closely to allow the collection of data that can be extrapolated to a mass culture situation. For this it is not only necessary to know or predict the actual environment in outdoor ponds but to also focus on the factors most likely to affect the growth rates and productivities of algal species. These factors, and their interactions, are reviewed in this chapter.

2.3 LIGHT

The specific problem addressed in species competition studies in mass cultures is competition with light as the primary growth limiting nutrient. Light is not a typical resource as it is not supplied with the media influent and it is not conserved, whatever is not immediately used is lost. Light, however, can be modeled satisfactorily with conventional saturation type growth kinetics (the P vs. I curve is similar to Figure 2.1). However, unlike most inorganic nutrients, there is often a detectable non-zero intercept of the P vs. I curve, with the intercept light intensity being the "compensation point", or the minimum light energy required to maintain the metabolism of the algae. It is thus, theoretically at least, for two algae to coexist with light as the sole limiting nutrient if their P vs. I curves intersect twice (This is, of course also possible for inorganic nutrients but in that case the maintenance nutrient level is generally so low as not to be detectable.)

Light comes in packets of photons which have a characteristic wavelength-energy density pattern. The pigment complex of the cell will absorb the incoming radiation according to its absorption spectrum and utilize it according to its action spectrum (the two being generally close but not identical). Different algae have different absorption patterns (for example see Figure 2.2) and different action spectra, that is photosynthetic rate as a function of wavelength. It is therefore the product of the light energy-wavelength relationship, the absorption spectrum and the action spectrum integrated over all wavelengths which results in the observed photosynthesis -irradiance relationship. Thus both light absorption and utilization efficiency are important.

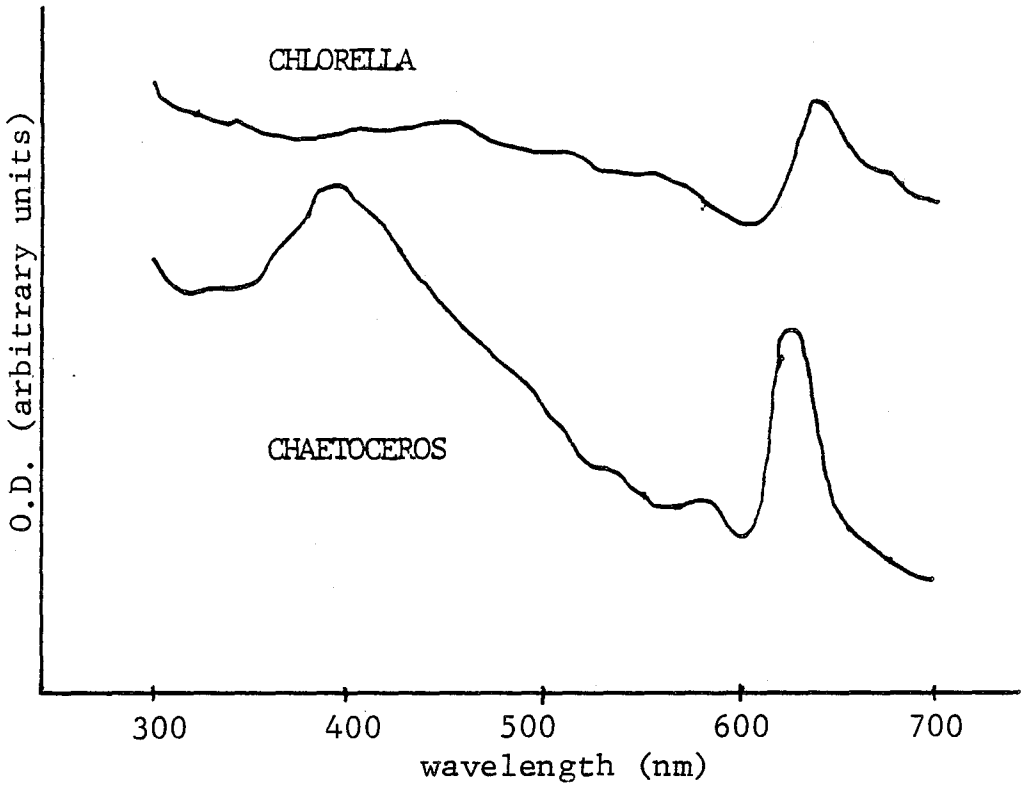
A consequence of this is that light acts not as a single nutrient but has the potential of behaving like multiple nutrients, such that more then one algal type can coexist under light limited conditions (Even if the P vs. I curves do not intersect twice). Indeed this can be observed in the case of the laboratory culture of Spirulina and Chlorella (28). A theoretical mathematical treatment of light as a multiple nutrient has been published (29). However, in practice, the coexistence generally is not co-dominance, the most efficient light capturing organism usually will make up over 90% of the total biomass, with the other algae capable, literally, of growing in the shadow of the dominant species, but representing a rather small fraction.

As stated, mass cultures, to maximize productivity, operate under light limited conditions, usually at cell densities of > 200 ppm, and with a standing biomass of 50 to 100 $g/m^2/day$. If light is the limiting nutrient for algal productivity, then, because of the law of the minimum the competition problem reduces to one of determining a given organisms ability to capture and utilize light under the conditions of the outdoor pond. It must be, however, again, be emphasized that light capture and utilization are not necessarily synonymous. Also, the reference here is to single individual cells, not the performance of a whole culture. A very competitive strain, which can rapidly become dominant in a culture may actually result in a decline in overall culture productivity, as explained next.

Light capture (absorption) by algal cells is dependent on the total pigment content of the cell, while light utilization (photosynthesis) is dependent on number and turnover rates of the photosynthetic reaction centers and the activities of the enzymes of the dark reactions (CO_2 fixation) in each cell. High pigment content in algae is due to a large amount of "antenna" chlorophyll and accessory pigments (biliproteins and carotenoids) and results in saturation of photosynthesis by low light intensities. This is a competitive advantage under light limited conditions. However at the surface of the ponds sunlight intensities are well in excess of saturating light intensities. Light is nevertheless absorbed by the pigment system. A large fraction of this incident light, whatever is above the saturating intensity, can not be used fast enough in photosynthesis, and is wasted as fluorescence and heat.

FIGURE 2.2 IN VIVO ABSORPTION SPECTRA OF MICROALGAE

Algae Spectra are not corrected for scattering.



Thus, as already recognized many years ago, productivity of algal mass cultures could be increased by simply using algal strains that have much lower antenna pigment concentrations, assuming that changes in other factors (e.g. compensation point) do not negate this advantage. Algae with lower antenna and accessory pigment contents would not shade out the underlying cells and not absorb more light then they need for maximal photosynthesis. However, such low pigment cells would have a disadvantage under conditions of light limitation, which apply throughout most of the pond depth profile (except in the top few cm or even mm). Because of the lack of the accessory pigments they would not be able to capture many photons in the dim depths of the ponds. They would individually grow slower then more pigmented cells at low light intensity. They would be quickly out-competed, by more pigmented cells.

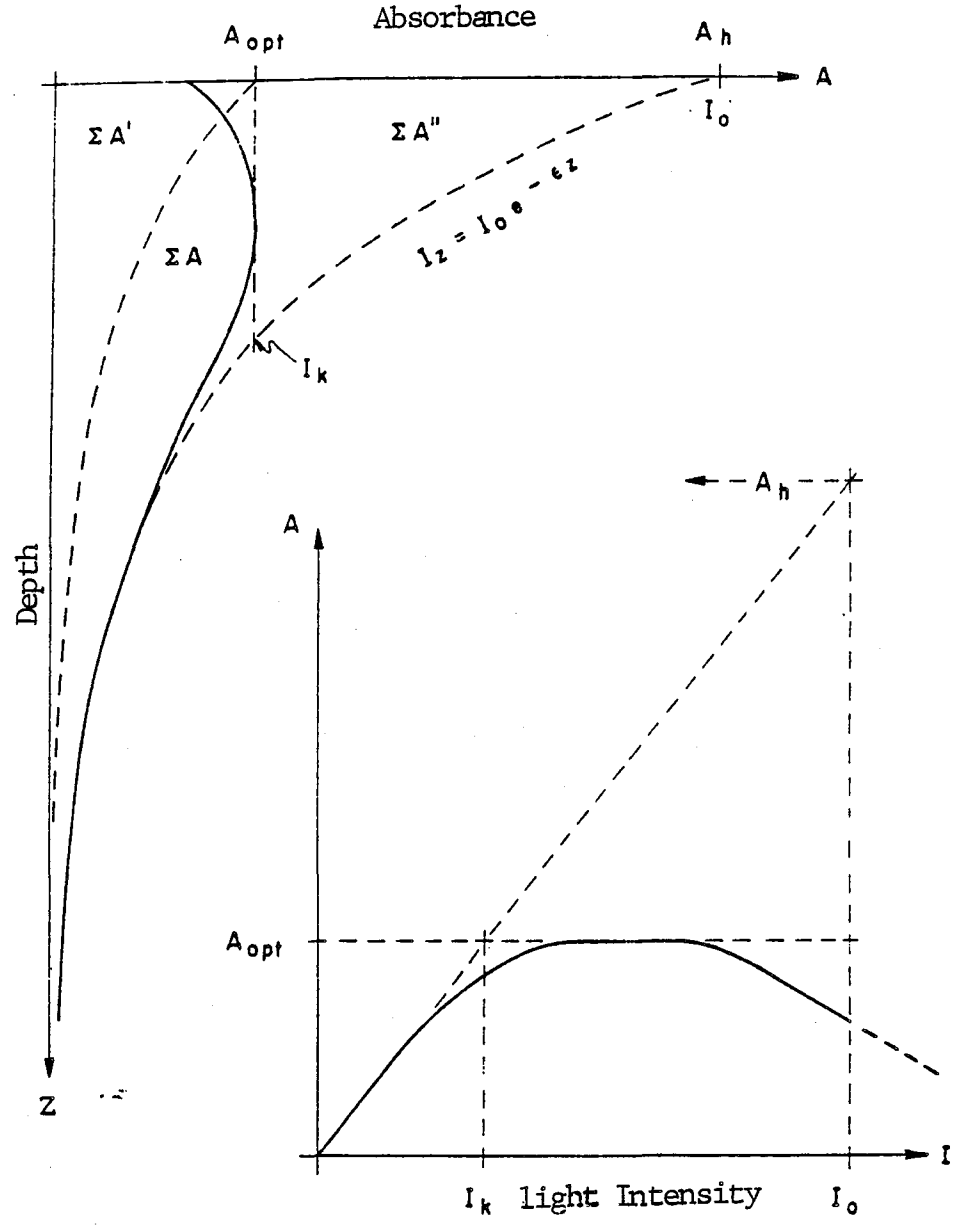
Only under dilute culture conditions would a reduction in accessory pigments be of advantage to the cells, to the extent that the additional metabolic investment into accessory pigment (and the effect of photoinhibition) would dictate. Indeed, there is a clear sun-shade adaptation phenomenon in most algae (30). However, use of dilute (low standing biomass) cultures would negate the objective of the mass culture, which is to maximize areal productivities, which can only be achieved by absorbing and utilizing as much light as possible. Dilute cultures would also have the additional severe disadvantage of requiring constant dilution and a large harvesting effort. If a strain of algae that naturally remains "sun" adapted even in light limitation, or has been genetically selected for reduced accessory pigment production were to be grown in outdoor ponds, it would be very quickly overtaken by shade adapted invading algae, which would have a significant advantage in terms of growth rate in such situations.

Therefore mass cultures must be relatively dense and will select for high pigment cells, which will underutilize incident light at the surface and shade the underlying cells. Mathematically this can be represented by the "Bush equation" which combines the absorption of an algal culture with the P vs. I curve.(31). (See Figure 2.3) (33). The overall result is a reduction in culture productivity, of as much as 60 to 70 % for a culture with saturating light intensities of 15% of sunlight, as compared to achievable productivities if the same amount of light could be delivered at a lower (below saturating) intensity to the culture.

One method for delivering sunlight in such an attenuated fashion is by producing a very high degree of turbulence, such that the algae will absorb the full sunlight intensity for only a small fraction of a second, sufficient only to trap one photon per photosynthetic unit (reaction center). This can be achieved in laboratory setting by either modulating the light into very short bursts (a few msec) (33) or by very rapid stirring of the cultures (34). Neither is practical in a production pond. The effect of longer periodicities on productivity is controversial, with both data for and against positive and negative effects (35-37).

FIGURE 2.3 PHOTOSYNTHESIS VS IRRADIANCE AND DEPTH RELATIONSHIP

(Source: Ref. 33)



From the perspective of this project, the key question is whether the specific light fluctuations likely to occur in ponds will have a significant effect on algal productivity and competitiveness and whether such fluctuations can be mimicked with sufficient accuracy in laboratory reactors. Equally important perhaps is how accurately the spectrum of sunlight, its intensity, and its diurnal, seasonal, and climatic (e.g. cloud cover) variability must be reproduced in the laboratory to allow conclusions regarding productivity and species competition that can be extrapolated, even in modified form, to outdoor systems.

In practice it would be very difficult (expensive) to faithfully reproduce both the spectrum (particularly UV) and the intensity of sunlight and it would be almost impossible to reproduce the light fluctuations due to pond mixing. Thus one of the assumptions made in this project is that a high intensity fluorescent light source (Duro-Test Vita Lite) delivering about 40% of full sunlight intensity and a magnetic/gas mixing system will not affect the overall results to a significant extent. (Of course, productivities must be corrected for the incident light intensity, applying the Bush equation to the P vs. I curve). In support for this view is that even if mixing effects in the 0.1 to 10 Hz range can be demonstrated they are likely to be small and more likely due to other factors then an increase in photosynthesis per se (e.g. decrease in O_2 tensions, reduction in photoinhibition, improved nutrient supply) which would not apply to the laboratory reactor, where these factors would be better controlled.

Thus, in conclusion, the outdoor light environment will not be reproduced very faithfully in the laboratory systems, either in terms of incident intensity, light quality, or light periodicities, because the problems involved greatly outweigh the likely benefits. Although strong arguments can be made for this, it points to the need for a verification and calibration of any results obtained with the laboratory system. This issue is discussed at the end of this section. The light environment will, however, be reproduced in terms of two critical issues: hours of sunlight (diel periodicities) and, at least in the future, the increase and decrease in light intensity during the daylight hours.

Of specific interest are the factors which affect the light-photosynthesis relationship. These are, among others, temperature, oxygen, pCO_2 and pH. Because of the fluctuating nature of these factors, the relevance of steady state measurements in predicting integrated growth responses is questionable. In the following sections each of these parameters will be discussed in terms of their general role in growth and metabolism, their selective pressure in mixed cultures, the time constants involved in their adaptation to new conditions, and their interactions with light (and each other). In most cases the literature only allows a cursory and general description of these topics.

2.4 TEMPERATURE

There are a number of ways in which temperature may affect the productivity of an organism and thus its relative competitive ability. Abiotically, changes in temperature affect ionic equilibria, pH, and gas solubility which affect different species differently. Direct biotic effects of temperature include structural changes in membranes, proteins, and other macromolecules, and the rates of enzyme reactions.

In plotting the growth rate vs. temperature, generally a more or less bell shaped curve is represented, with a certain optimum growth temperature. However, a closer inspection of such data, when available in sufficient detail (38) , reveals more fine structure (Figure 2.4): There is a broad region over which increases in temperature correspond to linear increases in the growth rate in an Arrhenius type of plot, indicating a constant energy of activation (and, presumably, the same limiting reaction). Usually, but not always, an inflection point is noted at low temperatures, indicating a change in energy of activation. Up to the temperature of maximal (e.g. optimal) growth rate, the growth of the organism is controlled by metabolic rates. Above the optimal temperature limit, usually within 2 to 3^{o}C, a decline of growth rate is noted which drops to zero very quickly. The reason for this rapid decline is not quite clear, however it is evident that some limiting reaction mechanism is affected.

The optimal growth rate temperature is usually measured under conditions of maximal growth rate (e.g. no nutrient limitations). However, temperature optima under conditions of nutrient limitation are different, because different metabolic activities have different temperature optima (and these, as may be expected, differ between species and strains). The effect of temperature on different metabolic pathways can result in countervailing effects: while increases in temperature enhance dark reaction enzyme activity (carbon dioxide reduction), they also increase the rate of respiratory processes(dark and light dependent respiration and glycolate excretion)(39). Thus the effect on productivity can be either positive or negative, depending on species.

Cultivation temperature may affect cell composition uptake rates, nutrient quota's, and photosynthetic performance(40-45). In general, the cell quota for protein (44), carbon, nitrogen, and phosphorous all increase with temperature changes away from the optimum (45). Temperature and nutrient limitation interact to a lesser extent as the limitation becomes more severe. Nutrient uptake rates are not necessarily correlated with the optimum temperature for growth. Rhee and Gotham (41) found for a N limited Scenedesmus sp. the optimum uptake temperature was 5-10 ^{o}C below that for growth. On the other hand, optimal temperatures for growth rate and nutrient uptake were similar for P limited A. formosa.

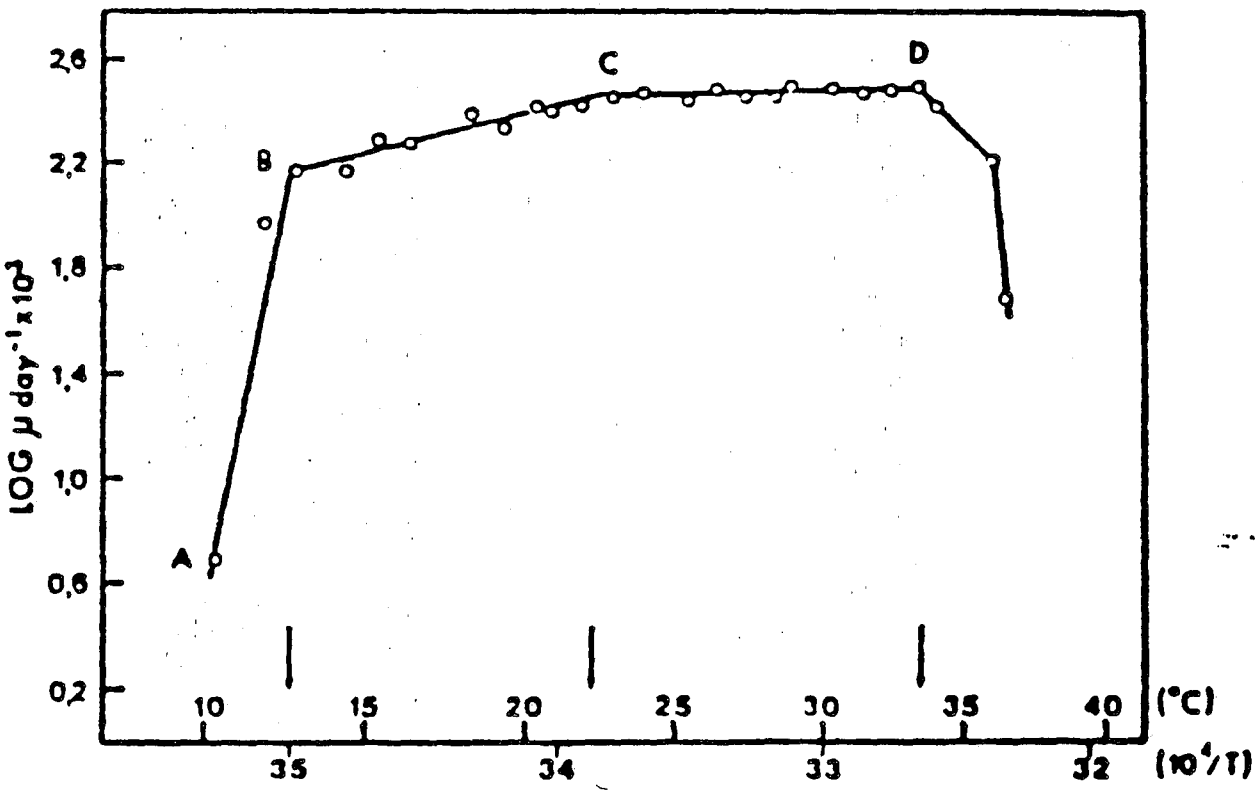


FIGURE 2.4 GROWTH RATE - TEMPERATURE RELATIONSHIP
(Source: Ref. 38)

Changes in chlorophyll tend to be species specific and are closely related to photosynthetic performance (38,43). For most species it appears that chlorophyll content increases with decreasing temperature (46). This and an increase in dark fixation enzymes(RUBP) compensate for the reduction in the rate itself, thus allowing the photosynthetic process to continue unchanged on a per cell or dry weight basis (46). At incident intensities much less than saturating, temperature should not affect photosynthesis (47) because it is being regulated only by electron capture. At intensities greater than saturating, temperature will be important by regulating the activity of the CO2 fixing enzymes. The overall result in terms of net CO2 fixation will be dependent upon the temperature mediated rates of light and dark respiration. In general this results in temperature effects on photosynthesis which are less then on other metabolic activities. However relatively few studies exist of temperature effects under light limited conditions of maximal photosynthetic productivity.

Of specific interest in algal mass culture is the effect of diurnal temperature fluctuations in ponds, where the min-max temperature differential may easily exceed 15^{o}C. These fluctuations are correlated with the diurnal light cycle, but dampened and delayed. This will have significant effects on algal productivity, however essentially no studies have been carried out on such diurnal temperature cycles. The reason is that in most natural environments (oceans, lakes, rivers) only seasonal temperature cycles, or temperature shocks are important. One recent study found that a six degree temperature differential (day night) actually increased growth rate (of Hantzschia) above the constant temperature control, but only within a rather narrow temperature range (48). The only other work found, goes back to the early studies of Tamiya (49) who carried out extensive studies during their development of a Chlorella production process. They reported that low night time temperatures significantly inhibited algal productivity by preventing cell division. This is corroborated from synchronous cultures, which show that there can be temperature sensitive stage during night time cell division (50). Obviously much more work is required in this area. One immediate question is to what extend the algae adapt physiologically to the diurnal temperature changes.

The existence of temperature driven changes in structural and/or rate processes defines the two adaptive responses observed in algae. Temperature adaptation is reviewed by Li (38). To summarize, temperature changes which occur within the normal physiological range of the organism (ie. those described by a linear ln u vs 1/T relationship), will result in an instantaneous response in the growth rate of the organism. Temperature changes outside of this range, will result in some damage to the cell, and an unpredictable outcome on growth.

In the U. S. mass culture of algae may well be more limited by the minimum night time temperatures to which the algae may be exposed then by limitations of solar radiation per se, or other

climatic factors. Thus the resistance of algae to low temperatures is of interest. Harris (46) in his review of phytoplankton ecology concludes that microalgae are essentially chilling resistant plants, experiencing no permanent damage at temperatures as low as a few degrees C. However there is some evidence suggesting otherwise. Exposure to low temperatures of an high temperature strain of Chlorella resulted in bleaching of chlorophyll on a time scale of 15 hours at 10°C (50 % reduction in chlorophyll a) (51). Also Rao et al. (52) investigated the sensitivity of cold shock treatment as a function of the previous growth temperature and found that while cells grown at 25 °C were tolerant to exposure at 0 °C, those grown at 40 °C were killed after 5 minutes. Thus it appears that loss of cell viability after exposure to low temperature will be dependent on the previous growth temperature. In ponds, low temperatures will only result in the winter or transition months, where the mean temperature will already be significantly reduced.

One of the few studies of temperature effects on species competition in outdoor ponds was carried out by Goldman and Ryther (53) who were studying temperature dependent species succession patterns in managed waste-water to aquaculture systems. Two, one meter deep, 15 square meter ponds were operated continuously with a combination wastewater-seawater feed, filtered to remove anything above 20 microns. Phytoplankton present in the feed were used as inoculum and the depth of the culture effectively dampened any daily fluctuations in temperature. These authors found that when the pond temperature remained below 25-30 °C diatoms dominated, above that, two chlorophytes were important. The organism Phaeodactylum tricornutum was the most common species and was displaced, in late spring, only after being predated upon by a "monad like flagellate". It was found to dominate in outdoor cultures over the temperature range of slightly less than 10 to 20 °C. However when laboratory competition experiments were conducted, it was not the dominant organism at 10°C and its dominance at 15 and 20°C is uncertain due to methodological problems (41). The apparent disagreement between laboratory and outdoor results is not surprising when considering the significant differences in environments (the laboratory cultures were, for example, exposed to continuous low intensity light). From productivity vs. temperature experiments on mixed cultures the authors concluded that productivity was insensitive to temperature.

A recent study from South Africa compared the productivity of algae cultures (no species was specified!) in open heated and unheated (as well as covered) circular ponds (54). The unheated ponds exhibited lower productivities, which, however, were rather low (< 20g/m^2/day in all cases). Indeed, except during winter time, when minimum temperatures dipped to 4°C in the unheated ponds, the differences in productivities were rather small, considering a 7 to 10 °C temperature difference (max and min) between ponds.

Tison et al.(55) have studied the influence of temperature fluctuations on species composition in cooling tower runoff from a nuclear power plant. In simulated ecosystems, they found that selection was based on the ability to survive at the extreme temperature swings(from ambient to >45 ^{o}C). Organisms that were commonly present in hot springs of similar temperatures were not found; therefore the fluctuation itself, or according to the authors, survivability at the low end of the temperature swing determined species composition.

In conclusion, it appears that for temperature fluctuations within the normal physiological range of an organism the growth response integrated over the pond temperature during the lighted portion of the day will be predictive of competitiveness. Respiratory losses at night will similarly be determined by integrating over the temperature range experienced for that portion. If there are temperature excursions outside of this range, a discontinuity exists - the effect of which is unknown. The optimal temperature determined under constant laboratory conditions should be considered a maximal tolerated temperature. It is often remarked that the actual niche of a particular algal species is at temperatures significantly below the optimal measured in the laboratory. One explanation is that, since the lethal (or at least zero growth rate) temperature is close to the optimal, the optimal temperature is not really optimal but the maximal temperature. Thus, to a first approximation the "optimal" laboratory temperature determines the suitability of the algal species to summertime outdoor cultivation conditions. Another area of concern is low night time temperatures, which may result in an upset of the normal cell division process and may affect productivity indirectly. Selection of algal species that divide during the daytime, or in the early dark phase (while temperatures are still relatively high) may be desirable.

2.5 OXYGEN

The presence of oxygen at levels far exceeding saturation in microalgae ponds (4,8) is due to high rates of photosynthesis, high cell densities, and the low gas transfer rates out of the pond. Oxygen is known to inhibit photosynthesis through a variety of mechanisms and therefore its high concentration represents a potential reduction in productivity. The earliest experiments of Warburg in the 1920's first demonstrated the inhibitory effect of 100 % O_2 on productivity. Glidwell and Raven (56) describe four oxygen consuming processes in algae; they are dark respiration, Mehler reactions, RUBISCO oxygenase, and glycolate metabolism. It is useful to briefly review each to learn of their possible role in determining productivity.

The consumption of oxygen as a result of normal catabolic metabolism is called dark respiration. Its consumption typically represents about 10% of the total oxygen evolved, it is temperature

(increasing exponentially with temperature)and growth rate dependent , but not oxygen dependent. Its rate is diminished in the light, apparently regulated by adenylate charge (46). Very low light intensities are required for inhibition. From short term incubations with labeled CO_2, it has been shown that no label appears in respired CO_2 therefore indicating a substrate far removed from recently fixed carbon (56). Because of its association with normal cell function and lack of sensitivity to oxygen it is not important in observed oxygen inhibition in ponds however is important in determining net productivity. It is commonly measured by short term incubations in an oxygen electrode.

Light and oxygen dependent respiration is called photorespiration which is directly associated with RUBISCO oxygenase activity and glycolate metabolism. The term is generally applied whenever a decrease in the oxygen concentration results in an increase in CO_2 uptake. When cells grown on several percent CO_2 with air are exposed to high levels of oxygen an immediate reduction in carbon fixation occurs with measurable amounts of carbon being excreted. The excreted carbon is glycolate and reflects an accumulation within the cell. Glycolate formation is due to the oxygenase activity of the CO_2 fixing enzyme RUBISCO (Ribulose bisphosphate carboxylase-oxygenase). RUBISCO is an bifunctional enzyme with affinities for both CO_2 and O_2. When catalyzing the oxygenation of RUBP (ribulose bisphosphate), glycolate is formed. Oxygenase activity is dependent upon high ratios of O_2/CO_2 which can either be caused by high O_2 or low CO_2 environments.

Surprisingly when cells grown on air levels of CO_2 are exposed to high O_2 tensions they do not show any of the common signs of photorespiration (decreased photosynthetic rate and glycolate excretion) (58). This is apparently the result of a "CO_2 Concentrating Mechanism" which enables cells to concentrate CO_2 at levels 2-3 orders of magnitude higher than the bulk concentration. The enzyme carbonic anhydrase, which catalyzes the equilibration of bicarbonate ion and CO_2, is central to the operation of this pathway (59). Glycolate metabolism in algae is catalyzed by glycolate dehydrogenase to glyoxalate -> glycine -> serine -> and PGA (60). When mutants of Chlorella containing excess amounts of the enzymes associated with the glycine-serine pathway were exposed to high oxygen, their rates of glycolate excretion were 3-4 fold less than the wild type.

Because of the fact that carbon is lost from the cell, photorespiration is often considered to be a wasteful process. It does however serve to reduce the immediate high oxygen environment and perhaps protect the cell from a more damaging problem with photooxidation of its pigments or delivering reductant without any available CO2. Thus while in the short term it is wasteful, over the long term it may be essential. After reviewing much of the literature on photorespiration, Raven and Beardall (61) concluded that RUDP oxygenase activity could not solely be responsible for the observed oxygen consumption. They suggested that a Mehler type reaction must be operative.

Despite the fairly extensive biochemical studies, relatively few studies deal with the effects of oxygen on the productivity of algal cultures, and even fewer have been carried out under conditions of relevance to algal mass cultures. Pope (62) studied the oxygen sensitivity of four different strains of microalgae and found that at low light(250 ft-c) oxygen inhibition was very small as compared to high light(3000 ft-c). A. nidulans fixed CO2 at a rate 20% of an oxygen free culture when exposed to only 60 % O2 in high light. Phaeodactylum tricornutum and Dunaliella tertiolecta grew at rates 70 and 50 % lower than the control culture when exposed to 100% O2. A Cricosphaera sp. was found to be insensitive to oxygen at all light intensities.

The question as to how reversible the features of O2 inhibition are following exposure to high oxygen concentrations is of obvious importance. Shelp and Canvin (58) found with Chlorella sp., that exposure at 50 % O2 was reversible after 20 minutes but a similar treatment at 100 % O2 was not. The process of respiration is a method for the cell to adjust its carbon balance. Respiratory process require substrate, therefore one would expect the size of the precursor pool to be important in determining the reversibility of oxygen inactivation.

While there has been no direct evidence of oxygen having a role as a selection force in open ponds, there is evidence that high oxygen tensions affect species differently. In Figure 2.5 is shown a summary of some experiments carried out by Weissman and Goebel (4) on six different strains of microalgae. They compared both maximum growth rate and productivity at either air levels or 100% O_2 in batch cultures exposed to continuous light. It is evident that high O_2 tensions appear to affect species differently with the greens being particularly sensitive, while the diatoms are not. These authors have found a good correlation between oxygen sensitivity in laboratory environments and poor competitiveness in outdoor ponds; however they have not found these strains to show particular sensitivity to oxygen when cultured outdoors.

In summary it is clearly evident that oxygen can inhibit photosynthesis in a selective manner. The mechanism through which this occurs can be a combination of photooxidation of pigments, photorespiration and Mehler type reactions. The degree of inhibition is more severe at high light intensities and temperatures. Because the pond environment will commonly attain high temperature, high light, and high oxygen during the afternoon, it is argued that oxygen sensitivity of an organism will be an important selective force in algal growth ponds.

2.6 pH/CO2

In weakly buffered systems, pH and dissolved CO2 are coupled through the bicarbonate equilibria as shown in Figure 2.6 for seawater in equilibrium with air (63) . Carbon dioxide consump-

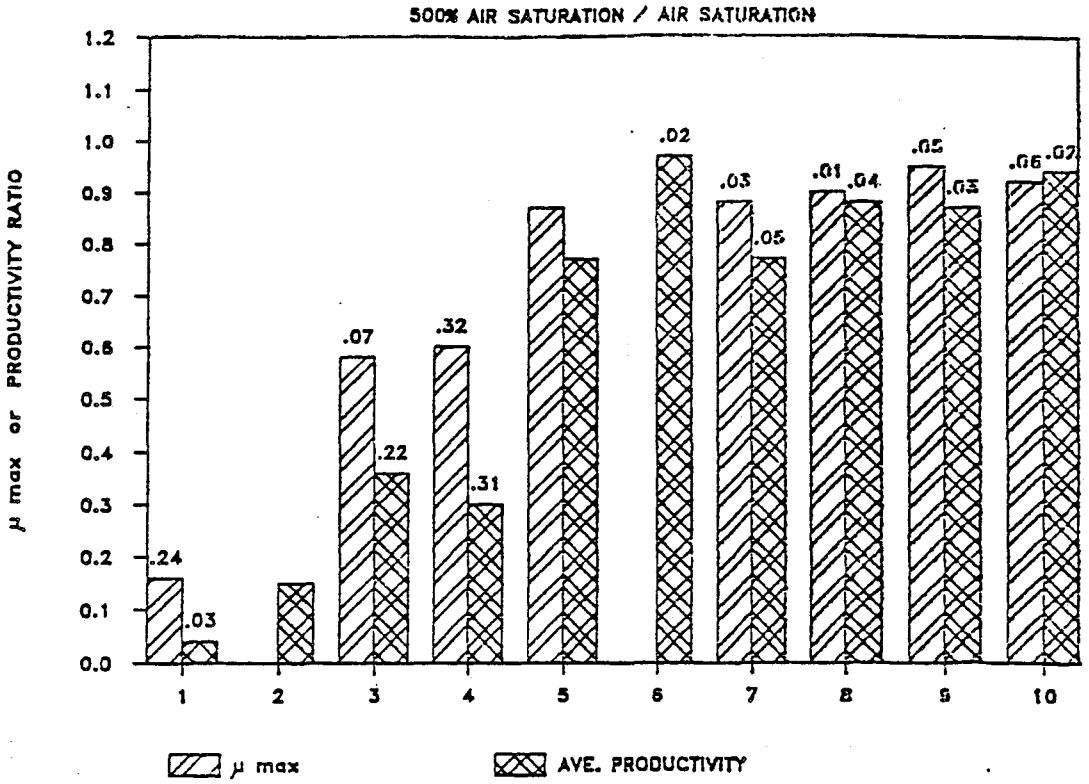


Figure 3-1. Oxygen Inhibition of Laboratory Algal Cultures

1. Nannocloropsis sp. 21
2. Oocystis sp.
3. Tetraselmis suecica
4. Chlorella ellipsoidea
5. Ankistrodesmus sp.
6. Scenedesmus quadracauda
7. Chaetoceros gracilis
8. Chaetoceros S/CHAET-2
9. Cyclotella sp.
10. Amphora sp.

FIGURE 2.5 SENSITIVITY OF MICROALGAE TO 100 % OXYGEN TENSIONS (Source: Ref. 4)

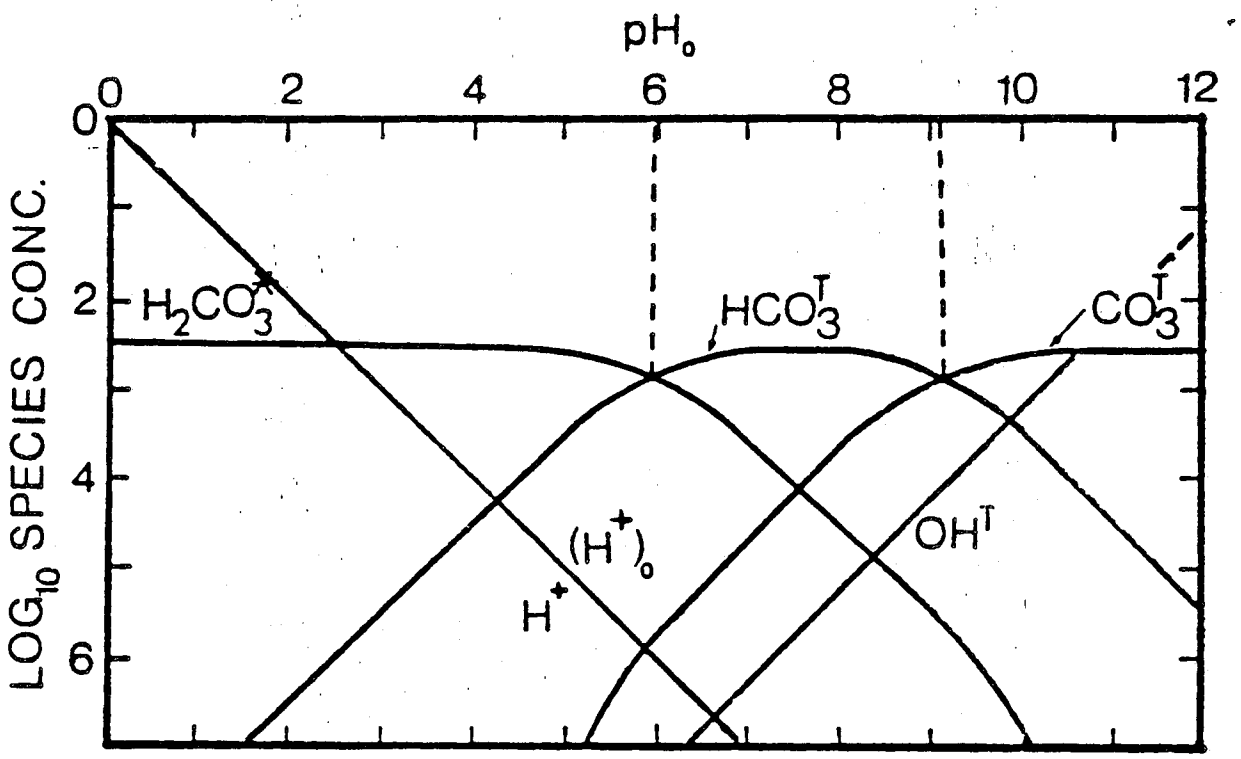


FIGURE 2.6 CARBONATE SPECIES IN SEAWATER AT EQUILIBRIUM
(Source: Ref. 63)

tion during the day, and evolution at night by algae are primarily responsible for the pH changes observed in ponds. The supply of CO2 is periodic, depending on the size of the pond (or the spacing between carbonation stations) and the mixing velocity (see Section 3.0) The CO_2 supply to the algae thus depends on the actual amount of CO_2 stored in the bicarbonate-carbonate buffer and the pH of the pond. Although the rate of H_2CO_3 formation from bicarbonate is nearly instantaneous, the actual conversion to free CO_2 is not. However algae (like most living organisms) possess carbonic anhydrase which is able to catalyze the hydration-dehydration reaction of CO_2. It is, however, unclear at what concentration CO_2 becomes limiting. The fact that pCO_2 drops to almost vanishing levels at high pH and that algae adapt to such low pCO_2 tensions by induction of an active bicarbonate uptake system suggests that this is of major import to the cell.

Thus the pH fluctuations that will be typically encountered in mass cultures must be constrained within the physiological and competitive limits of the organism being cultivated. Elucidations of these limits must be one objective of any screening program. It must be pointed out however that low cost production of algae will also constrain the allowable pH levels and fluctuations in terms of CO_2 losses from the ponds, alkalinity requirements etc. This issue is further addressed in the next section

2.7 THE FEASIBILITY OF SCALE DOWN

The entire discussion up to this section has been an attempt to demonstrate the importance of the fluctuating environment on strain productivity and dominance. We have focused on the fluctuating conditions because they represent the outdoor cultivation environment. The objective is to be able to reproduce the outdoor environment in its essential elements in laboratory cultures - e.g. to scale down the pond environment to a 1 liter laboratory reactor. In this section we discuss the reasonableness of the basic assumptions underlying the scale down approach.

Table 2.1 summarizes the key pond environmental parameters, their typical values for laboratory cultures, the range of the values for the outdoor system, the time period over which changes occur, and an estimate as to the feasibility of scaling that parameter. Temperature, dissolved oxygen, pH, and water chemistry (alkalinity) can all be scaled effectively. The first three by maintaining feedback control loops using heater inputs, and gas composition as the controlled variables and the last one by using media which matches site water composition.

Reproducing the appropriate light environment is somewhat more difficult. The incident light intensity, cell density, and mixing pattern will all combine to give the light distribution pattern for a culture. Algae simply don't average the light intensity for

TABLE 2.1

FEASIBILITY OF SCALING POND ENVIRONMENTAL PARAMETERS TO LABORATORY REACTORS.

PARAMETER	Units	TYPICAL LAB. VALUE (CONST.)	OUTDOOR RANGE	TIME PERIOD (hr)	SCALE DOWN
Temperature	oC	20 to 30	10-40	24	YES
Dis. Oxygen	ppm	6 to 15	0-40	24	YES
pH	logH+	7 to 9	7-10	>1	YES
Alkalinity	mM	0 to 50	0-50	none	YES
Light	μE/m2/s	<300	0-2000	24	?
Cell Density	mg/l	100-2000	50-500	minor	?
Mixing	-	fast	slow	const.	NO
Shear	-	high	low	const.	NO
Surface:Vol.	m-1	50	5	const.	NO
Biota(Invasions)	-	low	high	const.	NO

a given environment but respond more or less instantaneously to intensity changes. The photosynthetic processes of light absorption and charge transfer occur at time scales several orders of magnitude smaller than the time it takes for an alga to be carried into and out of the light even in very turbulent of systems.

As discussed above, algae are sensitive however to the light dark pattern and its frequency. Without a suitable model it is difficult to predict what the effect of the different mixing-light regimes will have on the photosynthetic response. At the mixing velocities (20 cm/s) and channel width (10 m) and depth (.2 m) common for a typical pond, a Reynolds Number of 80,000 is calculated. This is well into the turbulent flow regime and therefore a random light/dark periodicity can be assumed.

Another significant time constant is the daily light/dark cycle which varies between 9 to 15 hours light (and corresponding dark) periods. These can of course be easily reproduced in the laboratory as can, to a first approximation, the diurnal light cycle (e.g. the light intensity is raised and lowered gradually). The length of the dark night cycle can be important, as is amply demonstrated in the literature, for algal productivity (and presumably competition).

Certain parameters related to the light environment and culture mixing can not be scaled adequately in laboratory reactors: light intensity, light quality, light variance due to mixing (even if turbulent), shear effects, and secondary factors such as cloud covers. As discussed above, we consider that these parameters are of secondary importance and that valid conclusions can be reached in the absence of complete mimicry between the laboratory and mass culture systems. Indeed, there will always be differences that even the most sophisticated laboratory system would not be able to reproduce: surface to volume ratios, biotic contaminations, cell sedimentation, dispersion coefficients, etc. In the next section we develop a computer model of the pond system which is able to predict the abiotic environment of a mass culture system.

SECTION 3.0

MODELING THE ALGAL GROWTH POND ENVIRONMENT

3.1 INTRODUCTION

The cultivation of microalgae on a large scale requires that all incident light be utilized as efficiently as possible. The standing biomass (more accurately, standing pigments) must therefore be high enough to absorb all (98%+) of the incident light energy. Generally this will be about 20 to 30 g/m^2 of algal biomass (ash free dry weight). However, a higher standing biomass is desirable since it would entail less harvesting and dilution effort, a major consideration in system design optimization. The cultures must also be as shallow as possible, for the same reasons. The optimization of these two parameters, standing biomass and depth, is one objective of R&D of microalgal biomass systems.

Culture standing biomass is limited by productivity considerations. Although standing biomass vs. productivity is generally represented as a gaussian type curve with an optimum near half the maximal (non-light limited) growth rate (64,65), in actuality the maximal productivity range extends over a fairly broad range of dilution rates. As dilution rates, within this optimal productivity range, are inversely proportional to standing biomass, it is possible to optimize both the productivity and design considerations (minimum harvesting and hydraulic dilution) in algal mass culture. Indeed this is clearly apparent from batch cultures where there is a fairly long phase of arithmetic growth for most algal species. At some point, however, respiration becomes a significant fraction of primary productivity, at which point culture productivity declines. Respiration is, of course, affected by the pond environmental parameters (O_2, temperature, etc.), and their fluctuations, just as much as photosynthesis is.

Depth, the other primary design parameter under discussion, is limited by considerations of head losses (which are related to pond size and mixing velocities), carbon storage, and temperature profiles (4,66). Reduction of depth will result in each of these factors becoming more unfavorable, increases in depth require larger harvesting and water pumping efforts.

For the unaided mind, it is difficult to simultaneously consider all of the potential inputs (eg. climatic, design, operational, biological, etc.) required for optimization of depth and standing biomass (detention time), or even to predict the extent of their effects and interactions. This is, of course, also true of the many other environmental and design factors to be considered. In this context a model serves as a tool to study operating strategies, for predicting conditions for different site locations, and for determining the dynamic aspects of the physico-chemical environment. The development of such a model is presented.

3.2. POND DESIGN

Some of the early pond designs were first discussed in Burlew's book (1); other more recent reviews are also available (67,68). The general picture that emerges is that of many small scale systems of diverse designs reflecting a lack of concern for economics. As the system designs became larger (over several hundred m^2) they have tended to converge to the High Rate Pond (HRP) design.

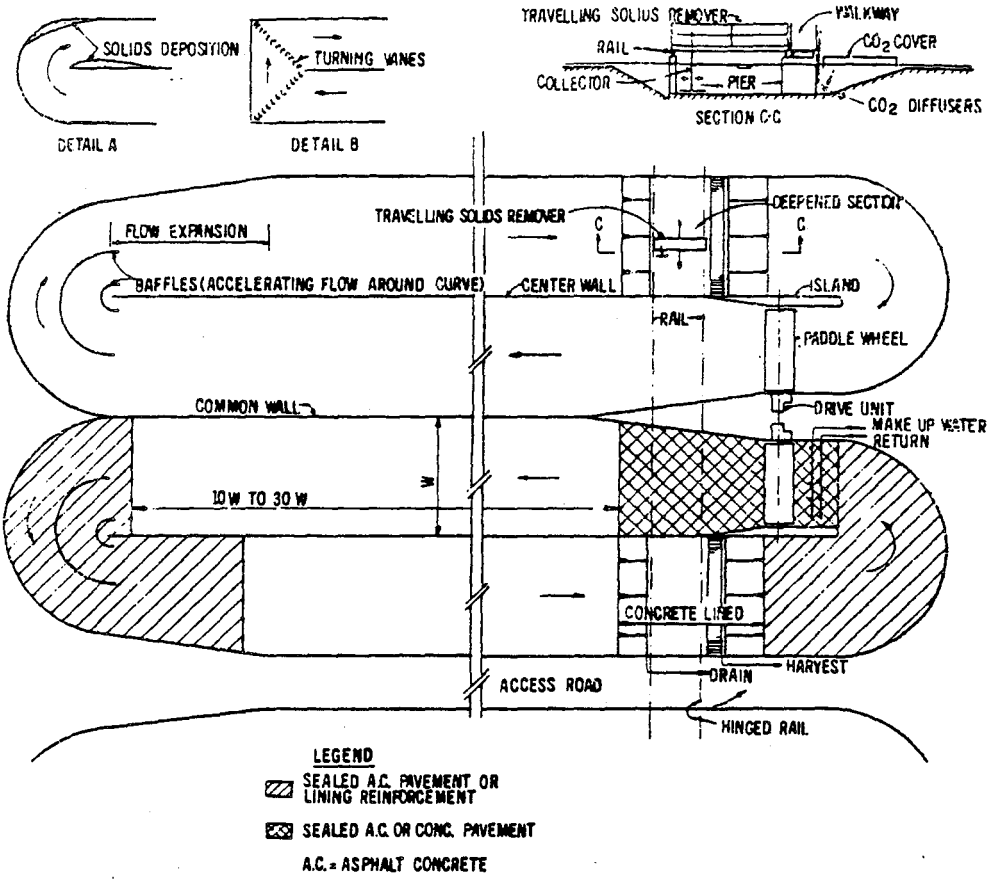
This design was first used by Oswald and colleagues in the early 1950's in their research on wastewater and scaled up to a 0.5ha system in the early 1960's. (69). A major modification was made about this time by the German group at Dortmund who introduced paddlewheels for mixing. The HRP pond design is characterized by its raceway track type of layout (Figure 3.1) (67) which allows it to be scaled to a large size, limited only by considerations of mixing (e.g. dispersion coefficient and mixing). As discussed elsewhere (66) these limitations are not severe up to a scale of several hectares for a single pond if mixing velocities are moderate (below 30 cm/sec) and depth are above 10 to 15 cm. These moderate mixing velocities and depths are also essential from the point of view of power requirements for mixing and silt suspension in unlined ponds (3) and for minimizing the temperature variations and maximizing carbon storage in ponds (see below).

Other designs involving shallower and faster mixed cultures have been proposed because of their alleged ability to increase biomass productivity (see 68 for a review). However no evidence for such increased productivity has been obtained. A number of circular systems were built in the 1960's in Japan, but that design has been essentially abandoned. Tubular reactors are likewise of little practical use at least in large scale, low cost systems, except, possibly, for inoculum production.

In summary, the basic algal production reactor design used by all recent commercial plants, and most pilot scale projects over the past two decades, is a paddlewheel mixed raceway type pond system. The ponds are operated at a depth of 10 to 30 cm and an aspect ratio of about 1:10 or larger. This design is both simple, flexible in operation, and can be easily scaled up from small experimental or pilot units to full scale systems of one hectare or larger. Before presenting the model we review the prior literature in this area.

3.3. POND MODELS

Models of pond reactors have been developed for a variety of applications including waste treatment (71,72), solar heating (73), and algae cultivation (74,75). There are many similarities in terms of the balance equations and empirical expressions in these model descriptions and even those not specifically dealing with algal growth ponds are relevant to the present effort.



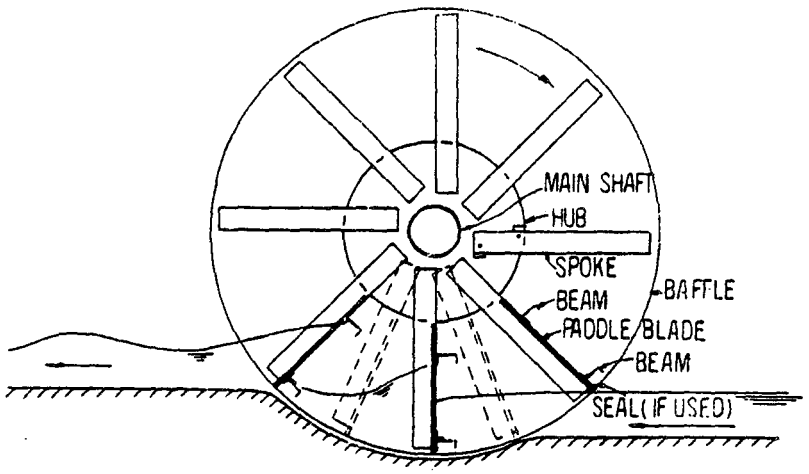


FIGURE 3.1 ALGAL GROWTH POND (a) AND PADDLEWHEEL (b) DESIGN (Source: Ref. 69)

Markl and Mather (74) presented calculations of oxygen and carbon dioxide balance equations for an 85 square meter pond located in Peru (as part of the German-Peruvian project). Their analysis was limited due to the small size of the pond, a questionably high kl_a value, and by their assumption that no exchange of carbon was occurring between the buffer and the carbon dioxide pools. Di Toro (76) has shown this to be an acceptable assumption below pH values of 7.0; however above this the error becomes increasingly large(50 % at a pH of 8.0) as the pH increases. Since most ponds operate at pH's above 8.0, this error is unacceptable. Their predictions for the need to replenish CO_2 (carbonation intervals) of less than 2 minutes is thus incorrect, by a large factor. This would severely constrain any pond system to only a very small size (<1,000 m^2). Additionally because of the high assumed kl_a value their calculations of maximum oxygen concentrations in the ponds were also quite low at 2.5 times air saturation.

Another pond model for algae cultivation was developed by Hubka for the Solar Energy Research Institute (82). The main emphasis of this work was to make predictions concerning areas of cost reduction in pond design. The objective of this model was thus quite different from the present one and it is not directly applicable to the model developed here. However it represents one of the few prior publications dealing specifically with modeling of an algal mass culture system.

Physical rate expressions are found in two separate models of waste stabilization ponds. Ferrara and Harleman (71) developed a dynamic model of a waste stabilization pond that included consideration of virtually all of the physical, chemical, biological, and hydraulic features. Inorganic carbon species and pH were predicted using alkalinity, bacterial respiration, algal uptake of CO2, and surface gas exchange as inputs and the equilibrium expressions of Stumm and Morgan (63). Dissolved oxygen was not treated. All rate and equilibrium expressions were temperature dependent with temperature being an input variable. The authors did carry out one case study using a set of empirical equations developed by Octavio et al. (77) and found good steady state agreement for a 1.2 m deep pond(for temperature predictions).

The model of Fritz et al. (70) was similar to the above except more attention was paid to the energy balance calculations. They developed an energy balance expression including terms for short and long wave radiation (solar and atmospheric), pond radiation, convective exchange, evaporation, and hydraulic flows. When tested against measurements recorded for a 1 m deep pond in New Mexico, the steady state temperature predictions were quite good.

Another model of a waste stabilization system was that of Incropera (72) who analyzed the efficacy of using waste heat generation in a closed loop exchange with a stabilization pond. The energy balance included the same flows as Fritz et al. (70) but also contained terms for conduction exchange with the pond surfaces

and the heat exchanger surface. Diurnal temperature predictions for the month of July gave a temperature variation of 2 ^{o}C for a 2 m deep pond. The model predicted that using waste heat effluent at 40 ^{o}C in a single pass heat exchanger, was capable of maintaining pond temperature above 25 ^{o}C for the entire year. Performance was very dependent upon fouling of the exchanger surface.

3.4 MODEL DESCRIPTION

3.4.1 Input Parameters

Table 3.1 summarizes the major input parameters to this model. The general growth pond input parameters of size, channel length to width ratio, depth, and mixing velocity, were mentioned above. The specific pond system that is modeled here consists of a one hectare raceway type pond with a channel to width ratio of 20. Mixing and CO2 supply are not specified as such, but would involve the use of paddlewheels for mixing (alternatively, air lift or screw pumps could also be used) and a sump (or any suitable system) for CO2 delivery (the carbonator).

We assume a single carbonator spanning the entire width of the channel and that in one pass through the carbonator all the CO_2 used by the algae since the prior pass could (and would) be recharged into the media. Utilization of CO_2 by the algae causes a pH rise in the ponds and a decline in the free CO_2 (CO_2 and H_2CO_3) level in the culture medium. Either or both these factors eventually leads to a decline in productivity. Here we assumed that this point is reached at the equilibrium partial pressure of CO_2 with the atmosphere, about 10^{-5}M. This is a species and strain dependent property which would need to be modified as more information became available.

To calculate CO_2 and O_2 mass balances a broad range of outgassing coefficients were used, as the actual values are relatively uncertain. The range of pH's experienced by the culture before and after CO_2 supply at the carbonation station (pH START and pH END), are inputs to the model. These, together with alkalinity determine the pond CO_2 storage capacity and, thus, overall pH fluctuations in the ponds. As any two (pH, alkalinity, CO_2 storage) fix the third, and as CO_2 storage capacity must equal or exceed that required by photosynthesis between carbonation events, the actual choice of variables is alkalinity or/and pH range. Constraints on the choices will be the loss of CO_2 due to outgassing, the cost of supplying the needed alkalinity (or the natural alkalinity present in the make-up water times the blow down factor) and, as indicated above, the tolerance and competitiveness of the desired algal species to the variations in pH and pCO_2. (The size, aspect, mixing velocities, and dispersion coefficient of the pond are additional parameters to be considered, but are either preset or ignored in this model, at its current state of development.)

Table 3.1 Summary of Input Parameters to ACP Model

VARIABLE	NAME	VALUE	UNITS
Geographical			
Latitude	ALAT	33.24	Degrees
Longitude	ALONG	104.32	Degrees
Elevation	ELEV	1103	meters
Climatic			
Air Temperature	TA	data file	oC
Wind Speed	WIND	data file	mph
Relative Humidity	RH	data file	%
Total Solar Radiat.	TOTRAD	10803-29624	KJ/m^2
Design			
Pond Size	AREA	10,000	m^2
Length:Width	LTW	20	-
Pond Depth	DEP	10-30	cm
Mix. Velocity	VEL	20	cm/sec
Physical			
Outgassing Coeff.	KLAPOND	$1-10x10^{-5}$	m/s
Alkalinity	ALK	0-0.030	moles/l
Starting pH	PHSTRT	7.0-9.0	pH
Ending pH	PHEND	7.5-10.0	pH
Biological			
Productivity	PROD	30	g/m^2-day
Carbon Yield	Yc	0.042	moles C/g algae
Oxygen Yield	Yo	0.042	" "

For the initial exercise of the model a site in Roswell New Mexico was selected. The primary rationale for this was the interest by the Solar Energy Research Institute in setting up a demonstration facility for algal mass culture at this site during 1987. The climatic data collected at the Roswell New Mexico Airport were obtained from the National Climatic Center, for the year of 1985 on magnetic tape. The data was used to calculate hourly averages of each climatic parameter - wind speed, air temperature, and relative humidity - for an average day for each month (Figure 3.2). Average values of the daily total solar radiation for Roswell for each month (averaged over the years 1940 to 1971) were obtained from Cinquemani et al. (78) and hourly solar radiation data calculated according to Collares-Pereira and Rabl (79). Finally the productivity of the algal culture was used as a model input, at 30g/m^2/day of ash free dry weight for the month of July, with constant hourly productivity over a 14 hour daylight period. This productivity input is conservative and based on recent results from outdoor ponds (Weissman, personal communication).

3.4.2 Model Subroutines

The model consists of nine separate subroutines which are flowcharted in Figure 3.3. The differential equations used for biomass, CO_2, O_2, and energy, used in this model, are given in Table 3.2. These were integrated by the program at a time interval of one second.

READIN/WRTOUT The READIN and WRTOUT routines carryout all I/O operations. Data is read in from a separate data file which is organized similar to the blocks of inputs shown in Table 3.3. Outputs are time, incident light intensity, air and pond temperatures, pH, oxygen, and algae concentration. These are printed at an interval prescribed by the user.

CLIMATE Hourly meteorological observations of air temperature, relative humidity and wind speed are interpolated to give instantaneous estimates of each required for solution of the energy balance(subroutine HEAT).

SOLAR Instantaneous estimations of the incident light intensity are generated by a semi-empirical formula (79). The required inputs are site latitude, Julian day, and the total radiation received over a single day (78). The amount of light actually absorbed by the culture will be reduced by back reflection and scattering. The transmission of the light into the pond is determined by applying Fresnell's equation for the direct beam component of the light and assuming 93% of the diffuse radiation will be transmitted (80). The 93% transmission term was obtained by integrating the Fresnell equation over the full sky assuming an even radiation intensity. Outputs of this subroutine are directed to subroutine GRATE and HEAT.

Figure 3.2 Diurnal Variation of Climatic Inputs for Roswell, New Mexico in July

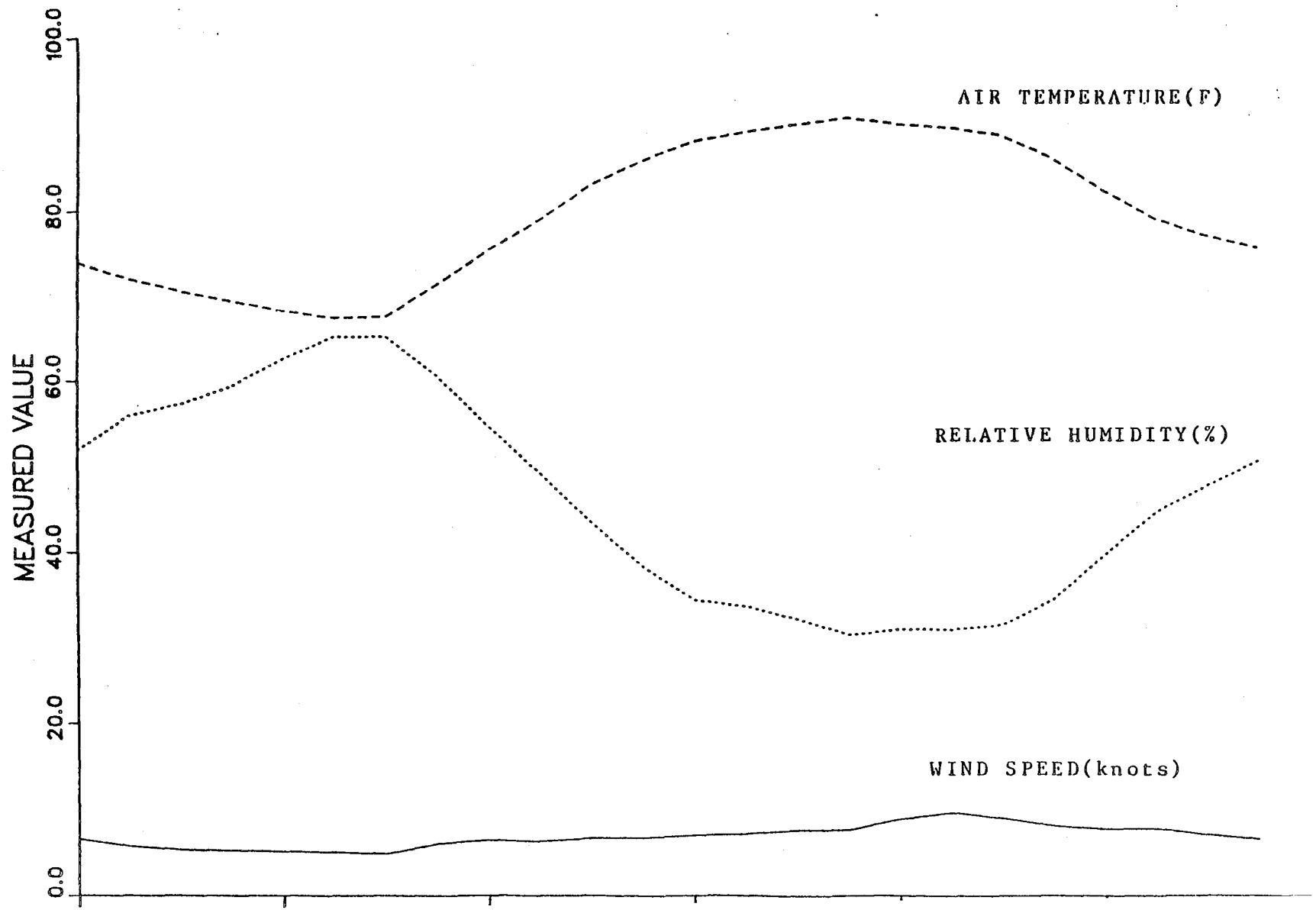


Figure 3.3 Algal Growth Pond Model Flowchart

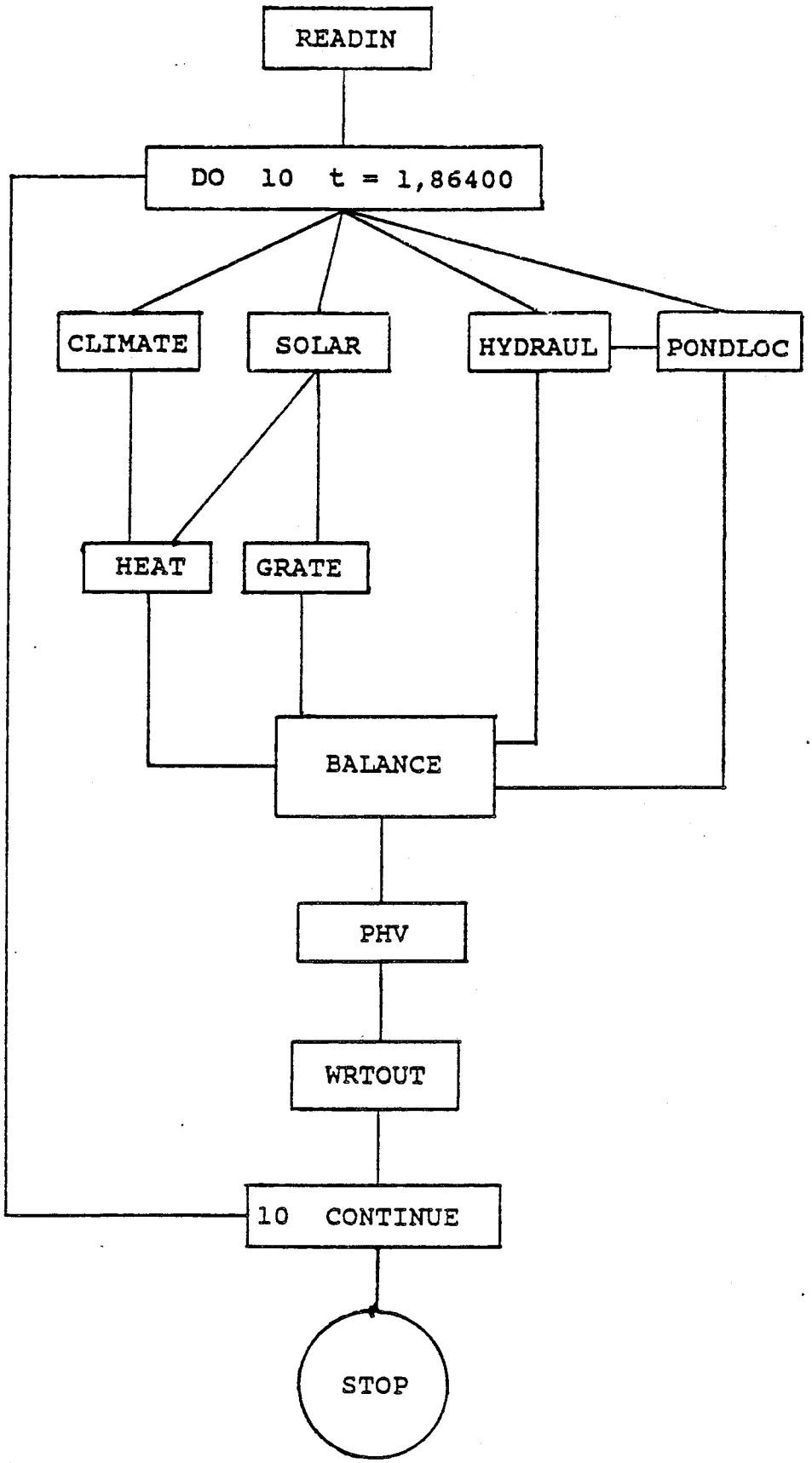


TABLE 3.2 MASS AND ENERGY BALANCE EQUATIONS

Algae:

$dX/dt = FX_1 + (u-D)X$
$u = u(T,I)$

Carbon Dioxide:

$dCO2/dt = FCO_{2i} - DCO_2 - kla/d(CO_2 - CO_2^*) - CUR$
$CUR = Yc \times u \times X$

Oxygen:

$dO2/dt = FO_{2i} - DO_2 - kla/d(O_2 - O_2^*) + OER$
$OER = Yo \times u \times X$

Energy:

$dT/dt = F(T_i - T) + Enet/(C_p \times rho \times d)$
$Enet = Elio + Elwr - (Eevp + Erad + Ecnd + Ecnv)$

Variables:

rho = density(g/m^3)
F = specific inflow rate(1/sec)
CUR = carbon uptake rate(moles/l/hr)
OER = oxygen evolution rate(moles/l/hr)
Cp = heat capacity(J/g/K)
d = depth(m)

Subscripts/Superscripts:

i = inflow
c = carbon
o = oxygen
lio = solar radiation term
lwr = long wave atmospheric radiation
evp = evaporation
rad = pond surface radiation
cnd = conduction
cnv = convection

HYDRAUL. This subroutine calculates any net exchange of media to or from the pond based upon a given input/output schedule and specific rates of inflow or outflow. New estimates of the volume and depth are calculated and any flow terms used in the mass/energy balances are initialized. Also as part of this routine the total amount of time required for recirculation based upon design specifications is determined as well as that for carbonation based upon carbonator size.

PONDLOC. This routine keeps track of the location of the fluid element and indicates whether it resides within the carbonator zone or the open pond. It also determines whether to inject CO2 (to lower the pH) based upon the culture pH and a predetermined upper pH value(PHSET). In its present configuration the pond pH value is simply shifted to PHSTRT when carbonation is required.

GRATE. A mean value of the growth rate is determined using pond temperature, incident light intensity, extinction coefficient, pond depth, cell density, saturating light intensity, maximum growth rate, and minimum, maximum and optimum temperature values. This is done by determining the mean light intensity at 1 cm depth intervals and integrating the calculated growth rates over the entire depth to obtain a mean estimate of the growth rate. No particular growth rate model is indicated here due to the fact that we have only considered cases of constant productivity in this analysis.

HEAT. The heat balance contains terms for solar radiation absorbed, net long wave radiation, radiation from the pond surface, evaporation, convection, and conduction. For the most part this analysis follows that described by Fritz et. al. (70) for predicting temperature profiles in waste stabilization ponds. The differences are in calculating the solar radiation term and the inclusion of conduction exchange with the earth surrounding the pond. Because of the small time intervals this was treated by the classical representation for heat transfer in a semi-infinite slab experiencing a step change in its surface temperature (81). Thermal conductivity and diffusivity values were used for that of a 50% clay/soil mixture that was saturated with water(k=3.3W/m/K and 1.3 x 10-6 m2/s (82). Inputs are the current estimates of air temperature, wind speed, and relative humidity from subroutine CLIMATE, the incident light intensity from subroutine SOLAR and the previous estimate of the pond temperature. Based on this, individual terms of the energy balance are calculated and the net heat exchange is determined (HNET). This is then passed to subroutine BALANCE to solve the energy balance equation.

BALANCE. The equations for algae, CO_2, oxygen, and temperature are integrated over one time interval using the IMSL subroutine DGEAR. Inputs, in addition to previous estimates of each state variable and the time interval, are growth rate, flow terms, kla, equilibrium concentrations for CO_2 and O_2 in seawater at the current temperature, and the net heat exchange term from subroutine HEAT. Outputs are the current estimates of the state variables.

PHV. Returns a current estimate of the pH based upon changes which occur in the total carbon concentration of the system. Specifically, the outgassing rate and carbon uptake rate are used to determine the net carbon lost from the system over one time interval. The carbon dioxide acidity is then determined by the difference between total carbon and alkalinity. Solution for a pH involves finding the zero of a 4th order polynomial in hydrogen ion concentration (83). The concentration of individual carbon species is then determined using the equilibrium expressions given in Stumm and Morgan (63). The 1st and 2nd dissociation constants and the ion product of water are all for seawater and temperature dependence is included.

3.5 RESULTS

3.5.1 Introduction.

Here is presented the initial results of a general Algae Pond Model (APM), as it applies to one specific site, Roswell, New Mexico, and a specific pond system design. Examples of model outputs for temperature, oxygen, and pH profiles are presented. Also, some validation measurements and experiments on the small ponds of Microbial Products, Inc. at Fairfield Calif. is presented.

3.5.2 Temperature

Solutions for the diurnal pond temperature were obtained for the month of July, using averaged diurnal ambient temperature data for that month, for pond depths of 10, 20, and 30 cm (Figure 3.4). It is a evident that there is a significant dampening effect due to depth of the pond which is a natural result of the increased thermal capacity of the system. For the worst case (10cm depth) a temperature amplitude of 30^{o}C is predicted over roughly a 10 hour period (heating phase) This amplitude is reduced by half for the 20cm case (T= 15^{o}C and by one third for the 30cm case (T=10^{o}C). While this specific case possibly represents an extreme because both heating (radiation inputs) and cooling (evaporative losses) rates are at a maximum during July, it nevertheless points out the very large diurnal fluctuations in pond temperatures possible.

In order to visualize the influence of evaporation rate on the temperature profile a series of simulations were run where the wind term in the evaporation rate expression was varied from zero to 15mph. The resulting evaporation rates ranged from 0 to 0.72 cm/day. (The model assumes no convection term for air and thus results in an infinite boundary layer above the ponds at zero wind speed). The predicted temperature profiles are shown in Figure 3.5.

Figure 3.4 Diurnal Temperature Profile for July at Roswell, New Mexico vs. Depth

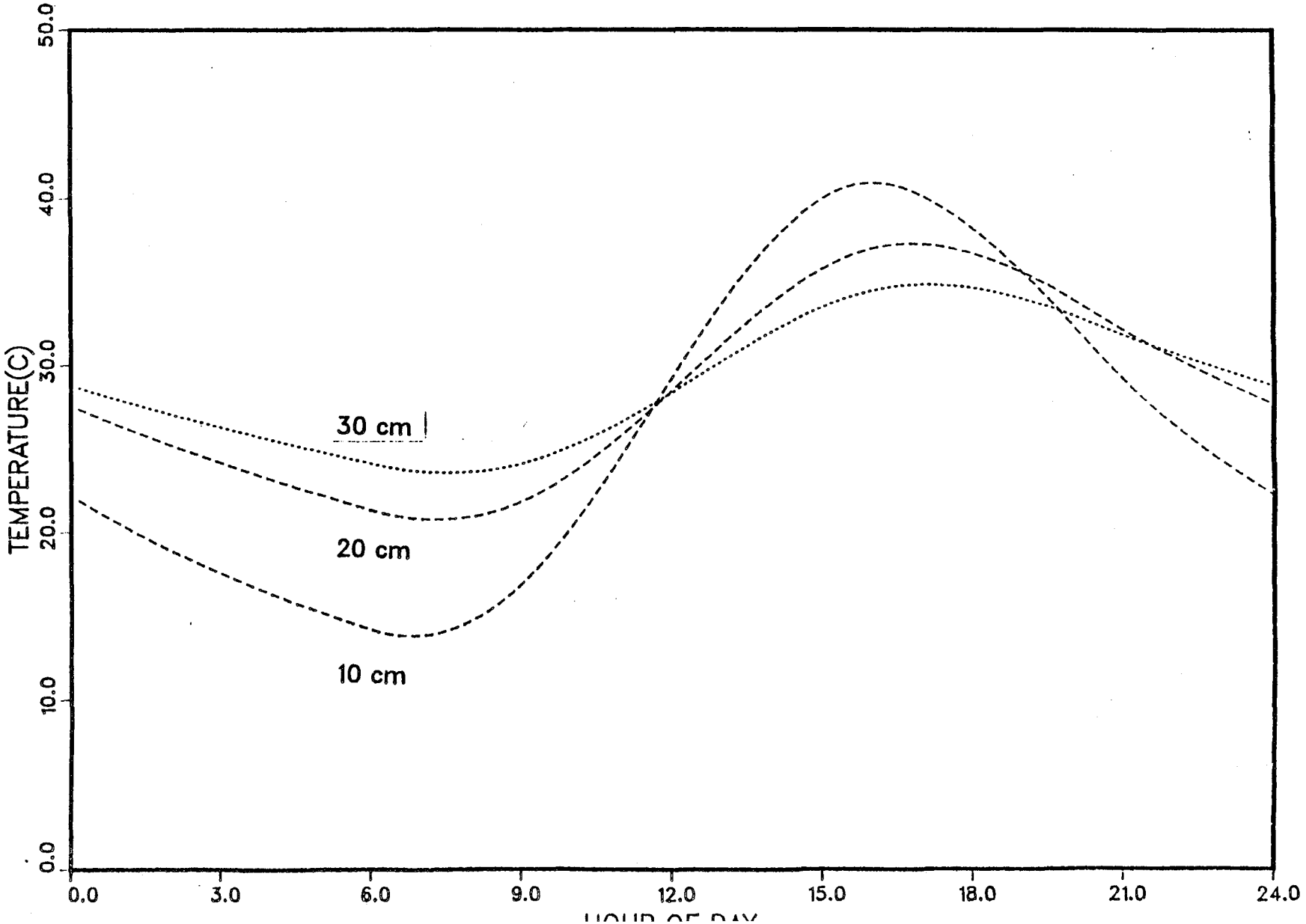
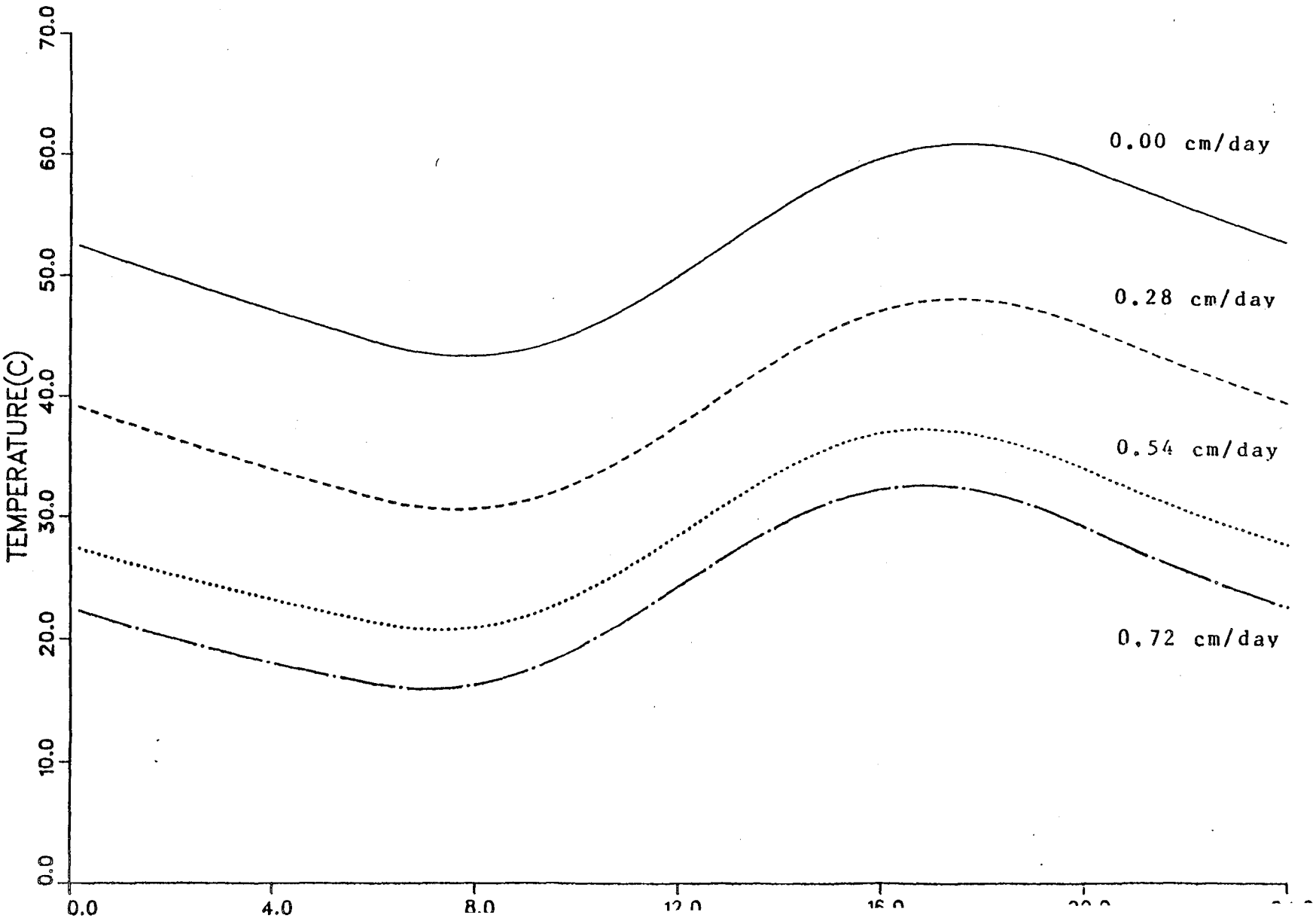


Figure 3.5 . Diurnal Temperature Profile for July at Roswell, New Mexico vs. Evap. Rate



The curve representing 0.54 cm/day was obtained from the averaged hourly wind speed data and corresponds to the 20cm pond depth case in the prior Figure (Fig. 3.5). Since the shape of the diurnal curves is similar in all cases, it appears that averaging wind speed on a daily rather the hourly, basis is not a major determinant of diurnal pond temperature profiles. The model does not contain a term for wind fetch over the ponds, thus wind direction is not a variable in it. The results suggest that evaporation rate is the major determining factor of the average and maximum-minimum diurnal pond temperature but does not greatly affect the amplitude of the temperature range as such.

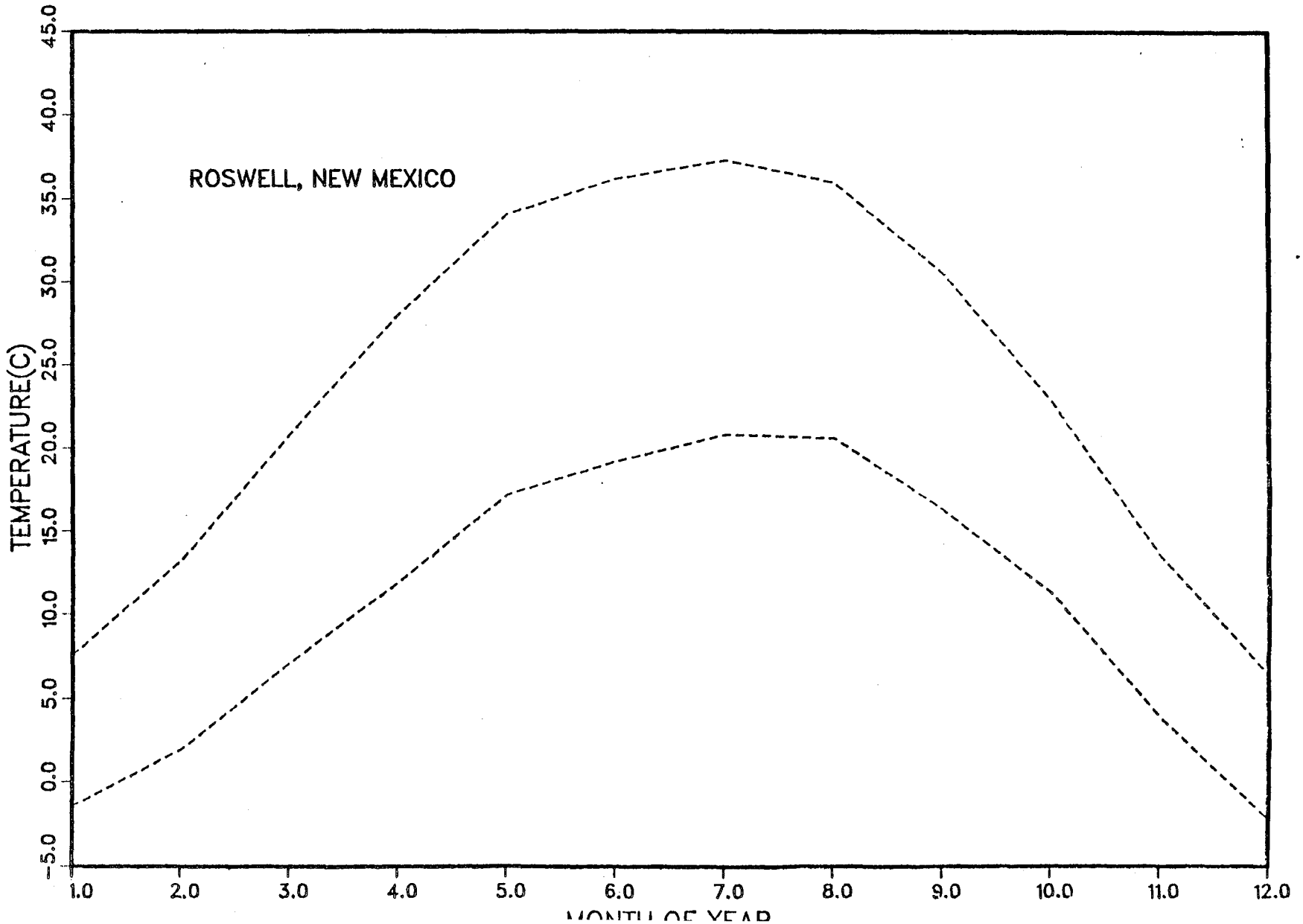
As an estimate of the reasonableness of the evaporation rate predictions the "average" rate of evaporation was calculated each month and an integrated value determined for the year. This calculation yields an evaporation rate of 140 cm/year, which is roughly 25 % lower than the rates published for Pan A evaporation for this location of the country (84). Although the predicted values for evaporation may be low, and thus the predicted temperature profiles may be slightly high, pan A evaporation rates can not be easily extrapolated to ponds and this issue requires further investigation.

Finally, temperature profiles were simulated for each month of the year in order to estimate the duration of, and limitations on, the growing season for this particular site. The results are shown in Figure 3.6 (again for a pond a of 20 cm depth). It is reasonable to expect that once average pond temperatures begin to fall below 20°C in the daytime and 10°C at night, significant declines in productivity will take place, possibly sufficient to make pond operations uneconomical. This would reduced the growing season to the march-october period, or about seven months of the year. However there is uncertainty regarding the actual temperature extremes that may be tolerated by potential candidate algal species and there may be techniques to better manage the heat budget to the ponds. Thus a growing season of eight to nine months may be possible at this site. Obviously, temperature responses of the algal species are of great interest.

3.5.3 Oxygen

Perhaps one of the most important environmental factors affecting pond performance is the oxygen buildup that occurs during the daylight hours as a result of photosynthesis. Depending upon productivity and outgassing (including at the carbonator) dissolved oxygen may reach up to 500% of equilibrium (with air) oxygen tensions (4). The outgassing coefficient is subject to great uncertainties, and will be influenced by a number of variables in pond design and operation (mixing velocity, depth, surface roughness, salinity, temperature, etc.). In principle the kl_a will depend on any physical or chemical factor that reduces the effective film transfer thickness. The Parkhurst Pomeroy correlation (85) predicts a value of $1x10^{-5}$ m/sec for a channel depth of 20cm, flowing at 20cm/sec.

Figure 3.6 Seasonal Distribution of Max/Min Pond Temperature for Roswell, New Mexico



Our measurements indicated that this value is roughly 3.4 times too low (see section 3.5.6). Other values in the literature range up to $9x10^{-5}$m/sec (74,85,86). Since it was difficult to assign a specific outgassing coefficient to predict O_2 profiles in the ponds, a range of coefficients was used to predict maximum O_2 tensions for a 20 cm deep pond (at the standard productivity of $30g/m^2/day$ as a function of time (starting from air saturation levels). It is evident from the results presented in Figure 3.7 that very high O_2 tensions can be reached, up to ten fold air saturation, in the case of the lowest kl_a assumed. Although such high levels have not been observed, and are unlikely to be reached in practice due to nucleation effects that lead to enhancement of oxygen desorbtion, oxygen levels of up to 500% of saturation are predicted by the model and have been observed in practice (4, see above).

3.5.6 Carbon Dioxide and pH

Culture CO_2 levels, and hence pH, will be dependent upon alkalinity, pond depth, outgassing coefficient, and productivity. Alkalinity and pond depth combine to determine the total carbon storage pool (for any given pH range), while productivity and outgassing determine the rate at which the pool is reduced. The efficiency of CO_2 utilization (e.g., the fraction of total CO_2 supplied incorporated into biomass by the algae) therefore will be highest where conditions restrict the degree of outgassing (i.e. at low dissolved pressures of CO_2, low turbulence).

This is shown in Figure 3.8 for three different alkalinities, at a kl_a of $3x10^{-5}$m/sec, a constant productivity of 30 $g/m^2/day$ and a 20 cm depth. This data was generated by using the pHV subroutine and assuming a final dissolved CO_2 level of $1x10^5$ M (the equilibrium CO_2 level with air). For a given alkalinity curve a lower value of the starting pH represents a larger total carbon storage capacity, and subsequently a longer time between carbonation stations (as shown in Figure 3.9).

It is evident that the utilization efficiency declines fairly significantly at the lower pH and higher alkalinity. At a pH of 7 and an alkalinity of 30 mM/l, the utilization efficiency would be only 63%. At the same pH for an alkalinity of 2 mM/l, the utilization efficiency would be 85%. At the high end (pH=8.0), there is much less of a differential in the efficiency and even 30 mMolar has a value exceeding 90%. This analysis illustrates the advantage, in terms of reducing potential CO2 losses, of operating at higher pH ranges (ie. pH > 8). This would be reflected in a smaller storage capacity which is demonstrated graphically in Figure 3.9.

The specific trade-offs between pH range and alkalinity will be determined by the relative optimization between the costs of CO2, capital costs for carbonators, and productivity reduction due to biological intolerance to pH extremes. A variety of pH-Alkalinity curves are presented in Figure 3.10, which correspond to differ-

Figure 3.7 Time Dependent Oxygen Profiles vs. Outgassing at a Productivity of 30 g/m^2/day

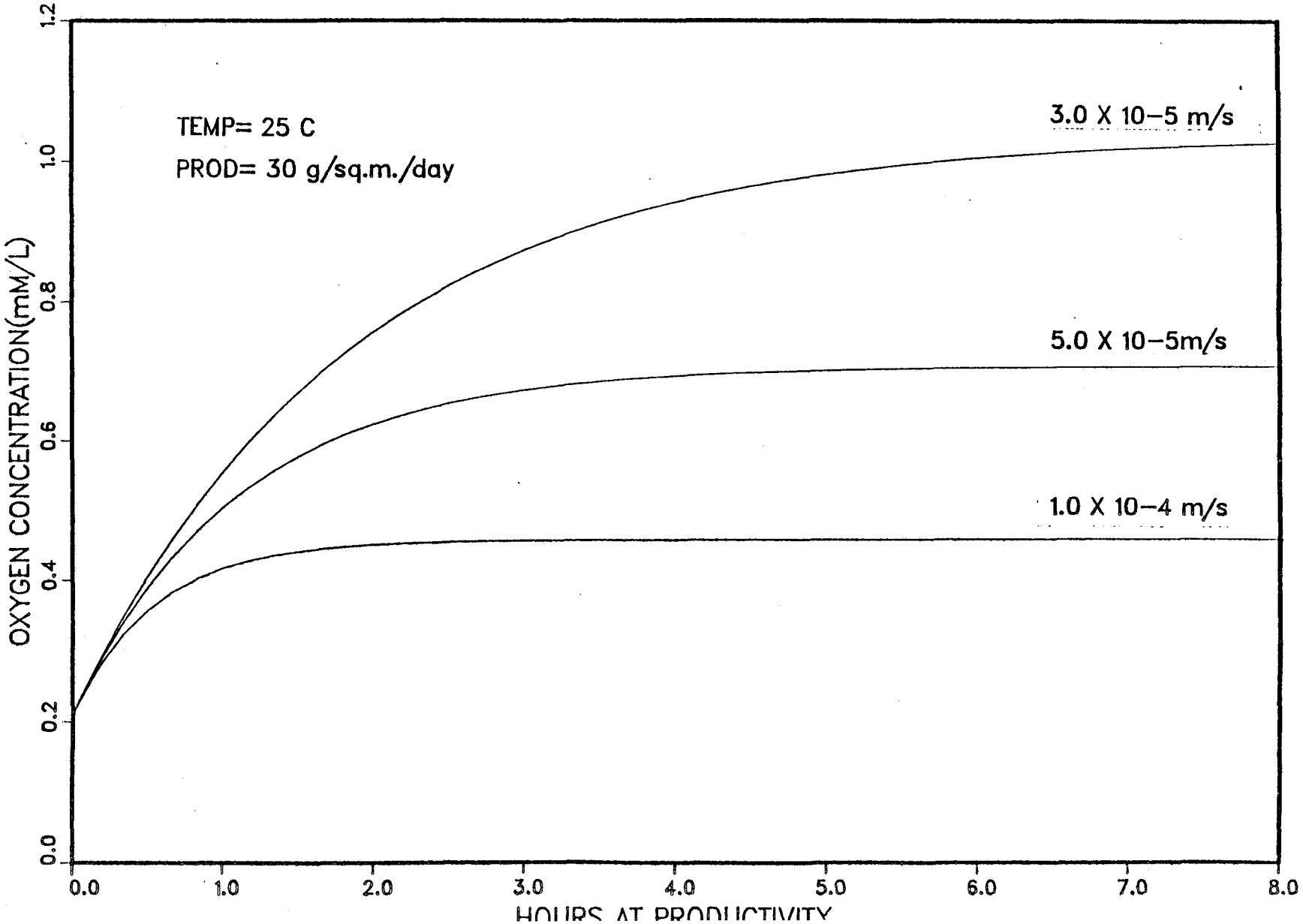


Figure 3.8 CO_2 Utilization Efficiency vs. Starting pH at 2, 10, and 30 mM alkalinity

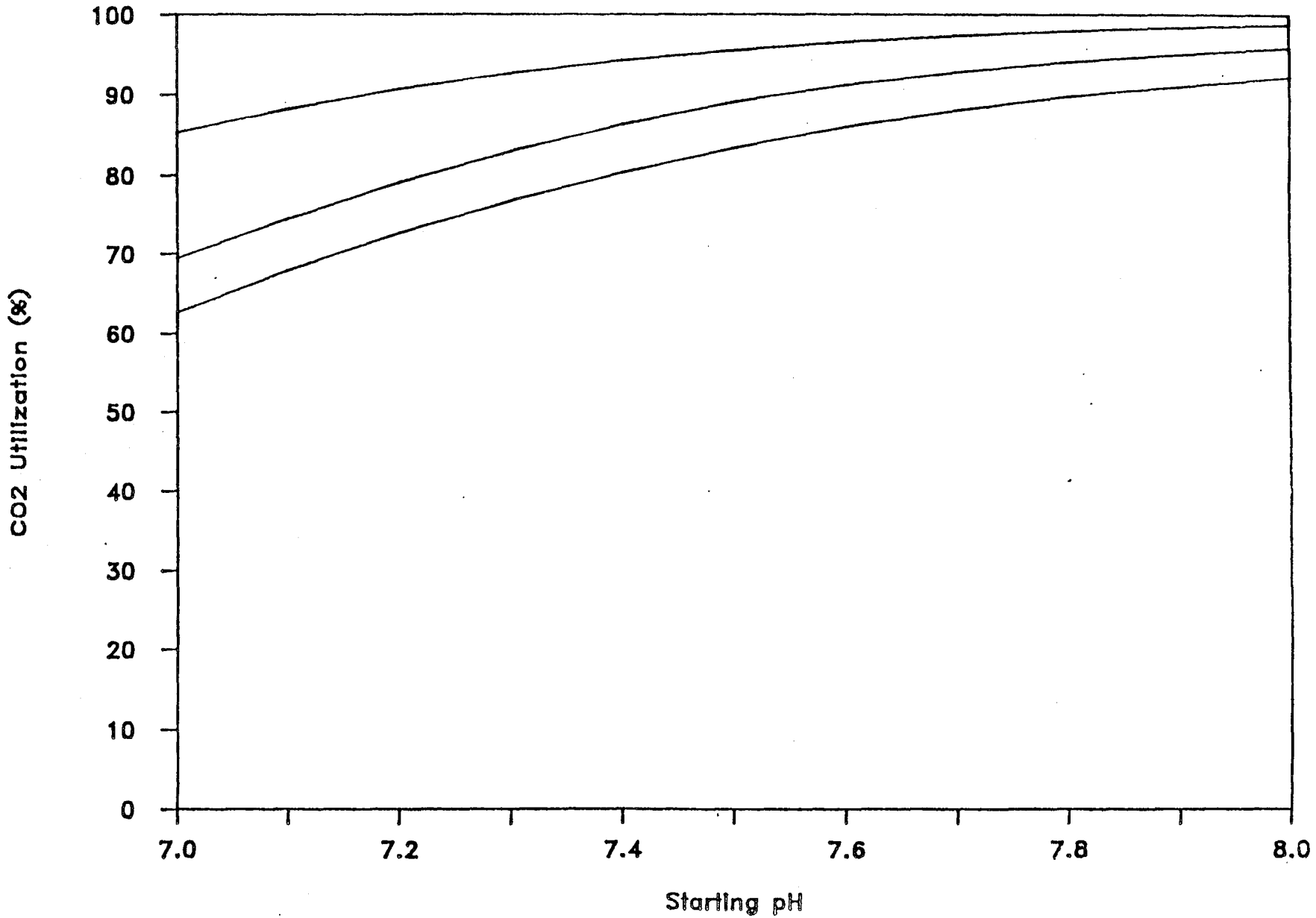


Figure 3.9 pH-Alkalinity Relationship vs. Starting pH for Carbonation Intervals of .5,1,2,4,8 hours

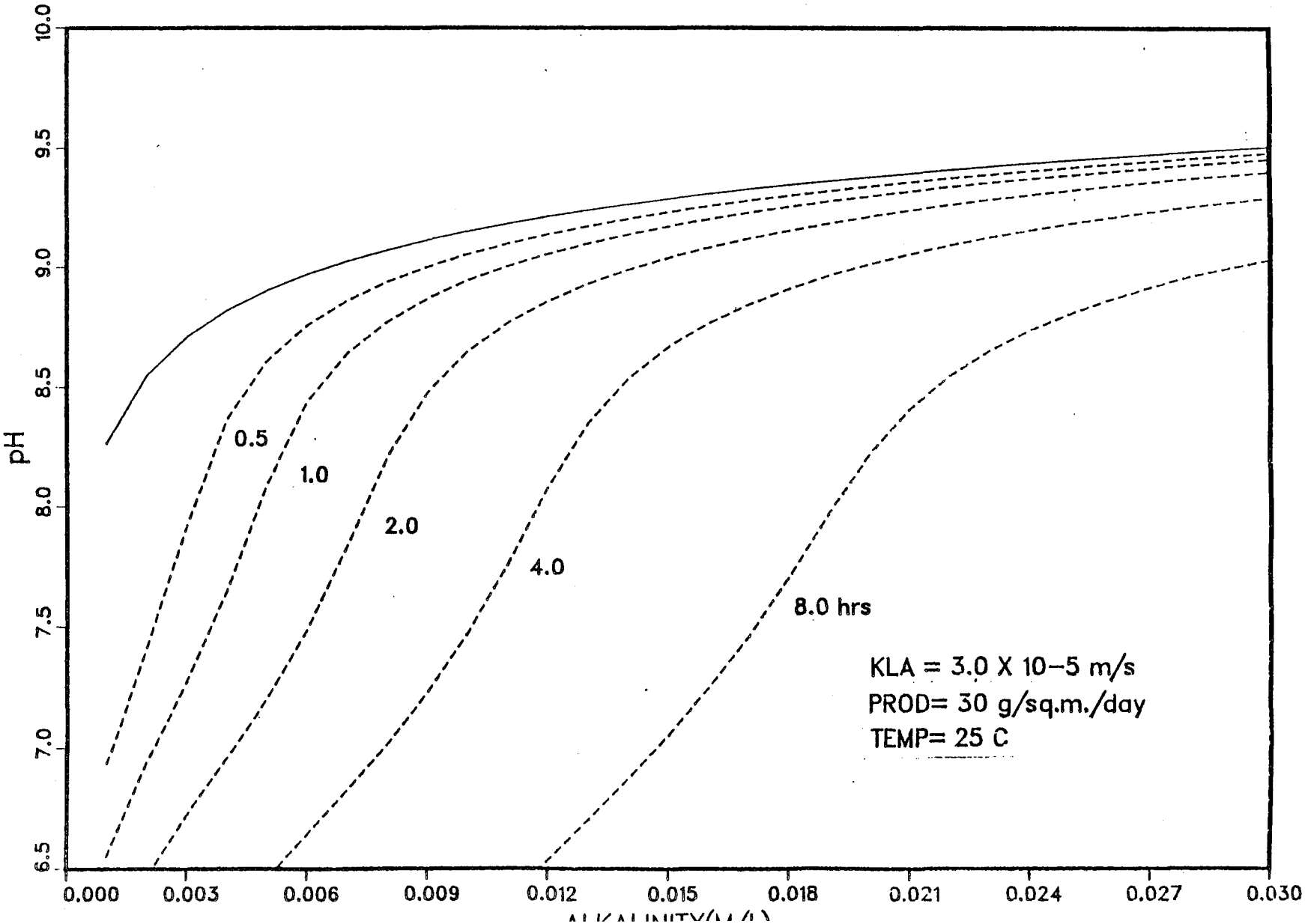
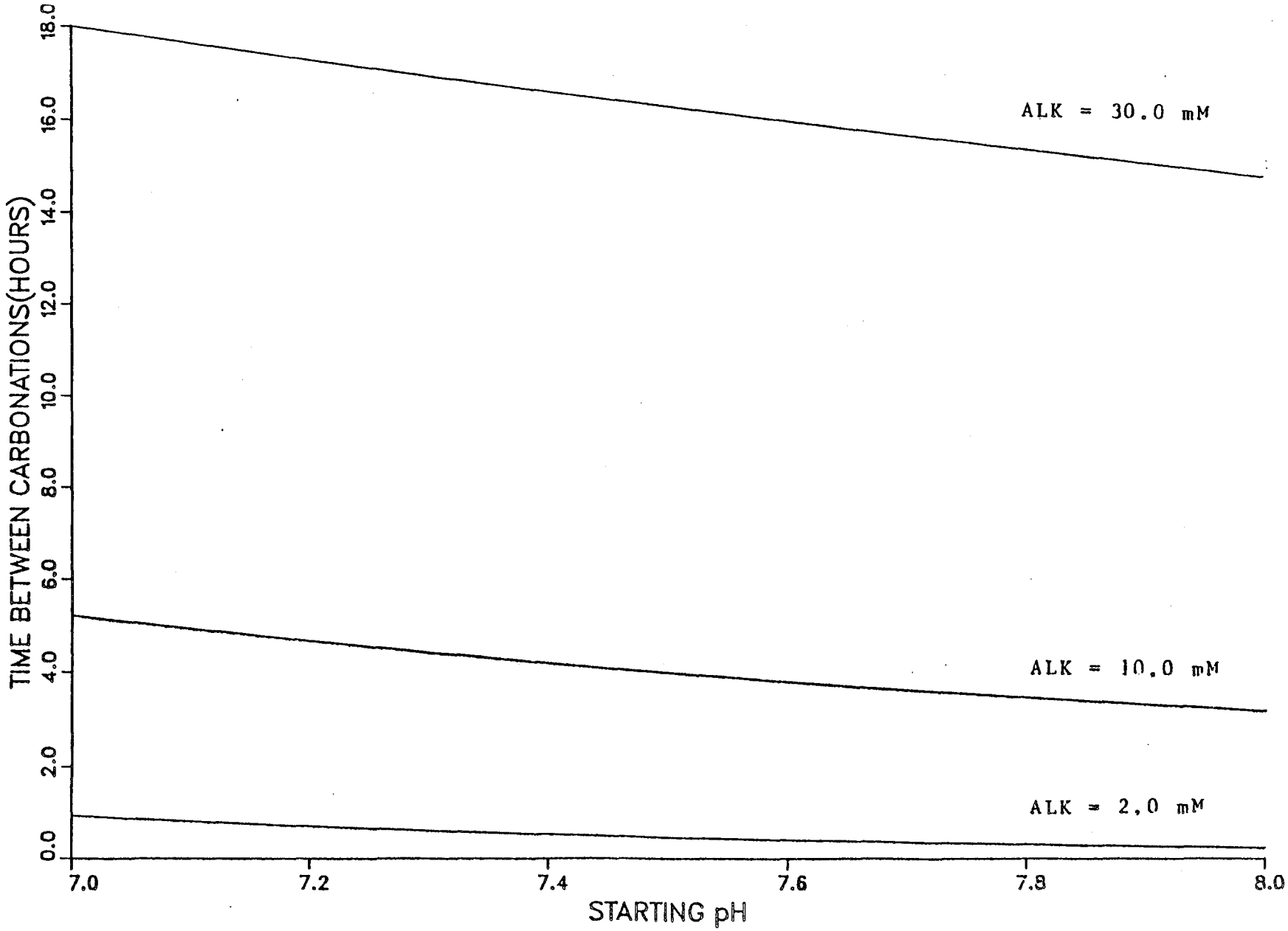


Figure 3.10 Time Between Carbonation Stations vs. Starting pH at 2, 10, and 30 mM alkalinity



ent cases of available carbon storage as measured by the time between carbonations. The single solid line (top curve) represents a dissolved CO2 concentration of 1 x 10^{-5} moles/l and thus indicates the pH endpoint for a given carbonation cycle. The resulting pH range at a specified culture alkalinity is easily predicted depending upon the frequency of carbonation. For cultures with low alkalinities, close to that of seawater(2mM/L), fairly large fluctuations will occur and recarbonation would have to be at intervals less than one hour. This would restrict the maximum size of one pond (with one carbonator) to about 1 ha.

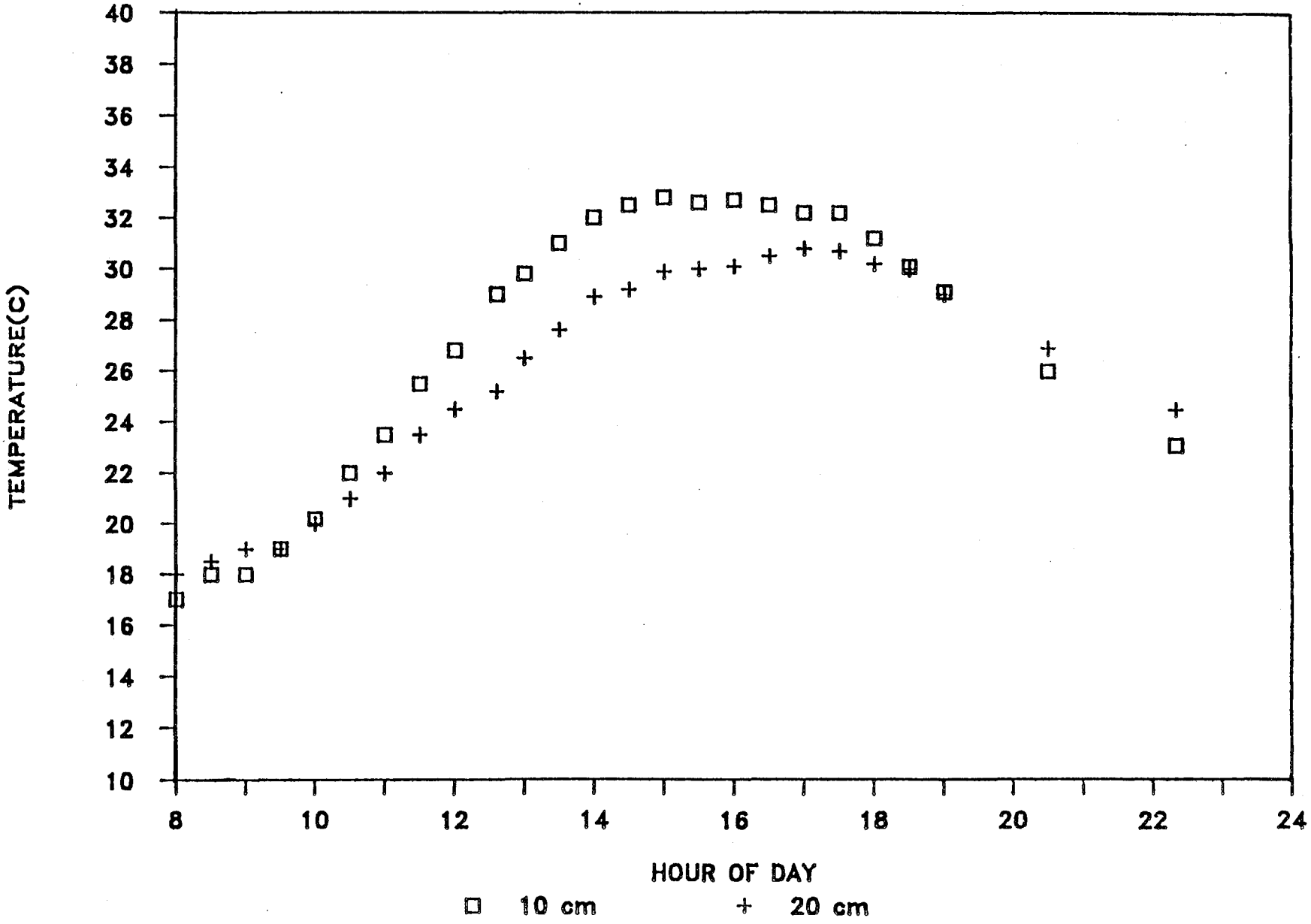
3.6 OUTDOOR POND MEASUREMENTS

The utility of any model is limited unless the data upon which it is based is physically sound and the predictions derived are reasonable in light of available information. For these reasons an effort(somewhat limited at this stage) was made to measure oxygen and temperature profiles in a small 1.2 m^2 experimental pond (pH was controlled between 7.5 and 7.8). In addition, experiments were performed to determine the mass transfer coefficient for the pond. These experiments were carried out in Fairfield California using the experimental ponds operated by Dr. J. C. Weissman of Microbial Products, Inc.

Dissolved oxygen was monitored over the course of the day at 15 minute intervals. The probe could not be left unattended because rapid fouling affected the readings. The pond was 15 cm deep, mixed at 30 cm/sec, and had a cell density of 200 mg/l of Chaetoceros sp. When measurements were begun at 8:30 AM (one hour after sunrise) the DO was already at 50% above saturation. Readings went off scale at 12:30 PM representing a DO of greater than 400 %. By 5 PM it was back on scale ending at about 100 % saturation by 7 PM (one hour after sunset at this site). The following morning a point was taken at 7 AM and it was slightly below saturation.

Shown in Figure 3.11 are the temperature profiles collected for two ponds operated at 10 and 20 cm. The mixing speed was 20 cm/sec. The initial points, starting at 8 AM were a little high because a heater was left on in the pond for the two previous hours. On the following morning(data not shown) the temperature at 6 AM was 14 ^{o}C and 16 ^{o}C for the 10 and 20 cm ponds respectively. The maximum temperatures were 32 and 30 degrees C and therefore the amplitude of the temperature fluctuations were 18 and 14 ^{o}C. There was roughly a four hour portion of the day, centered around 4 PM, where the temperature remained at its maximum for the 10 cm pond. The 20 cm pond slowly reached its maximum at 5:30 PM. The ambient temperature peaked at 37 ^{o}C from 3 until 5 PM, thus the shallower pond appears to have tracked the ambient temperature more closely. The small difference in temperature profiles as compared to that shown in Figure 3.4 indicates a problem with the model. Even though the environmental condi-

Figure 3.11 Diurnal Temperature Profiles for 20 cm Ponds for September 1986 in Pleasants Valley, CA



tions were not identical, they were similar enough to raise doubt concerning model performance, thus reinforcing the need for model validation. However, it must be pointed out that these small ponds have edge effects that may make a significant difference in the model.

Figure 3.12 is a plot of the rate of carbon loss from the pond as a function of the CO_2 driving force. The data represents two separate experiments carried out on a 1.2 m^2 pond over the pH ranges 6.77 to 7.59 at 15 cm depth. These experiments were performed by injecting CO_2 into freshwater of known alkalinity(determined to be 2.67 mM) and following the pH change as a function of time. Using the KLA.FOR program the pH values were converted into dissolved CO_2 and total dissolved carbon. The rate of change in total dissolved carbon was then plotted against the calculated average CO_2 driving force for that pH interval. Henry's law was used to calculate the equilibrium CO_2 concentrations. Its value as well as the dissociation constants and the ion product of water were corrected for temperature(26-30 C). The slope of the data shown in Figure 3.11 was .0136 +/-11.4% which translates into a kla value of 3.4 x 10^{-5} m/sec.

3.7 SUMMARY AND FUTURE WORK.

A model of a shallow, well mixed, raceway type algae cultivation pond has been developed which uses geographical, climatic, design, physical, and biological inputs to predict diurnal and seasonal variation of temperature, oxygen, and pH/CO2. Specific cases of diurnal temperature patterns and monthly max/min temperature profiles have been solved for a potential algae cultivation site in the U.S. Southwest. More general treatments of oxygen buildup and pH/CO2 behavior under a variety of conditions are also given. It has been shown that significant fluctuations in all of these variables may exist with periodicities and magnitude determined by operating/design assumptions. For example it was found that both evaporation rate and outgassing coefficient affected model predictions significantly, thus pointing to a need to measure these parameters under real operating environments. Finally the existence of a rather dynamic environment, as would be present in the system described here, indicates a need to better understand the biological response to these transients and its effect on both species productivity and dominance.

Future work on the model will focus on the energy balance section and validating predictions with available data sets. One such set has been collected by Dr. J. Weissmann of Microbial Products on the 1.2 m^2 experimental ponds at Pleasants Valley over this past summer. This data set includes maximum and minimum temperatures, daily evaporation rates, and daily insolation totals. These ponds were heated for several hours in the morning with a 500 W heating coil (to bring their temperature up to 25 C) and this will have to be taken into account.

One particular problem with the model is the accuracy of the evaporation expression. In general these equations tend to be specific to the body of water they originally correlated. Algae ponds aren't well represented by either shallow lakes and reservoirs or pan A estimates. To make the model more tractable to a given location, estimates of daily evaporation rates(for that location) will be used in the model. Daily evaporation rates will be differentiated into instantaneous (minute intervals) rates by solving the evaporation equation iteratively, using the vapor pressure gradient as representing the driving force. A solution will be obtained when the integrated evaporation rate equals the input value. A further search of the literature for a more appropriate rate expression, to be used in the absence of evaporation data, will also be undertaken.

After the temperature predictions of the model have been validated, an analysis of temperature profiles for two separate sites of different climatic conditions will be performed. This analysis will include the monthly distribution of max/min temperatures and the diurnal variation of temperature as a function of depth and season. A final area of investigation will be to determine to what extent dilution rate "profiling"(over the course of the day) can improve the temperature balance of the pond. For example diluting the pond during the middle of the day may decrease the maximum temperature of the pond (assuming makeup media is at a lower temperature).

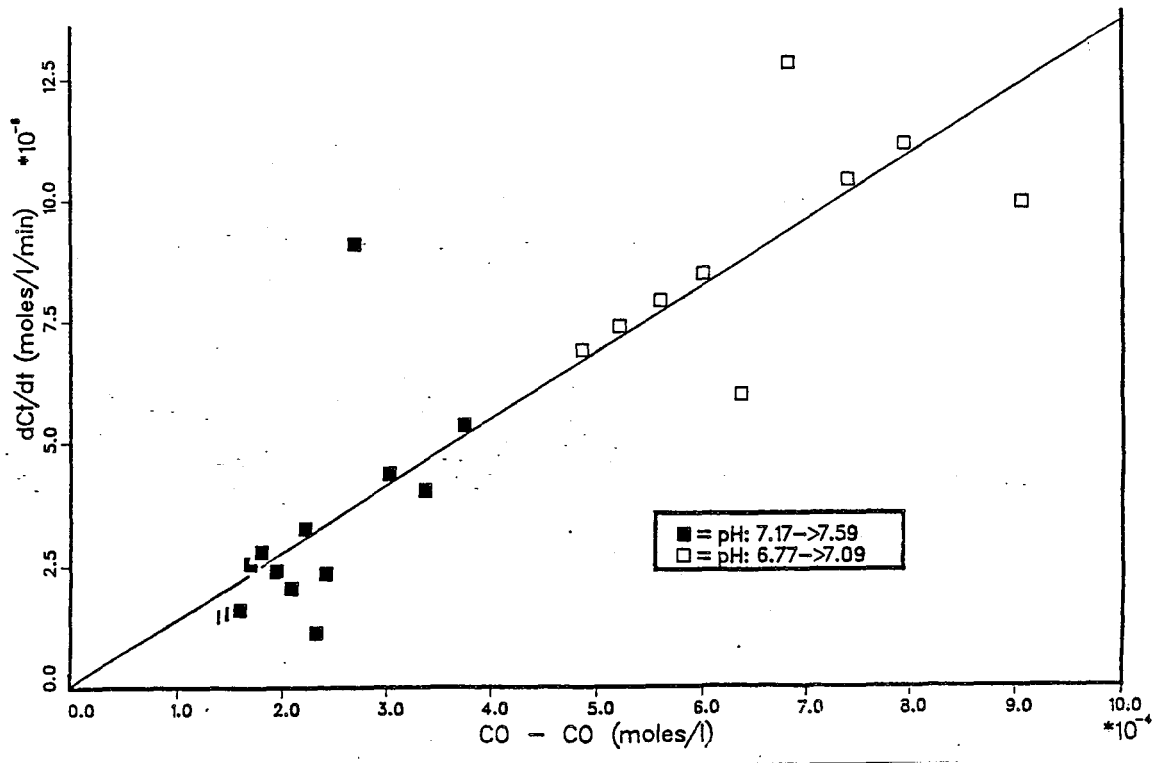


FIGURE 3.12 PLOT OF RATE OF CARBON LOSS VS. CO_2 DRIVING FORCE FOR A 15 cm POND MIXED AT 30 cm/sec

SECTION 4.0

MICROALGAE COMPETITION STUDIES IN LABORATORY REACTORS

4.1 INTRODUCTION

A series of species competition experiments were carried out with mixed cultures in order to estimate the selective importance of the pond environment. Three experiments will be presented here; another experiment (testing the relative competitiveness of several species of algae) is in progress and will be reported in the Final Report. We chose to investigate transient conditions of pH, temperature, and dissolved oxygen in factorial design experiments. In these studies we relied upon an experience base of pond conditions (rather then the results from the model presented in Section 3.0) in order to assign culture conditions.

Each experiment consisted of combining either 3 or 4 different algal strains at equal cell densities (100 ppm) and following their individual cell concentrations as a function of time. Overall culture dry weight and dilution rate were also recorded. Determination of success at a particular condition was measured by equilibrium cell concentrations, which at the minimum was taken to be 21 days (>12 residence times). The experimental design and the final results of the species competitions are summarized in Table 4.1. In the following sections the results of each experiment will be discussed and then a final summary section and future work plan will be presented. Details of the experimental apparatus and the methods are presented in the Appendix.

4.2 FLUCTUATING TEMPERATURE AND OXYGEN

In this experiment the effect of fluctuations in oxygen, temperature, and light on the competitiveness of Chlorella, Chaetoceros, and Cyclotella was investigated. A summary of the experimental conditions and results is given in Table 4.1a. In Figure 4.1 are shown the dry weight curves for each culture and in Figures 4.2a-d the cell count results.

One general observation for each of the cultures was that while significant cell concentration differences began to appear in the cultures between roughly 6 to 9 days(4-6 residence times) the final "equilibrium" result was not apparent until after 18 days (12 residence times).

For culture 131, kept at air levels of oxygen and constant temperature, a codominance between the Chlorella and Chaetoceros was observed. The about 3 fold decline in Chlorella between day 9 and

TABLE 4.1 SUMMARY OF EXPERIMENTAL CONDITIONS AND RESULTS FOR THE CONTINUOUS MULTISPECIES CULTURES

FLUCTUATING TEMPERATURE AND OXYGEN MIXED CULTURE EXPERIMENT

(Cell counts in million/ml at beginning and end of experiment)

#	TEMP oC	LIGHT E/m2/s	OXYGEN	CHLORELLA Beg.	CHLORELLA End	CHAETOCEROS Beg.	CHAETOCEROS End	CYCLOTELLA Beg.	CYCLOTELLA End
1	30	550	AIR	4	36	2	17	0.3	.06
2	30	550	AIR/O2	5	3	2	30	0.6	0.9
3	20-32	550	AIR/O2	4	7	1.5	33	0.3	0
4	20-32	550+SPOT	AIR/O2	4	94	1	72	2	0

FLUCTUATING pH EXPERIMENT

(Cell Counts in million/ml at beginning and after

#	ALK mM	pCO2 %AIR	pH	CHLORELLA Beg.	CHLORELLA End	CHAETOCEROS Beg.	CHAETOCEROS End	ANKISTl Beg.	ANKISTl E
1	1	.35	7.1	2	13	2	14	2	0
2	1-10	.35	7.1-8.2	3	14	2	21	3	0
3	10	.35	8.2	2	11	2	15	3	0
4	10	.04-4.0	7.1-8.2	3	16	2	14	3	0

DILUTION RATE AND SPARGER DESIGN EXPERIMENT

(cell counts in million/ml at beginning and after 18 days)

#	DILUTION RATE(1/d)	SPARGER	CHLORELLA beg	CHLORELLA end	CHAETOCEROS beg	CHAETOCEROS end	ANKIST. beg	ANKIST. end	NAVICULA beg	NAVICULA end
1	1.23(.05)	fritted	1.8	13	1.0	9.6	.95	.03	.59	.20
2	0.83(.01)	"	2.0	60	1.0	.19	.91	.02	.59	0
3	0.33(.03)	"	2.1	110	.88	.76	.94	.54	.59	.17
4	0.73(.02)	bubbler	2.1	13	.91	22	.94	.16	.61	.58

Figure 4.1 Dry Weight Curves for Cultures 131-134:
Fluctuating Temperature and Oxygen

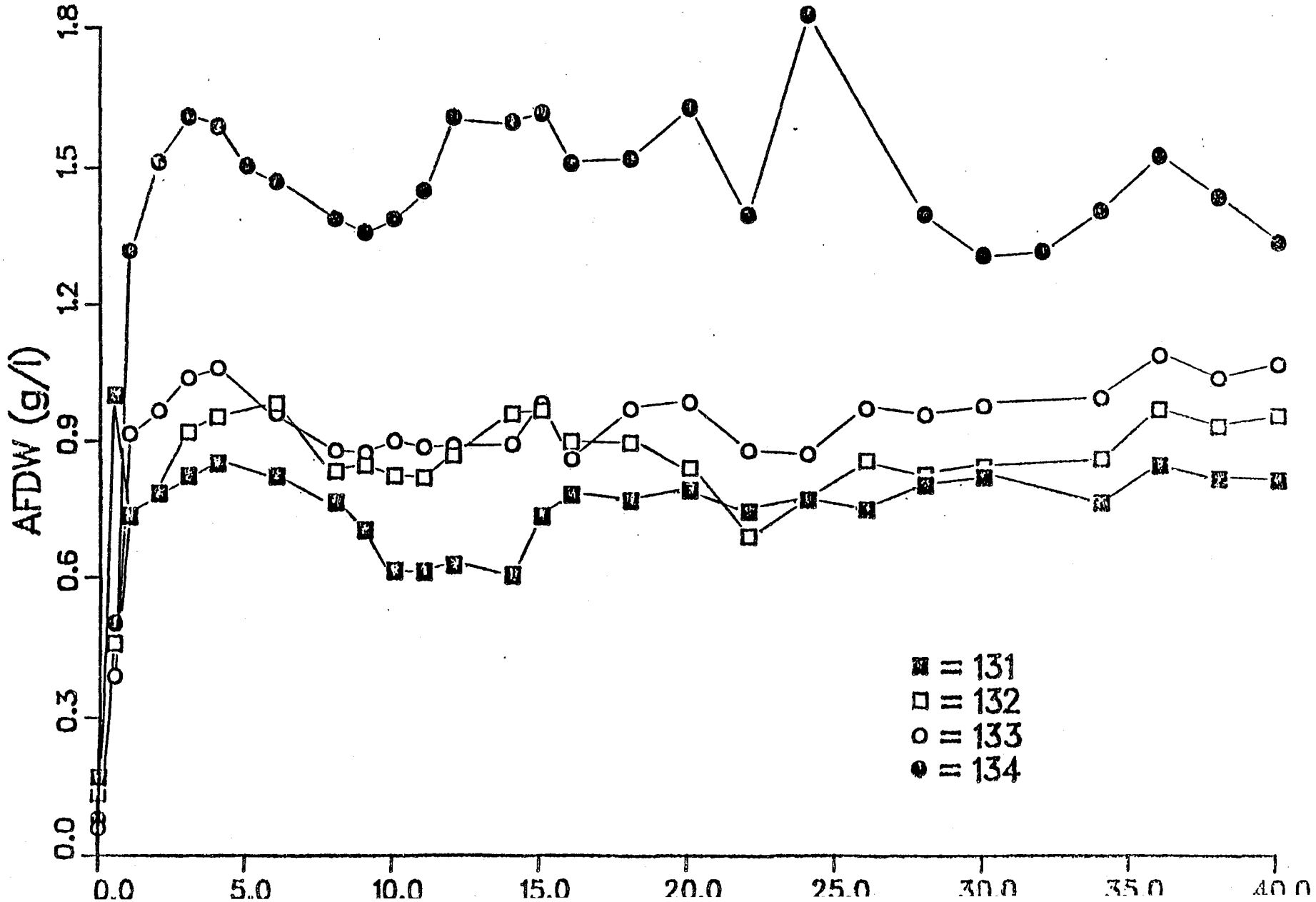


FIGURE 4.2a CELL CONCENTRATION FOR LOW OXYGEN AND CONSTANT TEMPERATURE CONDITIONS (CULTURE 131)

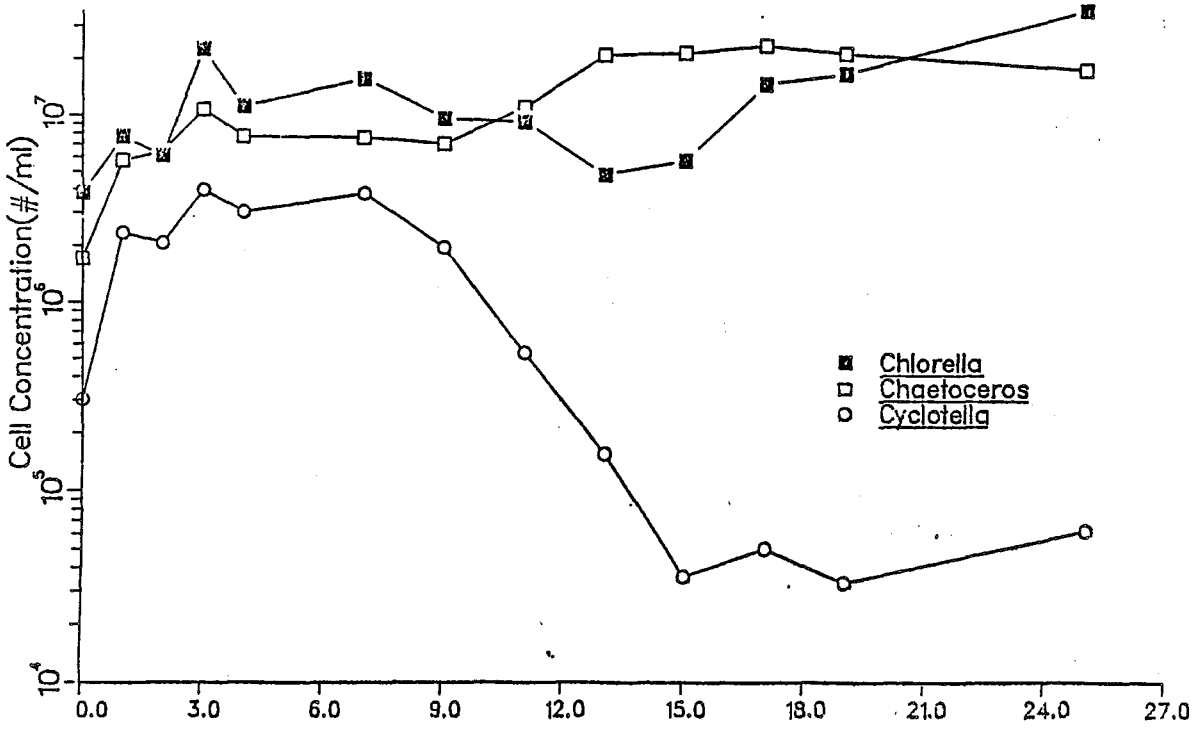


FIGURE 4.2b CELL CONCENTRATION FOR FLUCTUATING OXYGEN AND CONSTANT TEMPERATURE (CULTURE 132)

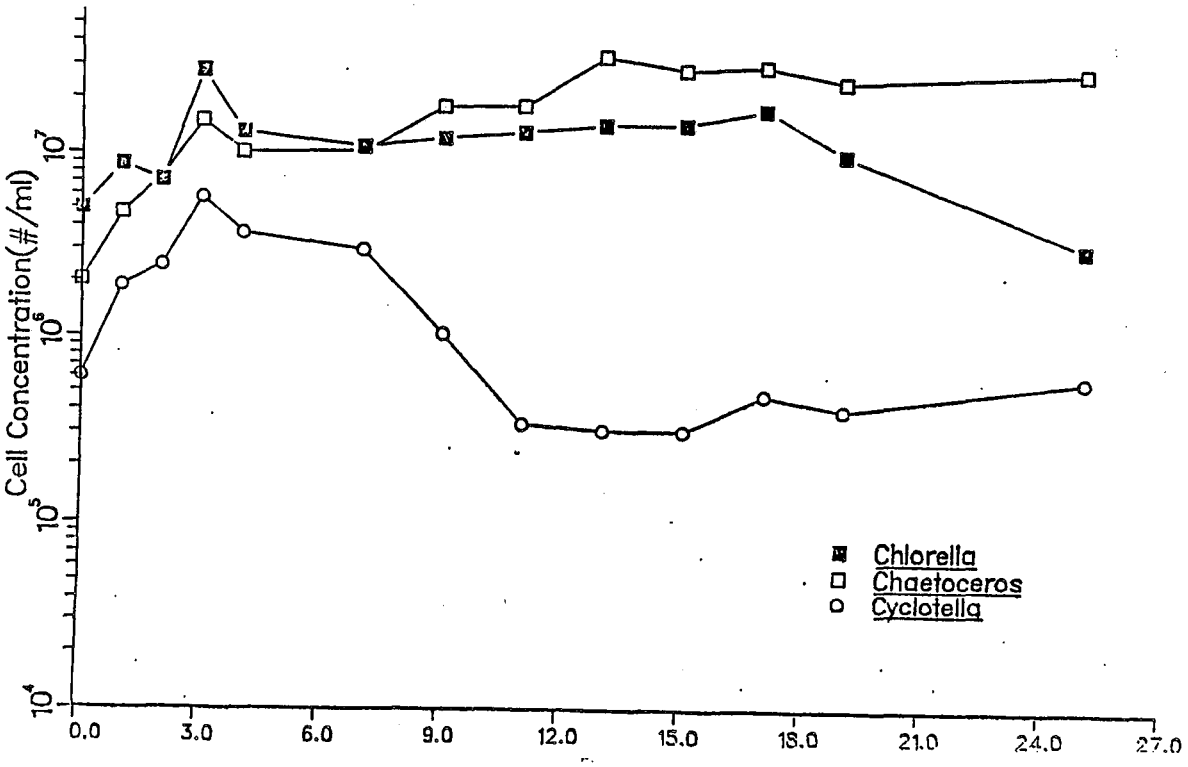


FIGURE 4.2c CELL CONCENTRATION FOR FLUCTUATING OXYGEN AND FLUCTUATING TEMPERATURE CONDITIONS (CULTURE 133)

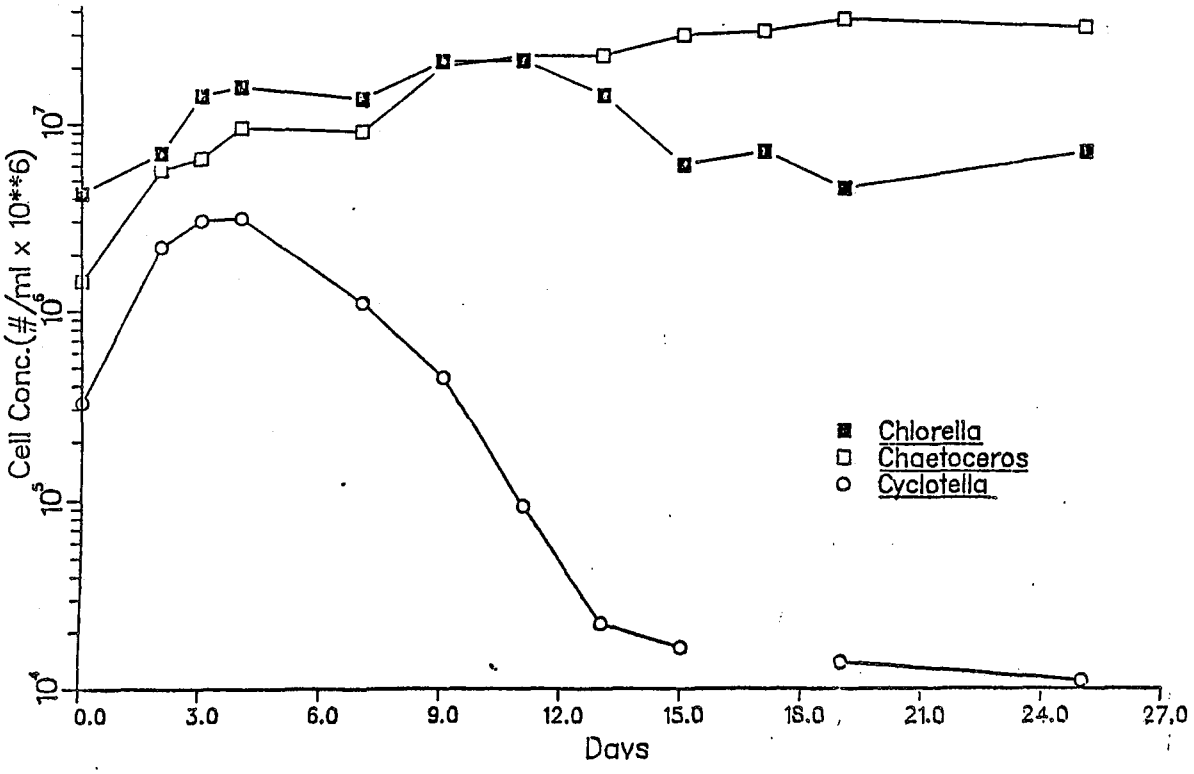
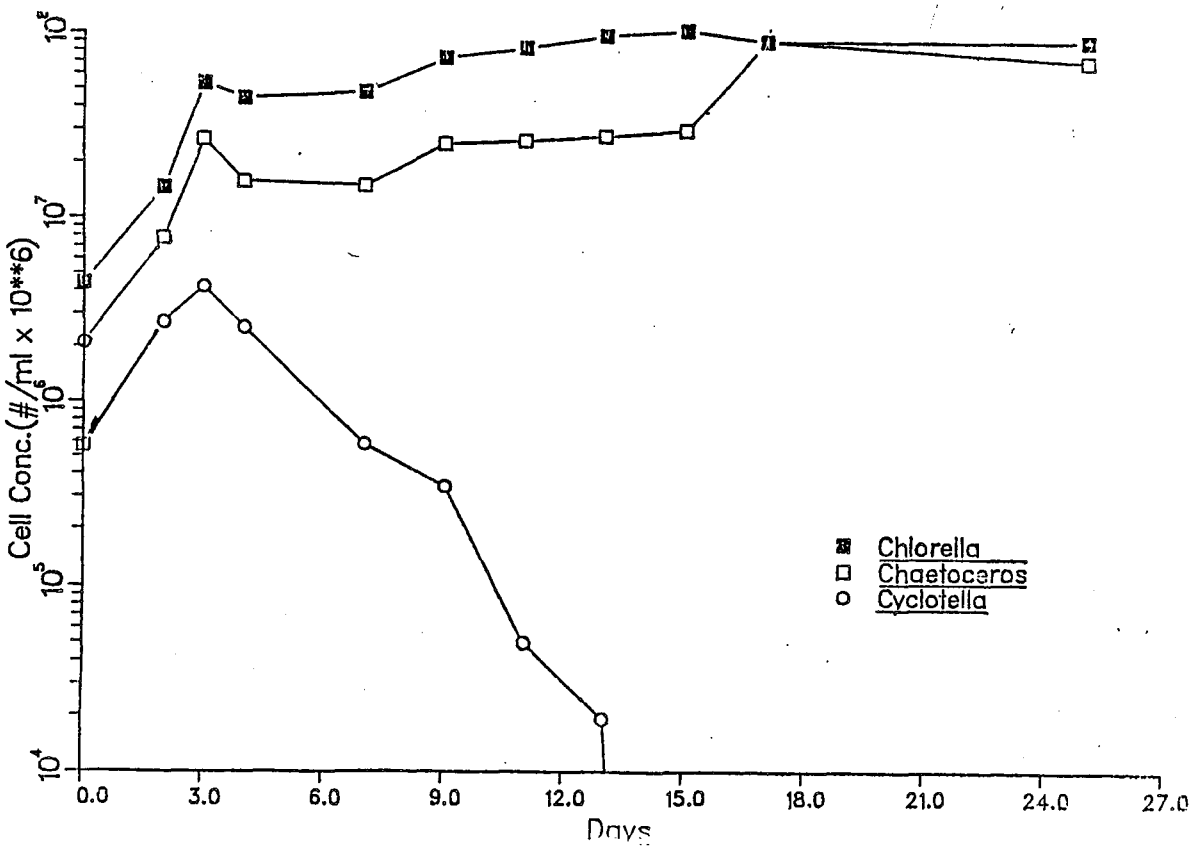


FIGURE 4.2d CELL CONCENTRATION FOR FLUCTUATING OXYGEN AND TEMPERATURE AND HIGH LIGHT CONDITIONS (CULTURE 134)



-99-

15 is believed to have been the result of a wall growth problem (which is specific to Chlorella). Chaetoceros increased three fold during this period. The exchange of reactor vessels at this point (day 15) resulted in an increase in total biomass and the gradual increase in the Chlorella concentration. By day 25 of the experiment it was roughly equal in biomass density as the Chaetoceros. All this occurred while no outward changes of the culture were noted. A cell count taken on day 45 (the final day of the experiment) gave cell concentrations of 34, 16, and 0.2 million cells per ml for Chlorella, Chaetoceros, and Cyclotella respectively. These results agree closely with those for day 25.

One clear observation of culture 131, was that the Cyclotella did poorly under this condition declining from day 7 (at 3.8 million cells/ml) to a final steady state cell concentration of about 0.05 million cells/ml by day 15. Thus under these conditions Cyclotella is not competitive with either Chaetoceros or Chlorella and that these two species are about equally favored.

Culture 132 tested the effects of a periodic high oxygen environment, while maintaining a constant temperature as in 131. For this condition, the Chaetoceros clearly dominates over both the Chlorella and Cyclotella. After day 18, there is an additional increase in the Chlorella concentration which by day 45 was at 2.2 million cells/ml. The Cyclotella, while still being a poor competitor did roughly an order of magnitude better than in the low oxygen case(where Chlorella had been more competitive). A similar pattern as in culture 131 is seen: a rapid increase in all cell counts, but with Chlorella favored followed (day 7-10) by a crash in Cyclotella and a subsequent rise in Chaetoceros, which remained dominant.

Culture 133 was designed to evaluate, in addition to high oxygen tensions, the effect of a fluctuating temperature (on a diurnal cycle). Similar to culture 132, Chaetoceros is the dominant species after 10 days of growth with cell concentrations more or less the same as in culture 132. Chlorella growth is again depressed presumably due to the high oxygen tension. The major influence of the temperature fluctuation was the disappearance of Cyclotella, which may be an indication of this organisms sensitivity to the temperature regime.

Culture 134 was designed to determine the combined effect of high light, high oxygen and fluctuating temperature. Cell densities were considerably higher in this culture reaching over 100 million cells per ml. Under this condition both Chlorella and Chaetoceros did very well. As compared to culture 133 (without the high intensity spot) Chlorella was significantly more competitive, occupying approximately 25-35 % of the biomass compared to less than 10 % in culture 133. Cyclotella disappeared from culture after 13 days(as in culture 133) so the higher light environment had no measurable effect on it.

The codominance between Chlorella and Chaetoceros, seen in all cases studied, suggest competition for different resources. This would result if the two had different spectral absorption patterns and therefore be utilizing separate regions of the radiation spectrum. While it is difficult to measure an organisms absorption pattern accurately , because of light scattering, a semi-quantitative result can be obtained when neglecting the scattering effects. This has been done and is shown in Figure 4.3 Even though the units are somewhat arbitrary, distinctly different patterns are obtained for each strain. Additionally, the effect of the high light (in culture 134) was to favor Chlorella, which one would expect due the red spectral emphasis of the quartz spot lamp. Thus this experiment, while not conclusive, does support the contention that codominance may exist between strains with different absorption patterns.

4.3 FLUCTUATING pH

For the most part, pH changes in engineered systems are the result of CO2 gas transfer and photosynthesis, while alkalinity remains constant. Deleterious effects of the pH fluctuations could either be the result of changes in the concentration of dissolved CO2 or the hydrogen ion concentration itself. In this experiment, pH fluctuations at one hour intervals, were applied by either step changes in the alkalinity(.5 to 10 mM) or by varying the pCO2 concentration from air levels(.035%) to 3-4 %. Mixed cultures containing the organisms, Chaetoceros, Chlorella, Cyclotella, and Ankistrodesmus were tested. A summary of the experimental conditions and results is shown in Table 4.2. Cell counts for each condition are shown in Figures 4.3a-d.

The most general observation that can be made from these results is that Chaetoceros was dominant for all conditions and Ankistrodesmus was noncompetitive for all conditions. The fact that Chlorella was not dominant is inconsistent with all other experiments tested at low oxygen and this dilution rate. The reason for its lack of competitiveness is unclear, but may be the result of the larger proportion of Cyclotella present in these reactors. There were no significant differences in productivity between reactors-they were all 400 mg/l/day. Furthermore there were no obvious differences in species outcome for the varied pH environments, perhaps signifying the lack of pH sensitivity of the organisms tested within the range experienced.

4.4 DILUTION RATE EXPERIMENT

The objective of this experiment was to test the sensitivity of the experimental design to changes in dilution rate and sparger design. Dilution rates of 1.23, 0.83, and .33 day^{-1} were established on cultures containing Chlorella, Chaetoceros, Ankistrodesmus, and Navicula. The results for each dilution rate are shown in Figures 4.4a-d and a summary of experimental conditions and results are given in Table 4.1c.

FIGURE 4.3a CELL CONCENTRATION VS. TIME FOR pH = 7.1

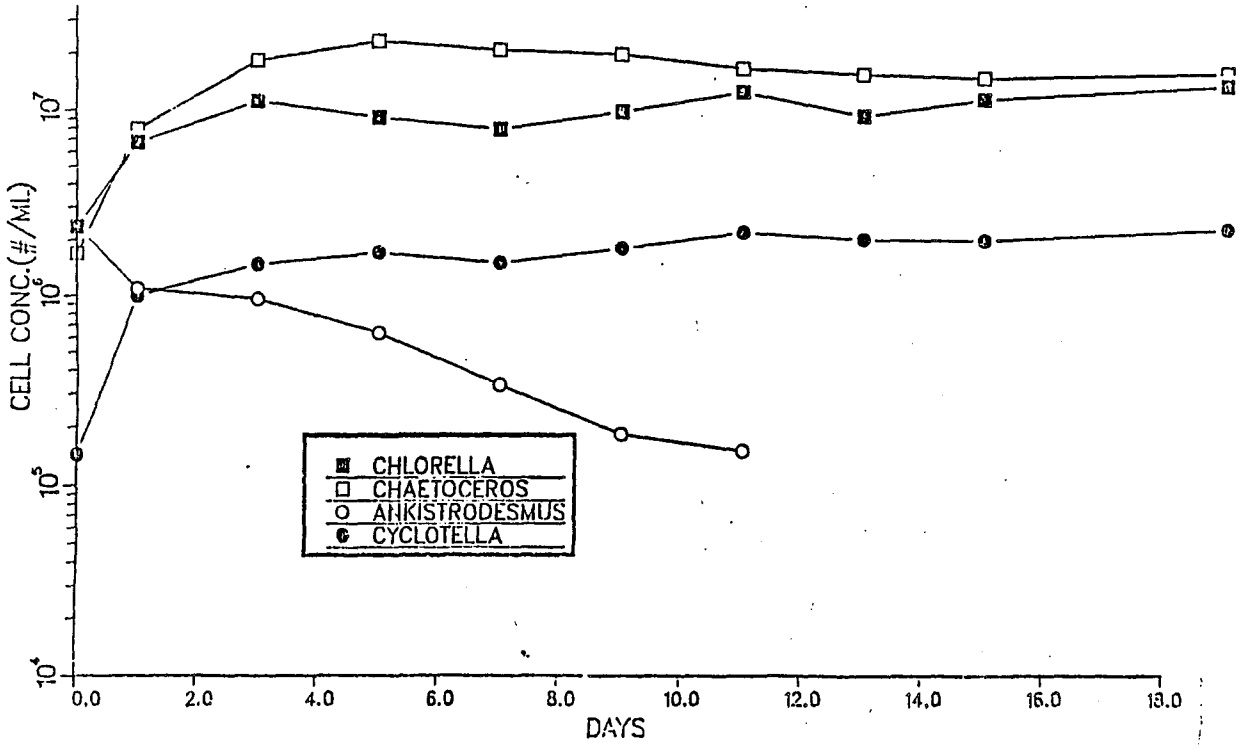


FIGURE 4.3b CELL CONCENTRATION VS. TIME FOR FLUCTUATING ALKALINITY

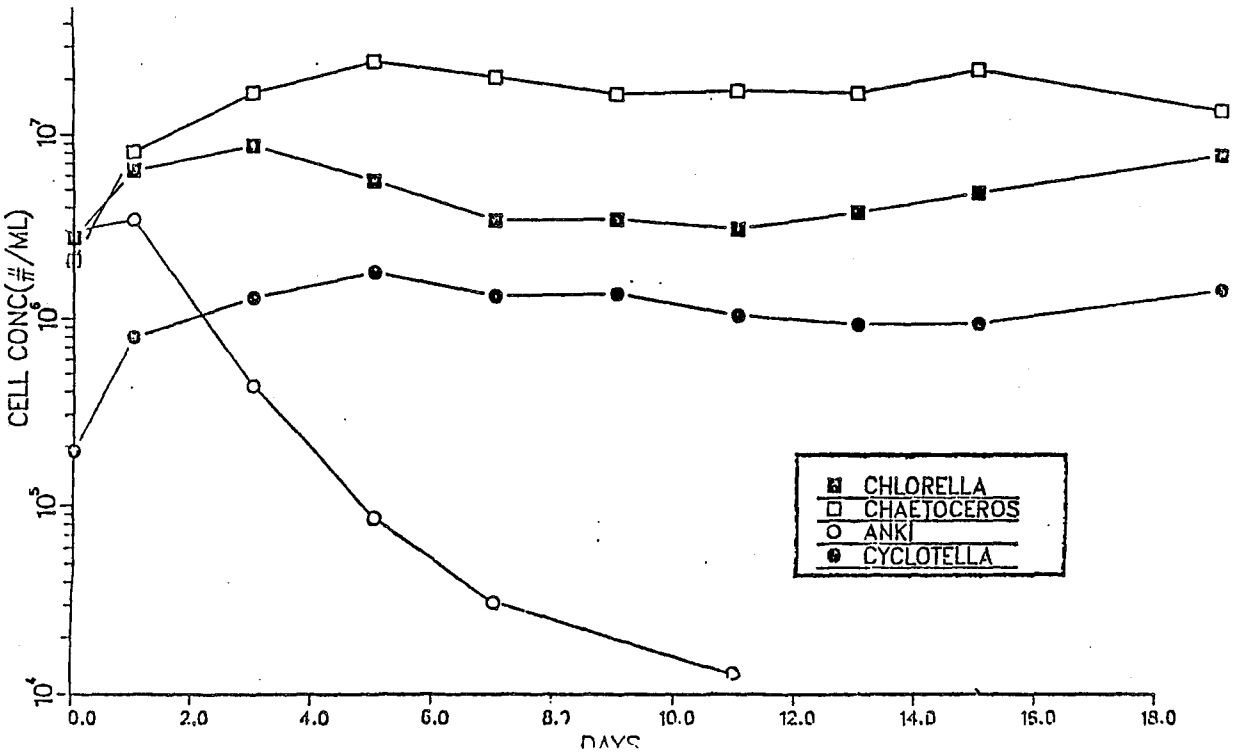


FIGURE 4.3c CELL CONCENTRATION VS. TIME FOR pH = 8.2

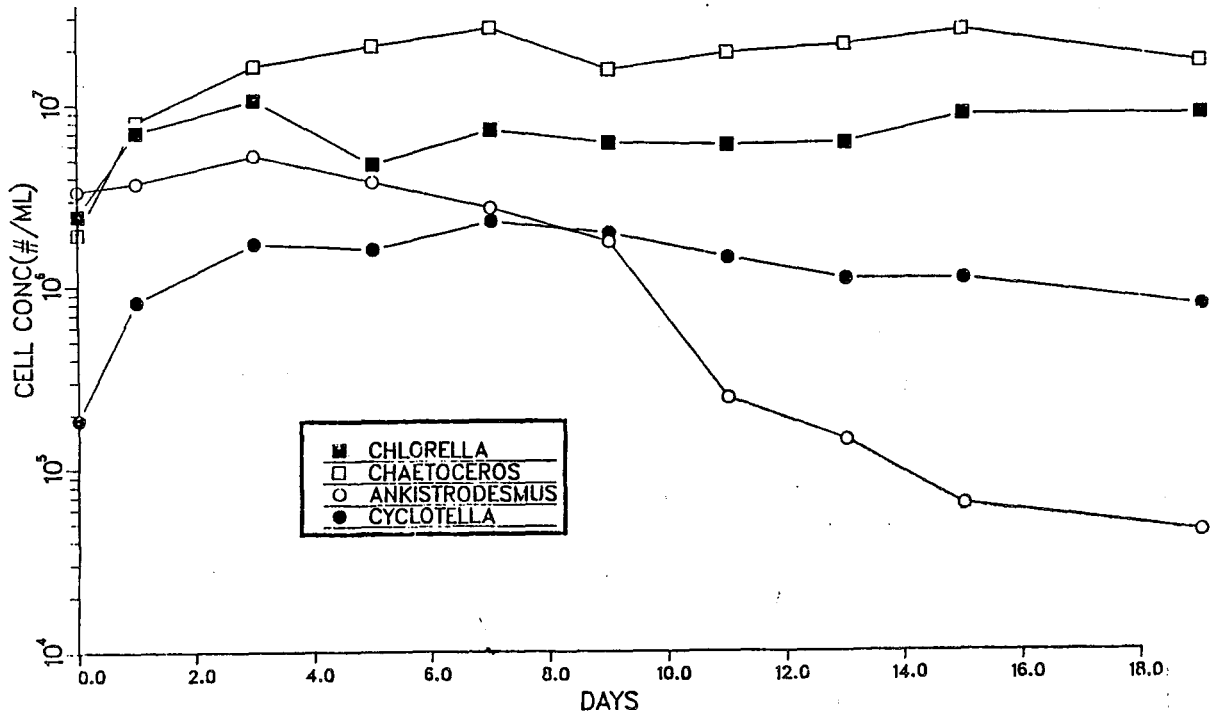


FIGURE 4.3d CELL CONCENTRATION VS. TIME FOR FLUCTUATING pCO_2

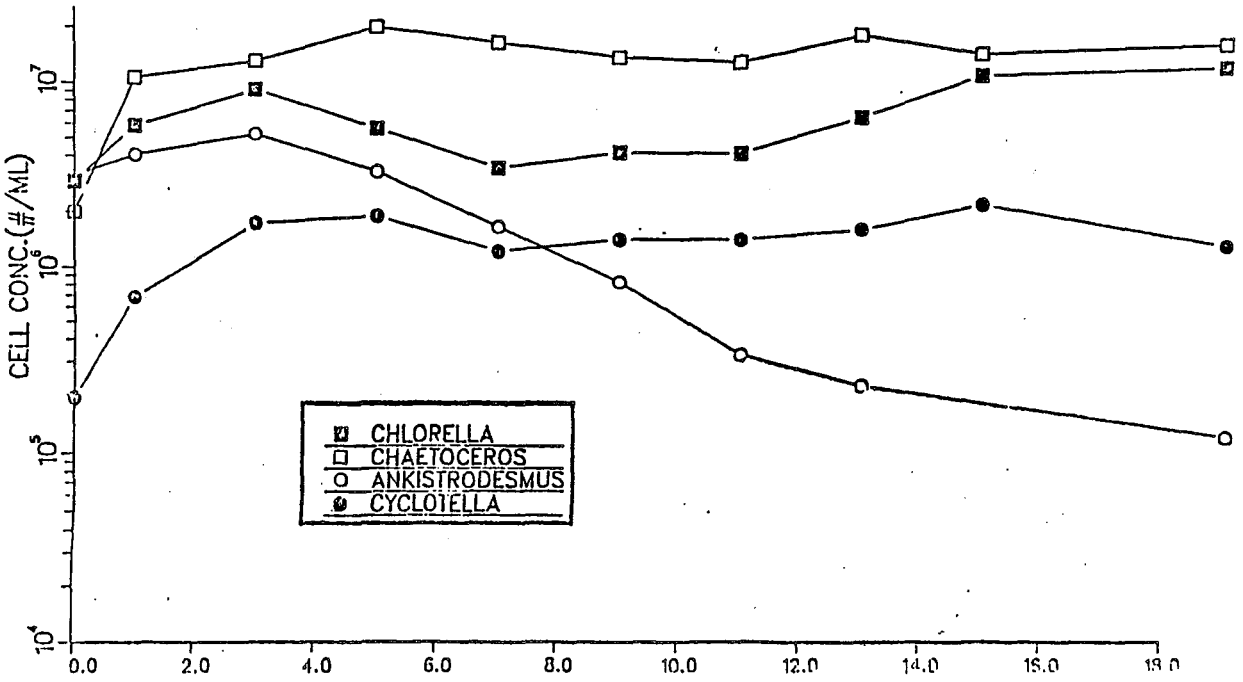


FIGURE 4.4a CELL CONCENTRATION AT A DILUTION RATE OF 1.23/day

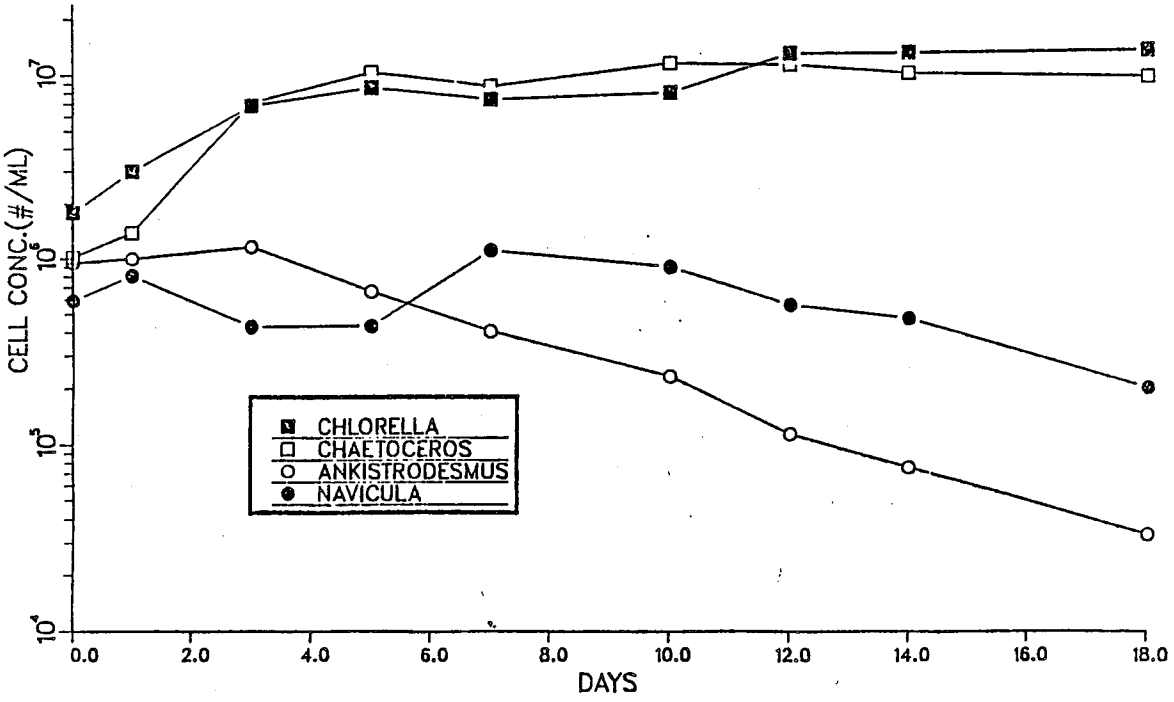


FIGURE 4.4b CELL CONCENTRATION AT A DILUTION RATE OF 0.73/day WITH A GAS BUBBLER

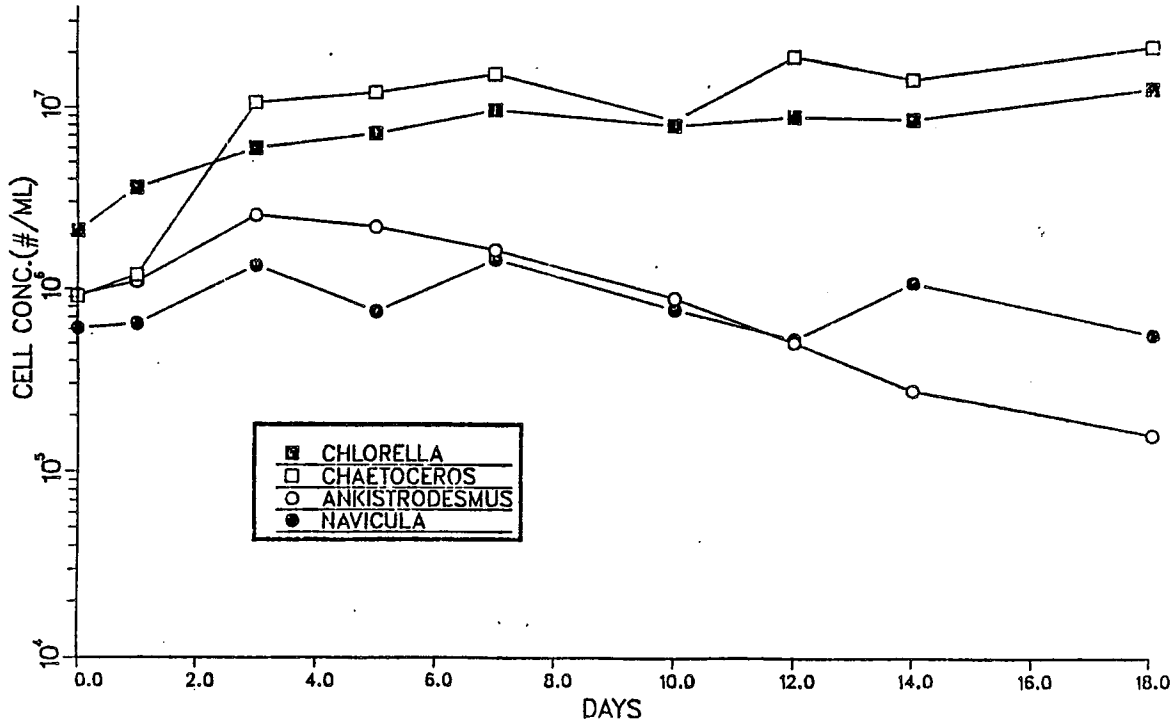


FIGURE 4.4c CELL CONCENTRATION AT A DILUTION RATE OF 0.33/day

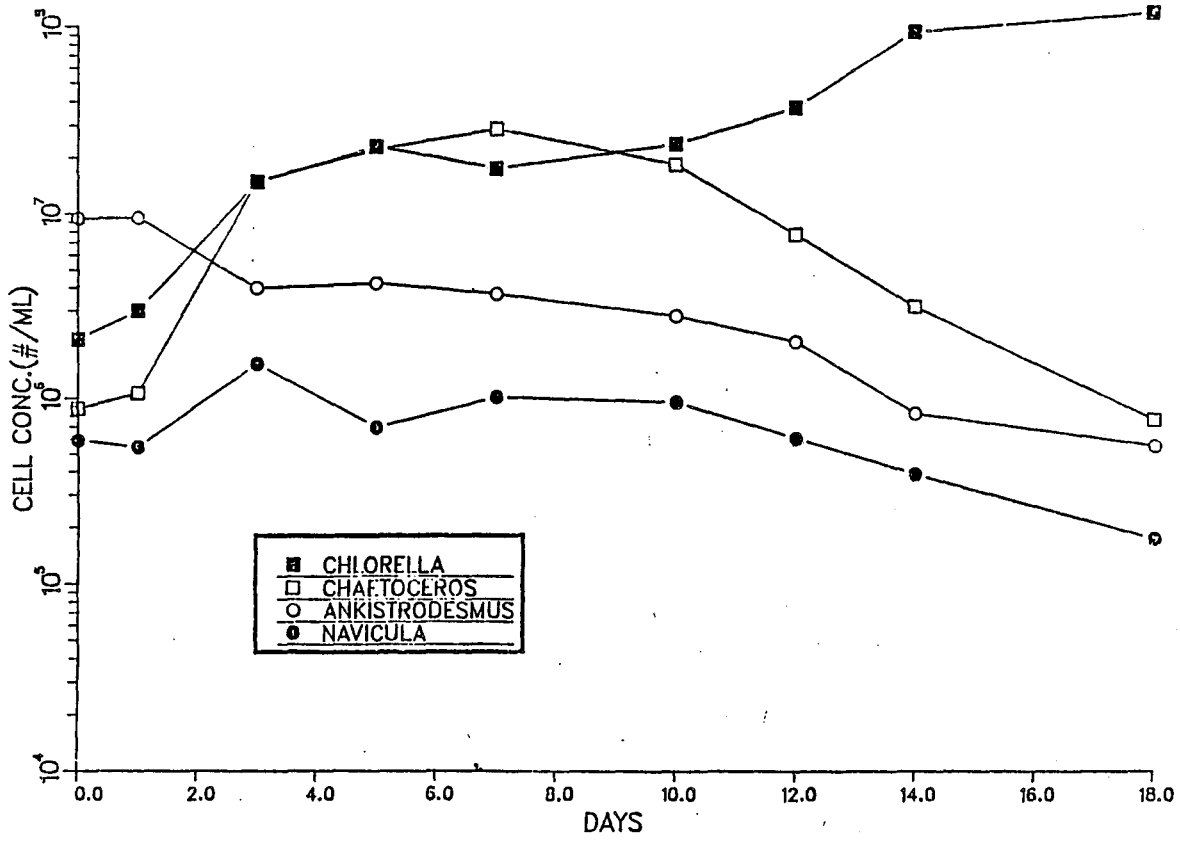
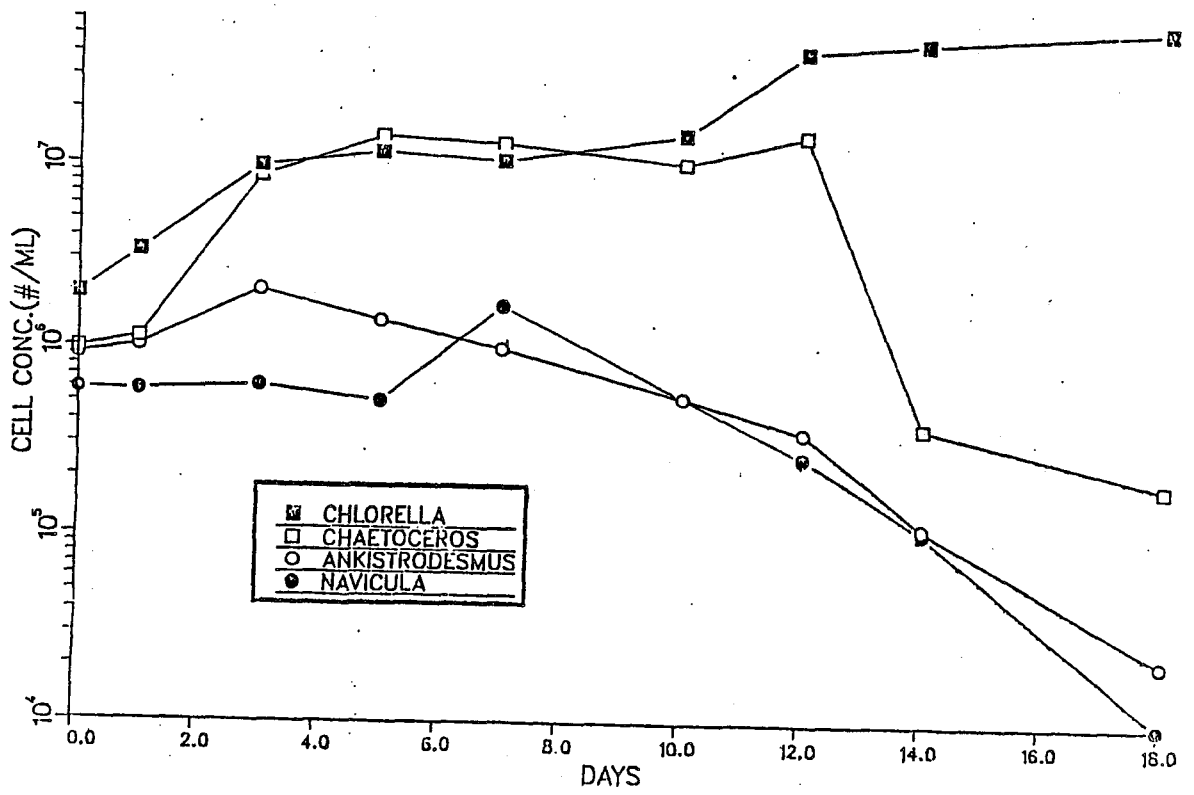


FIGURE 4.4d CELL CONCENTRATION AT A DILUTION RATE OF 0.83/day



Chlorella and Chaetoceros codominated with Chaetoceros maintaining as larger proportion of the biomass(ca. 60%). The other strains, Navicula and Ankistrodesmus, competed very poorly both declining in cell number. At a dilution rate of 0.83 day^{-1}, Chlorella was clearly the dominant organism after 10 days of growth. Chaetoceros while competitive in the early stages of the experiment, was diluted significantly following the sharp increase in Chlorella. The overall patterns of for Navicula and Ankistrodesmus were similar to that observed at the higher dilution rates. At the lowest dilution rate (0.33 day^{-1}), a pattern quite similar to that observed for the intermediate dilution rate was obtained. Chlorella again became dominant after roughly ten days of growth. Chaetoceros competed well initially, but declined significantly after 10 days. Ankistrodesmus and Navicula again competed poorly.

In planning this experiment it was expected that changes in dilution rate would only affect the amount of time required to obtain the steady state result. These results clearly show that this is not the case. Most importantly differences between the fastest (1.23 day^{-1}) and intermediate (0.83 day^{-1}) dilution rates were significant and would have led to opposite conclusions regarding strain dominance. Moreover the transition from Chaetoceros dominated growth to Chlorella dominated occurs after the same period of time for the low and intermediate dilution rates. Thus overall culture age appears more important than the growth rate of the culture itself in establishing the amount of time required for a steady state result.

The reason for the differences observed between the highest dilution rate and the lower dilution rate cultures may be explained by differences in the degree of light limitation. At the highest dilution rate, the cell density hovered a little above 400 ppm, while at the intermediate and low dilution rates the cell densities were roughly 600 and 1100 ppm respectively. Therefore it is implied that the rate of pigment synthesis or light utilization efficiency is greater in Chlorella than Chaetoceros.

Coincident with the dilution rate experiment, a fourth culture was used to compare the effects of sparger design on the dominance patterns. In previous work it has been observed that a significant amount of cell debris can accumulate above the gas liquid interface presumably stripped out of culture by the high gas liquid interfacial area. It was of interest therefore to determine whether the sparger itself was applying an additional selective pressure. In the test culture(#4) a gas bubbler was used instead of the standard course sintered glass cylinders usually used. The gas bubbler consisted of a 1/8 in. ID glass tube with one end closed and formed into a small sphere with 6-7 1mm holes along its perimeter. Bubble sizes are roughly 1 order of magnitude larger than those from a sintered cylinder. The dilution rate was 0.73 day^{-1} and therefore is comparable to the 0.83 day^{-1} culture. From Figure 4.4d it is clear that Chaetoceros

remained dominant in this culture for an extended period of time, in contrast to what was observed in the cylinder sparged culture. The Chlorella maintained a relatively constant cell concentration, with no sudden expansion in cell number being observed. There was no foaming and very little cell material deposited above the liquid level.

The results from this experiment indicate that light limitation influences species dominance patterns. This is logical and suggests that future experiments operate only under light limitation it correlates with growth conditions in outdoor ponds. Within light limited growth, differences in dilution rate appeared to have little effect on actual cell concentration patterns. Thus little bias is introduced into the dominance patterns as a result of dilution rate variations. Gas sparger design appears to have a significant effect on species outcome presumably due to differences in the shearing forces exerted upon a culture. Because open ponds experience primarily a low shear environment, with periodic high shear at the gas transfer stations, the gas bubbler is considered most representative of the pond environment, although power densities are well above those found in mass culture ponds.

4.5 CONCLUSIONS AND FUTURE WORK

It has been demonstrated that fluctuations in oxygen, temperature, and, to a lesser extent, pH fluctuations are all important features of microalgae cultivation systems in terms of both productivity and species dominance patterns. In light of the discussion in Section 2.0, a new approach will be pursued in the remaining experiments under this contract (currently underway) and future research in this project. Specifically, future experiments will emphasize more work with monoalgal cultures, with mixed culture experiments being designed primarily for validation of monoculture results.

The basic hypothesis to be tested is that there is little if any interaction between algal cultures (e.g. allelopathy or other interferences) and that mixed culture results can be predicted from unialgal cultures. Thus, dominance in mixed cultures should be predictable from the productivity (in terms of light absorbance units) of the cultures. This should be established, at least on a preliminary basis by the current, ongoing, experiments in which several species are being operated under both unialgal and mixed culture conditions.

In the current and next few sets of experiments planned two general conditions will be tested: the standard, non fluctuating, condition and a simulated outdoor condition reflecting the results from the AGP model. The currently ongoing experiments are designed to allow the selection of three species which will be used for future experiments and which have a high promise for outdoor cultivation.

These experiments involve the operation of five monoalgal continuous cultures at a detention time of 0.7 day^{-1} under conditions of fluctuating temperature (nighttime dropping down to 15°C, daytime maximum of 32°C), high O_2 (8 hours of 100% O_2 gas phase during the daylight hours), fluctuating pH (7.5-8.5, 1hr cycles), and a high light intensity illumination on a 14hr light:10 hr dark cycle. The light intensity to the culture is increased in four steps over a 2 hour period at the start and end of the 14/10 hr day-night cycle. The maximum intensity reaches 700-800 E/m2/s using Vitalite VHO fluorescent lamps. These lamps reproduce the true solar spectrum better than any other on the market. One additional flask is used as a mixed culture control, with all five strains inoculated at the same time. The first run will be completed in December and the second in early January.

The data should allow establishment of which culture is the most productive and competitive (which should be the same one) under these conditions. A final selection of the three algal species to be selected for further work will be carried out based not only on the data from the above reported (and the ongoing) experiments but also on the information available from other SERI subcontractors and the SERI researchers. Work in the coming year will concentrate on the three selected algae.

The objectives of the future research are: 1) To validate the fact that monoalgal cultures will consistently be predictive of competitiveness in mixed culture experiments; 2) To test the physiological limitations of the selected algae in terms of the seasonal extremes in the outdoor ponds for a selected location (Roswell New Mexico, where SERI will locate its algal mass culture systems Demonstration Project) based on the results of the AGM; and 3) To determine which individual factors (pH, pO_2, daylength, temperature) have the greatest influence on culture productivity.

The reactor system, as configured has the capability of running six cultures simultaneously. The experimental design will test the growth of the three strains as a function of both fluctuating and steady state conditions, in both unialgal and mixed cultures. The experiments will be carried out at a dilution rate of 0.7 $days^{-1}$ and last for approximately 10 residence times, thus requiring two weeks. Together with start-up time and allowances for failed experiements one run per month can be carried out. Daily measurements will include O.D., dry weights, and, where required, cell counts (primarily for mixed culture experiments). Twice or three times a week pigments and total dissolved organic carbon (TOC) will be measured. After each culture has reached steady state, dark respiration, and the maximum rate of photosynthesis will be measured and cells will be harvested for protein, carbohydrate, and CHN. Three strains will be tested following this protocol with a total of four runs planned, including the mixed culture experiments and replicates. The results of these experiments will allow the determination of factors most influencing productivity and competitiveness (and whether these are synonymous). Further experiments will involve changing the conditions to those expected from seasonal extremes.

SECTION 5.0

LIPIDS FROM MICROALGAE

5.1 INTRODUCTION

Historically microalgal lipids have been of interest since World War II when von Witsch proposed them as a source of vegetable oils and beta carotene (88). Even before the war the relationship between nitrogen nutrition and lipid content of algae was already recognized (89). After the war the study of microalgae lipids was pursued by groups in Germany (90), England (91), and, the U.S. (92). The report by Sphoer and Milner (92), that, Chlorella could accumulate up to 80% of its dry weight in the form of lipids, attracted considerable attention and their procedure, involving nitrogen starvation, was even patented (93). However, since the early 1950's the study of algal lipids has been primarily associated with taxonomic or biochemical studies of metabolism (94,95).

The energy shortage of the mid 70's resulted in a renewed interest in algal lipids as a source of fuels (96) and prompted the DOE to establish a program to develop microalgae technology, which has been under SERI management since 1979. A major focus of the SERI/DOE program has been to identify high lipid yielding strains and develop the technology for producing an algal derived liquid fuel. This chapter begins with a literature review of lipids in general, the algal lipids and fatty acids in particular, nitrogen deficiency as a promoter of lipid formation in algae, and, models of nitrogen deficient growth is also presented. The results of the experiments carried out are then presented followed by discussions of their significance.

5.2 REVIEW OF LIPIDS AND NITROGEN LIMITED GROWTH OF MICROALGAE

5.2.1 Lipids from Microalgae

Lipids are a class of molecules that, together with proteins and carbohydrates, broadly define overall cellular composition. Operationally they are defined as any cellular compound extractable in an organic solvent such as chloroform, or a mixture of chloroform and methanol. Their specific solubility properties are dependent upon the relative proportions of polar and nonpolar residues in the lipid molecules. Thus, some lipoproteins and glycolipids found in cells can be extracted into nonpolar solvents due to their lipid residues or side-chains. In the present context lipids are defined as all cell constituents that are extracted into the chloroform-methanol solvent mixture of the Bligh-Dyer method. (See the Appendix).

Lipids can by classified as both polar and non-polar. The polar lipids are comprised of the phospholipids (phosphotidyl choline, PC; phosphotidyl serine, PS; phosphotidyl glycerol, PG; phosphotidyl inositol, PI; and phosphotidyl ethanolamine, PE) and glycolipids (mono and digalactosyldiglyceride, MGDG, DGDG; and sulphoquinosyl diglyceride, SL) and are functionally associated with membrane structure and fluidity. Nonpolar lipids are primarily mono, di and triglycerides but also certain pigments, vitamins, wax esters, sterols, and cyclic and acyclic hydrocarbons.

The triglycerides have been considered an energy storage reserve due to their high specific caloric value(9.3 kcal/g) as compared with protein and carbohydrate(5.1 and 4.5 kcal/g respectively). Indeed, triglycerides are clearly an energy reserve in higher organisms, and even in algae there is evidence of triglyceride breakdown during periods of energy limitation (96). From the perspective of fuel production the microalgal triglycerides, because of their prevalence and similarity with the vegetable oils, are of greatest interest. Hydrocarbons, such as those produced by Botryococcus braunii would also be of interest. However they are produced in quantity by only by that one species which grows slowly and can not be mass cultured. Thus, we emphasis in our discussion only the glycerides and fatty acids from microalgae.

The algal fatty acid containing lipids are composed of PC, PE, PG, PI, MGDG, DGDG, SL, and triglycerides. Free fatty acids (FFA) are usually present at roughly 1-5% of the total lipid fraction. Saponifiable lipid make up roughly 30-40% of the total lipid. Fatty acid composition has been studied quite extensively since the 1950's and has been reviewed by several authors (95,97,98). All classes of algae contain large proportions of saturated C16's, however the remainder are very much class specific. Green algae (Chlorophytes) have the bulk of their fatty acids as saturated and unsaturated C18's. Their composition is very similar to that of vegetable oils. Diatoms (Bacillariophyceae) contain some monounsaturated C18's and also unsaturated C20's (20:4,20:5) and saturated C14. Golden-Brown algae (Chrysophytes) have lipid patterns similar to the diatoms, and also contain unsaturated C22's. The unsaturated C20's and C22's (omega-3 fatty acids) are of considerable current commercial interest because diets high in these have been linked to reduced heart and other diseases.

5.2.2 Factors Affecting the Lipid Content of Microalgae.

Factors such as temperature, light intensity, and, most markedly, nutrient supply have been shown to affect composition of microalgae overall lipid composition and content. The effect of light intensity on fatty acid and lipid composition has been studied by several authors. Constantopoulos and Bloch (99) found that in Euglena gracilis lipid content paralleled light intensity which was the result of decreasing chlorophyll content and its associated lipids. They also found that the degree of unsaturation decreased and in particular the palmitic acid content of the chloroplast lipids declined sharply while on a whole cell basis it changed very little.

Opute (100) found that the diatom Nitzschia palea changed its unsaturated to saturated fatty acid ratio from 2 to 1.3 between roughly 2% to 0.2% sunlight intensity. He also found that in low light very little triglycerides were formed and attributed this to the preferential synthesis of the necessary structural lipids in an energy limited environment. In the same study, he compared heterotrophic vs autotrophic grown cultures of Navicula muralis and found that the dark grown cells had roughly twice the amount (on a relative basis) of saturated fatty acids as compared with light grown cells. He concluded as others (101) that there was some functional coupling between photosynthetic processes and the degree of unsaturation of the fatty acids.

Supporting this was the work of Pohl and Wagner (102) who reported that when cultures of Euglena gracilis were grown with sufficient nitrogen in the light, the fatty acids were highly unsaturated and the primary lipids were MGDG, DGDG, PG, and SL while dark grown , low nitrogen, or DCMU treated light grown cells had a higher percentage of saturated fatty acids and a higher content of the nonpolar lipids PC, and PE. In reviewing this area Pohl (96) concluded that the formation of polyunsaturated fatty acids and chloroplast lipids are favored by increasing light intensity.

Fatty acids appear to increase in unsaturation as temperature decreases. It has been suggested in the literature that this is due to the need to maintain mobility of the lipoprotein complexes for proper cell function. Patterson (103) determined the fatty acid distribution for a high temperature strain of Chlorella at seven temperatures between 14 and 38°C. He found that the degree of unsaturation did increase with decreasing temperature until 22°C after which unsaturation decreased. It may be that temperatures below 22°C was too far out of the organisms normal metabolic range and the response was of a secondary nature. In addition he noted that the total lipid content did not change as a function of temperature. Materassi et al.(104) studying two strains of Scenedesmus, reported that saturated fatty acids increased 18 to 32% in going from 20 to 35 °C.

In synchronized cultures, storage compounds such as carbohydrates and lipids tend to accumulate on a per cell basis during the lighted portion of the growth period. Sundberg and Nilshaman-Holmvall (105) have described this for Scenedesmus by electron microscope observations. Shifrin and Chisholm (106) have found a similar pattern for synchronized Ochromonas polymorpha by direct measurements. Their data show however that while changes occur on a per cell basis, very little change in the lipid fraction occurs for the total population(on a weight basis).

Of all environmental parameters, nutrient deficiency appears to be the most effective means for inducing changes in the lipid content and composition. Silicon limitation in diatoms (107) and severe P deficiency (108) are reported to increase lipids in microalgae. These are of minor importance, however, compared to the general increase in lipids observed upon nitrogen deficiency.

5.2.3 Nitrogen Deficiency and Lipid Content

Increases of over 100% in lipid content are commonly reported in response to limiting the nitrogen supply to algal batch cultures. The first recognition that nitrogen limitation increased the lipid content of algae was made by von Witsch (89) who observed a positive relationship between culture age(and diminished culture nitrogen) and lipid content. At the same time Spoehr and Milner (90) demonstrated that a nitrogen starved Chlorella reached 86% lipid while containing only 5% in exponential cultures. Many other reports of lipid induction by nitrogen deficiency are found in the literature in the 1950's and 1960's. A selected overview of the literature is presented in Table 5.1.

More recently, Shiffrin and Chisholm (106) studied 30 different species of marine and freshwater chlorophytes and diatoms and found that for log phase cultures diatoms contained on average 36% lipid as compared to 19% for chlorophytes (ash free basis). Cultures exposed to nitrogen starvation resulted in changes of -40% to +320% their log phase values after one week of starvation. Most notable was that while the diatoms had high log phase lipid contents they responded rather poorly to nitrogen starvation. On average there was very little change in lipid content. The greens however displayed a wide variety of responses ranging from a slight reduction in cellular lipid (Dunaliella) to increases of 2 to 3 times log phase values (Chlorella vulgaris). Some methodological problems with this study limit its value: inoculum sizes varied and, due to experimental design, cultures only grew to 2-3 times their inoculum density; the nitrogen mass balances (as calculated from the reported data) show large losses or gains in many of their cultures, thus the actual nitrogen status of these cultures is uncertain; and the lipid extraction methodology resulted in the inclusion into the lipid fraction of the solvent interface fraction, which would tend to increase lipid contents (Tornabene, personal communication). The data collected did not allow conclusions regarding lipid productivity.

Much of the current work on lipid induction in algae is being carried out under the support of the SERI. Table 5.2 summarizes some of the work collected by investigators associated with this program. In general the data supports earlier studies on the importance of the nitrogen nutrition on lipid content. However the key issue of lipid productivity is still an open problem.

5.2.4 Nitrogen Deficieny and Lipid Productivity

While nitrogen starvation in batch cultures (called herein nitrogen deficiency, as compared to nitrogen limitation in continuous cultures) increases lipid content in many microalgal species, particularly the green algae, it is also correlated with a reduction in biomass productivity. In order to evaluate a microalgae based lipid production process, both content and productivity will be important. Indeed, an optimization between the two will be required: high content is desirable from the perspective of efficient extraction technology and high productivity is

Table 5.1 Algal Lipid Contents from Selected Literature Reports

SPECIES	LIPID CONTENT NS	ND	REFER.
Chlorella pyrenoidosa	20(80)	35(17)	91
" "	18(?)	65(?)	91
" "	25(?)	40(?)	89
" "	20(?)	70(?)	109
" "	25(?)	35(4)	110
" sp. strain A	20(log)	45-53(17-26)	111
" strain 10-11	19(log)	18-26(5)	111
Bracteacoccus minor	25(?)	33(?)	112
Chlorella vulgaris	27-33(?)	54(?)	112
Nitzchia palea	22(log)	39(7-9)	100
Chlorella pyrenoidosa	14(log)	36(7-9)	106
Oocystis polymorpha	13(log)	35(11)	106
Monollanthus salina	41(log)	72(11)	106
Nannochloris sp.	20(log)	48(11)	106
Scenedesmus obliquus	26(log)	47(22)	113
Chlorella vulgaris	24(log)	64.5(28)	113

NS - nitrogen sufficient, ND - nitrogen deficient; numbers in parenthesis represent days of batch or logarithmic growth.

Table 5.2 Lipid Content of SERI Culture Collection Algal Strains

SPECIES	GROWTH CONDITION	LIPID CONTENT NS	ND	SOURCE
Amphora sp.	B,B	4.1	14	Benemann, 1986
Ankistrodesmus	B,BW	24	40	BenAmotz, 1984
Boekilovia sp.	B,BW	28	36	Benemann, 1986
Boekilovia sp.	C(?),BW	23-29	42	SERI
Botryococcus braunii	B,FW	44	54	BenAmotz, 1984
Chaetoceros gracilis	B,SW	15	28	Benemann, 1986
Chaetoceros sp.	B,BW	--	33	SERI
Chlorella sp.	B,FW	10	34-48	Lien, 1985
Chlorella ellipsoida	B,BW	16	21-30	SERI
Cyclotella sp.	B,BW	13	42	Tadros, 1985
Isochrysis(Tahitian)	B,SW	7	26	BenAmotz, 1984
Monoraphidium sp.	B,BW	21	25	Benemann, 1984
Nannochloropsis sal.	B,SW	29	60	Tornabene, 1984
Nannochloropsis sp.	B,SW	28	53	Benemann, 1984
Navicula sp.	B,BW	32	58	SERI
Nitzschia sp.	B,SW	27	--	Thomas, 1983
Nitzschia dissipata	B,SW	26	66	Tadros, 1985
Oocystis pusilla	B,BW	10	--	SERI
Phaeodactylum tri.	C(.25),SW	20	23	Thomas, 1983
Tetraselmis ap.	SC,SW	18	15	Laws, 1981

FW, BW, SW represent fresh, brackish and seawater respectively; B, C, SC batch, continuous and semicontinuous modes of growth.

the primary concern in the economic feasibility of these systems. Although the specific considerations in such an optimization can not yet be specified, as they will depend on many factors that are not at present quantitated (extraction technology and costs, CO_2 costs, by-product values, etc.), it is clear that a detailed understanding of the factors impacting on both lipid content and productivity are of central importance to this project.

The issue of lipid productivity was already clearly stated over thirty years ago, when it was pointed out that the production of lipids would likely be most economical with nitrogen sufficient cells having a low lipid content rather then by nitrogen deficient cells which, despite their much higher lipid content, had such a low productivity that total lipid yields were negligible (114). This particular issue seems to have been essential overlooked since then.

For example, Shiffrin (98) commented that the organism Monalanthus salina would be"...a worthy prospect for further consideration..."(as a lipid producer). However the data (106) reveals that while M. salina achieved a lipid content of over 70% its lipid productivity was practically zero after nitrogen depletion. On the other hand a Scenedesmus sp. had the highest lipid productivity for all of the species studied before and after nitrogen deficiency, however this organism was not even mentioned as a good lipid "producer".

Recently, Lien and Roessler (unpublished) reported results for five species undergoing nitrogen starvation in batch culture. They measured biomass and lipid productivities as well as the photosynthetic efficiencies for various cellular components. In two of the five strains, Boekilovia sp. and Cyclotella, lipid productivity decreased after nitrogen limitation. In Cyclotella, biomass productivity dropped from 400 to 20 mg/l/day. The other organisms studied showed moderate (Chlorella ,25%; and Ankistrodesmus, 17%) and substantial (Nanno Q, 90%) increases in lipid productivity. Photosynthetic efficiency and biomass and lipid productivity all appeared to be closely coupled. Significantly, photosynthetic efficiency actually increased for Nanno Q for the early stages of nitrogen deficiency, while decreasing between 20 and 90% for the other strains.

Our own, independent, studies have also demonstrated the relative uniqueness of Nanno Q in lipid production (11) and again illustrated the variability which exists between species in their response to nitrogen deficiency. This variability exists not only between species but also within species, as Opute's study of 40 species of Chlorella demonstrated: some exhibited only carbohydrate storage and others lipid storage in response to nitrogen limitation. Thus any survey for high lipid producers can not rely on using representative strains of different species.

The biochemical basis for lipid storage is poorly understood. However, it must have an evolutionary and selective basis. One suggestion has been that a high lipid content would allow algae

to regulate their buoyancy. However this can be dismissed, (perhaps Botryococcus is an exception) on the basis of hydrodynamic arguments. Lipids do not sufficiently alter cell buoyancy to affect their settling behavior in most cases. Thus the best explanation is that lipids indeed serve as an energy reserve. Since lipids are much more concentrated energy forms then carbohydrates (almost nine fold on a hydrated weight basis) there is an advantage to store lipids for long term utilization. However this has not yet been demonstrated experimentally.

5.2.5 Nitrogen Metabolism and Nitrogen Limitation

Nitrogen deficiency in algae causes a variety of responses dependent upon species, growth history, and severity of the depletion. Breakdown of chlorophyll is the most visible sign of nitrogen limitation, although this can also be observed in response to other environmental stresses. The storage of carbon is a major and universal response to nitrogen limitation. This may be partially the result of a simple overflow metabolism of carbon fixation into cellular components not containing nitrogen. A rapid reduction in protein content and a redistribution of enzymes also occurs although there is little information concerning specific enzymology.

Thurston and Rulands (115) have found specific protein decay rates of 12-40% in N starved cultures of Chlorella. On a culture basis protein synthesis continues until the nitrogen supply is exhausted. Activity of the enzymes of the nitrate assimilation pathway are all increased rapidly due to nitrogen depletion. Coupled with this is an increase in specific rates of uptake of nitrogen which occurs both by a reduction in cell nitrogen and an increase in the absolute rate of assimilation (115). The increasing enhancement in Vmax correlates well with decreasing growth rate (116). One interesting point is that nitrogen limitation results induction of enzymes (e.g. nitrate reductase, hydrogenase) which normally require an inducer without any inducer (or inducing condition) being present either before or after nitrogen limitation(117).

Chlorophyll content decreases as a function of cellular nitrogen; correspondingly per cell or per weight estimates of maximum rates of photosynthesis also decrease (118,119). On a per weight chlorophyll basis however, Pmax values are unchanged. Thus the generalized reduction in photosynthesis does not appear to be due to a loss of specific proteins involved in carbon fixation. Finally the light intensity at which photosynthesis is saturated remains unchanged as a function of deficiency (118).

In some strains carbohydrate levels can reach above 70% without reduction in culture productivity (Weissman and Benemann in preparation). This occurs relatively rapidly(< 1day). More slowly(> 1day), lipid content increases anywhere from 0 to 200 % of healthy cultures. This has already been discussed and will not be elaborated on here.

Until now this discussion has not clearly differentiated between nutrient limited (continuous, e.g., chemostat) and nitrogen deficient (e.g. starved, batch) cultures even though there are significant differences between them, as discussed next.

A nutrient limited culture is one growing in an external environment of constant, but insufficient levels of a limiting nutrient. The resulting intracellular levels of that nutrient will be dependent on its extracellular concentration and, in turn, specify the actual growth rate at which the organism grows. Operationally this is achieved in inverse order: the hydraulic dilution rate of the continuous culture imposes a growth rate on the organism, since slower growth leads to wash out and faster growth to population (cell density) increases until growth is balanced with dilution rate. Indeed, initially upon start-up of a chemostat, cell density increases until competition for nutrient is severe enough to reduce the incoming (in the dilution media) nutrient concentration to a level low enough to support growth only at the rate at which it is supplied (e.g. the hydraulic inflow). The chemostat is essentially a selfregulating growth rate control mechanism.

In practice there is considerable initial fluctuation in cell density during a start-up, as overshoots (and subsequent undershoots) result from the organisms attempting to adapt their metabolism to the external environment (which, of course, is subjected to similiar, but inverse, fluctuations in limiting nutrient concentrations.) These oscillations however dampen out in short order (a few detention times) and thereafter balanced growth (= dilution rate) is maintained.

Only when the influent nutrient concentration is high enough to support such a cell density that some other factor becomes limiting (e.g. oxygen supply or, in case of algae, light) do extracellular nutrient levels increase above the low levels normally present in a chemostat. Indeed, extracellular nutrient levels are often so low as to be below detection (by any but the most sophisticated analytical methods) levels. This is particularly true for algae growing on phosphate or nitrate as limiting nutrients below their half maximal growth rates (eg. at external nutrient concentrations below the K_s).

In essence, the chemostat (or its variations, turbidostats, etc.) sets the growth rate of the culture and thereby the equilibrium extracellular nutrient concentration, which matches cell growth rates and dilution rates. The nutrient concentration in the feed will set the overall cell density. Of course, the metabolic (genetic) capabilities of the organism itself determine the exact extracellular concentration at any particular detention time (growth rate).

In contrast, a starved batch culture does not have the ability to adjust its cell density to cope with declining nutrient supply nor is it receiving a continuous supply of the limiting nutrient. Therefore continued cell growth in batch cultures, once the

external supply is exhausted, occurs at the expense of the limiting nutrient stored in the cells. The metabolic consequences become increasingly more severe as a function of time after nutrient depletion. Growth rate continuously declines as intracellular nutrient concentrations drop and become zero (maximum standing biomass) at a certain minimum intracellular concentration (% of the biomass) of the nutrient.

One significant difference between these two modes of growth lies in the ability of the chemostat cultures to adapt to the nutrient limitation. We consider continuous cultures to be adapted to their environment, as, by definition in a steady state situation, there is constant growth rate equal to the hydraulic dilution rate. Thus such adapted cells partition and prioritize the use of the limiting nutrient among the various metabolic requirements according to its availability, resulting in an overall growth rate that reflects their genetic and metabolic capabilities.

An important point to remember here is that in mixed cultures in nutrient limited continuous culture systems, efficient nutrient utilization (e.g. low q - the nutrient quota, as % nutrient in the biomass, or any other convenient unit) is not important in determining the outcome of species competition. An efficient uptake system (e.g. low K_s, the nutrient concentration at half maximal growth rate) is the determinant of success in a competition between two or more species in a nutrient limited chemostat. In the case of an intermittent nutrient supply a high V_{max} (maximal nutrient uptake rate) and possibly a low K_m (half saturation concentration of nutrient for V_{max}, which could be different from K_s) may become critical, their relative importance depending on nutrient supply fluctuation frequencies, amplitudes, and time delay constants. (See Section 2.) The maximum growth rate (u_{max}) is only of significance in the case of competing organisms with intersecting u vs S curves (Figure 2.). Thus maximal growth rate determinations could be of value, but only in the context of also knowing K_s (under balanced growth situations). The main conclusion is that in continuous cultures frugality is not important, efficiency in uptake is what counts. The same arguments hold in the case of light, where a low I_k is more important than a high P_{max}.

Batch cultures, by contrast, have little if any chance to adapt to the transient conditions they experience. There is a relatively rapid transition from fast, nutrient non-limiting growth to severe nutrient deficiency. Thus competition for a limiting nutrient never becomes an issue in a mixed culture.

In the case of nitrogen, the difference between the maximum (nutrient sufficiency) and minimum (extreme deficiency) N contents (or cell quota) is about three to five. Thus a batch culture doubles in biomass about twice before growth ceases completely. Since biomass does not correspond to cell numbers in such conditions, and since the critical nitrogen content (cell quota or % N of the biomass at which point growth rate is first reduced) is only a factor of about two to three above the minimum

nitrogen content, the transition between maximal growth and zero growth rates often takes place in the period of one cell generation, or even less. Thus there is only limited time for the cultures to adjust physiologically to the new situation. In any event, the adaptations that will take place are clearly quite different from those of a continuous culture, even one at very long detention times. Thus kinetic models of nutrient limited cell growth developed for continuous cultures are not applicable to batch growth.

5.2.6 Kinetics of Nitrogen Limited Growth

The most popular model of nutrient limited growth is that of Monod (120) which relates uptake rate to a hyperbolic function of the limiting nutrient. This has been discussed above and in Section 2.0. During nutrient sufficient growth the maximum uptake rate for the nutrient equals the growth rate, but this is not true during nutrient limited growth when V_{max} is much higher then u_{max}, and the K_s can differ from K_m. Furthermore V_{max} is very sensitive to the measurement protocols, as the initial kinetics can be complex. Thus, the relationships between growth and nutrient uptake during nutrient deficient balanced growth are everything else being equal, not straight forward.

The Monod model, with its close identification with the Michaelis-Menten equation, has been proven both popular and adequate for many batch and continuous culture studies. The Monod expression, in combination with a biomass balance and yield coefficient for the limiting nutrient allows the determination of overall culture productivity for a range of nutrient inputs and hydraulic detention times, the two major control variables in conventional microbial reactors. However, Monod kinetics are only applicable under conditions where the assimilation of nutrient is directly proportional to the production of biomass (balanced growth) and for within a range of growth rates in which the yield coefficient does not change and for which the basic Michaelis-Menten assumptions apply (e.g. $E \ll S$). Therefore, even in balanced growth, Monod kinetics may not be applicable at very slow growth rates (long detention times) where nutrient concentrations are below $0.1K_m$ (eg. $u < 0.1u_{max}$). Furthermore, if the nutrient concentrations fluctuate (or other environmental factors are not at steady state) and if adaptation to nutrient levels are not instantaneous, then Monod kinetics may also not be applicable, even though the culture may appear to be in balanced growth. These caveats significantly restrict the usefulness of this approach, particularly to microalgae which are subject to large variations in environmental parameters over seasonal, diurnal, and even shorter time periods.

In general it has been found that nutrient limited growth is more accurately represented by internal, not external, concentrations of nutrient (121,122). Droop (123) introduced the cell quota (nutrient content per cell) concept, already used in different form with higher plants for many years, which was an outgrowth of his observation with vitamin B12 limited algal growth.

Droop proposed that the growth rate (u) obeyed the following relationship to the cell quota:

$$u = um(1-qm/q) \qquad 5\text{-}1$$

(where q_m is the minimum cell quota, q the actual cell quota, u_m the extrapolated maximum growth rate at infinite cell quota for that nutrient). This equation adequately correlated his data. Others (118,124) have corroborated this relationship.

Goldman and McCarthy (125) noted, however, that the utility of equation 5-1 is limited by the fact that the maximum growth rate in this expression (u_m) is actually not the true maximum growth rate (u_{max}) (under the experimental conditions) but is a function of the prevailing culture conditions in the absence of nitrogen limitation. They have proposed the following relation:

$$u_m = u_{max}(1 - q_m/Q), \qquad 5.2$$

where q_m and Q are the actual (for the prevailing conditions) true minimum and maximum cell quotas respectively, and u_{max} is the true growth rate. Values for q_m and u_{max} can be determined in batch cultures under exponential growth using what are presumed to be optimal conditions. Goldman (126) noted that the reason the Droop equation describes Vitamin B12 deficiency is the result of the algae storing large amounts of the vitamin and being able to grow below its u_m over a wide range of intracellular concentrations of that nutrient (> one order of magnitude). On the other hand, nitrogen content varies much less (usually from about 10 to 2%) during nitrogen limitation. Thus the transition from nitrogen sufficiency (maximum growth) and nitrogen starvation (zero growth) is much more rapid. The same difficulty is observed with Si limitation. In general, the adequacy of applying the cell quota theory diminishes as a function of the plasticity of the nutrient pool (eg. carbon limited growth cannot be modeled this way), as already pointed out by Droop (121).

A more detailed model was developed by Grenny et al. (127) who recognized that the entire nitrogen pool was not necessarily an indicator of growth rate, but certain compartments within that pool. They divided intracellular nitrogen into inorganic, and organic forms, and protein, and extracellular nitrogen into inorganic and excreted forms. Growth was considered dependent on both intracellular and extracellular inorganic nitrogen. Correlation with the data of Caperon and Meyer (122) was good, but not significantly different from the Droop model. While introducing structure into nitrogen limited growth models is desireable, this representation overlooked one important feature of uptake kinetics: the energy dependence of most of the transport and reduction reactions. Essentially this model is similar to Droops's in that the parameters are still dependent upon the particular growth environment. Furthermore these models do not recognize explicitly the effect of nutrient storage, which results in some nutrient quota variations not affecting growth rate and in time lags during nutrient limitation.

One final note on uptake kinetics concerns the kinetic response under transient conditions of nutrient supply. Cunningham and Maas (23) studied transients of dilution rate (hence growth rate) in nitrogen limited continuous cultures. They found that while cell quota and growth rate were still coupled, the response in growth lagged by roughly 10 hours behind changes in cell quota. In general it is known that growth (photosynthetic biomass production) under nutrient limited conditions is actually inhibited for a period after the addition of the limiting nutrient.

5.3 LIPID INDUCTION AND PRODUCTIVITY: RESEARCH OBJECTIVES.

Due to the economic limitations inherent in any fuel production process the key issue in the production of lipids by microalgae is the overall productivity of the process, suitably optimized. To a first approximation it can be assumed that lipid productivity should be maximized for a biomass containing about half of fixed energy in the form of extractable and utilizable lipids. Allowing for inevitable losses, about 35 to 40% of the dry weight of the algae should be total lipids. This is higher than the lipid content found, with few exceptions, in algae grown under nitrogen sufficient (light deficient) conditions.

One approach could, therefore, be to investigate the exceptional, constitutively high lipid, algal strains. However, as the case of Botryococcus makes clear, high lipid content would entail relatively poor competitiveness in outdoor cultures. Too large a fraction of photosynthetic reductant is diverted to the production of unproductive biomass away from cell growth. In a light (energy) limited environment this would make such cells highly uncompetitive. Only in batch cultures, as long as a high enough inoculum were to be present initially, could a culture with a high lipid content be produced in outdoor ponds subject to contamination. Production of such an inoculum would, however, be limiting.

Thus investigation of lipid formation rates under nitrogen deficiency, and the underlying kinetic features, required the identification of a suitable strain that could serve as model for the process that is being developed. Specifically the model strain would have to exhibit relatively rapid and a high degree of lipid induction, with high overall biomass and lipid productivities during the initial stages of nitrogen deficiency. Toward this end, eight strains of microalgae from as many genus [Ankistrodesmus, Boekelovia, Chaetoceros, Chlorella, Cyclotella, Isochrysis galbana(Tahitian), Nannochloropsis sp.(Q strain), and Thallasiosira sp.]. were screened. They include examples from the chlorophyceae, eustigmatophyceae, and bacillariophyceae. Screening involved measuring lipid (and biomass) productivity under conditions of batch growth with a limiting amount of nitrogen, such that the nitrogen would become limiting in the early to mid linear growth phase (eg. after light became limiting). The results were reported in last years Final Report (11).

Of the eight strains, Nanno Q proved to have a high lipid content under both nitrogen sufficient (30%) and deficient (50%+) conditions and, most important, the highest lipid productivity after transfer to a nitrogen deficient condition. Thus it was selected for more detailed studies on the kinetics of lipid production during this transition. It must be emphasized that this organism is only a model system with which to demonstrate the basic feasibility of lipid production with microalgae. It is unlikely to have any potential for outdoor mass cultivation. Thus what is learned with it will need to be applied to other strains, which have been proven to be suitable for outdoor cultivation.

The approach taken by this project was to separate the main biomass production phase from the lipid production phase, using nitrogen deficiency as the environmental factor that controls algal lipid biosynthesis and content. The objective thus is to determine how to best optimize such a two stage process in which lipid induction takes place after a light limited growth phase. It is important to note that to maximize overall lipid productivity the lipid induction phase must also be operated under light limitation. The conventional kinetic models of algal growth are not applicable under such conditions of dual nitrogen and light limitation. This defines a second objective of our research: to determine the effects of dual nitrogen and light limitations on lipid induction. Of course, an overall goal is to demonstrate experimentally that the two stage approach is preferable to a single stage continuous culture, limited for either light or nitrogen (or both).

In the following sections we report our experimental data on batch and continuous, light and nitrogen limited, cultures of Nanno Q. We conclude with a summary and discussion of our results.

5.4. LIPID PRODUCTIVITY IN NITROGEN DEFICIENT BATCH CULTURES OF NANNO Q

5.4.1 Introduction

Biomass and lipid productivities were investigated in batch cultures containing between 25-105 mg/l nitrogen (added as nitrate) with either one or two side illumination of 250 $E/m^2/s$. The objective of these experiments was to determine how nitrogen quota (%N of afdw), lipid content, and lipid and biomass productivity, varied as a function of total light input into the cultures. The experimental protocol is given in the Appendix.

5.4.2 Growth-Rate Cell Quota Relationship

In order to demonstrate that light did indeed affect the nitrogen quota - growth rate relationship, cultures were grown at 45 mg/l nitrogen in the media with either single or double sided light inputs. The growth rates, as calculated from daily productivi-

ties, are shown in Figure 5.1. It is clear that nitrogen quota is a function of the light supply rate and therefore of the biomass density at which nitrogen deficient growth is established. For example a nitrogen content of 5 % of afdw, at one side illumination, will result in a growth rate of 0.18/day vs 0.33/day for two side illumination. This is a demonstration that cells which are experiencing nitrogen deficiency can not simply be described by a single limiting nutrient approach. Thus the interpretation of the experimental results must account for the manner in which light regulates the growth rate in nitrogen deficient (batch) cultures.

5.4.3 Chlorophyll a Content vs Cell Nitrogen

In Figure 5.2 the data of percent chlorophyll a vs. percent nitrogen (as calculated by mass balance) is summarized. Nitrogen sufficient, light limited cultures contained up to 4.3 % chlorophyll a while deficient cultures went as low as 0.4 %. The chlorophyll a values were correlated tightly with cell nitrogen below nitrogen values of 6 %. Above this the previous light environment appeared to control the chlorophyll content as evidenced by the scatter at the high nitrogen values.

5.4.4 Lipid Content vs Cell Nitrogen

Lipid content as a function of calculated cell nitrogen is shown in Figure 5.3. While there is a high degree of scatter, a general trend between lipid content and cell nitrogen is observed. Maximum lipid contents were slightly greater than 50 %. Two points at a cell nitrogen of 6% show a lipid content of 50-53% - they appear to be anomalous. Nitrogen sufficient lipid contents averaged about 28 %. The scatter in the correlation can be a reflection of either the assay or the culture environment or both. There is no systematic difference between external light environments, however much of the variation can be accounted for by variation in culture densities and hence the trajectory of the culture, e.g. variations in light-nitrogen limitations during the growth of the culture.

5.4.5 Lipid Productivity vs Cell Nitrogen

Running three day averages of lipid productivity were calculated for N deficient batch cultures at two side illumination. These results are presented in Figure 5.4 as a function of cell nitrogen. Maximum lipid productivities were greater than 150 mg/l/day occurring at roughly 5-6 % cell nitrogen, while nitrogen sufficient productivities were less than 100 mg/l/day. The relationship appears to be independent of cell density except for the 25 mg/l case. One point worth noting however is that all cultures (except for the 25 mg/l culture) achieved a maximum lipid productivity of 150 mg/l/day. A similar, but lower, productivity maximum (at 5-6% N) was obtained for the single side illuminated cultures (data not shown).

FIGURE 5.1 GROWTH RATE - CELL QUOTA RELATIONSHIP DURING N DEFICIENCY AT ONE AND TWO SIDE ILLUMINATION

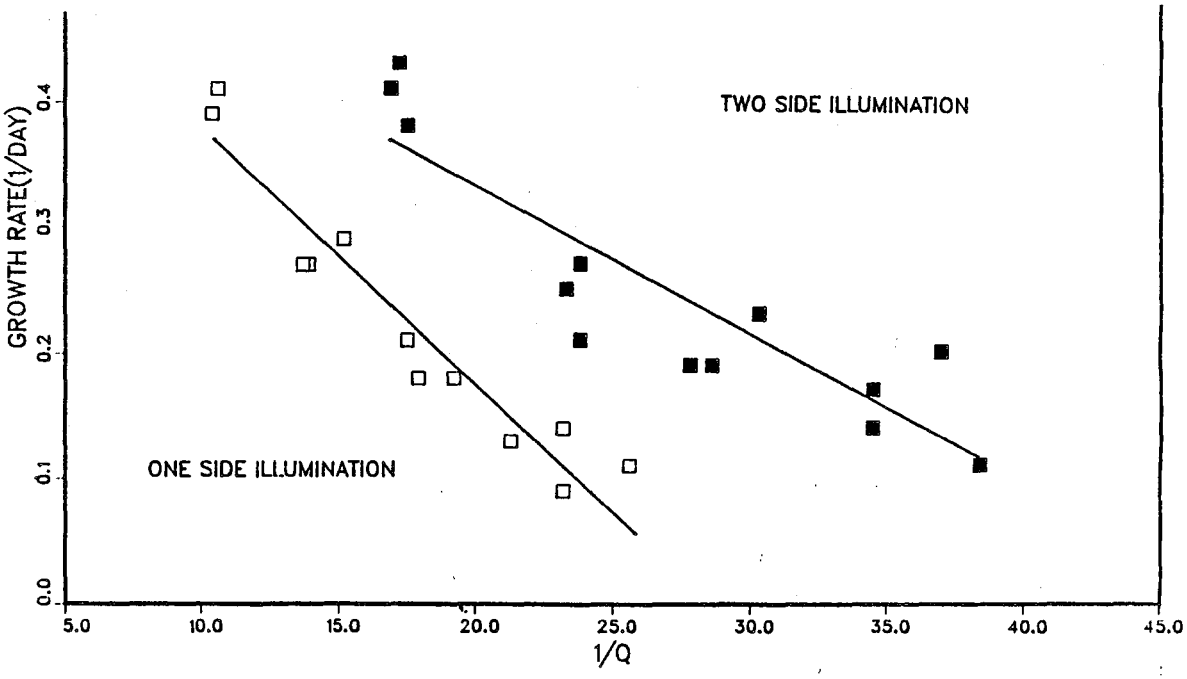


FIGURE 5.2 CHLOROPHYLL a CONTENT VS. CELL NITROGEN

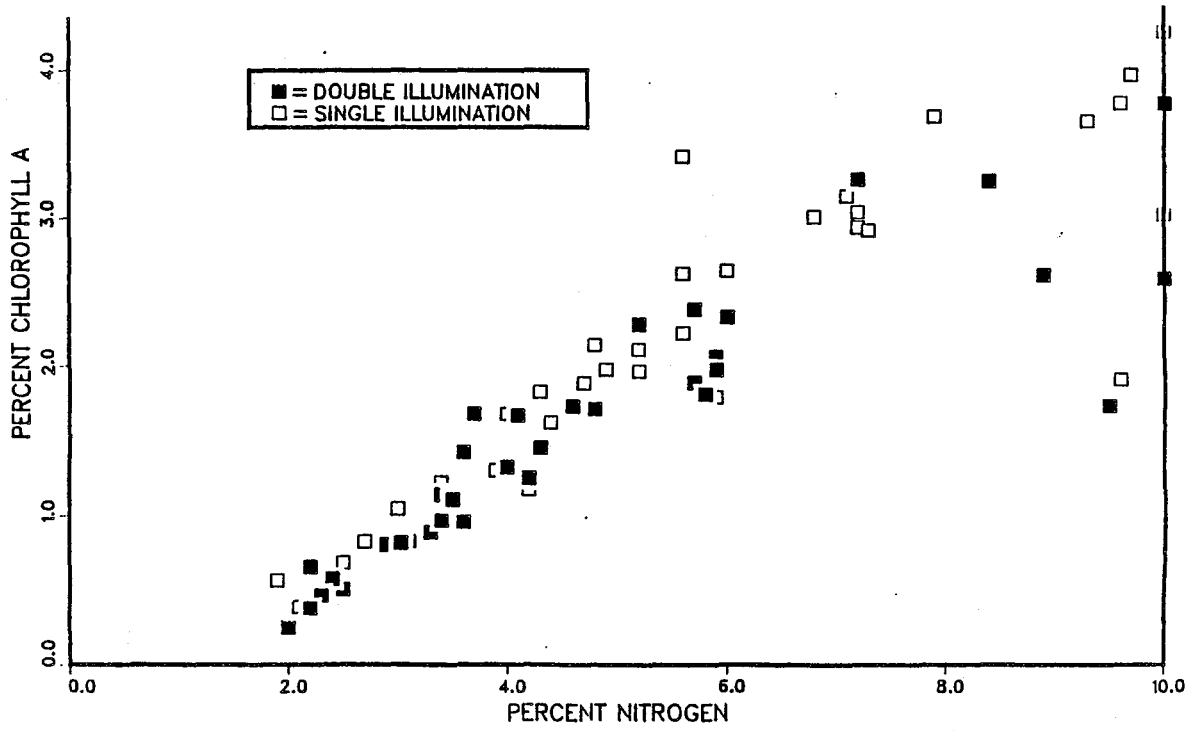


FIGURE 5.3 LIPID CONTENT VS. CELL NITROGEN

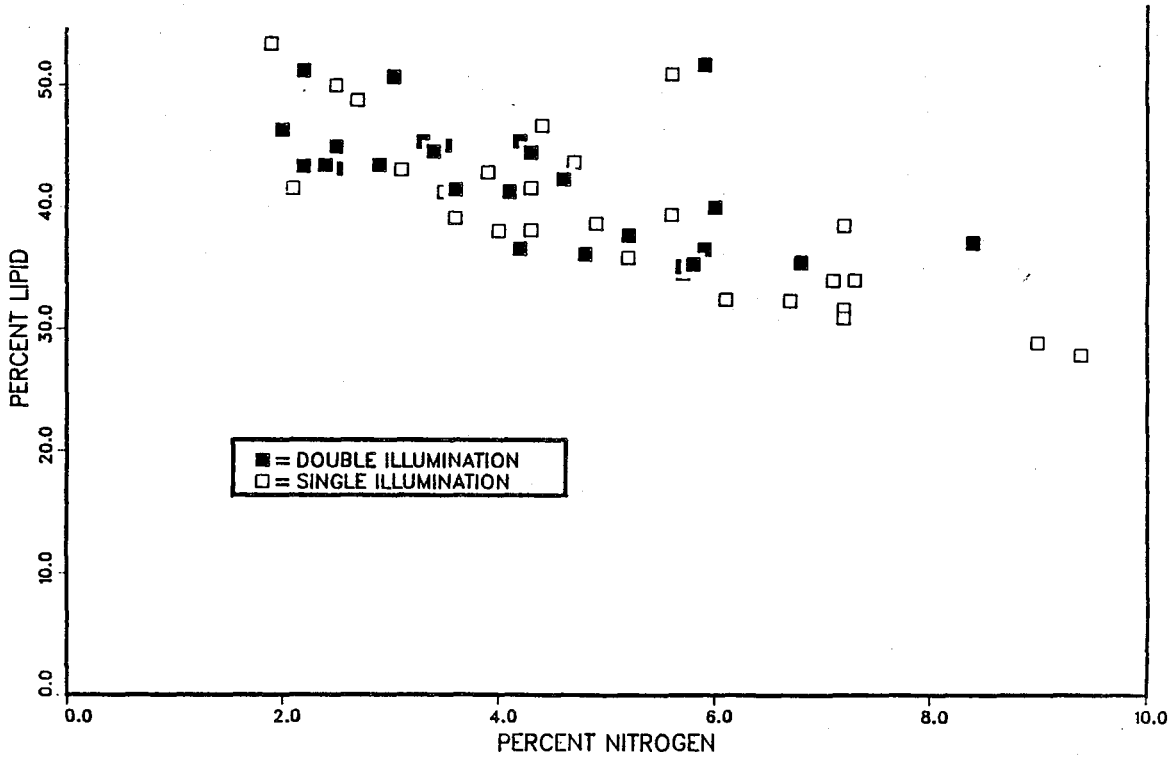
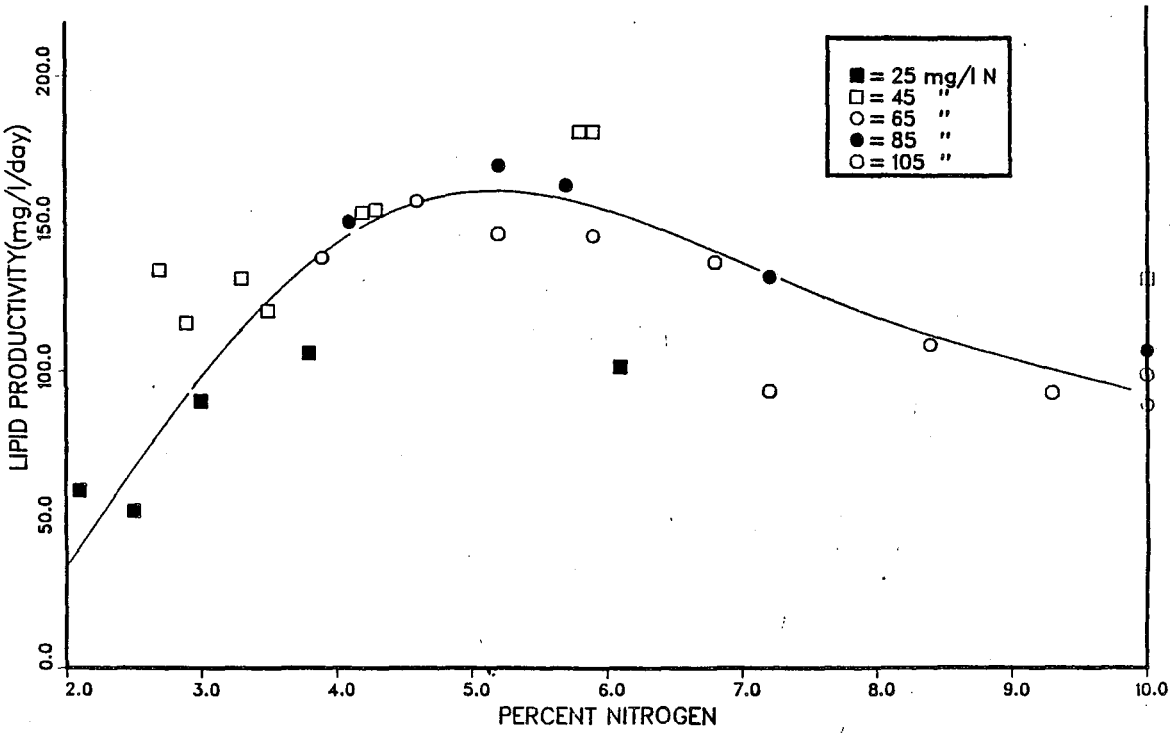


FIGURE 5.4 LIPID PRODUCTIVITY VS. CELL NITROGEN



5.4.6 Heat of Combustion vs. Cell Nitrogen

Cultures were grown up in sufficient quantities (400 mg/sample) and harvested at different intracellular levels of nitrogen in order to correlate heat of combustion, cell nitrogen, and degree of reduction. The data is shown in Table 5.3 and the heat of combustion vs cell nitrogen results are plotted in Figure 5.5. As expected, the heat of combustion and degree of reduction both increase with decreasing cellular nitrogen. The degree of reduction is defined as the number of electrons available for oxidation (129). When calculated, ignoring the contribution of the N source, the degree of reduction is found to correlate many organic compounds, as well as biomass generally, with the equation:

$$h_c = 27.5G \text{ kcal/mole.}$$

Where, $G = 4 + H - 2O$, H is the moles of hydrogen and O is the moles of oxygen (128). The final column in Table 5.3 shows close agreement with the coefficient(27.5 vs 27.6) and therefore is a suitable method for determining h_c for nitrogen deficient Nanno Q. The correlation between cell nitrogen and h_c is quite good and would also be a useful predictor of the heats of combustion.

5.4.7 Light Shift Experiment

In order to test the idea that nitrogen deficient lipid productivity may be in part regulated by the cells ability to carry out photosynthesis, an experiment was performed where the supply of light to the culture was increased, by a factor of two, after the onset of N deficiency. This was accomplished by switching the culture from one side to two side illumination (of the same intensity of light). The analogy to an open pond culture would be to double the pond area for the second stage, that is the pond area used for lipid induction would be twice that of the biomass production ponds).

A plot of biomass yield is shown in Figure 5.6. Light limited linear growth starts around 300 mg/l which occurred between days 2 and 3. The light shifted culture therefore experienced roughly one day of light limitation before it was switched to two sided illumination on day 4. The effect of this one day of light limited growth is demonstrated in Figure 5.7 showing the culture chlorophyll concentrations. The single side illuminated and the shifted cultures reached a maximum chlorophyll at 18 mg/l vs the double side illuminated culture at 13-14 mg/l. Chlorophyll following this maximum initially declined but then leveled off. This constant concentration during a period of increasing biomass concentration indicates that on a per culture weight basis chlorophyll was decreasing. Continued growth would eventually dilute the chlorophyll to such a degree that growth would cease completely.

In Figure 5.8 lipid yield is plotted. It is apparent that lipid yield is the same for the double side and light shifted cultures by day 8 (880 mg/l). While the single illumination is roughly half that(400mg/l). Thus an additional 1-2 days of light energy

TABLE 5.3 DEGREE OF REDUCTION, % NITROGEN, AND HEAT OF COMBUSTION FOR NANNO Q CULTURES

Sample	%N	γ	h_c exp(kcal/mole)	h_c exp/γ
209	9.44	4.94	134.8	27.29
230	8.09	4.97	137.6	27.69
214	8.32	5.002	138.9	27.77
211	5.63	5.028	141.0	28.04
210	6.36	5.039	140.3	27.84
212	5.03	5.051	142.2	28.15
215	4.71	5.131	142.5	27.77
231	3.60	5.140	142.3	27.68
216	3.10	5.18	144.8	27.95
217	3.20	5.24	140.7	26.85
218	2.96	5.24	141.2	26.94

$x = 27.63 \pm .43$

FIGURE 5.5 HEAT OF COMBUSTION VS. CELL NITROGEN

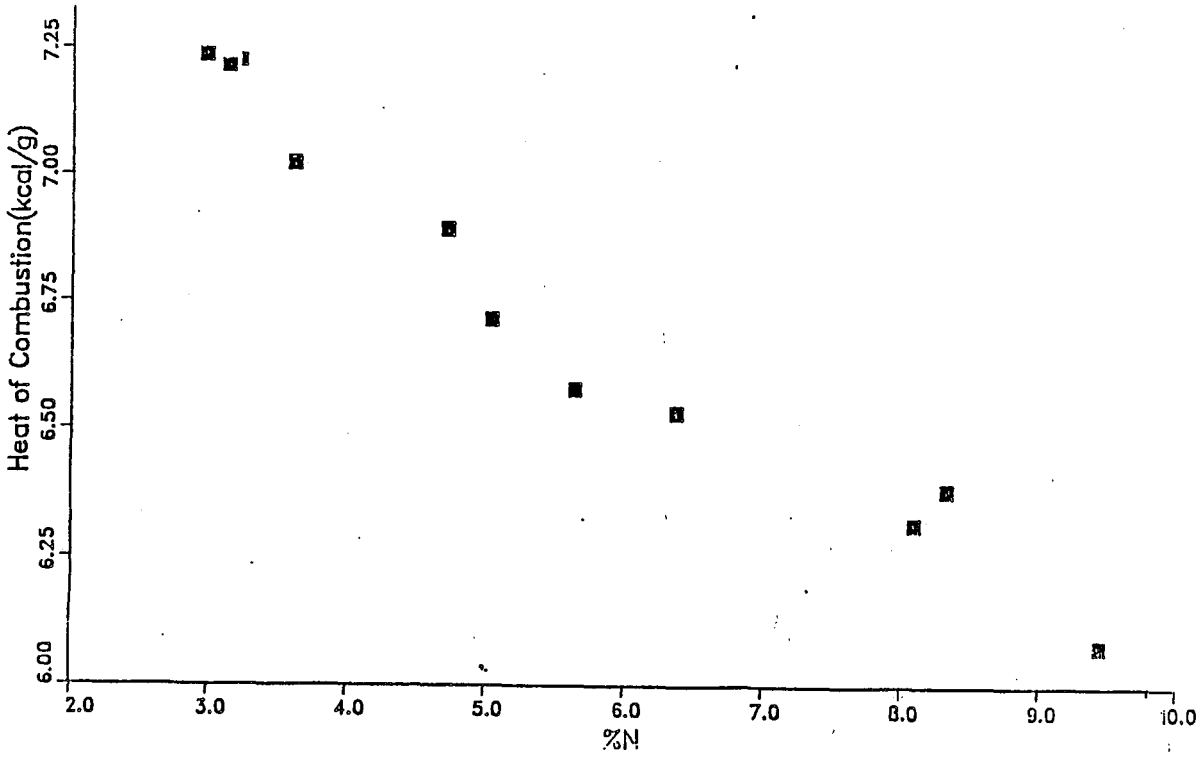


FIGURE 5.6 BIOMASS YIELD DURING LIGHT SHIFT

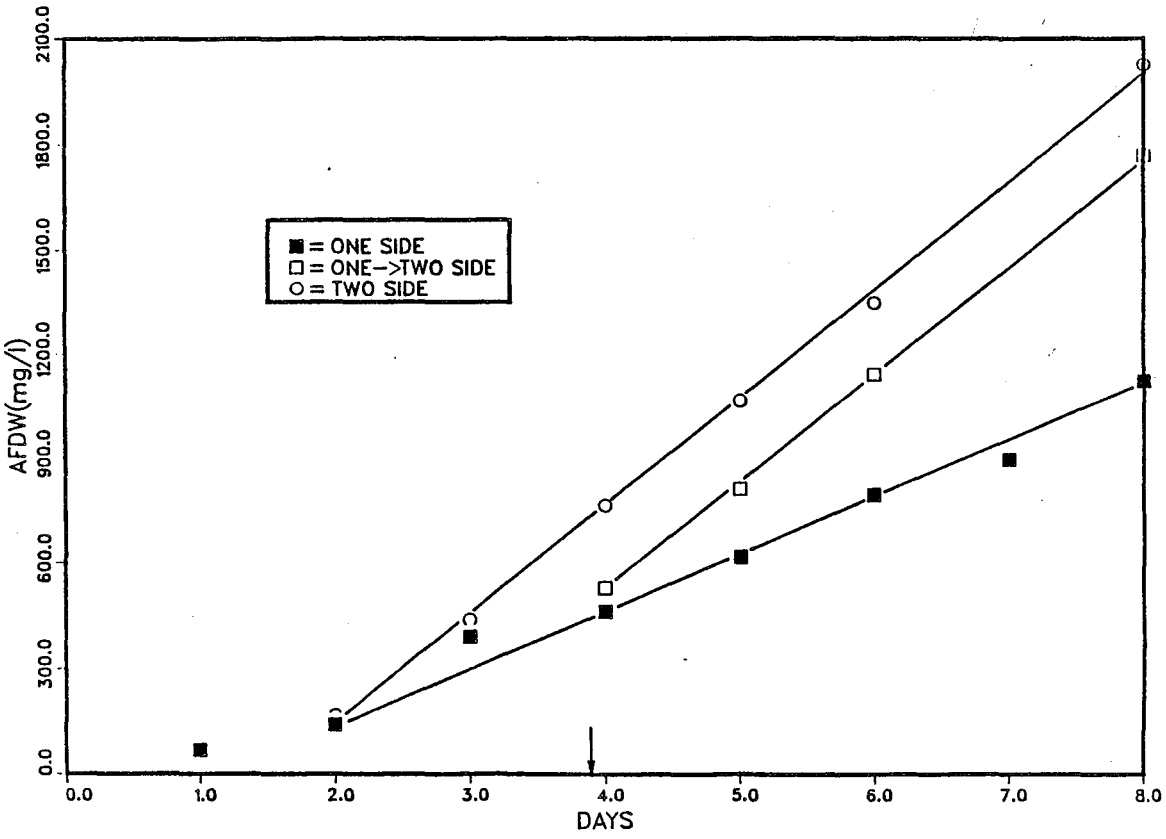


FIGURE 5.7 CHLOROPHYLL a YIELD DURING LIGHT SHIFT

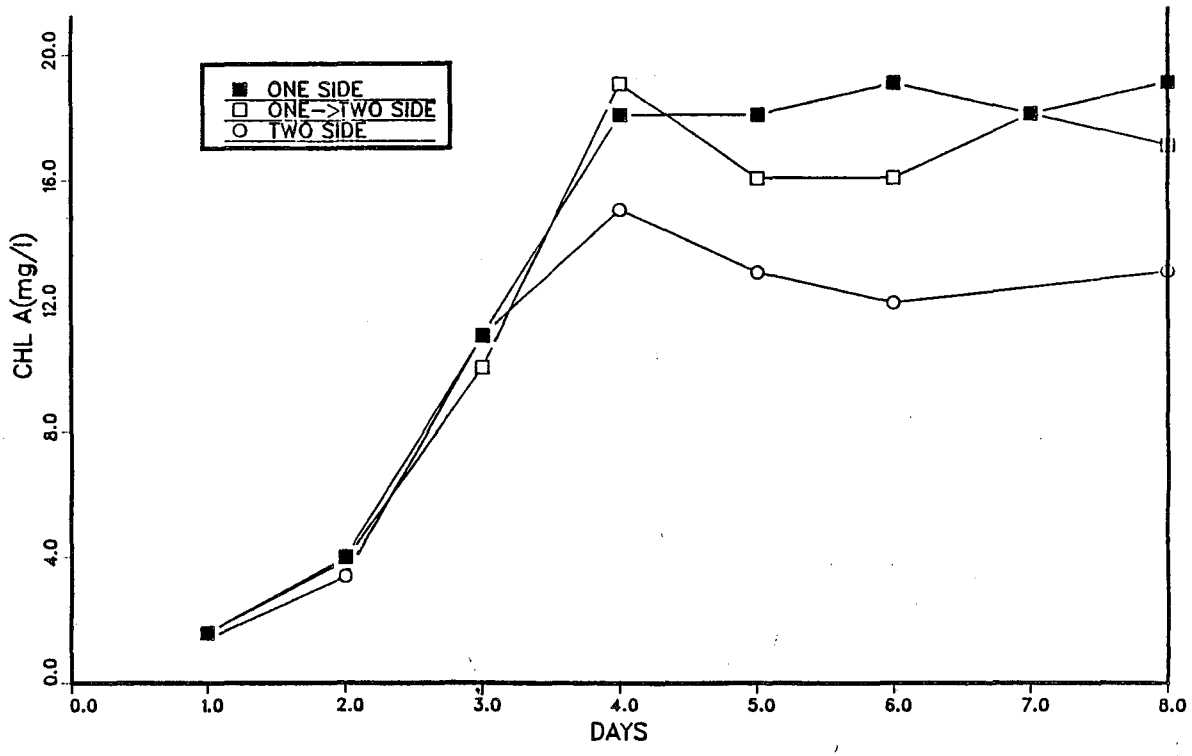
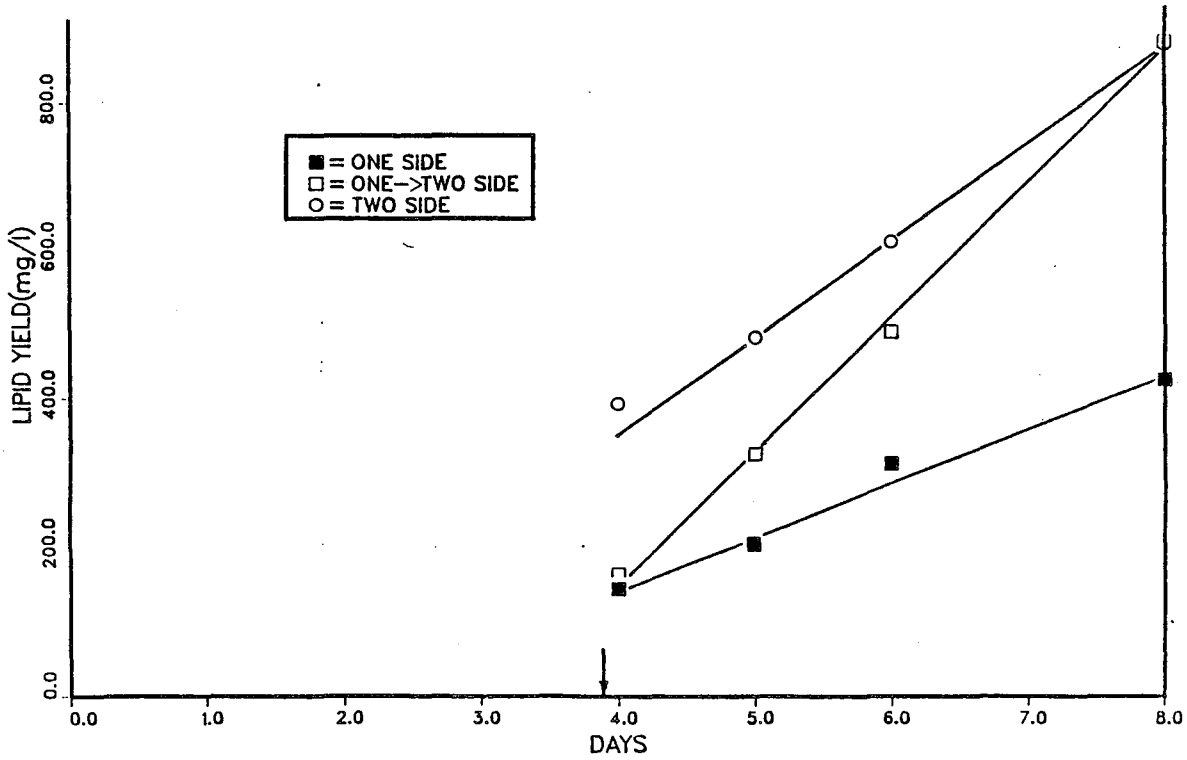


FIGURE 5.8 LIPID YIELD DURING LIGHT SHIFT



(from one side) was unnecessary to attain the same lipid yield. The lipid productivity was 180 mg/l/day in the shifted culture as compared to 150 mg/l/day in the two side illuminated culture. This experiment was repeated with virtually identical result (data not shown).

5.4.8 Discussion and Conclusions

It must be recognized that although lipid induction by microalgae is a widespread phenomenon among certain classes of algae a high lipid productivity during lipid induction in response to nitrogen limitation appears to be (unlike carbohydrate productivity) the exception rather then the rule. However, too little data presently exist to allow quantitation of this extrapolation. Thus, selection of Nanno Q as a model system for lipid induction was based simply on the fact that both our work [and that by the SERI researchers (Lien and Roessler, unpublished)] demonstrated that this organism is suitable for such studies.

Nitrogen deficient batch culture experiments with Nanno Q were carried out at initial nitrogen concentrations of 25 to 105 mg/l. This translated into "stationary" biomass concentrations ranging from 500 to 2400 mg/l. A combination of single and/or double illumination with 250 $E/m^2/s$ was used to investigate the interaction of light and nitrogen on culture performance. It was demonstrated that light supply (total area exposed), or the severity of the light limitation (cell concentration at the onset of nitrogen limitation) affected the organism requirement for nitrogen and thus the growth rate- nitrogen quota relationship. This a logical consequence of the energetic requirements for nitrogen metabolism. One interesting question is whether this relationship would have changed if another nitrogen source (ammonia, urea) which require less metabolic energy had been utilized.

Heat of combustion, lipid, and chlorophyll a all correlated with the nitrogen quota. Severely deficient cultures of Nanno Q had a heat of combustion of 7.5 kcal/g vs. 6.0 kcal/g for light limited cultures. Lipid content ranged from 28 to over 50 % with increasing nitrogen deficiency. Chlorophyll a content was found to correlate with cell nitrogen below 6 % and in that region was independent of light supply. Lipid productivity was demonstrated to have a maximum of 150 mg/l/day at 5 to 6 % cell nitrogen and was apparently independent of light supply and cell density (when initial nitrogen concentrations exceeded 25 mg/l). The lower boundary for maximizing lipid productivity appears to occur coincident with that of biomass productivity. That is, a high enough cell density must first be established, prior to nitrogen deficiency, such that light limited growth and biomass productivity was maximized.

Within the nitrogen levels used the upper boundary for lipid productivity had not been reached. It could be as high as the cell density at which light limited productivity begins to decline (due to maintainance requirements). The implication is that in mass cultures, where cell density must be maximized to reduce

costs for the harvesting process, nitrogen limited cultures could operate at a high a cell density, before productivity losses due to respiration become significant.

Finally, it was demonstrated by the three experiments that for nitrogen deficient cultures, an increase in the light supply (doubling the exposed surface area) results in overall lipid productivities significantly greater than those obtained for cultures kept at the higher light supply on a constant basis. It is reasoned that this may be due to an increased concentration of chlorophyll, in the shifted culture, thus enabling a higher relative rate of electron capture and, therefore, supply of reductant. This result suggests that an optimum process design should include an initial period of light limitation to increase culture chlorophyll, followed by a second stage, after nitrogen deficiency has begun, where the light supply would be increased. In a pond environment this could be done by decreasing the culture depth or density (by dilution) of the second stage thereby making the pond areas for the second stage larger then the first stage. The overall effect would be to increase lipid productivity, as the total pond area for both stages would be minimized relatively to the alternative of keeping the light input constant.

5.5 LIPID PRODUCTIVITY IN NITROGEN LIMITED CONTINUOUS CULTURES OF NANNO Q

5.4.1 Introduction

In the above experiments, the lipid and biomass productivities, nitrogen content, degree of reduction, heat of combustion, as well as compositional changes were investigated in nitrogen deficient (starved) batch cultures of Nanno Q, demonstrating the limits of efficiency of converting light energy into lipid for batch cultures. Once conclusion form this work was that some degree of light limitation is advantageous in order to maximize lipid formation. The work reported on in this section had the following objectives:

1. Determine whether lipid biosynthesis can be increased in continuous cultures by nitrogen limitation and compare productivities between batch and continuous cultures;
2. Investigate the effects of dual light and nitrogen limitation on lipid formation in continuous cultures; and
3. Determine lipid formation rates in nitrogen starved (batch) cultures derived from preconditioned nitrogen limited (continuous) cultures.

5.5.2 Experimental design

Conventional chemostat experiments designed for determining nutrient limited growth kinetics are normally carried out at a single feed concentration of the limiting nutrient and varying dilution rates. The culture therefore adjusts its cell density to

reflect the nitrogen supply rate (at $u<u_{max}$ and $K_m<<S$, where S is the influent nutrient concentration). Thus each different imposed growth rate is limited at different external nutrient concentrations and at different cell densities. For microalgae, if the light intensity is not significantly above the saturation value (I_k), some degree of light limitation will exist, becoming more severe as a function of cell density. Thus, unlike for heterotrophic systems, cell density is important.

In ecologically oriented research this problem is avoided by use of very low nutrient concentrations in the feed and, therefore, very low (<10 mg/l) algae concentrations in the culture. This minimizes light absorption (<10% of incident light) and avoids variations in the light absorbed per algal cell. However, in mass culture type of work, the effects under conditions of complete light absorption (>95%) are of interest. Even if it is argued that at incident light intensities below I_k light absorption per se is not an important variable, the practical constraints of providing sufficient sample size for the various required analysis suggest that dense cultures be used.

Dense nutrient limited continuous cultures will superimpose light limitation on nutrient limited growth. This results in two simultaneous variables, light and (in this case) nitrogen limitation. This problem can be overcome by operating the chemostats at (more or less) fixed culture densities, as estimated by chlorophyll concentration. This has the advantage of enabling operation at higher cell densities which decreases the volume requirements for sampling, but has the disadvantage of requiring manual adjustments of feed nitrogen levels. A combination of the two above mentioned approaches, constant feed supply and constant cell density, was used in the experiments reported on in this section.

Using the Droop equation and estimates of u_{max} and the minimum nitrogen quota from previous experiments, feed rates and nitrogen concentration required to maintain an approximately constant cell density were estimated. Below we report on biomass and lipid productivity in nitrogen and/or light limited continuous cultures of Nanno Q operated at a hydraulic detention time of 0.17 to 0.9 day^{-1}. The data reported is preliminary and does not represent all the completed experiments. Nitrogen and some lipid analysis remain to be completed.

5.5.3 Light Limited Growth

Data was collected for seven distinct steady state conditions in which growth was limited by light not nitrogen supply. A summary of this data is shown in Figures 5.9-5.11. It was found that regardless of dilution rate, lipid content remained relatively constant at about 30% (Fig. 5.9). This compares quite closely with what is observed for nitrogen sufficient batch cultures (see figure 5.3). Chlorophyll a content as a function of dilution rate is shown in Figure 5.10. Maximum chlorophyll a reached around 4 % and began to decrease above 0.5/day. Biomass and lipid productivity are plotted as a function of growth rate in Figure 5.11.

FIGURE 5.9 LIPID CONTENT AS A FUNCTION OF DILUTION RATE FOR A LIGHT LIMITED, NITROGEN SUFFICIENT CONTINOUS CULTURE OF NANNO Q

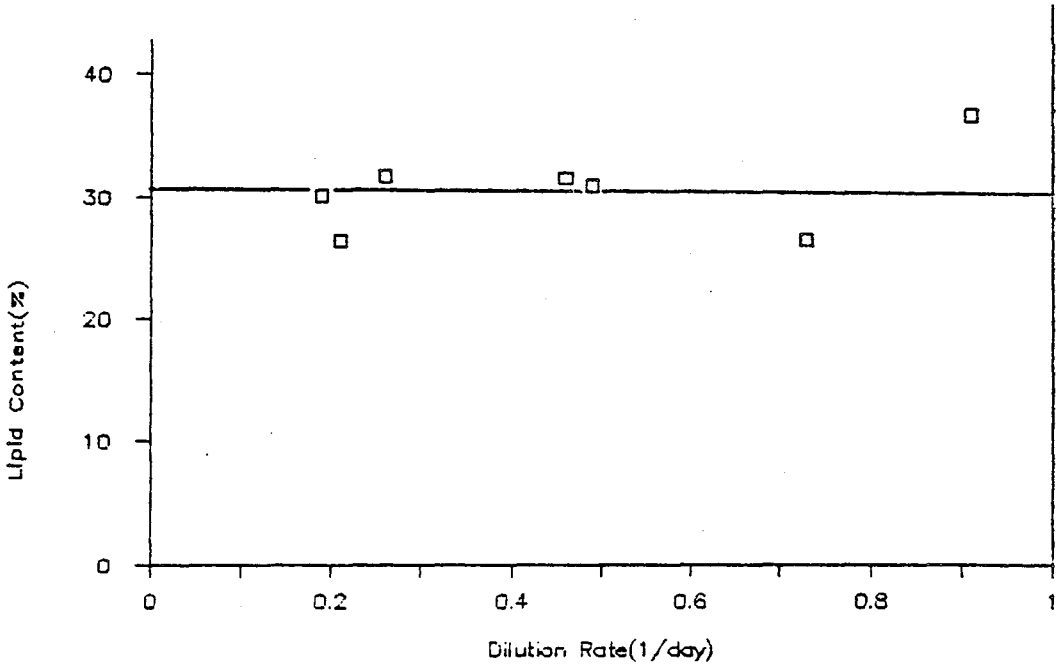
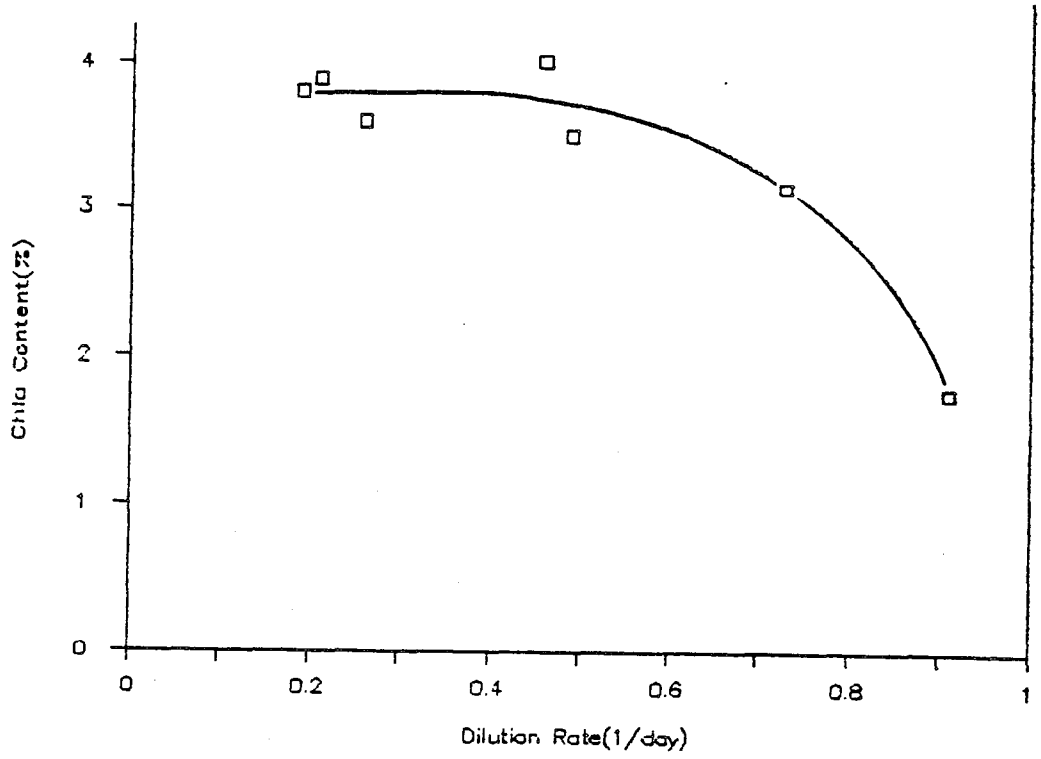


FIGURE 5.10 CHLOROPHYLL a CONTENT AS A FUNCTION OF DILUTION RATE FOR LIGHT LIMITED, NITROGEN SUFFICIENT CONTINOUS CULTURES OF NANNO Q



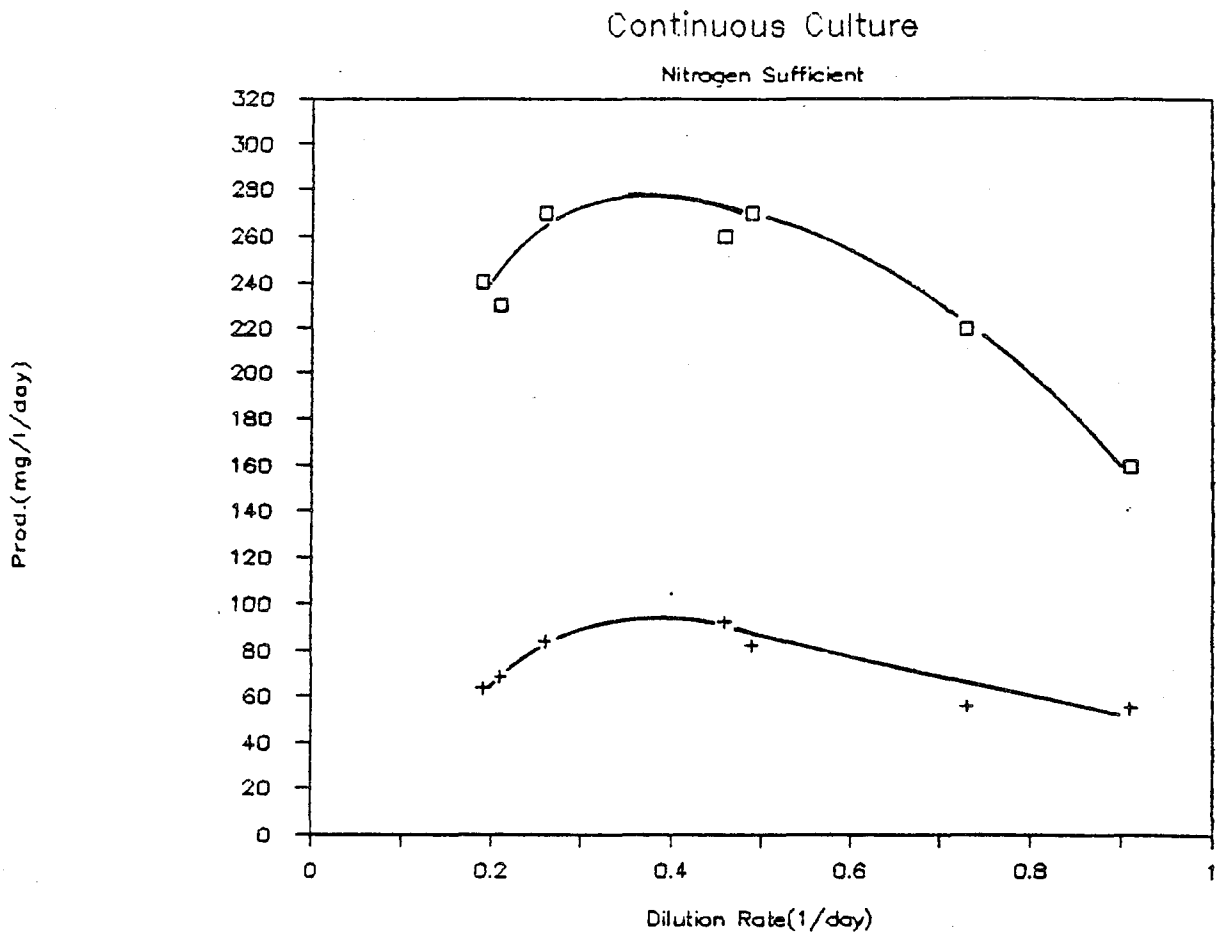


Figure 5.11 Biomass and Lipid Productivity in Light Limited Continuous Cultures of Nanno Q.

They both display roughly the same optimum, somewhere between 0.25 and 0.45/day. Maximum biomass productivity reached 280 mg/l/day which is less than the 350 mg/l/day observed for light limited batch cultures. Maximum lipid productivity was 80-90 mg/l/day, slightly less than the 100 mg/l/day achieved in nitrogen sufficient batch cultures.

5.5.4 Nitrogen Limited Growth

In one experiment, nitrogen limited cultures were established at low cell densities by varying the nitrogen concentration in the feed. This data is shown in Table 5.4. By adjusting the feed nitrogen and hence the cell density it was possible to eliminate any compounding effect of decreasing light supply - on a per cell basis as growth rate decreased - that would have resulted if a constant nitrogen feed concentration was used. Growth rates ranging between .28 and .81/day at cell densities less than 200 ppm were investigated. Cell nitrogen has not been determined as yet, the numbers reported in Table 5.4 are calculated on a mass balance basis assuming negligible abiotic nitrogen. Lipid content was significantly increased over nitrogen sufficient cultures. It varied between 44 and 36 % for dilution rates of 0.28 and 0.81/day respectively, or between 5 and 7 % cell nitrogen. These values are similar to those observed for batch cultures.

TABLE 5.4 STEADY STATE RESULTS FOR NITROGEN LIMITED CULTURES OF NANNO Q. (Number in parentheses are 2x st.dev.).

Dilution Rate	%N	Density mg/l	P_{bio} mg/l/day	Percent Lipid	Percent Chl a	P_{lip} mg/l/d	P_{chla} mg/l/d
0.28(.02)	5.1	150(20)	42(6)	44(12)	.58(.08)	19(5.6)	.25(.05)
0.52(.07)	4.6	200(20)	100(20)	43(9)	.86(.32)	43(10)	.82(.05)
0.81(.06)	7.1	170(20)	140(14)	36(3)	1.7(.3)	50(7)	2.4(.4)

Another experiment, conducted at higher cell densities and with lesser degree of nitrogen limitation was carried out on cultures that were previously nitrogen sufficient. The complete history for these cultures is given in figures 5.12a-d. The arrow is an indication of the start of nitrogen deficient media inflow, the two marked regions indicate the collection of points comprising the steady states. Because nitrogen analysis are incomplete, it is only possible to discuss these results in terms of their general response to nitrogen limitation(Figures 5.13 -5.15).

The cultures displayed some variety in their responses but where clearly nitrogen limited due to their decreased rates of chl a synthesis. Biomass productivity decreased in three of the cul-

FIGURE 5.12 a ASH FREE DRY WEIGHT HISTORY FOR CULTURE 266

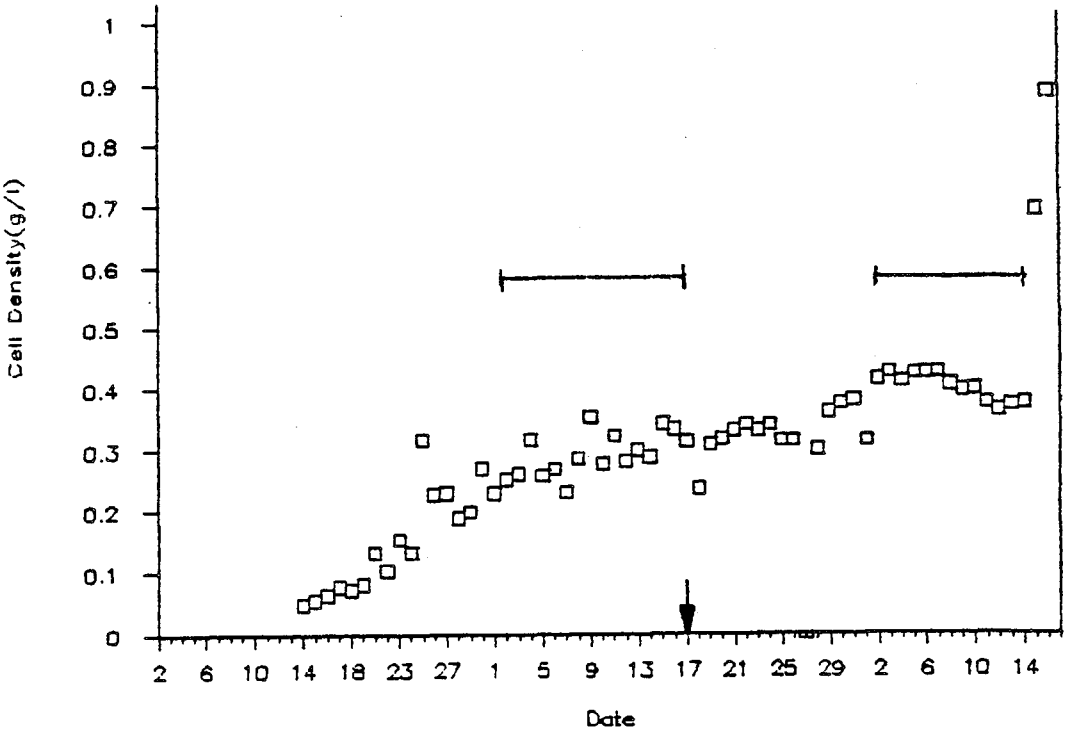


FIGURE 5.12b ASH-FREE DRY WEIGHT HISTORY FOR CULTURE 267

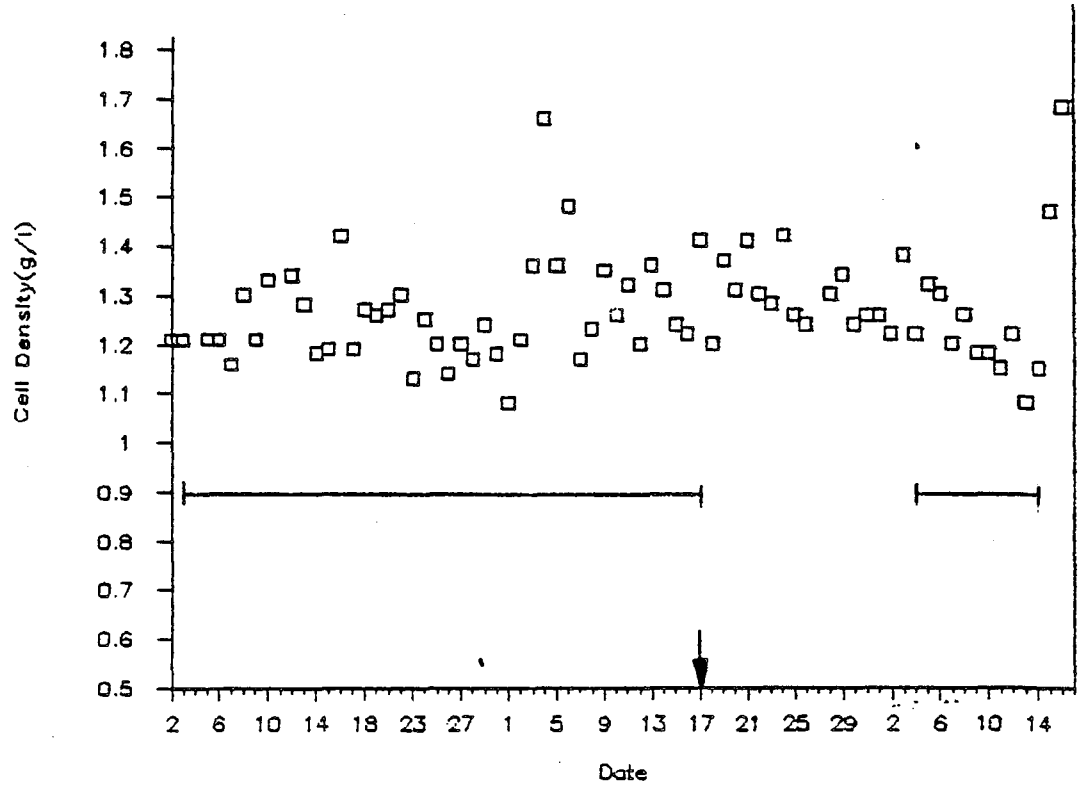


FIGURE 5.12c ASH-FREE DRY WEIGHT HISTORY FOR CULTURE 268

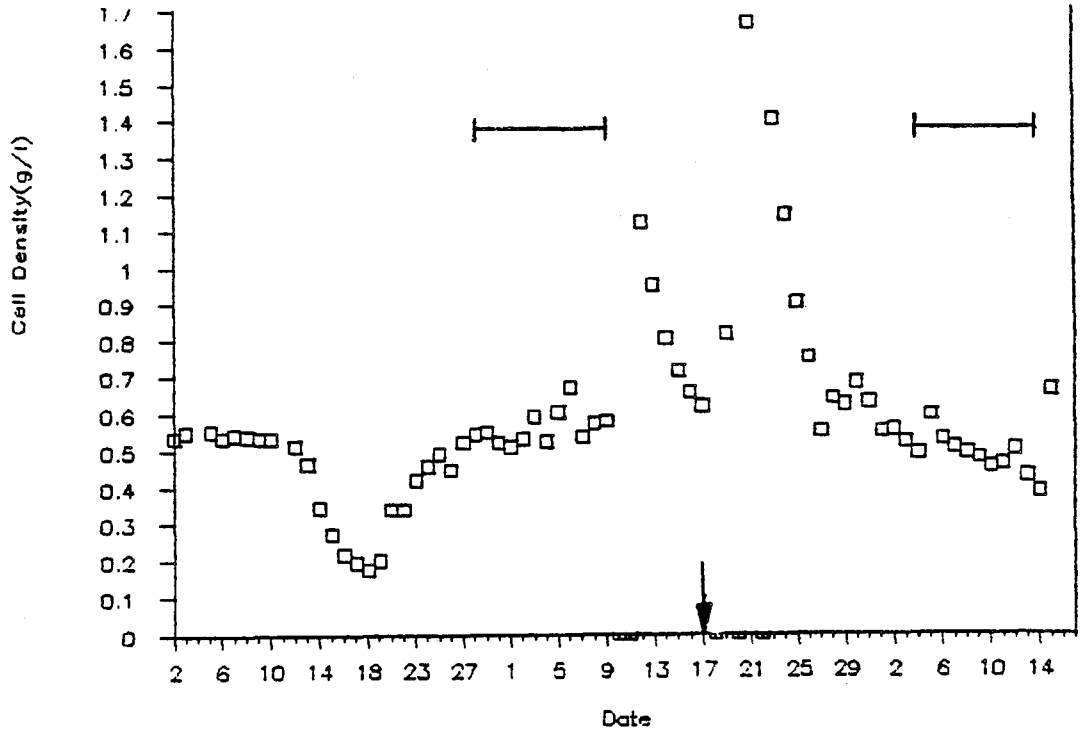


FIGURE 5.12d ASH-FREE DRY WEIGHT HISTORY FOR CULTURE 269

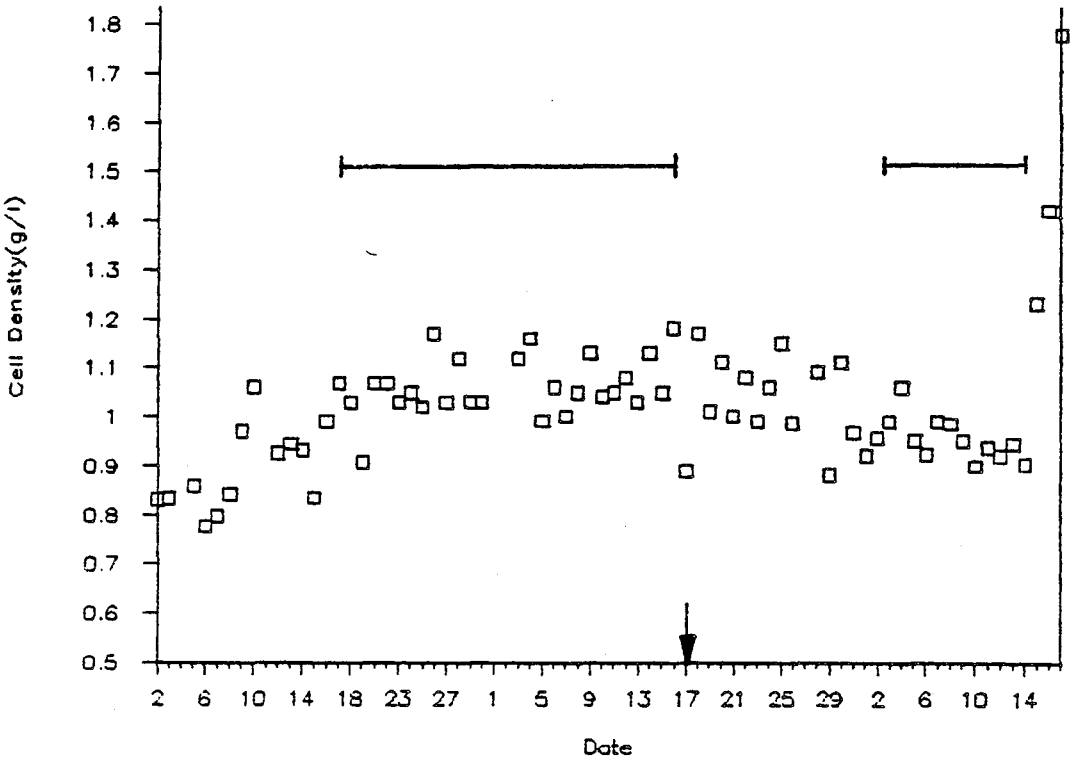


FIGURE 5.13 A COMPARISON OF CHLOROPHYLL a PRODUCTIVITY IN N SUFFICIENT AND DEFICIENT CONTINUOUS CULTURES

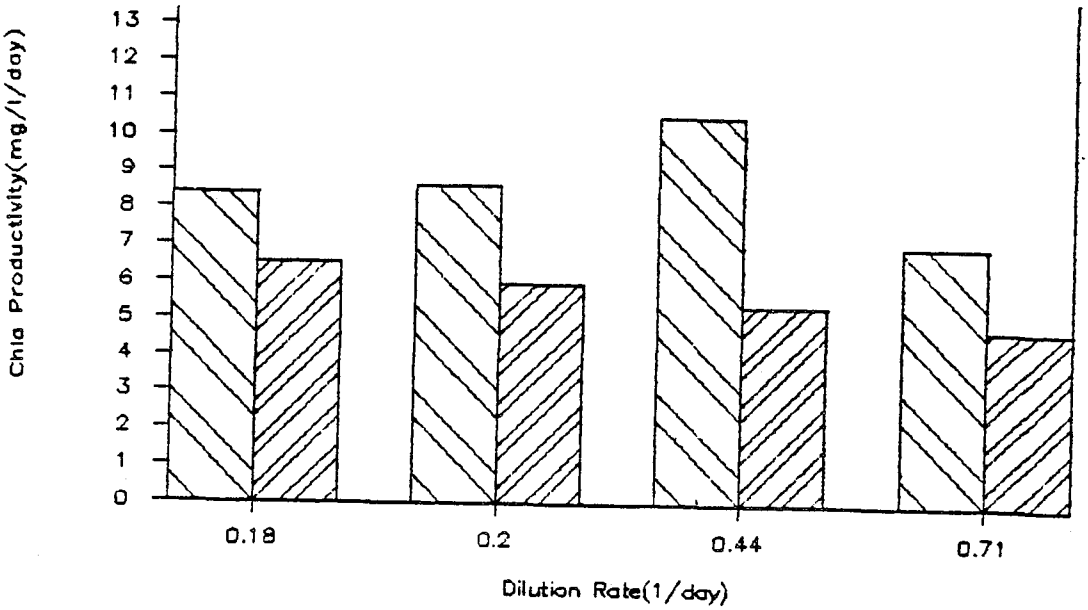


FIGURE 5.14 A COMPARISON OF BIOMASS PRODUCTIVITY IN N SUFFICIENT AND DEFICIENT CONTINUOUS CULTURES

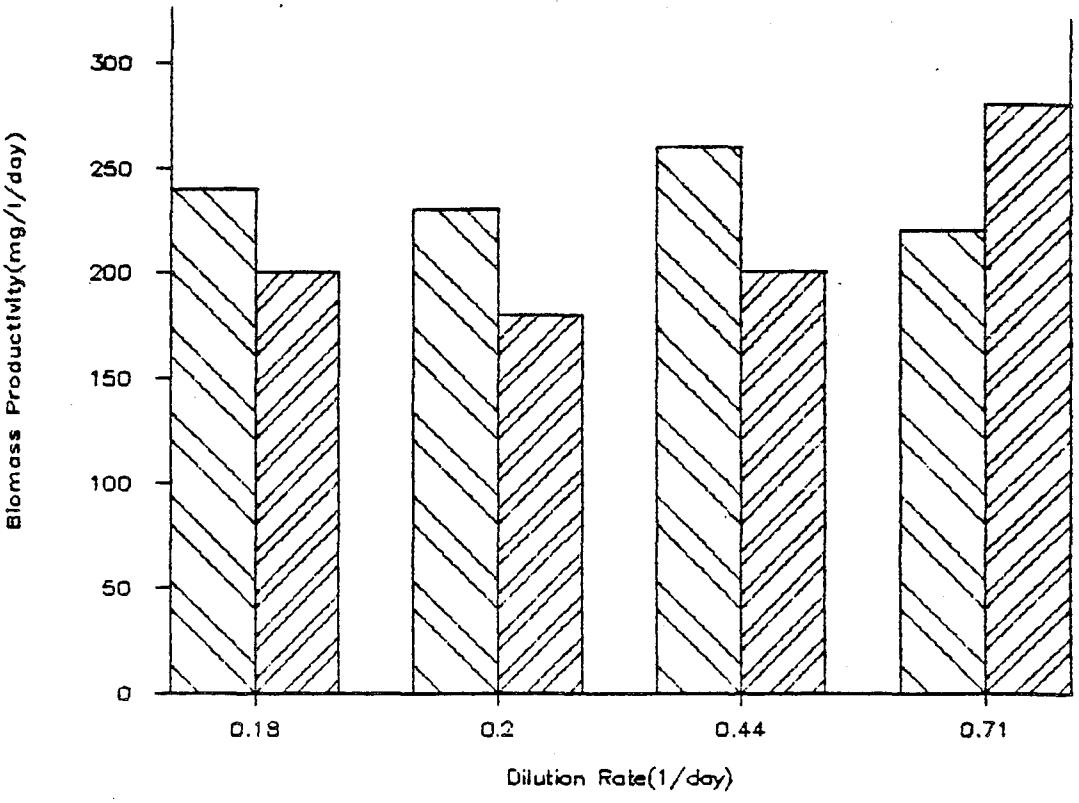
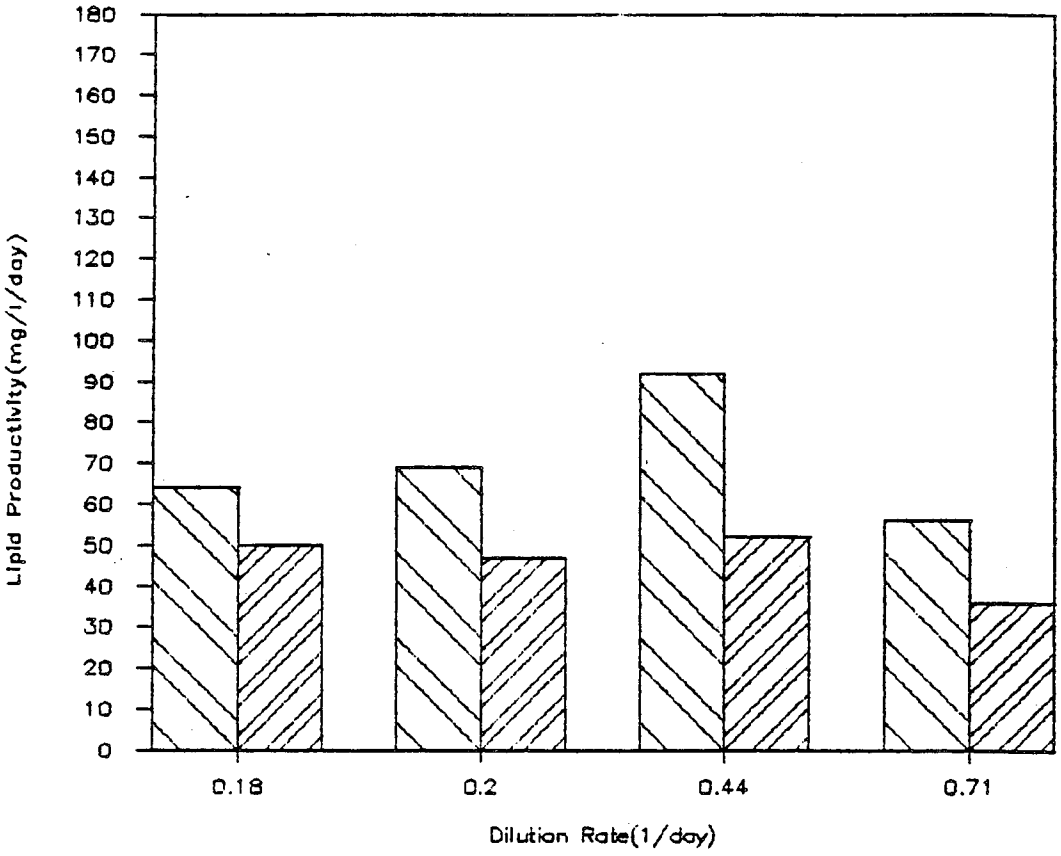


FIGURE 5.15 A COMPARISON OF LIPID PRODUCTIVITY IN N SUFFICIENT AND DEFICIENT CONTINUOUS CULTURES



tures, however increased in one culture operated at the highest dilution rate (0.71/day). This result, while appearing anomalous, may in fact be an indication of a unique physiological state. Evidence supporting this view is the significantly lower lipid content this culture possessed as compared with the other N limited cultures (13 vs 25 %). Additionally another N limited culture in a separate experiment displayed a biomass productivity of 290 mg/l/d, with a small Chlorophyll a productivity (3.2) and again a very low lipid content (9 %) (data not shown).

It can be argued that nitrogen limitation may aid in the reduction of accessory pigments and thus increase the light utilization efficiency for the entire culture by a decrease in the extinction coefficient. This of course would have to be coupled with no significant reduction in enzyme levels. The fact that lipid productivity is substantially less in these two cultures is significant and indicates a metabolic adjustment which allows a higher carbon fixation rate. These two cases are by no means sufficient to draw any general conclusions from, however do suggest a worthwhile area of future investigation.

Finally, lipid productivity decreased in all cultures relative to the nitrogen sufficient case as shown in Figure 5.15. The maximum productivity for both the sufficient and deficient cases was coincident with maximum biomass productivity and was only 50 mg/l/day. One point of uncertainty is the lower lipid contents achieved in these cultures(ca. 25%) as compared with those in Table 5.4 (ca. 40%).

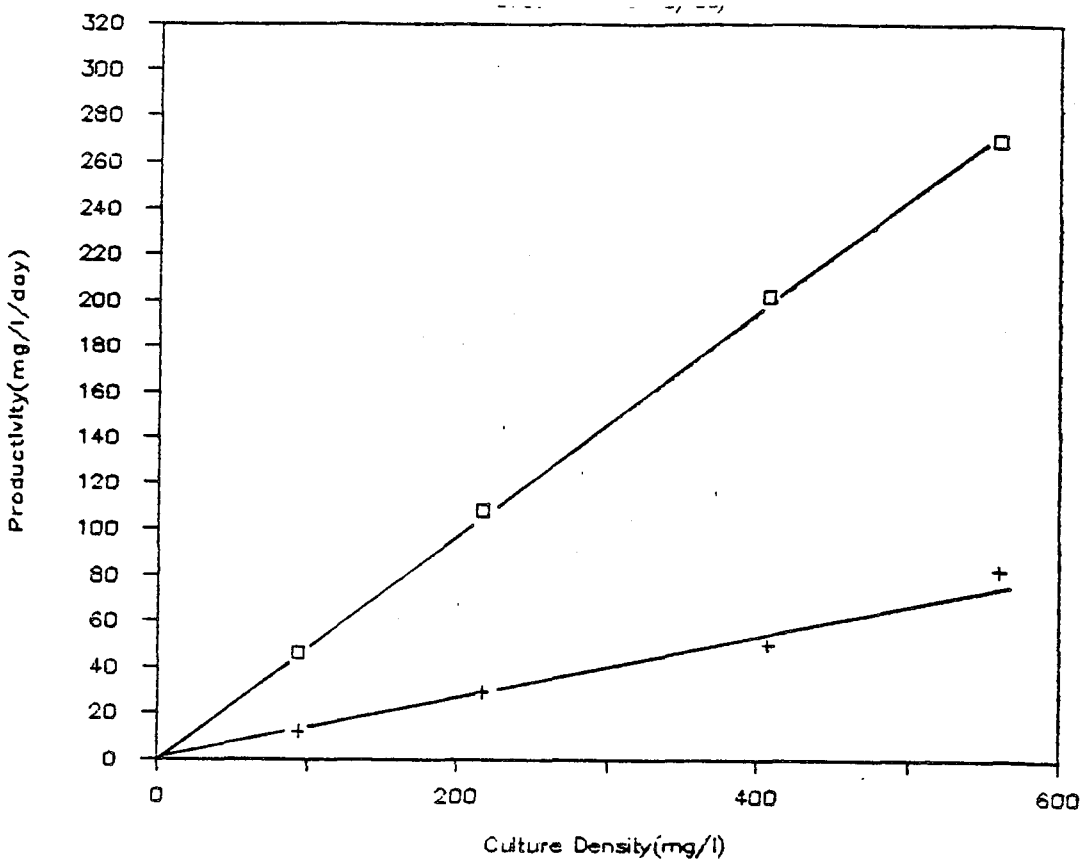
5.5.5 Nitrogen Limited Growth vs. Cell Density

In order to test the idea that cell density may affect the nitrogen limited productivity of lipid (by affecting the lipid content), cultures were operated at the same dilution rate but with varying nitrogen supply rates (feed nitrogen concentrations). In theory, this experimental design allows cell density to be an independent variable, with the nitrogen quota being the same in all cultures. Figure 5.16 is plots biomass and lipid productivity for three nitrogen limited cultures (AFDW < 420 ppm) and one nitrogen sufficient culture (AFDW = 570 ppm). The first three biomass productivity points should, by definition, fall on a straight line. The observation that the final point also is predicted by the same line(ie. u = 0.5/day) is an indicator of experimental consistency. The curve for lipid productivity is also linear, therefore indicating little, if any, change in lipid content as a function of cell density. Thus nitrogen limitation appears to decrease lipid productivity relative to nitrogen sufficient and light limited cultures operated at the same dilution rate.

5.5.6 Continuous to Batch Induction of Lipid

The process alternative for a microalgal lipid production which we favour would use a first stage pond, operated at maximum biomass productivity, followed by a second stage for lipid induction.

FIGURE 5.16 BIOMASS AND LIPID PRODUCTIVITY IN N LIMITED CONTINUOUS CULTURES AT 0.5/day DILUTION RATE AS A FUNCTION OF STANDING BIOMASS



The first stage in principle should be operated at a point as close to nitrogen limited growth as possible. This may involve either (semi)batch or continuous cultures. It is therefore of interest to study the batch induction kinetics following a pre-adaptation to nitrogen limited continuous culture and to compare these to the prior results with lipid induction and productivity in batch cultures. This was done for eight separate continuous cultures, representative results from two of these are presented here.

Biomass and lipid yield are shown as a function of time for two cultures experiencing batch growth following nitrogen limited culture (Figures 5.17 and 5.18). The top curves represent a lower dilution rate and hence a higher cell density in the continuous cultures. The conditions of preadaptation were 0.17 and 0.69/day (dilution rate) and calculated nitrogen contents of 7.5 and 5.1% respectively.

In all cultures studied thus far, a period of significantly reduced lipid production occurs following batch induction as evidenced by the near zero slope for the two days after induction (Figure 5.18). Steady state lipid productivities were 50 and 36 mg/l/day and 31 and 22 mg/l/day for the following three day period. Biomass productivity was similar to that obtained for the nitrogen limited cultures and roughly the same for both the high and low dilution rate cases. The constant rate however is impressive as towards the final day of the experiment cell nitrogen should have been close to its minimum.

The reason for the period of essentially zero lipid productivity following preadaptation to nitrogen limitation is unclear. It may be that the cells ability to adapt to the changing nutrient condition is impaired by the previous period of nitrogen limited growth by the reduction/elimination of some precursor. Regardless of the underlying physiological principle, these results indicate that the preadaptation condition can not be overlooked in process development studies.

5.5.6 Discussion

The results from the continuous culture studies indicated that nitrogen limitation does not result in an increase in lipid productivity. Since apparently contradictory results were obtained concerning N deficient lipid content as a function of cell density, any conclusion must await future analysis of nitrogen content. Light limited cultures had optimum lipid and biomass productivities at detention times between 2.5 and 4 days and were higher than nitrogen limited cultures. In two cases nitrogen limitation resulted in higher biomass productivities and this was coupled with a significant reduction in lipid synthesis. Lastly it was shown that very low rates of lipid induction were observed following nitrogen limited growth.

FIGURE 5.17 BIOMASS YIELD VS. TIME IN BATCH CULTURE FOLLOWING PREADAPTATION TO NITROGEN LIMITATION IN CONTINUOUS CULTURES (Culture was shifted from continuous to batch mode on day 14)

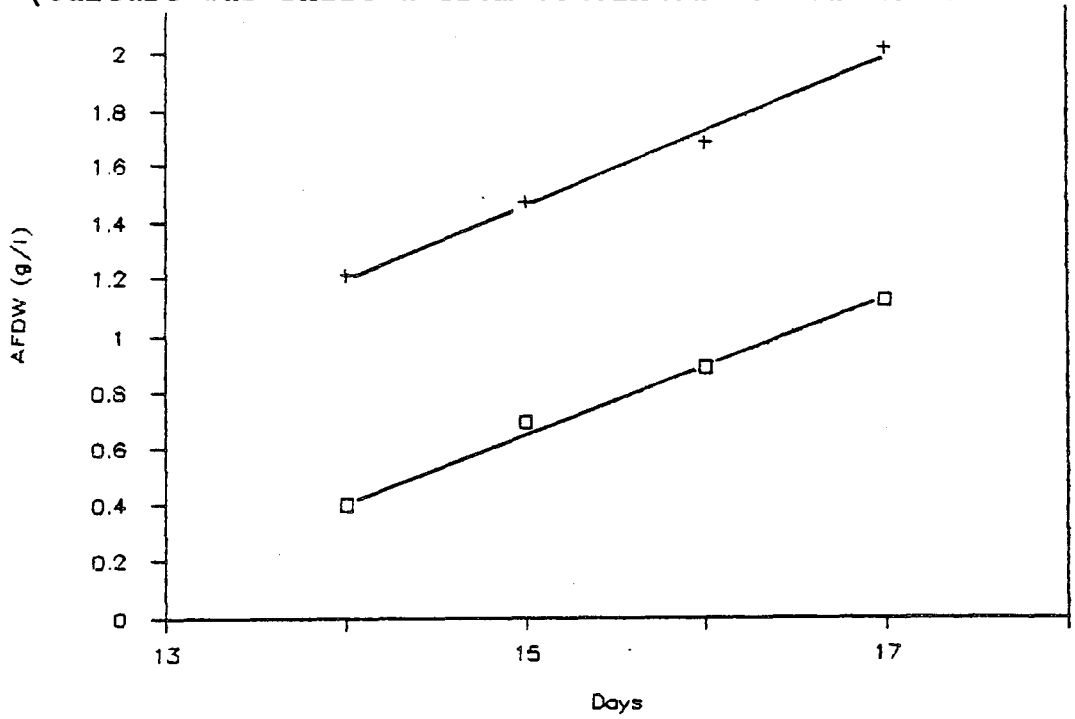
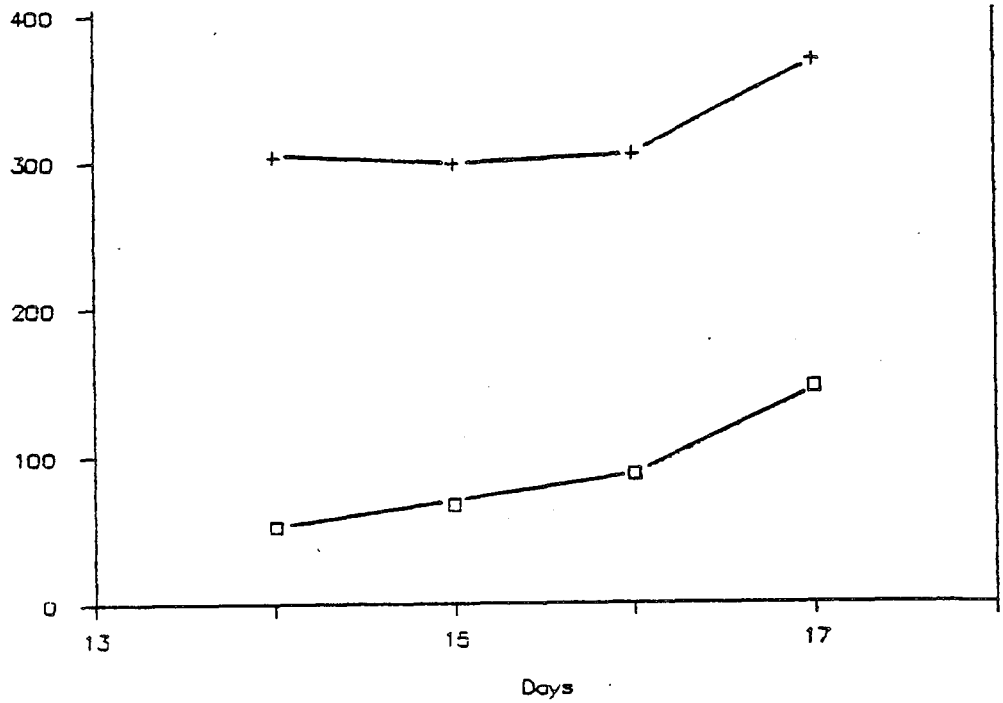


FIGURE 5.18 LIPID YIELD VS. TIME IN BATCH CULTURE FOLLOWING PREADAPTATION TO NITROGEN LIMITATION IN CONTINUOUS CULTURES (Culture was shifted from continuous to batch mode on day 14)



5.6 SUMMARY AND CONCLUSIONS

Table 5.5 summarizes the lipid productivity results as a function of the variety of culture environments tested. The general conclusion can be made that lipid productivity was greater under all environmental conditions in batch rather than continuous cultures. The maximum lipid productivity, for all conditions, occurred in batch cultures experiencing a transient increase in their light supply rate during nitrogen deficiency. A more detailed discussion of these results will be presented in the technical publications.

Table 5.5. Comparison of Maximum Observed Lipid Productivities (mg/l/day) for a Variety of Growth Conditions.

Condition	Batch	Continuous
Nitrogen Sufficient	100	90
Nitrogen Deficient	150	50
Light Shift	180	--
Continuous to Batch	--	20-30

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APPENDIX. MATERIALS AND METHODS

I.1 Strains of Microalgae

All strains referred to in this report have been obtained from Dr. Bill Barclay as part of the SERI culture collection. Further information concerning each can be obtained through the SERI culture collection catalogue (Barclay,1986).

I.2 Dry Weights

Dry weights were determined by centrifuging a volume of culture corresponding to 10 mg of ash free dry weight for 20 minute at 15000 rpm(some pellets required longer times). Supernatents were decanted and the tubes were rinsed with distilled water in order to remove residual drops of media(being careful to avoid the pellet). The pellets were then removed by using several small washes of distilled water(1-3 mls each) into preashed, preweighed aluminum drying boats. Water was evaporated from the boats over a hot plate(60-70 C) for about 20 minutes. The boats were subsequently dried in a forced air convection oven at 103 C for two hours, weighed and then ashed in a muffle furnace at 505 C for 30 minutes and weighed again. The ash free dry weight represented the difference between the dried and ashed weights.

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

I.3 Protein Assays

Proteins were assyed by the Folin Lowry Method (1971). The procedure involved centrifuging 10 ml samples of cultures, resuspending the pellet in 5 mls of distilled water and digesting 4.5 ml of that volume with 0.5 ml 1N NaOH in a boiling water bath for 1 hour. This digestion time was found to give the maximum protein content in a time course experiment. After cooling, two aliquots of the digestion mixture were diluted with 0.5 ml 1N NaOH. Alkaline CuSO4 solution and Folin reagents were added sequentially and this mixture was read at 650 nm on a Bausch and Lomb spectrophotometer. Protein content was determined by comparison with Bovine albumin standard (Sigma Chemical).

I.4 Carbohydrates

Carbohydrates were determined by the phenol-sulfuric acid method (Herbert,1971). The procedure involved centrifuging 10 mls of culture, resuspending the pellet in 5 mls of distilled water, and digesting 0.5 mls of that in 4.5 mls of 1.1 N HCl for one hour at 100 C. Sample volumes were removed from the digestion mixture and diluted into distilled water to 1 ml. One ml of 5 % phenol solution was added and the test tube was mixed on a vortex mixer.

Immediately following this 5 mls of sulfuric acid were added and the tube mixed again. Samples were left at room temperature for 20 minutes to develop color and were then read on a "Spect 20" at 488 nm. Carbohydrate content was determined by comparison against a glucose standard (Sigma Chemical).

I.5 Chlorophyll a

One to ten mls of culture were filtered under vacuum onto a 4.7 cm glass fiber filter. The retained cells and filter paper were suspended in 10 mls of methanol and sonicated for 30 seconds. This mixture was then placed in a 65 C water bath for 3 minutes, followed by one minute in an ice bath. The material was filtered through a 2.4 cm glass fiber filter and the filtrate was collected and read on a B and L spectrophotometer against a MeOH blank at 665 nm. Chlorophyll a concentration was then determined using an extinction coefficient of 77.6 abs/g chl a. The assay was carried out in dim light and extracts were kept in the dark until being read(< 10 minutes).

I.6 Lipids

Lipid analysis was carried out along the lines as described in Tornabene and Ben-Amotz(1983). Forty to fifty mg's of cells were harvested from culture by centrifuging at 4000 RPM for 20 minutes. The pellets were then resuspended in 10 mls of distilled water. These samples were than frozen until ready for analysis. Upon analysis, they were thawed and 25 mls of MeOH was added, followed by 12.5 mls of CHCl3. The samples were swirled a few times and allowed to sit overnight. The following day the samples were centrifuged at 1000 RPM for 10-15 minutes. The supernatent was transferred to 250 ml separatory funnels trying to transfer as little of the pellet as possible. A second extraction of the cell pellet was performed for 3 hours. This was then centrifuged for 15 minutes at 1000 RPM and transferred to the separatory funnels. The 10:5:4 MeOH:CHCl3:H2O solvent ratio was then adjusted to 10:10:9 by adding CHCl3 and H2O, and the mixtures were swirled. Phase separation was allowed to occur overnight. The lower CHCl3 fraction, containing lipid, was then drawn off into a round bottom flask. Additional CHCl3 was added to the separatory funnels to wash the partitioning phase. Two hours were allowed for phase separation and the CHCl3 phase was again drawn off and into the round bottom flask. The CHCl3 phases were then concentrated by rotoevap to about 1-2 mls. The concentrate was transferred to preweighed weighing flasks using CHCl3 and evaporated to dryness under a stream of Nitrogen. The flasks were stoppered immediately and transferred to a dessicator. The stoppers were then cocked slightly and the dessicator was aspirated for 15 minutes followed by vacuum pump for another 15 minutes. The vacuum was released by pumping in Nitrogen. At this point the weighing flasks were sealed and weighed. The difference between the dried weight and the original preweighed flask was due to lipid. Following weighing samples were resuspended in CHCl3 and stored in the freezer.

I.7 CHN Analysis

Analysis of carbon, hydrogen, and nitrogen was performed on a Model 240 Perkin-Elmer CHN Analyzer.

1.8 Cell Counts

A 1 ml sample is withrawn from the reactor and exactly 10 drops from a long neck Pasteur pipet of Lugols solution are added to immobilize the algae. These samples are then either read immediately or stored in the refrigerator until analyzed. Counts were performed using a Brightline Hemacytometer and a Officine Galileo Microscope at 520X magnification. Somewhere between 500 to 1000 cells were counted for each measurement. The major source of variability in the measurement comes from the slide preparation. For this reason at least four individual preps were performed, typically counting 200 cells per field. Each field contained 1 x 10^{-4} mls of sample - cell concentrations were corrected for the addition of the Lugols solution.

1.9 Protocol for Lipid Induction Studies

Batch Cultures:The batch lipid induction studies were carried out in 1 liter Roux bottles, innoculated with previously adapted batch grown cultures to a final cell density of around 50 mg/l. The initial nitrate concentration was dependent upon the experiment, the amount being calculated by consideration of the desired cell concentration when deficiency would begin(@ roughly 8 % nitrogen). All experiments were conducted using a 1 % CO_2/Air gas stream with gas bubblers as spargers. All cultures had 5 mM bicarbonate alkalinity and were grown in either MSW or MSW/2(half strength) media (see Table A.1). Culture bottles were illuminated from either one or two sides with 40 W Vitalite Fluorescent lamps. The intensity at the reactor surface was 250 E/m^2/s. The exposed surface was carefully controlled by keeping the cultures planar surface perpendicular to the light beam and by covering all other surfaces (sides and top) with black cloth or electrical tape. These precautions are necessary if calculations of energetic efficiency are to be carried out. Culture temperature fluctuated between 26 - 29 ^oC with the light/dark cycle.

Daily measurements of the culture included pH, OD, dry weight, and Chlorophyll a(in later experiments). Dry weight samples were taken by sampling directly from the culture bottle using a sterile pipet. Lipid, protein, carbohydrate, and CHN were sampled for after the onset of nitrogen deficiency.

Continuous Cultures: Nitrogen limited continuous cultures were carried out in 0.8 l working volume reactors as shown in figure A.2. Environmental conditions were identical to that used in the batch cultures; similarily the culture flasks were taped with black tape to allow an accurate calculation of the light energy recieved by the culture. Dilution was carried out only during the lighted portion of the day(shifted by 1/2 hour). Effluent was collected into darkened containers kept in an ice bath. All

samples were taken from these containers- the accuracy of which was verified by sampling both from the effluent and directly from the culture vessel for dry weight and chlorophyll a(see figure A.3). Measurements of pH, effluent volume and dry weight were recorded daily, at steady state, additional samples of chlorophyll a, CHN, and lipid were taken.

I.10 System Design for Species Competition Studies

The system design for the planned scale down experiments is shown diagrammatically in figure A.1. Dotted lines indicate transfer of signals to and from the Data Aquisition System (DAS). Only one reactor is shown for clarity - the system as designed operates with four reactors in parallel (for reactor design see Figure a.2). All four reactors are immersed in a temperature bath which is controlled by a combination of a cooling loop and immersion heater. Power to the immersion heater (200W) is from a power output board that is interfaced with the DAS. In this way temperature can be controlled by turning power to the heater on or off. Temperature is sensed using a type J thermocouple immersed within the bath. Cultures are sparged with a mixture of CO_2 and air, with the capability of diluting/substituting the air for N_2 or O_2. Gas Flow and composition are regulated by Mass Flow Contollers (Tylan Corp.). Effluent gas from each culture is measured by a mass flow meter (Tylan Corp.). Following this the gas stream is split by adjustable rotameters such that roughly 2/3 of it goes to an Infrared CO_2 Analyzer (IRGA) and the remainder to a Paramagnetic Oxygen Analyzer (POA). The concentration ranges for both analyzers must be calibrated every 12 hours.

Culture pH is monitored by routing probe signals through a high impedence multiplexer(custom design) and these signals are then sent to the DAS. Probes are calibrated, using a BASIC program called CALPROBE, which operates very much like microprocessor based pH meters. In experiments conducted over a period of one month, recalibration of the pH probes on a 2-3 day interval was adequate to maintain accuracy. The data aquisition system is a System 520 from Kiethley with the following features: 16 single ended low level A/D, 32 single ended high level A/D with cold junction compensation for thermocouple use, 5 channels of 0-5 VD D/A, 32 channels of Digital I/O, and 16 channels of switched controlled 120 VAC output.

I.11 Protocols for Mixed Cultures Experiments:

Fluctating Temperature and Oxygen Experiment: Four cultures(131-134) were inoculated at roughly equivalent cell densities of Chlorella B.L., Cyclotella, and Chaetoceros (Hawaiin strain) to give a combined density of 100 ppm. Dry weight and cell counts were followed for a period of 40 days. A summary of the experimental conditions is given in Table A.2. All cultures were expoosed to a 14/10 hour light/dark cycle. For cultures 132-134, the cultures were sparged with pure oxygen for eight hours during the "afternoon". Cultures 133 and 134 were subjected to a temperature cycle that varied between 20C during the dark period and 32 C

during the light period. The time constant for these temperature changes was roughly one hour.

The light intensity incident on culture 134 was increased by shining a 150 W flood lamp mounted approximately 8 inches from the culture. A one inch thick water filter was placed in between for heat absorption. All cultures recieved 550 $E/m^2/s$ on one side from VHO Sylvania "Daylight" flourescent lamps. The cultures were diluted during the illuminated portion of the day(shifted 1/2 hour) at a rate of .67 day^{-1}. The media used was MSW/2 with 5mM alkalinity as bicarbonate. Dry weight samples, as well as pH and OD's were taken daily and cell counts every other day.

pH Fluctuation Experiment: In this experiment the temperature was controlled between 28-29.5 C, the light intensity was 550 E/m^2s and air levels of O_2 were used for sparging. Equal cell densities of Chlorella, Chaetoceros, Ankistrodesmus, and Cyclotella were innoculated into each reactor(221-224) at a total concentration of ca. 100 ppm. Sampling schedules were similar as to that described for the fluctuating temperature and oxygen experiment. The control of a pH setpoint was better than .05 pH units. pH was measured with either a "pencil" thin combination electrode (Fisher) or a larger fermentation grade electrode from Phoenix Electrode. A special high impediance op amp circuit was constructed similar in design to that described by Terry and Hock (1985) which has the capability of recieving ten input signals. pH control was either by alkalinity or pCO_2 adjustments. In the case of alkalinity, either 2N Na_2CO_3 or 1N HCl were dosed to the culture by peristaltic pumps(Masterflex). In the case of CO_2, changes in the gas concentration were made by a 0-100sccm mass flow controller(Tylan Corp.). Specifically a PD controller was used to adjust voltage signals to the desired setpoint. All cultures utilizing alkalinity control were sparged with .35% CO_2/air mixture. The media fed to the low pH (#221) and fluctuating alkalinity (#222) cultures had an alkalinity of 1mM $NaHCO_3$. The high pH (#223) and fluctuating pCO_2 (#224) cultures were fed MSW/2 with 10 mM alkalinity.

Dilution Rate and Sparger Design: Methodology and experimental conditions were similar to the previously discussed experiments except for the following differences. Cultures 250-252 were run at different dilution rates ranging from .33 to 1.22 per day. Culture 253 was operated at a dilution rate of .73 and was sparged with a bubbler rather than the commonly used(as in all previous experiments) glass fritted cylinder (course grade). A summary of conditions (with standrad deviations) is given in Table A.4.

Table A.4. Summary of Conditions for Dilution Rate Experiment

Culture	O_2	Temperature	I(E/ms)	pH	u(day^{-1})
250	Air	28-29.5	550	7.63(.10)	1.23(.05)
251	Air	28-29.5	550	7.65(.14)	0.83(.01)
252	Air	28-29.5	550	7.82(.14)	0.33(.03)
253	Air	28-29.5	550	7.78(.13)	0.73(.02)

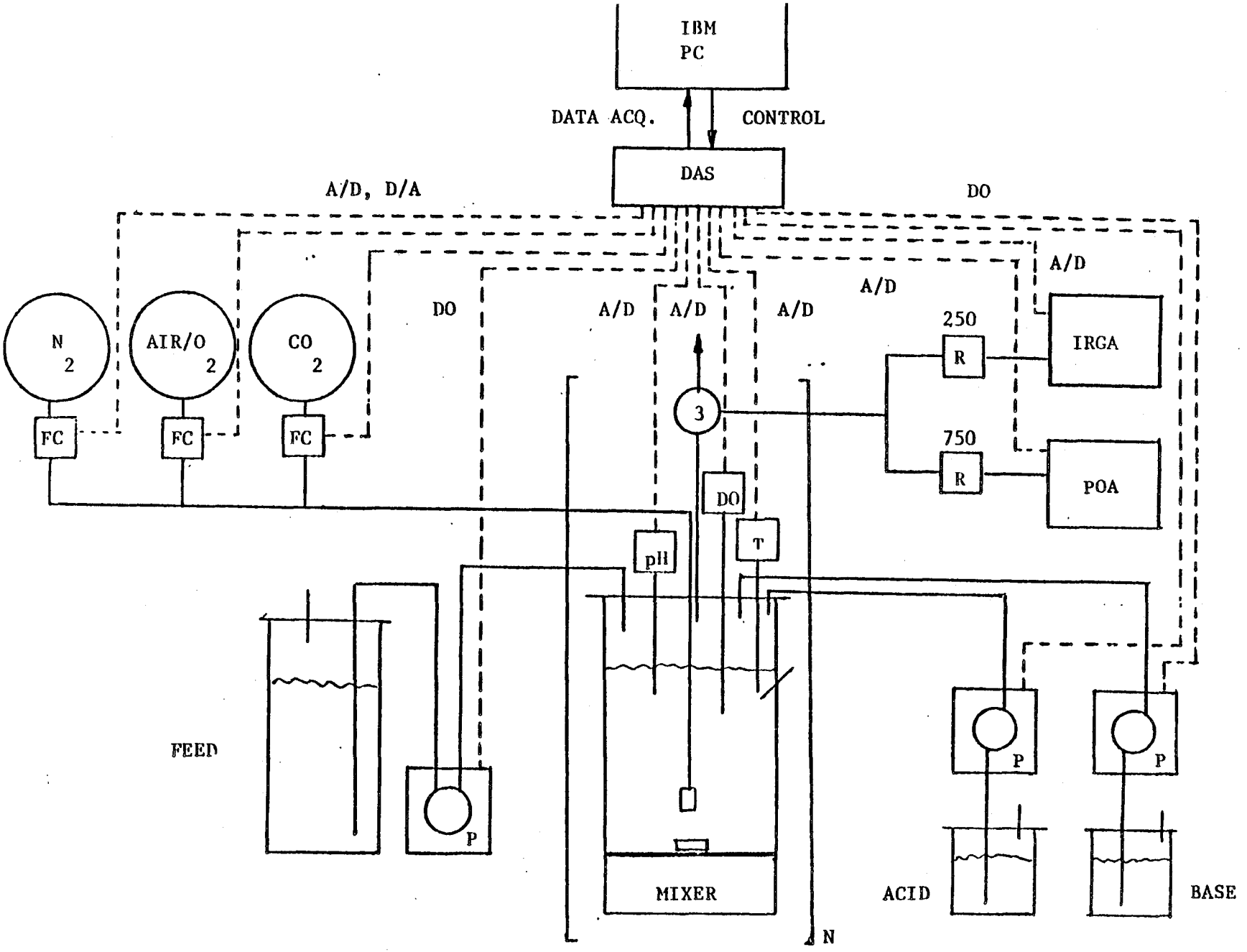

FIGURE A.1 System Layout for Scale Down Experiments.

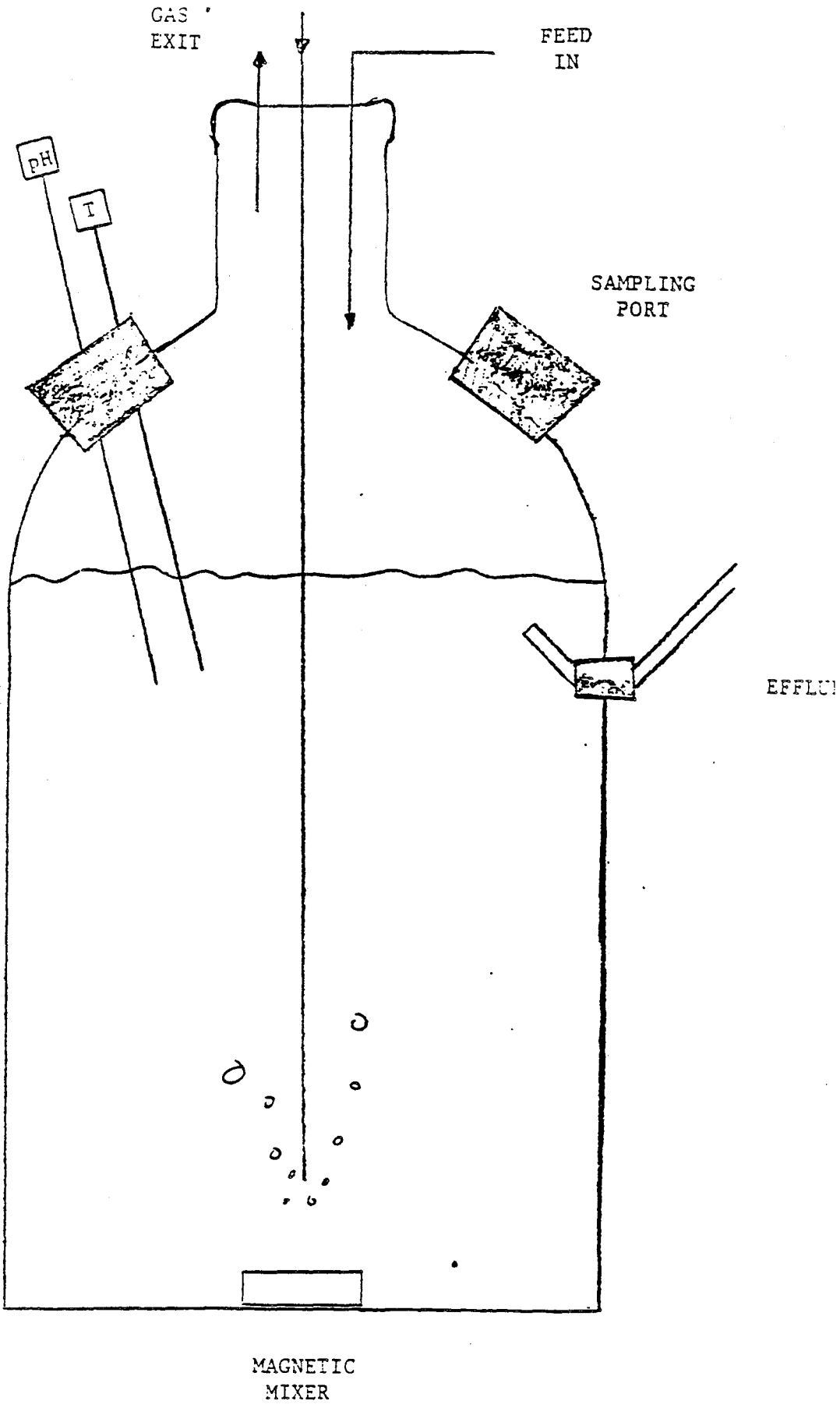

FIGURE A.2 Continuous Culture Roux Bottle