INFORMATION TO USERS

This dissertation was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

University Microfilms

300 North Zeeb Road
Ann Arbor, Michigan 48106

A Xerox Education Company

LIGHT-LIMITED GROWTH AND CELLULAR COMPOSITION OF *DUNALIELLA TERTIOLECTA* CULTURED WITH AMMONIA AND NITRATE

by

BRUCE SHARFSTEIN

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1975

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

October 1, 1975
Date

Chairman of Examining Committee
Professor O. Roels, City College

January 7, 1976
Date

Executive Officer
Professor Louis G. Moriber

Prof. T. Malone — City College (Institution)

Prof. G. Posner — City College (Institution)

Prof. J. Rachlin — Lehman College (Institution)

Prof. E. Carpenter — SUNY, Stony Brook (Institution)

Prof. J. Ryther — Woods Hole Oceanographic Institution (Institution)
(Served as reader)

_____ — _____ (Institution)

_____ — _____ (Institution)

The City University of New York

ABSTRACT

LIGHT-LIMITED GROWTH AND CELLULAR COMPOSITION OF *DUNALIELLA TERTIOLECTA* CULTURED WITH AMMONIA AND NITRATE

by

Bruce Sharfstein

Adviser: Dr. Oswald A. Roels

The growth rates, and cellular concentrations of ATP, particulate "carbon", particulate "nitrogen", chlorophyll a, protein, carbohydrate and lipid, were measured for *Dunaliella tertiolecta* grown in light-limited continuous culture in nitrate and ammonia medium.

Cells cultured in ammonia medium had consistently higher hourly growth rates at all levels of light intensity, the difference increasing from 22% at the highest intensity to 62% at the lowest intensity. Growth in ammonia medium was shown to be dependent on I_{abs}/unit chlorophyll, the response of cellular chlorophyll a to changing light intensity, and the energy demands of division. At the highest intensity studied, nitrate-grown cells showed a similar response to light, while at lower intensities, light-limited growth was

primarily mediated by the light-dependent component of nitrate assimilation. A theoretical model of light-limited growth was developed based on these results.

Nitrate- and ammonia-grown cells showed similar levels of intracellular chlorophyll a, lipid, and ATP. Nitrate-grown cells showed a tendency to synthesize 40% more protein/division than ammonia-grown cells. It was suggested that it is this additional synthesis, rather than the energy differences in uptake and assimilation of nitrate and ammonia, which account for the observed differences in growth rate at light intensities of 0.018 ly/min where the light-dependent component of nitrate assimilation is saturated. Further, it was suggested that this additional protein synthesis might be related to the synthetic machinery and enzymes involved in the reduction of nitrate to ammonia by the cell.

A consideration of potential strategies for the operation of an effluent mariculture system was developed, based on the experimental results.

ACKNOWLEDGEMENTS

I wish to thank Dr. O. A. Roels, Dr. Tom Malone and Dr. Gerald Posner for their guidance and encouragement during the preparation of this thesis. I also wish to acknowledge the financial support provided by the Rockefeller Foundation and by a Jessie Smith Noyes Foundation fellowship. In addition, I would like to acknowledge the technical assistance rendered by Miss Kiki Nelson, Mrs. Nadia Kostyk, Dr. Susan Williams and Mr. Richard Devine.

TABLE OF CONTENTS

TABLES

FIGURES

1 INTRODUCTION

1.1 Objectives

The two major objectives of this work are to establish the effects of ammonia and nitrate as sole nitrogen source on the growth rate and cellular composition of *Dunaliella tertiolecta* in continuous culture under varying conditions of light intensity and photoperiod, and to apply this information to a consideration of potential strategies for the operation of an effluent mariculture system.

1.2 Rationale

In June of 1970, work was started on the development of a biological means of tertiary sewage treatment to remove dissolved inorganic nitrogen and phosphate from secondarily treated sewage effluent prior to its discharge into the New York Estuary. Nutrients would be removed from secondarily treated sewage by phytoplankton, which, in turn, would be filtered from the effluent by shellfish. Shellfish excretory products, and any remaining nutrients from the secondary effluent would then be removed by racks of agar and carrageenan producing fixed algae. By reclaiming the inorganic nitrogen and phosphate in the secondary effluent through a managed system, a previously unexploited nitrogen source might be converted to a usable food source, and the input of nutrients to the estuary reduced, thereby eliminating a

potential cause of eutrophication.

In March 1972, the project was moved to the Tallman Island Pollution Control Plant, situated on the East River, in Queens County, New York City, and an intensive study of the nutrient composition of the secondary effluent and of the nearby estuarine receiving waters was initiated. Results of this study are summarized in table 1-a. Ammonia is the major inorganic nitrogen source in both the secondarily treated sewage effluent and in the East River Water. Consequently, ammonia concentrations in the mixture of secondary sewage effluent and East River Water used as a substrate for the growth of phytoplankton cultures in our effluent mariculture project at the Tallman Island Pollution Control Plant are considerably higher than the combined nitrate and nitrite components of this medium (Sharfstein, Roels, Garside, Williams, Kostyk 1972). Further, since Sawyer and McCarty (1967) report that in secondary sewage effluents with an inorganic nitrogen composition similar to that found at Tallman Island, the non-refractory organic nitrogen fraction is approximately 1/100 of the ammonia concentration, ammonia is probably the major nitrogen source available at Tallman Island.

New York City is currently experimenting with a secondary sewage treatment process that utilizes oxygen rather than air to induce bacterial growth and sludge formation. The use of ozone rather than chlorine, as the final step of treatment, has also been considered. Both of these processes can accom-

TABLE 1-a

NUTRIENT CONCENTRATIONS IN TALLMAN ISLAND SECONDARILY TREATED SEWAGE EFFLUENT AND IN THE NEARBY EAST RIVER

Secondary Effluent

µg-atoms/l.

nutrient	mean[1]	range[2]
nitrate	9	1-30
nitrite	2.0	1-15
ammonia	1,160	46-1670
orthophosphate	129	28-230
silicate	81	6-220

East River Water

µg-atoms/l.

nutrient	mean[1]	range[2]
nitrate	17	8-40
nitrite	4	1-15
ammonia	83	20-440
orthophosphate	26	21-70
silicate	27	2-75

[1]Mean of nutrient values is the arithmetic average of all values recorded for a nutrient over a particular sampling period.

[2]Range of nutrient values represents the single highest, and single lowest value recorded over a particular sampling period.

plish considerable nitrification (Wilde, Sawyer, and McCarty 1967), producing an effluent rich in nitrate and nitrite and low in ammonia. Since these modifications of the secondary sewage treatment process are under consideration, it is advisable to determine whether or not such nitrification is desirable from the point of view of effluent mariculture.

Lui and Roels (1972), Bongers (1956), Syrett (1956, 1962), and Eppley et al. (1971) have noted various changes in μ max and cellular composition when phytoplankton are grown on ammonia as compared to nitrate. Since effluent mariculture will probably be a light-limited process, maximum attainable growth rate will be a major determining factor in the overall efficiency of the system, since phytoplankton growth rate is probably the step which will govern the rate at which biological tertiary treatment can proceed.

Preliminary investigations of shellfish nutrition by Castell and Trider (1974) have shown that dietary composition affects the growth rate and physiological state of *Crassostrea virginica*. Thus changes in phytoplankton cellular composition induced by the nature of the available nitrogen source may ultimately influence the growth of shellfish in an effluent mariculture system.

Incident daily irradiance (400-500 and 600-700 nm) at the Tallman Island Pollution Control Plant varies seasonally over the range 131-71 ly/day.

Variations in light intensity and photoperiod have been shown to affect nitrogen uptake (Eppley et al., 1971),

photosynthetic rates and chlorophyll a content (Harris and Lott 1973), division rates (Hobson 1974), ATP concentration (Bornefeld and Simonis 1974), and lipid content (Constantopoulos and Block 1967) in a variety of phytoplankton species. A study of the effects of changing light energy flux on phytoplankton grown in ammonia and nitrate-rich medium is essential since seasonal changes in phytoplankton growth rate and efficiency of nitrogen uptake may have considerable impact on the ability of an effluent mariculture system to process secondary effluent during certain times of the year, while changes in phytoplankton cellular composition will influence shellfish growth and physiological state in the system.

Although laboratory conditions can never adequately mimic conditions in a natural system, if the trends in growth rate and cellular composition shown by *Dunaliella tertiolecta* in response to available nitrogen substrate, and changing light energy can be quantified, they may be used as inputs to a model predictive of the problems and potentials of an effluent mariculture system operating in New York City.

1.3 Scientific Background

The effects of ammonia or nitrate as nitrogen sources on phytoplankton growth and cellular composition probably have their basis in the energy requirements and specific biochemical pathways involved in the assimilation of these two forms of inorganic nitrogen.

Uptake of nitrate is followed by a series of enzyme mediated reducing reactions. In the first of these, nitrate is reduced to nitrite by nitrate reductase. Nitrite is further reduced to another intermediate form of nitrogen by the enzyme nitrite reductase (Eppley and Rogers 1970), and this intermediate is ultimately reduced to ammonia through an undetermined number of additional reducing reactions (Lui and Roels 1972). The ammonia thus produced is then converted to amino nitrogen by one of a number of amino acid dehydrogenases (Batt and Brown 1974). These reductive pathways are inactive in cells grown on ammonia medium (Morris and Syrett 1963) and uptake of ammonia is followed by direct amination. Syrett (1962) has shown that the energy requirement for the conversion of nitrate to ammonia is 74 Kcal/mole of NO_3 reduced while the energy required for the subsequent amination step is 10 Kcal. Thus in terms of "nitrogen" assimilation, growth on an ammonia substrate should provide the cell with a net energy saving of 74 Kcal for each mole of ammonium ion converted to amino nitrogen.

Possibly due to the toxicity of ammonia to algal chloroplasts (Winkenbach, Grant and Bidwell 1972), cells grown in nitrate medium accumulate intracellular nitrate in the order of 40 m moles/liter cell volume while ammonia grown cells show little intracellular storage of inorganic nitrogen (10 m moles/liter cell volume) (Eppley and Rogers 1970), although Lui and Roels (1972) have noted an intracellular pool of soluble amide nitrogen in the diatom *Biddulphia aurita*.

As a result, growth rate in N-limited nitrate culture is related to internal nitrogen/unit population, while in N-limited ammonia culture, growth rate is related to the external "nitrogen" concentration (Caperon and Meyer 1972).

Assimilation of nitrate also shows a direct dependency on light, for both the nitrate and nitrite reducing stages of the process (Eppley and Coatsworth 1968), although in fact there may be two independent mechanisms for nitrate reduction, one located within and one outside of the chloroplast (Grant 1968). In *Dunaliella tertiolecta*, the light dependent component of nitrate assimilation reaches saturation at 1000 foot candles (0.018 ly/min), while carbon fixation does not become saturated until an intensity of 1800 foot candles is reached (Grant 1967). Interestingly, despite the fact that light affects the nitrate and nitrite reducing stages of nitrate assimilation, the rate limiting step in the assimilation of nitrate in the light is the formation of cellular nitrogen (amination) (Grant and Turner 1969). A possible explanation for this effect is offered by the results of Batt and Brown (1974). They found lowered amino acid dehydrogenase activity (GDH, AspDH and AlaDH) in nitrate cultured cells of the blue-green alga *Anabaena cylindrica* attributable to cofactor competition with the other reductive processes involved in nitrate and nitrite assimilation.

The sum of the biochemical differences noted between the assimilation of nitrate and ammonia may be reflected by

differences in cellular composition or growth rate in cells cultured in ammonia or nitrate medium. As already noted, Lui and Roels (1972) and Syrett (1956, 1962) have shown that ammonia-grown cells have a higher amide and amino nitrogen content than cells of the same species grown on nitrate. Based on cell carbon data, Caperon and Meyer (1972) suggested that when *Dunaliella tertiolecta* is grown on ammonia, "Cell division takes place with successively smaller amounts of non-essential carbohydrates and lipids as the supply rate of ammonium ion increases." Eppley et al. (1971), working with logarithmic phase batch cultures of natural phytoplankton populations, found that cultures enriched with ammonia had higher particulate carbon, particulate "nitrogen", ATP, protein and lipid contents per cell than cultures enriched with nitrate. Carbohydrate content per cell was slightly lower for ammonia than for nitrate-enriched cultures; chlorophyll a content per cell was also lower in the ammonia-enriched cultures.

Bongers (1956) showed that algal growth under conditions of light limitation was better with ammonia than with nitrate, while when light was not limiting, algal growth was the same with either nitrate or ammonia as the substrate.

Similarly, Paasche (1971) found that when cells of *Dunaliella tertiolecta* were grown with short photoperiods of bright light, the use of ammonia rather than nitrate as the nitrogen source led to a 30% reduction in the time required to double cell matter, and a 10% reduction in the time

necessary to complete a cycle of division.

On the other hand, under conditions of nitrogen limitation, Caperon and Meyers (1972) found that maximum specific growth rate in batch cultures was the same whether ammonia or nitrate was the nutrient substrate, while Eppley et al. (1971) found that in ammonia and nitrate enriched natural phytoplankton populations grown at 20% of full sunlight, division rates for eight species of diatoms were slightly lower in ammonia than in nitrate cultures.

1.4 Experimental Design

Continuous culture was chosen as the best technique for studying the relationship between light energy and growth on ammonia or nitrate. "Steady state" continuous cultures are not subject to the constant fluctuations in growth conditions characteristic of batch cultures, and are most representative of the type of phytoplankton culturing best suited to conditions in an effluent mariculture system. Temperature, salinity, and media composition were representative of mean conditions in our greenhouse-enclosed phytoplankton cultures at the Tallman Island Pollution Control Plant, Queens County, New York.

It is difficult to simulate natural conditions of illumination in the laboratory. In addition to characteristic seasonal changes in incident intensity, the quantity and spectral distribution of natural light changes during the day in response to changes in solar altitude, and from day

to day in response to changing sky conditions. Natural incident light intensities are generally an order of magnitude higher than those obtainable by artificial illumination with fluorescent lamps. Also, the natural photoperiod is not fixed seasonally, but changes continuously throughout the year. The experimental light intensities were therefore set in three approximately equal steps starting with the highest intensity obtainable in the culture chamber used.

Experimental photoperiods were chosen to correspond to the summer maximum, the winter minimum, and the annual mean, 15, 9, and 12 hours, respectively.

Ammonia and nitrate continuous cultures were run simultaneously at each of the light intensity-photoperiod combinations studied. The first phase of each run was the adjustment of culture flow rates to match the maximum attainable growth rate possible under the light and nutrient conditions used. Two replicate runs at each light intensity-photoperiod combination were done, primarily to confirm that steady state at the maximum flow rate had been attained.

Although μ max was established at all three light intensity-photoperiod combinations, only the high light-long photoperiod, and medium light-medium photoperiod cultures were sampled for cellular composition analyses, since maximum flow rates in the low light-short photoperiod nitrate cultures were so small (17.8%/day) that periodic removal of samples of sufficient size for the required analytical work would have seriously altered steady state conditions.

Following the establishment of maximum attainable flow rate in the high light-long photoperiod, and medium light-medium photoperiod, continuous cultures, cultures were allowed to acclimatize to steady state conditions for 2-3 days. A 52-hour sampling period was then initiated. Samples were taken every four hours during this period, encompassing two complete light:dark cycles. Samples from these continuous cultures were analyzed for intracellular carbohydrate, protein, chlorophyll a and ATP; total particulate carbon and nitrogen; and residual medium nitrate, nitrite, ammonia and orthophosphates. Total cell-density, numbers of dividing cells, and pH were also monitored. Since each sampling run was replicated, data for a total of four complete light:dark cycles are available for each nitrogen substrate.

Subsequently, it was decided to measure intracellular lipid to obtain a more complete picture of phytoplankton cellular composition, and nutritional value for shellfish. A separate set of high light-long photoperiod continuous cultures, and a separate set of medium light-medium photoperiod continuous cultures were run to produce cells for this purpose. In addition, intracellular carbohydrate analyses were run on cells from these cultures to determine whether they reproduced the cultures used for the analysis of all other parameters.

2 MATERIALS AND METHODS

2.1 *Dunaliella tertiolecta*

Dunaliella tertiolecta is a pear-shaped, marine-flagellated chlorophyte, 12-14μ in length. It is a euryhaline tide pool species, and has proved to be one of the most successful species under culture in our effluent mariculture system at the Tallman Island Pollution Control Plant, Queens County, New York City.

The cultures of *Dunaliella tertiolecta* used in these experiments were originally obtained from Mrs. Helen Stanley of the Woods Hole Oceanographic Institute.

Two axenic stock lines, one in nitrate medium, and one in ammonia medium have been maintained in this laboratory for the past two years.

2.2 Culture Conditions

All cultures were grown in a Sherer Controlled Environment Chamber. The temperature controller was set at 22°C but regulation was poor and temperatures fluctuated from 20-24°C on the air input side of the chamber and 21-25°C on the exhaust side of the chamber.

Illumination was provided by General Electric, cool white 40-watt fluorescent tubes. Two complete sets of fluorescent tubes were used. These were alternated with each

replicate run to eliminate differences in light intensity caused by deterioration of the bulbs with use.

Three light intensity, photoperiod combinations were used (Table 2-a). The difference between high and medium light intensities was .011 ly/min while the difference between medium and low light intensities was .0067 ly/min. Uniformly spaced intervals of light intensity would have been preferred but could not be attained since intensity regulation could only be accomplished by turning on or off various combinations of bulbs in sets of two.

TABLE 2-a

LIGHT INTENSITY, PHOTOPERIOD COMBINATIONS

Photoperiod	Light Intensity ly/min
15 hours	0.022
12 hours	0.011
09 hours	0.0043

2.3 Media Compositions and Preparation

Hudson Canyon Surface Sea Water was used for preparation of all stock and experimental media. Water was collected aboard the R.V. ATLANTIC TWIN and the R.V. COMMONWEALTH in the following manner:

Beginning at a distance of 100 miles south-east of New York City, surface water samples for phosphate determination were taken at intervals of 10 miles. When the phosphate concentration of the sea water was less than the lowest standard distinguishable by eye (less than 1μ eq PO_4/l), water collection was started. Care was taken that no ship effluents were discharged during collection, and that the collecting hose was kept out of contact with the ship's hull. Water was collected with an epoxy-headed pump and nylon hose. Pump and hose were rinsed for 10 minutes before collection was started. Water was pumped into 15-gallon polyethylene drums which had been rinsed three times with tap water, and three times with the sea water. Phosphate concentration was checked at the beginning of pumping, midway through the pumping, and at the end of collection. Upon return to the laboratory, water was transferred into pre-rinsed, 50-gallon polyethylene drums and stored at 5°C. Water was analyzed at this time for inorganic nitrate, nitrite, ammonia and phosphate (Table 2-b).

For use in media, Hudson Canyon Surface Sea Water was diluted to a salinity of 20°/00 with distilled water. This salinity was chosen to correspond to the mean salinity of

TABLE 2-b

COMPOSITION OF HUDSON CANYON SURFACE SEA WATER

Nutrient	µg-atoms/l.
nitrate	0.76
nitrite	0.09
ammonia	0.24
total inorganic nitrogen	1.09
phosphate	0.11

the secondary sewage effluent and East River water mixture used to culture phytoplankton at our Effluent Mariculture Field Station in Queens County, New York. Similarly, the nitrogen content of the media was adjusted to 180 µeq/l of either nitrate or ammonia which corresponds to the mean total inorganic nitrogen content of our sewage-based phytoplankton growth medium. Phosphate content of the medium was adjusted to 22.5 µg-atoms P/l, giving a molar nitrogen to phosphate ratio of 8:1 and establishing nitrogen as the potentially limiting macronutrient. Trace metals plus ferric chloride and EDTA, vitamin B_{12} and thiamine were added in excess approximately corresponding to Guillard's F/3 medium (R.R. Guillard and Ryther 1962). The complete medium composition is shown in table 2-c.

To avoid precipitation or complexing of nutrients during autoclaving in sea water, the following media making technique was used. The Millipore filtered Hudson Canyon Surface Sea Water: distilled water mix was autoclaved and cooled. Nutrient stocks were made up aseptically in one-liter quantities and proportioned into parafilm-capped vials or screw-capped culture tubes in quantities required to make either one liter of medium (for general use) or nine liters of medium (for use in continuous culture media reservoirs). Proportioned nutrient stocks were stored frozen. Prior to use, they were thawed, autoclaved, and added to the presterilized and cooled saline water.

Ammonia nutrient stocks were adjusted to a pH of 3.5

TABLE 2-c

MEDIA COMPOSITION

Nutrient	Quantity/liter of medium
nitrate	180μ eq
or	
ammonia	180μ eq
phosphate	22.5μ g-atoms
vitamin B_{12}	0.330μ g
thiamine	62.5μ g
ferric chloride	9.4 mg
EDTA	2.72 mg
$CuSO_4 \cdot 5H_2O$	6.12μ g
$ZnSO_4 \cdot 7H_2O$	13.75μ g
$CoCl_2 \cdot 6H_2O$	6.25μ g
$MnCl_2 \cdot 4H_2O$	112.5μ g
$Na_2MoO_4 \cdot 2H_2O$	3.94μ g

with concentrated HCl before autoclaving to insure the stability of the ammonium ion during sterilization.

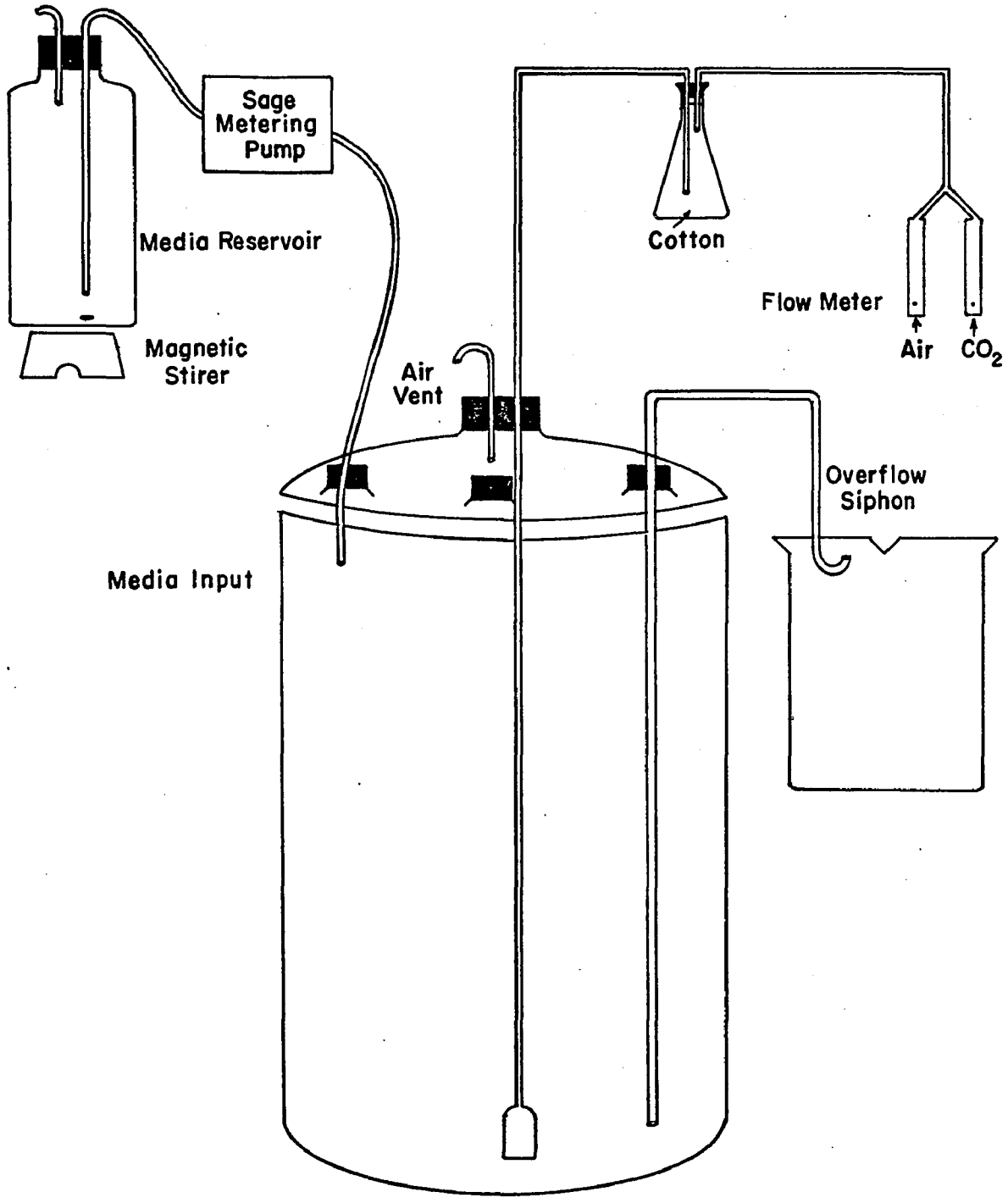
2.4 The Continuous Culture Apparatus

The continuous culture vessels were constructed from four-liter glass bottles with clamp on glass lids. Lids were equipped with a central 1.5"-diameter collared port, and three peripheral 1"-diameter collared ports.

Culture medium, stored in nine-liter pyrex solution bottles, was introduced to the continuous cultures through one of the peripheral ports via a Sage 375 A variable speed metering pump. Level control and culture overflow was provided by a siphon and "U" tube. Air plus 1.5% technical grade CO_2 supply for agitation and pH regulation, was metered through two Gilmont flow meters, passed through a saturated solution of $ZnCl_2$ to strip any gas-borne ammonia contamination (Caperon and Meyer 1972), and through a sterile cotton filter to limit air-borne bacterial contamination. A "U"-shaped vent tube and clamp allowed air escape when open, and a positive pressure means of sampling through the overflow siphon when closed (Figure 2.1).

The Continuous Culture Vessels were installed in a Sherer Environmental chamber to provide regulation of temperature and light regime. Since it could not be verified that light and temperature conditions were identical throughout the chamber, the position of the nitrate and ammonia vessels was alternated between replicate runs.

Figure 2.1 Continuous culture apparatus


Sage
Metering
Pump
Media Reservoir
Magnetic
Stirer
Cotton
Flow Meter
Air
CO_2
Air
Vent
Overflow
Siphon
Media Input

2.5 Inoculation of Continuous Culture and Establishment of Steady State

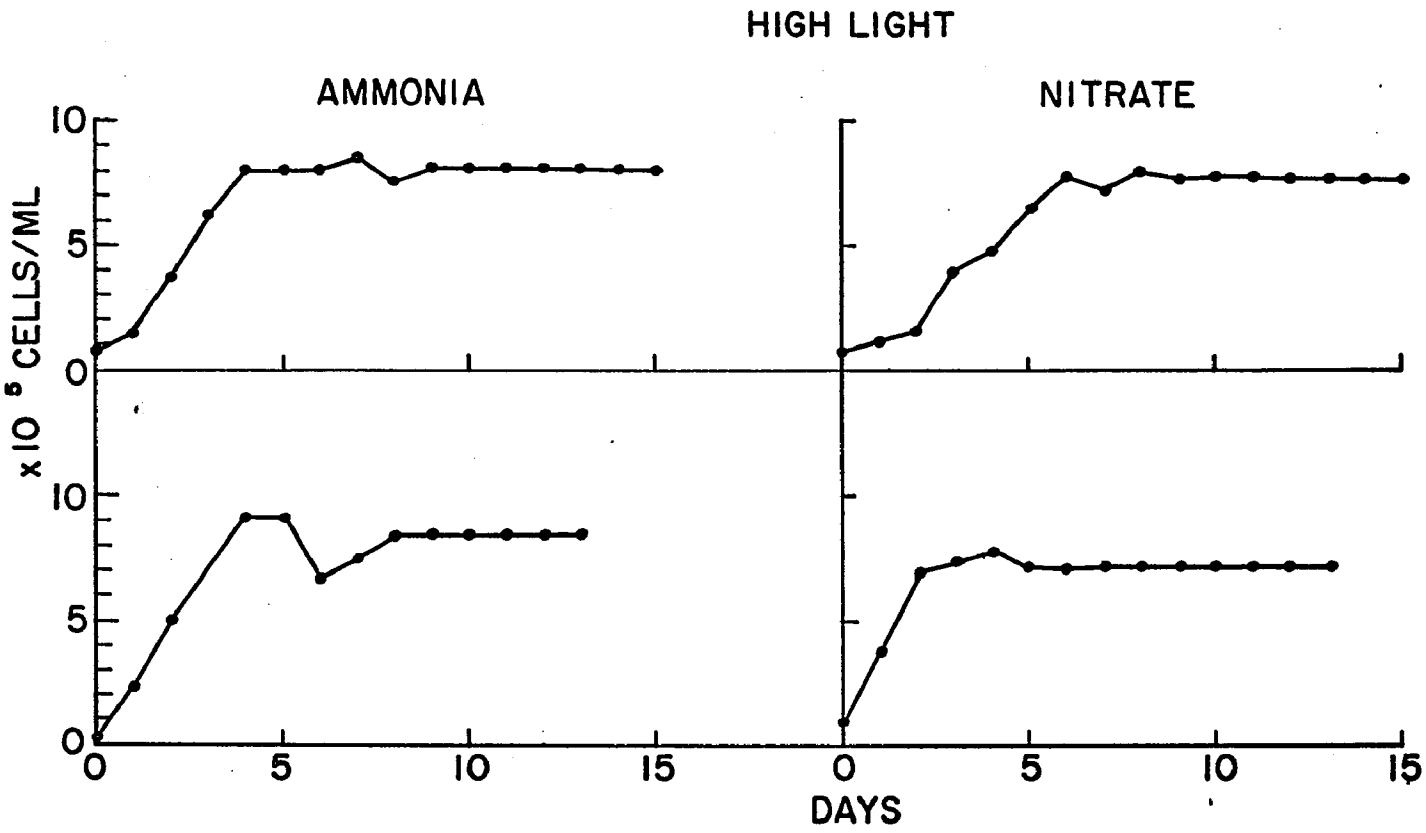
Prior to inoculation, the culture vessels were dry, sterilized and filled with 3,500 mls of sterile growth medium under aseptic conditions. One-half liter of inoculum, grown in one-liter aspirator bottles, was introduced via a length of sterile tubing through the air vent.

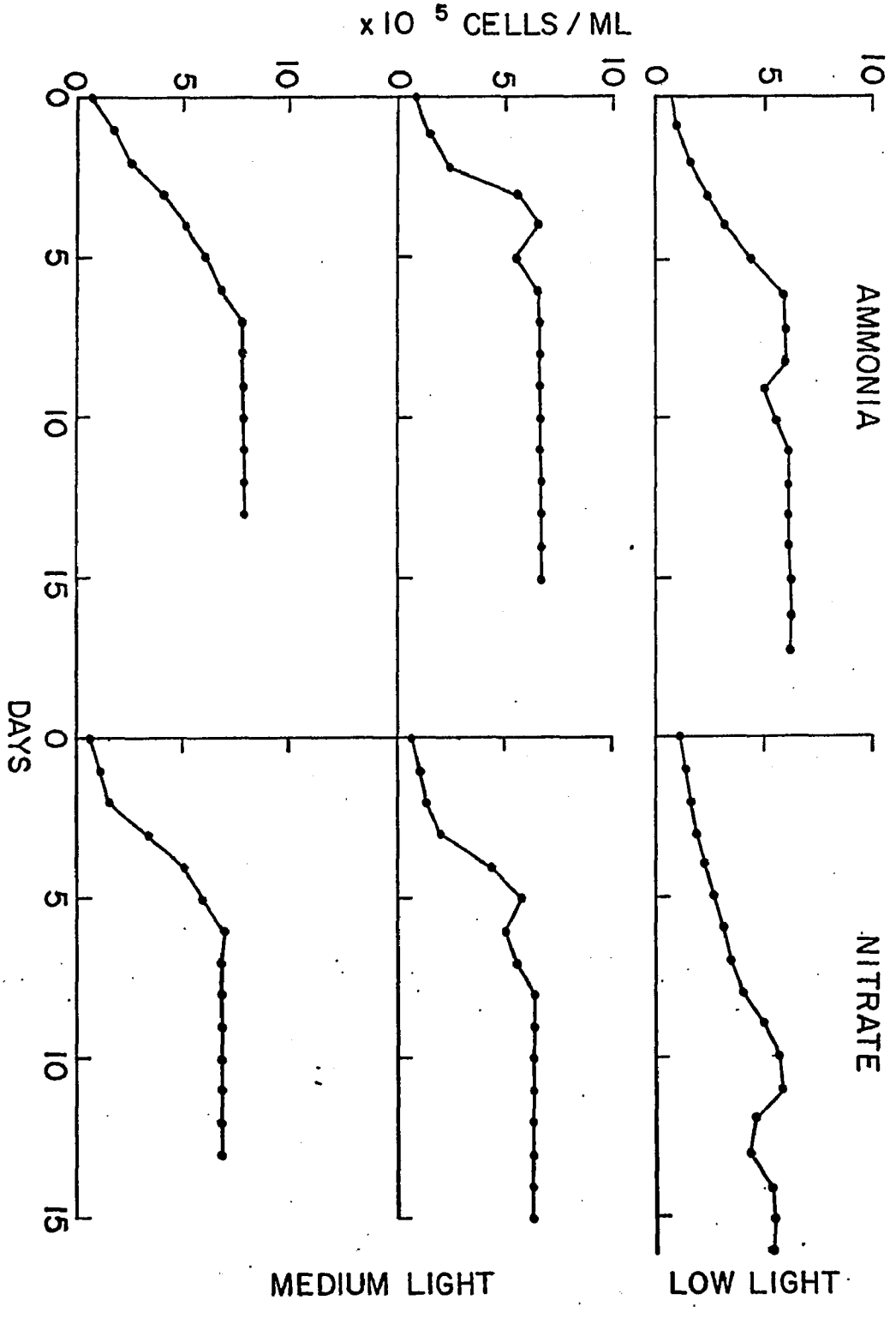
After inoculation, the cultures were incubated until cell densities neared 6-8 x 10^5 cells/ml. Continuous flow was then begun and cell densities were measured twice daily. Flow rate was adjusted daily in response to the trend shown by the three preceding cell counts. By initially increasing or decreasing the flow rates in large increments and then reducing the adjustment increments, steady state at the maximum attainable flow rate for the conditions of light intensity, photoperiod, temperature and nutrient regime being used was obtained (Figure 2.2).

Steady state was considered to have been attained when "changes in cell concentration were relatively small (less than 1 x 10^4 cells/ml) and without trend for three or four days" (Caperon and Meyer 1972) and at this point, sampling was initiated. It should be noted that I modified the above definition of steady state by limiting it to the cell density at a given hour (usually 09:00) each day since under conditions of alternating light:dark cycles there is a diurnal fluctuation in cell density of approximately 2 x 10^5 cells/ml. This is probably related to the development of synchrony in cell

Figure 2.2 Steady state conditions

Cell density at 09:00 vs time in days.


HIGH LIGHT
AMMONIA
NITRATE
x10 5 CELLS/ML
10
5
0
10
5
0
0
5
10
15
0
5
10
15
DAYS


x 10 5 CELLS / ML
0
5
10
0
5
10
0
5
10
AMMONIA
NITRATE
DAYS
0
5
10
15
0
5
10
15
MEDIUM LIGHT
LOW LIGHT

division.

To check the stability of the "steady states" attained, Williams (1971) R_x and r_x indexes were used.

1) $R_x = \frac{\text{mean of the first three cell counts}}{\text{mean of the last three cell counts}}$

if: $R_x = 1$, culture conditions are stable

$R_x > 1$, parameter decreases with time

$R_x < 1$, parameter increases with time

2) $r_x = 100 - (100 \cdot R_x \cdot (T_2/t))$ Where: T_2 = doubling time interval studied

r_x measures the average percent change/generation. Table 2-d presents R_x and r_x values for the experimental cultures, using daily values of cell density at 06:00 hours.

As a standard of comparison for the R_x index, if flow rate exceeds culture growth rate by 2%/day, R_x after nine days would equal 0.89. If flow rate is 2%/day less than culture growth rate, R_x after nine days would equal 1.13. Thus all the experimental cultures were in very stable steady state.

2.6 Sterility Checks

Since the behavior of individual cells of *Dunaliella tertiolecta* was being studied by treating the entire cell population as a single organism, it was necessary to maintain axenic cultures at all times. Sterile or aseptic technique was used throughout. In addition, stock cultures were plated out once a month to check for bacterial contamination, and continuous cultures were plated out on a daily basis when an

TABLE 2-d

ANALYSIS OF THE STABILITY OF STEADY STATES

Culture	%/day turnover	9 days[1] - R_x	r_x
high light ammonia 1	83.5	1.00	0.0%
high light ammonia 2	83.5	1.00	0.0%
high light nitrate 1	69.8	1.00	0.0%
high light nitrate 2	67.7	1.02	0.42%
medium light ammonia 1	67.1	1.00	0.0%
medium light ammonia 2	65.7	1.01	0.19%
medium light nitrate 1	49.2	0.99	0.29%
medium light nitrate 2	47.5	1.00	0.0%
low light ammonia	47.0	1.00	0.0%
low light nitrate	17.8	0.98	0.25%

[1]Nine days is the shortest time for which steady state was maintained in any of the continuous cultures.

experiment was in progress.

To check for contamination by both marine and terrestrial, autotrophic and heterotrophic bacteria, culture samples were streaked onto saline, and fresh water based 1% peptone agar with added inorganic nitrate or ammonia and phosphate, trace metals mix, B_{12} and thiamine. Rather than mixing all components of the bacterial growth medium prior to autoclaving, nutrient stocks, saline or fresh water, and agar dissolved in a small volume of water were autoclaved separately, and combined under aseptic conditions. This technique is reported to give better bacterial growth and to be suitable to a wider range of species (K. C. Haines personal communication).

Inoculated petri dishes and blanks (inoculated with sterile distilled water) were incubated for 72 hours at 22°C and a light intensity and photoperiod corresponding to that of the phytoplankton culture from which the sample was drawn.

When contamination was detected in a continuous culture, the culture was disposed of, and any data generated from the culture was discarded. This occurred only once and was traced to a contaminated stock tube.

2.7 Sampling Procedure

Samples for media composition, and cellular composition analyses were collected from high light and medium light continuous cultures only, since maximum attainable flow rates in the low-light nitrate cultures were so small (17.8%/day) that

periodic removal of a sample large enough for the required analytical work would have severely altered steady state conditions.

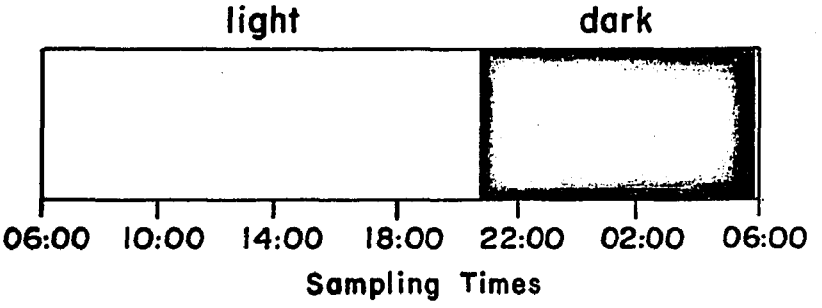
Samples were collected from high light cultures and medium light cultures once every four hours over a 52-hour period. The actual hours of sampling are as follows: 06:00, 10:00, 14:00, 18:00, 22:00, 02:00. Two full light periods were included in each sampling run. (Figure 2.3)

At each sampling time, 50 ml of culture was wasted to the overflow collecting beakers to remove the stagnant culture that had been standing in the overflow siphon since the preceding sampling. A sample from each culture was then collected into a clean beaker by closing the air vent clamp and forcing the sample out through the overflow siphon. Generally, 350 ml of sample was collected from each culture. In medium light-nitrate cultures, with maximum attainable flow rates of from 47-48% daily turnover, every other sample was reduced to 280 mls so as to maintain a continuous flow through the culture vessel. Consequently, in these cultures, pH was monitored only every eight hours.

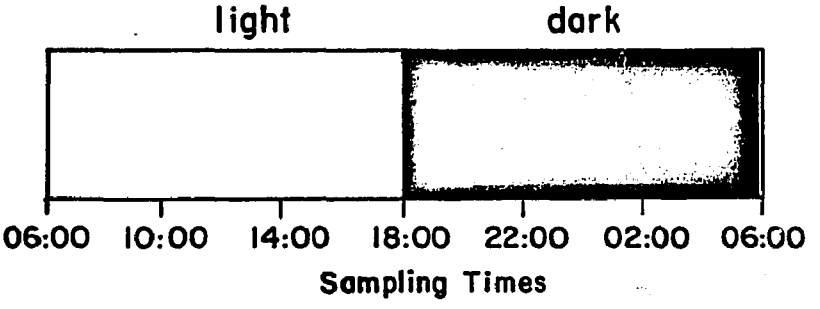
Total cell density/ml, dividing cells/ml, and pH were measured at each sampling period. Samples for chlorophyll a, carbohydrate, protein, total particulate "carbon" and "nitrogen", and residual medium nitrate, nitrite, ammonia and phosphate were collected, prepared and stored for later analysis. Since even short exposure to light can initiate rapid changes in phytoplankton physiology (Arnon D. 1960), all dark period

Figure 2.3 Light:dark cycles and sampling times.

High Light - Long Photoperiod



Medium Light - Medium Photoperiod



samples were collected and prepared for storage under dimmed room illumination. Collection and treatment of a full set of samples from one sampling period required 2-2.5 hours. To minimize the effects of this time lag between collection and fixing of samples, a standard order of sample-splitting and preparation was always followed. Thus, the aliquot to be used for analysis of any given parameter always stood outside the culture for approximately the same length of time. In addition, before removing an aliquot from the whole sample, the sample was sitrred vigorously to minimize the effects of settling.

To eliminate the possibility of cross-contamination, samples from ammonia and nitrate cultures were prepared using completely separate sets of glassware. All sampling glassware was rinsed three times with distilled water between uses.

2.8 Analytical Procedures

2.8.1 Chlorophyll a

Chlorophyll a was measured fluorometrically with a Turner fluorometer according to techniques modified from Strickland and Parsons (1968). Due to the relatively high cell densities attained in the experimental cultures, and the concentration of chlorophyll a in *Dunaliella tertiolecta*, it was possible to eliminate the cell concentrating vacuum filtration steps recommended by Strickland and Parsons (1968).

At each sampling time, 1 ml of culture was pipetted to a centrifuge tube and immediately frozen in an acetone:dry ice

bath. Analyses were always done immeditately at the end of a sampling run so that the maximum length of storage of any chlorophyll a sample was approximately 52 hours. To verify that storage for this length of time did not cause deterioration of chlorophyll a, twelve 1 ml-samples from an ammonia-grown batch culture of *Dunaliella tertiolecta* were collected at the same time and frozen in an acetone:dry ice bath. Two samples were thawed immediately and analyzed for chlorophyll a. The remaining samples were thawed and analyzed two at a time at 12-hour intervals over a 60-hour period (Table 2-e). No significant change in chlorophyll a concentration was noted.

Originally, 1 ml of culture was pipetted to a centrifuge tube, and 9 mls of reagent grade acetone was added to make a 90% acetone mixture. The tube was then shaken briefly and centrifuged at 14.7 g in a clinical centrifuge for 10 minutes. The supernatant was pipetted into a cuvette and read in the fluorometer.

Linearity and reproducibility of this method was checked by analyzing replicate samples of 0.5 ml of culture made to 1 ml with Hudson Canyon sea water:distilled water mix, 1 ml of culture, and 2 mls of culture concentrated by centrifugation and resuspended in 1 ml of Hudson Canyon sea water:distilled water mix. Chlorophyll a concentrations were plotted, and linear regression lines calculated (Figures 2.4 and 2.5). Both reproducibility and fit were poor. Spread for ammonia replicates was as high as 12.4% and mean percent deviation from the regression line was 20.9%. Spread for nitrate repli-

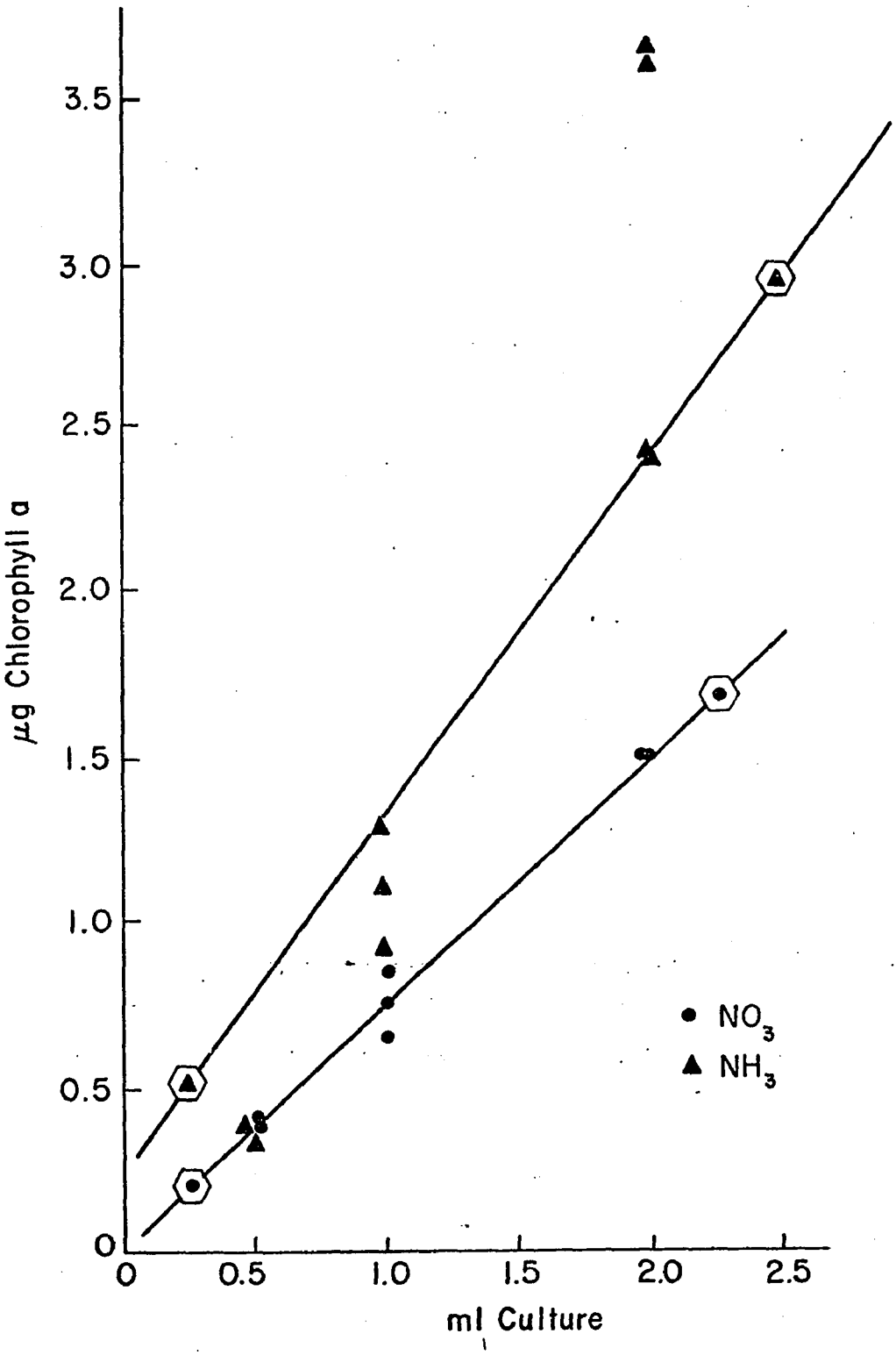
TABLE 2-e

EFFECTS OF SHORT-TERM STORAGE ON CHLOROPHYLL A CONCENTRATIONS

Hours stored	µg chl. a/ml	mean µg chl. a/ml
0	0.246	0.254
	0.262	
12	0.254	0.254
	0.254	
24	0.230	0.242
	0.254	
36	0.262	0.262
	0.262	
48	0.246	0.250
	0.254	
60	0.247	0.251
	0.254	

Figures 2.4 and 2.5 Chlorophyll *a* methods
Samples not ground before extraction

µg chlorophyll *a* vs mls culture


3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
μg Chlorophyll a
0
0.5
1.0
1.5
2.0
2.5
ml Culture
NO3
NH3

cates was as high as 12.5% and mean percent deviation from the regression line was 4.14%. This was probably caused by incomplete and inconsistent extraction of the chlorophyll a, as evidenced by a green color in some of the extracted precipitates. To solve this problem, an effective means of disrupting the cells was required. The culture was placed in a tissue grinder with 2 mls of acetone and ground for six strokes while immersed in an ice bath. The resultant acetone-water mixture was quantitatively transferred to a graduated centrifuge tube and made up to 10 mls with acetone. The centrifuge tube was then shaken briefly, and spun down in a clinical centrifuge at 14.7 g for 10 minutes. The supernatant was then pipetted into a cuvette and read in the fluorometer. Although this modification did not substantially change the concentration of chlorophyll a per ml, it did reduce the maximum percent spread of ammonia replicates to 4.2% and the mean percent deviation from the regression line to 3.9%; maximum percent spread of nitrate replicates was reduced to 0.9% and the mean percent deviation from the regression line to 1.14% (Figures 2.6 and 2.7). This method of grinding during extraction was adopted for use in all experimental samples.

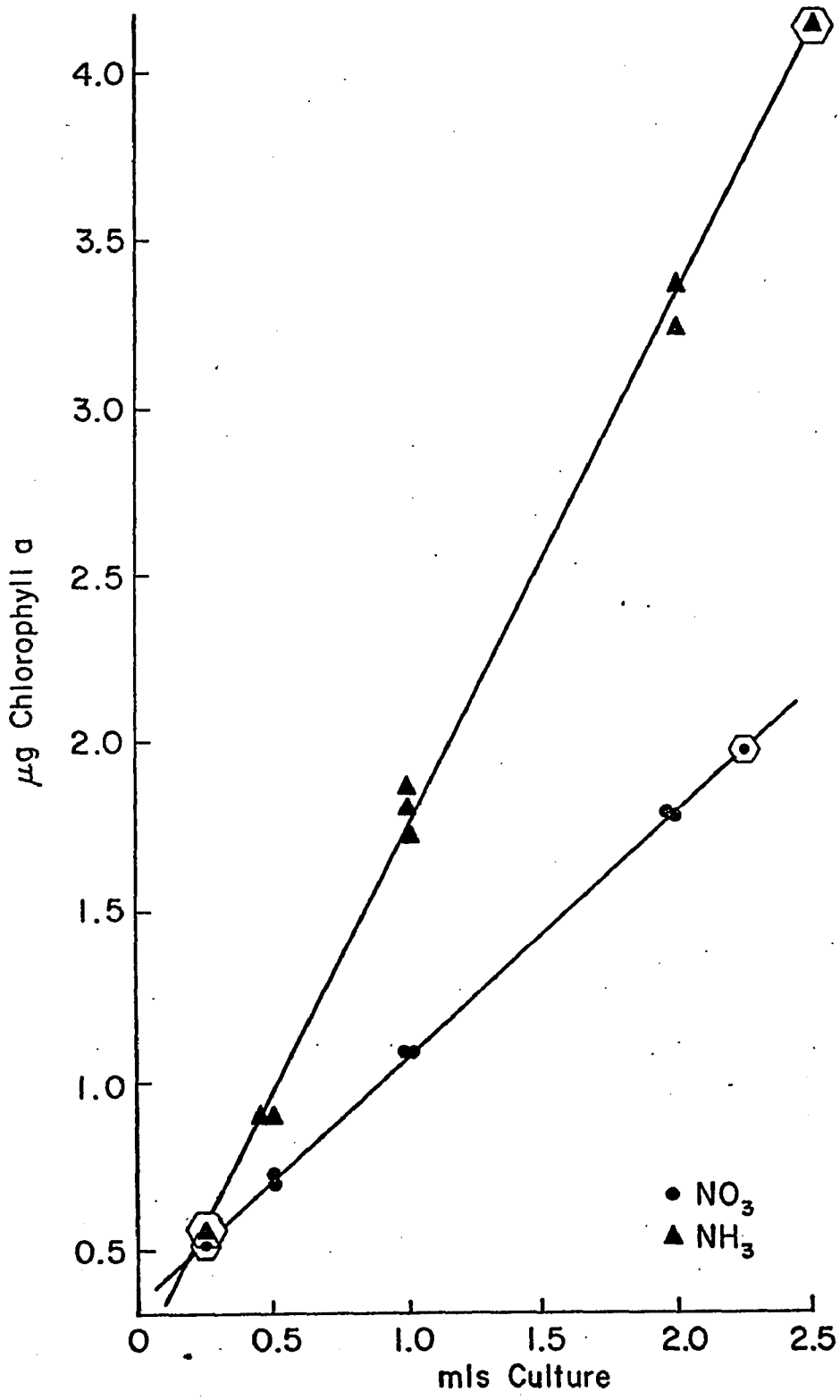
2.8.2 Carbohydrate

Total cell carbohydrate was measured by the sulfuric acid-indole technique of Dische and Popper as reported by Colowick and Kaplan in Methods in Enzymology (1957). The

Figures 2.6 and 2.7 Chlorophyll a methods

Samples ground before extraction

µg chlorophyll a vs mls culture


4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
µg Chlorophyll a
0
0.5
1.0
1.5
2.0
2.5
mls Culture
NO3
NH3

analysis consists of digestion of the sample with concentrated (37.5%) sulfuric acid, followed by color development with an indole, absolute methanol solution at 100°C. Samples were read at 480 nm in a 1 cm cell. The method is sensitive for pentose, hexose and heptose sugars in the range 5-25 µg/ml. Glucose was used as the standard. Reproducibility of standards and linearity were uniformly good (Figure 2.8).

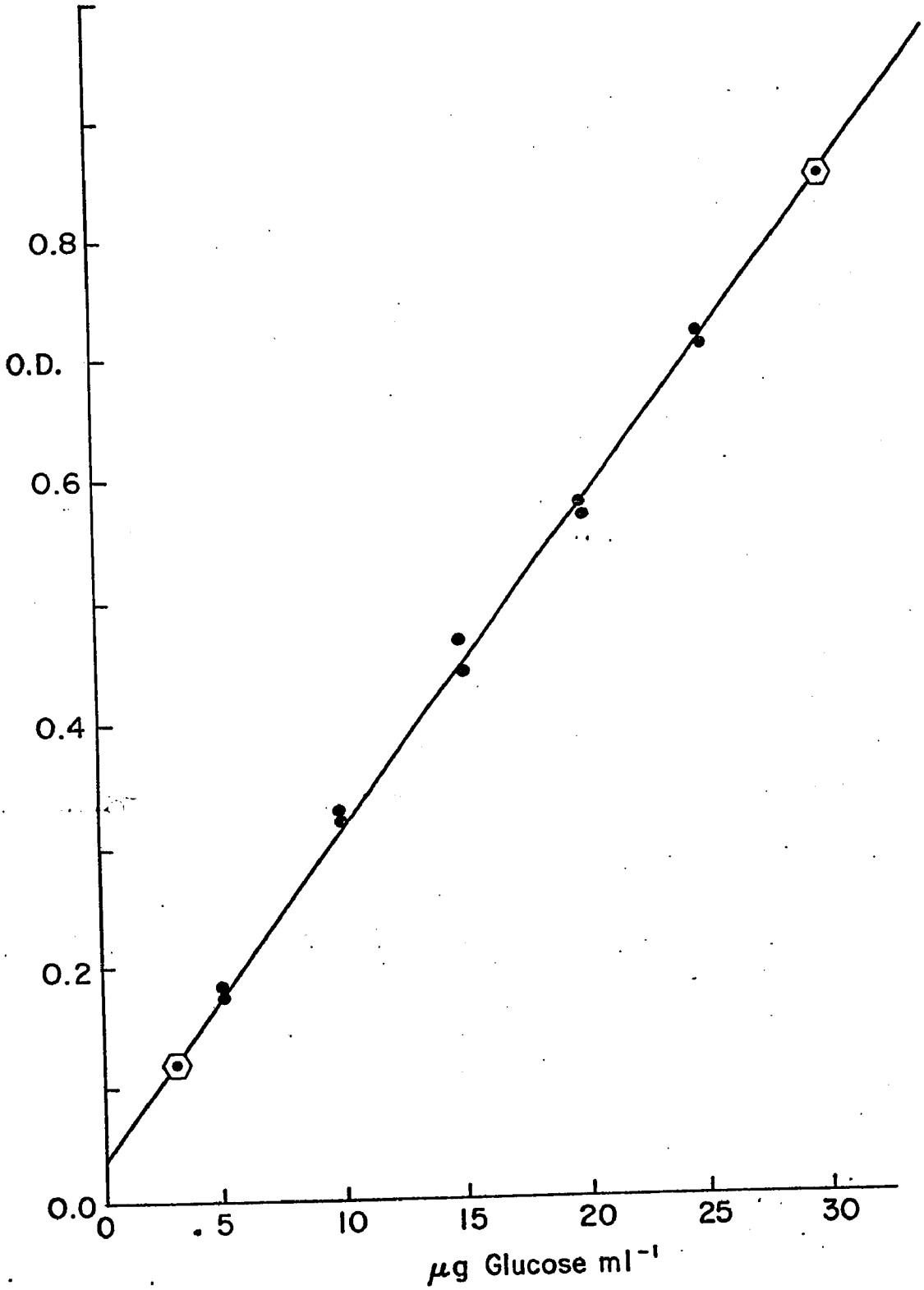
Unconcentrated 1 ml samples of *Dunaliella tertiolecta* culture could be analyzed directly since the range of total carbohydrate was usually within the sensitivity of the method. In addition, the method is not subject to salt error, and the sulfuric acid addition effectively dissolves all particulate material.

Analyzing unconcentrated cell suspensions introduces the possibility that extracellular dissolved carbohydrate is being measured along with intracellular material. However, analysis of continuous culture filtrates showed no measurable color development and would seem to confirm that primarily intracellular carbohydrates were being measured.

Samples for carbohydrate analysis were pipetted volumetrically into a capped culture tube, frozen in an acetone:dry ice bath, and stored frozen at -20°C until analysis.

Figure 2.8 Carbohydrate standard curve

O.D. vs µg glucose ml^{-1}


0.8
O.D.
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
μg Glucose ml⁻¹

2.8.3 Protein

Total cell protein was measured by the Folin-Ciocalteu phenol reagent technique of Lowry (1951). The Folin-Ciocalteu reagent was a pre-diluted commercial preparation supplied by the Fisher Scientific Company. The method consists of treating a whole cell sample with a solution of 2% w/v Na_2CO_3, 0.01% w/v $CuSO_4 \cdot 5H_2O$ and 0.02% w/v sodium potassium tartrate in 0.10N NaOH followed by color development with the Folin-Ciocalteu phenol reagent. Samples were read at 750 nm in a 1 cm cell. The only modification of the original method was to centrifuge the sample, at 14.7 g in a clinical centrifuge for 10 minutes, after color development. This was necessary since a small amount of undissolved cell debris tended to remain in suspension and caused falsely high absorbance readings. Linearity and reproducibility of standards (Figure 2.9), and of centrifuged samples, was consistently good.

Culture was prepared for analysis in the following manner. A 2 ml aliquot of cell suspension was centrifuged at 14.7 g in a clinical centrifuge for 10 minutes. All but 0.1 ml of the supernatant was drawn off with a syringe and pasteur pipet mounted on a stand (Figure 2.10). The pellet and remaining supernatant were brought to 2 mls with distilled water and thoroughly mixed on a vortex mixer. This dilution was sufficient to prevent a salt-induced precipitate from forming during analysis. A 0.4 ml sample of the diluted cell suspension was transferred to an ungraduated centrifuge

Figure 2.9 Protein standard curve

O.D. vs μg protein ml^{-1}

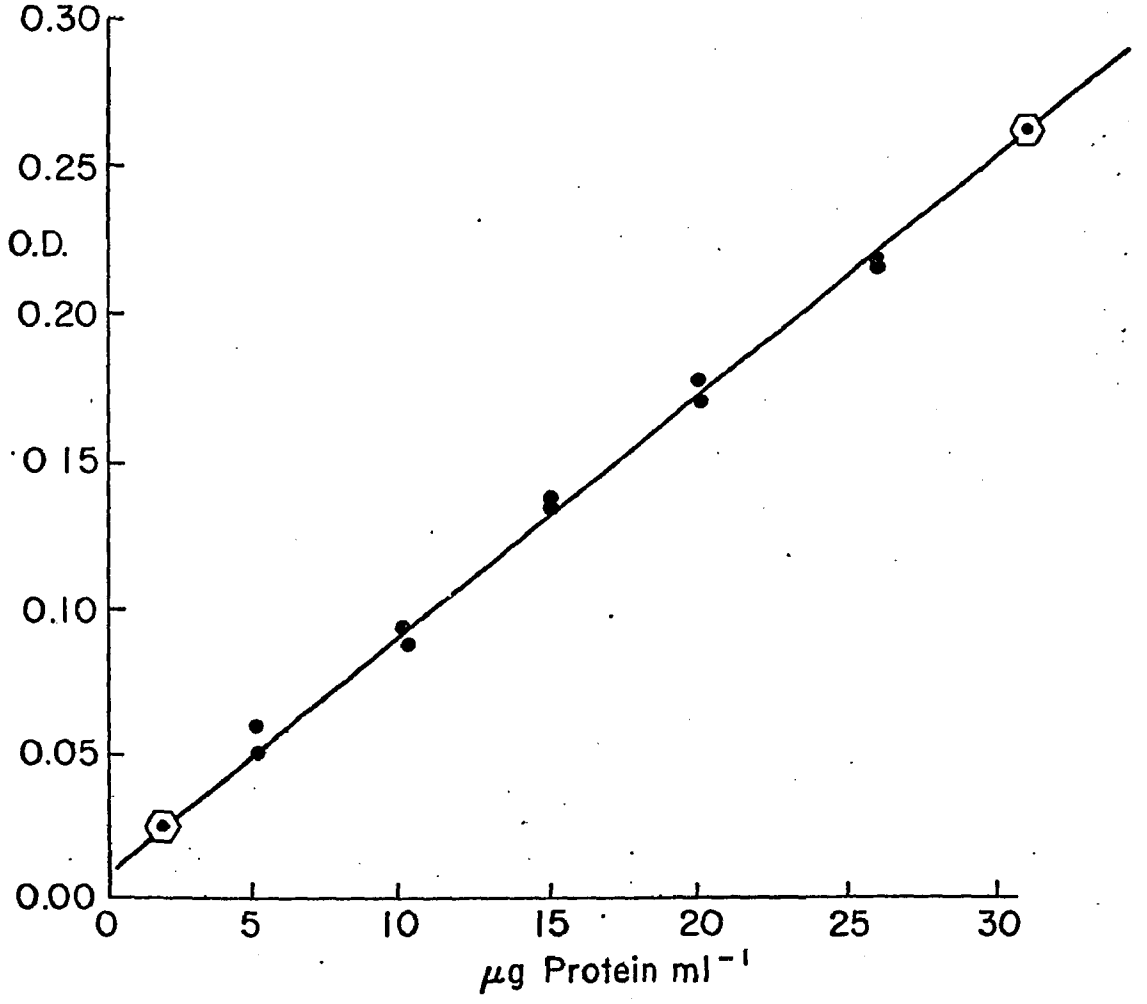
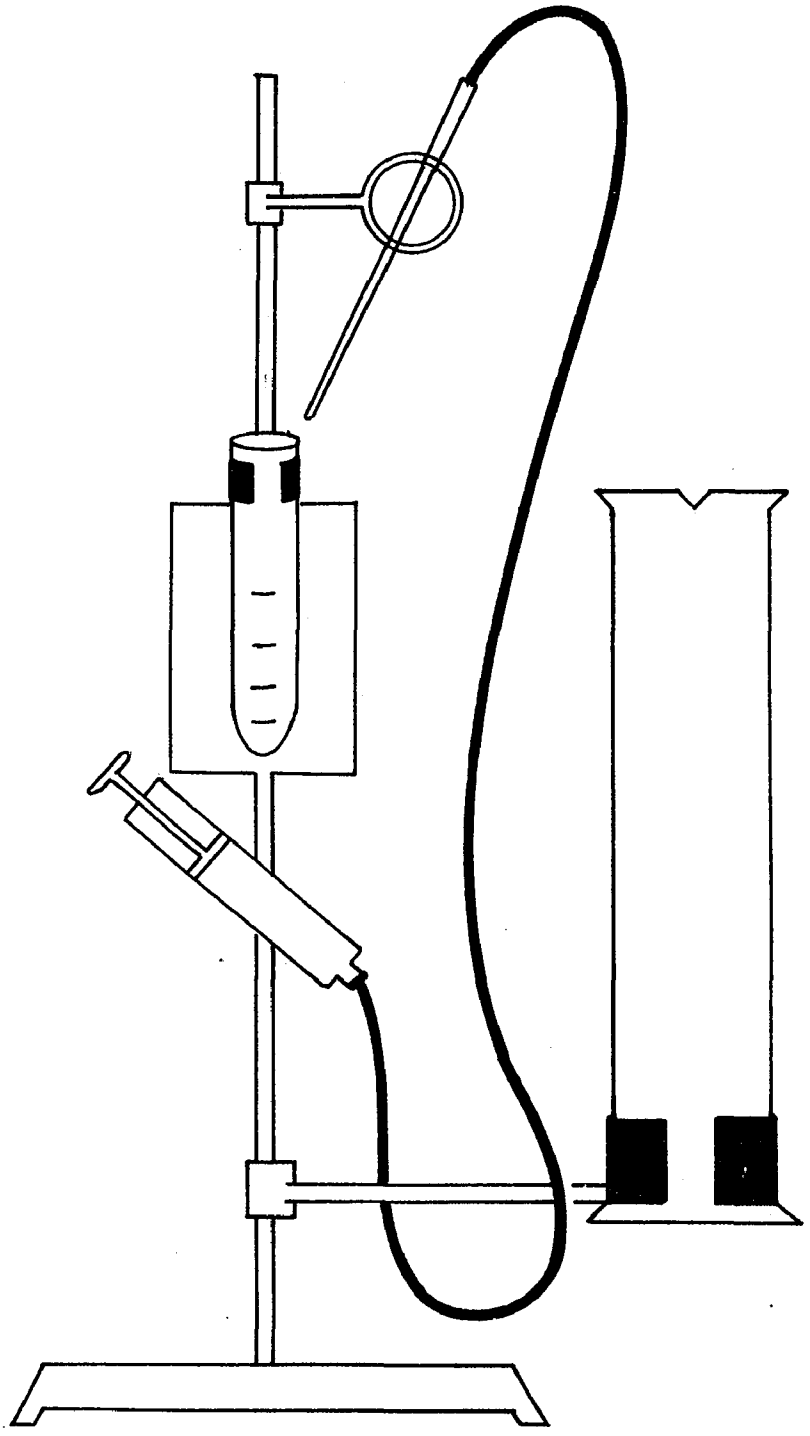

0.30
0.25
O.D.
0.20
0 15
0.10
0.05
0.00
0
5
10
15
20
25
30
μg Protein ml⁻¹

Figure 2.10 Apparatus for drawing off supernatants



tube, capped with parafilm and quickly frozen in an acetone: dry ice bath. Samples were stored frozen at -20°C, in the dark, for analysis.

2.8.4 Cell Counting

Cell counts of continuous cultures were done at each sampling period. Both total cells/ml and number of dividing cells/ml were counted. Counting was done with a Spiers Levy Eosinophil counting slide and a Wild Heerbrug phase contrast microscope. Counts were done at 200x. At this magnification, the field was large enough to view an entire square (1 of 10 in each chamber of the slide) of the counting chamber and still provided enough detail to resolve major cell structures such as chloroplasts and flagella. Thus it was possible when counting to check the morphological condition of the cells.

Since *Dunaliella tertiolecta* is a highly mobile organism, cells had to be fixed before counting. Powdered merthiolate was used as the fixative. Two ml of culture was transferred to a 5 ml beaker. A pasteur pipet was dipped in merthiolate and culture was drawn through the merthiolate three times to provide adequate mixing. The cell suspension was then immediately transferred to the Eosinophil counting slide. Observations of freshly fixed samples showed that it took approximately three minutes for all cells to stop moving, and an addition five minutes for all cells to settle into the plane of focus. After an additional 20-30 minutes, marked

cell elongation became apparent, and cells began to lyse. Counts were therefore begun 10 minutes after fixation, and when more than one sample was to be counted each sample was fixed after the preceding sample had been counted.

For counts of total cells/ml, 150-200 cells in each of the four chambers of the Spiers Levy slide were counted giving a total count per sample of 600-800 cells. Counting error was generally under 5%. When counting error exceeded 5%, a fresh sample was prepared and counted. For number of dividing cells/ml, the same squares that had been used for the total cell count were re-scanned. In counting the number of dividing cells/ml, a standard point of reference had to be established. Although it was frequently possible to observe equatorial plate formation in anaphase cells, this could not always be done with great certainty. The only unmistakable division phase proved to be telophase. At this time, *Dunaliella tertiolecta* elongates and becomes obviously "dumbbell" shaped. This "dumbbell" appearance was chosen as the criterion for a dividing cell. Nevertheless, some telophase cells were probably missed since the "dumbbell" shape would not be observable in cells that had settled with their anterio-posterior axis perpendicular to the counting slide.

2.8.5 Total Particulate Carbon and Nitrogen

Total particulate "carbon" and "nitrogen" were measured with a Hewlett Packard CHN analyzer. Samples were collected

on 0.3 μ mesh, 47 mm diameter glass fiber filters which were heated at 450°C for one hour prior to use to burn off any residual organics.

Fifty ml of continuous culture cell suspension was volumetrically pipetted to a millipore filter funnel, and filtered through double glass fiber filters at a vacuum of 3" Hg. Filters were then washed with three 10 ml aliquots of a low "nitrogen" isotonic sodium sulfate solution to remove any residual dissolved "nitrogen" from the culture medium without lysing cells. Filters were separated, transferred to plastic petri dishes, and stored upright in an evacuated dessicator at -20°C.

Generally, only the upper of the two filters was analyzed. However, in several instances, flawed upper filters allowed some cell material to pass to the lower filter as evidenced by a spot of green coloration. These lower filters were analyzed and the values obtained were added to the values obtained from the upper filter. In all cases where this was done, combined values fit the trend established by adjacent non-combined samples.

2.8.6 Residual Medium Inorganic "Nitrogen" and Orthophosphate

Residual medium nitrate, nitrite, ammonia and orthophosphate were measured by automated techniques adapted from the manual chemistries described by Strickland and Parsons (1968). Nitrite is determined by treating the sample with a solution of sulfanilamide and the diazonium ion is coupled

with N-(1-naphthyl)-ethylenediamine to give a pink azo dye. Nitrate is reduced to nitrite by passing the sample through a column of copperized cadmium filings. Nitrite is determined as above. Ammonia is determined by treating the sample in alkaline citrate with sodium hypochlorite and phenol to produce a blue indophenol dye. Orthophosphate is determined by reaction with ammonium molybdate followed by appropriate reduction to form phosphomolybdenum blue. Particulate-free media for these analyses was prepared by vacuum filtration at 3" Hg through 0.3 μ mesh 47 mm diameter glass fiber filters. Three 10 ml aliquots of continuous culture cell suspension were passed through the filter to rinse the filter flask, and discarded. One hundred mls of cell suspension was filtered, and split into polyethylene and glass sample bottles. Prior to filling, each bottle was rinsed with three 5 ml aliquots of filtrate. Glass bottles were refrigerated until analysis, polyethylene bottles were stored frozen at 20°C. Water for nitrate and orthophosphate analyses was taken from the glass bottles since nitrate is stable under refrigeration and phosphate is absorbed on polyethylene. Water for nitrite and ammonia analyses was taken from frozen samples since neither compound is stable under refrigeration.

2.8.7 Lipid

Particulate lipid was measured colorimetrically according to a technique described by Strickland and Parsons (1968). The particulate lipid contained in a whole-cell phytoplankton sample is converted to soluble soap by refluxing with alcoholic KOH. Fatty acids are then extracted and a blue color developed by reaction with pinacyanol reagent (Eastman Co.) and mono-bromo benzene. Samples are read at 620 nm against a stearic acid standard and alcoholic KOH blank.

Strickland and Parsons (1968) recommend concentrating cells for analysis by vacuum filtration onto a $MgCO_3$ coated millipore filter followed by resuspension of the $MgCO_3$ - cell mixture into 15 mls of filtered sea water. Further concentration is accomplished by centrifugation of the sample and removal of all but 1 ml of the supernatant. When this technique was tried with dense cultures of *Dunaliella tertiolecta*, it was found that complete resuspension of the $MgCO_3$-cell mixture could not be accomplished with a wash of only 15 mls. However, preliminary analytical runs on these incompletely resuspended samples indicated that 25-50 mls of a dense *Dunaliella tertiolecta* culture contained adequate lipid to be within the range of sensitivity of the method. Since a relatively small culture volume was suitable, sample concentration could be done by centrifugation alone.

Forty ml of culture was transferred to an ungraduated centrifuge tube marked at the 1 ml level. The culture was

centrifuged at 5.8 g for 20 minutes, all but 1 ml of the supernatant was drawn off with a pasteur pipet and syringe, and the pellet was resuspended in the remaining supernatant. Pellet and supernatant were transferred quantitatively to a 15 ml graduated centrifuge tube, and made up to 15 ml with sterile, filtered 20 0/00 salinity water. The sample was again centrifuged at 5.8 g for 20 minutes and all but 1 ml of the supernatant was drawn off. The pellet was resuspended in the remaining supernatant, alcoholic KOH was added, and the refluxing was begun.

Linearity was good, and results were reproducible to within ±0.73 µg lipid/ml (Figure 2.11). Lipid per ml was calculated by the following equation:

$$\mu g \text{ lipid/ml} = \frac{(\text{sample} - \text{blank})\ F}{\text{ml sample}^{-1}}$$

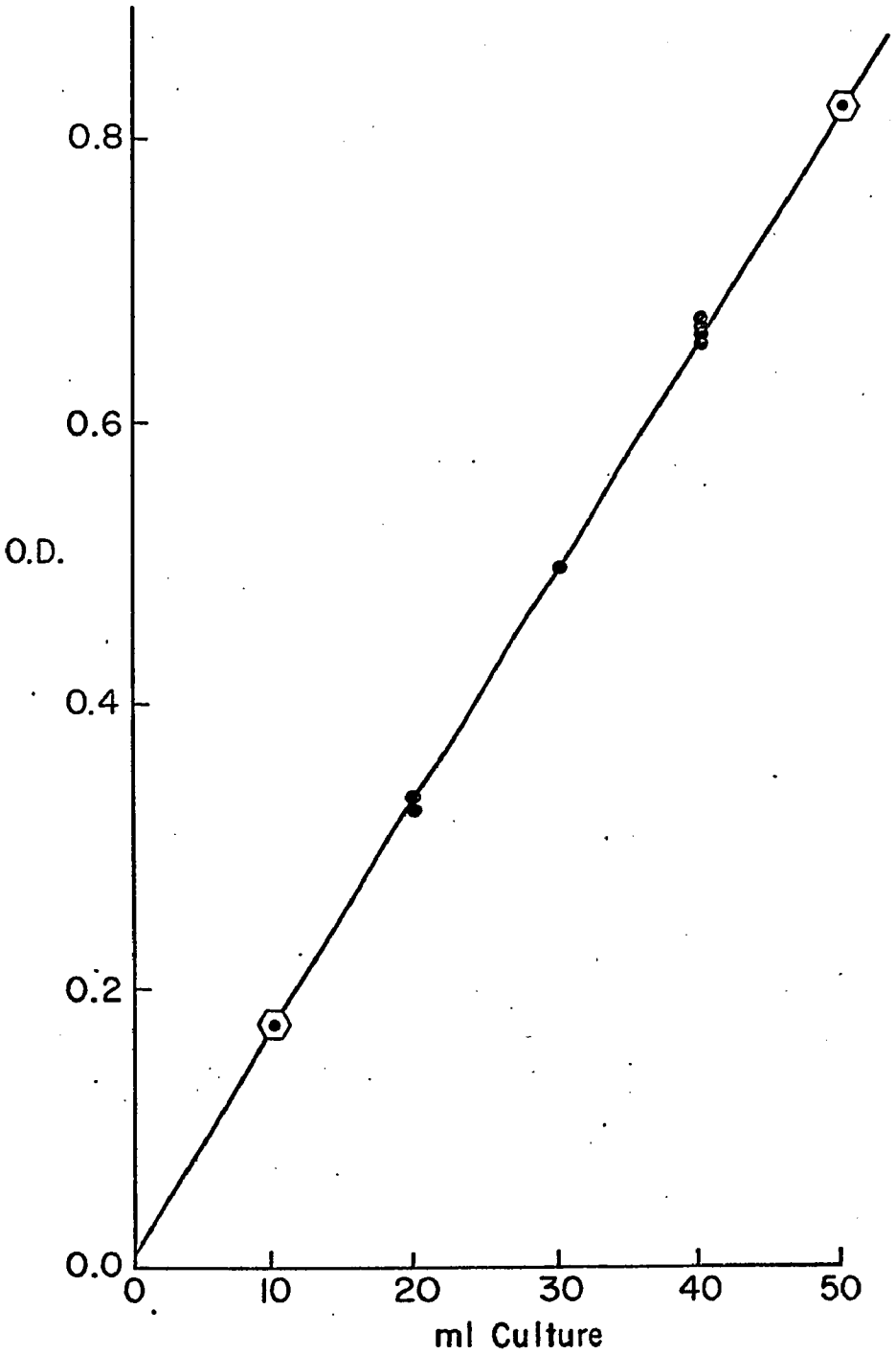
$$F = \frac{300}{E_s - E_b}$$

E_s = extinction of a 300 µg/ml stearic acid standard

E_b = extinction of an alcoholic KOH blank

Since the analytical method is extremely sensitive, and glass is not easily washed lipid free, a complete set of glassware was reserved for lipid analysis. Initially, glassware was washed in hot chromic-sulfuric acid, and rinsed three times with glass-distilled water and three times with reagent-grade acetone. Subsequently, glassware was rinsed with reagent-grade acetone between uses.

Figure 2.11 Lipid internal standard curve
O.D. at 620 nm vs mls culture


0.8
0.6
O.D.
0.4
0.2
0.0
0
10
20
30
40
50
ml Culture

Due to the volume of culture required for lipid analysis, samples could not be collected from the same continuous cultures used to monitor other intracellular components. Separate sets of high light-long photoperiod and medium light-medium photoperiod cultures were run to produce cells for lipid analysis. Intracellular carbohydrate was also measured in the lipid continuous cultures to determine whether these cultures were comparable to the cultures used for the analysis of all other parameters. Lipid analyses were run immediately after sample collection.

2.8.8 ATP

Samples for ATP analysis were prepared and stored according to the methods of O. Holm-Hansen (1966). Ten mls of culture were filtered onto a 0.45 μ millipore filter at a vacuum of 3" Hg. The filter was then rolled and immersed in 5 mls of sterile boiling Tris Buffer pH 7.34 in a screw cap culture tube. The sample was boiled for seven minutes and then quickly frozen in an acetone:dry ice bath. Frozen samples were stored for analysis at -20°C.

ATP decays very rapidly following cell death unless ATP-consuming enzyme systems are inactivated (O. Holm Hansen, 1966). Therefore, the speed with which filtration and immersion in boiling Tris Buffer is accomplished is of great importance. Tubes of Tris Buffer were kept constantly at a boil in a 100°C water bath, and by positioning all needed equipment close at hand, the process of filtration and immersion

was kept to under two minutes. A second difficulty in preparing samples for ATP analysis is that both cations and anions interfere in the reaction. For samples in full strength sea water, it is recommended that residual sea water represent no more than 1/20 of the final Tris Buffer sample volume (JRB photometer operating manual). Since the experimental culture medium has a salinity that is approximately 60% of that of full strength sea water, the maximum permissable volume of media that could be carried over into 5 mls of Tris Buffer was 0.15 mls. To ascertain whether this condition was being met, repeated 10 mls volumes of culture were filtered through millipore filters and the filtrate was collected in a graduated centrifuge tube. All samples gave a filtrate recovery of at least 9.8 mls.

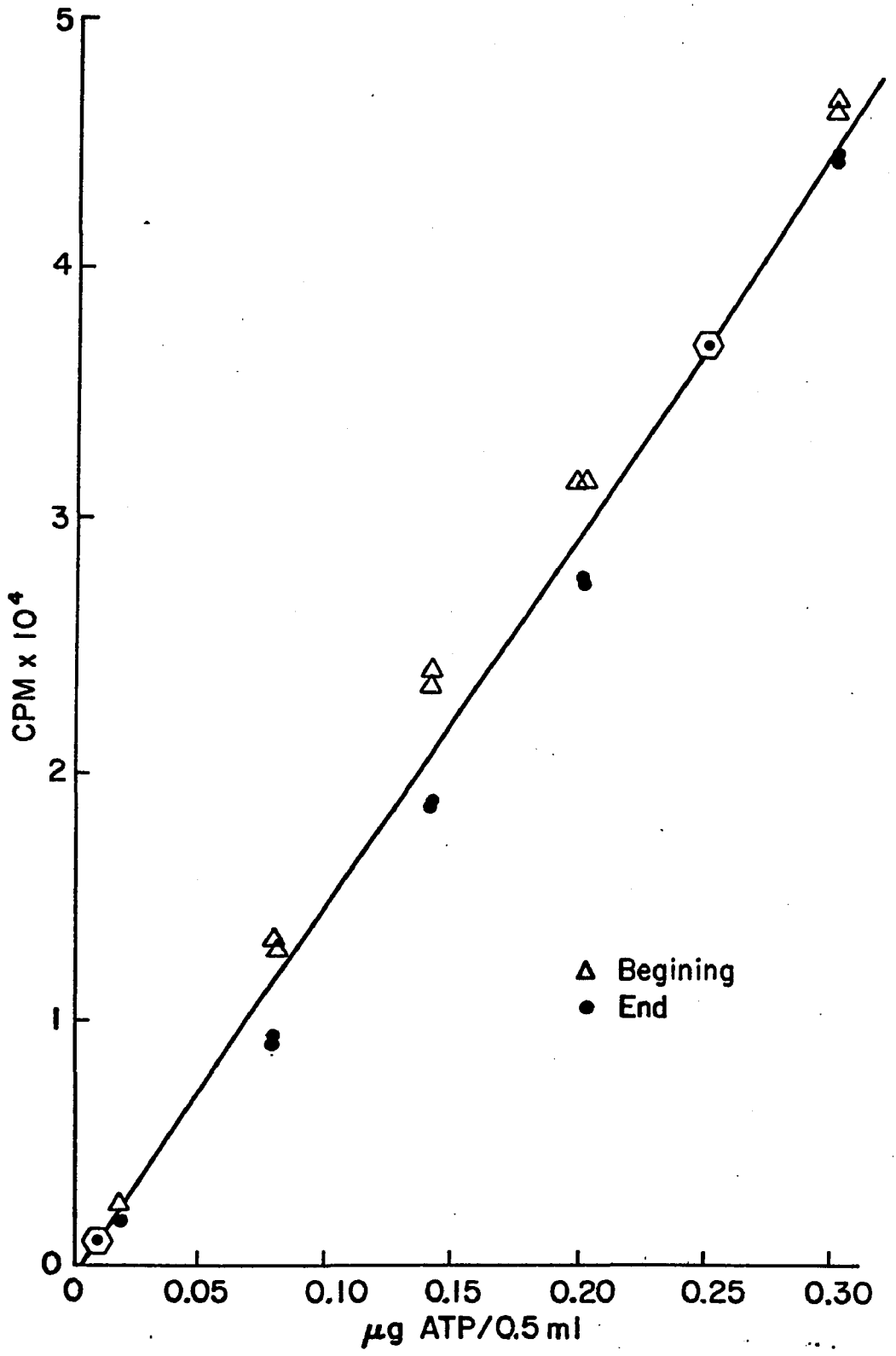
ATP samples were analyzed with a JRB ATP photometer. The method is based on measuring the amount of light emitted when ATP is added to a crude preparation of luciferin and luciferase derived from ground, lyophilized firefly lanterns (Sigma FLE-50). Since the preparation contains some residual ATP, the analysis consists of first measuring background light emission over a six-second period, which is automatically integrated to 60 seconds. The sample is then introduced and the light emission is measured over a 60-second period. Background emission is then subtracted from sample emission. The effects of residual ATP are further minimized by allowing the luciferin-luciferase preparation to stand for several hours after hydration. Both Holm Hansen (1966) and

the JRB photometer operating manual recommended that the enzyme preparation be allowed to stand 2.5-4 hours before use. However, even after standing four hours, the enzyme preparation gave initially high backgrounds. Time course studies on background light emission were run and enzyme background was found to stabilize at a low level five hours after hydration.

Standard curves were run on the disodium salt of equine ATP (Sigma) diluted with sterile Tris Buffer pH 7.34. The standard curve covered the range 0.02-0.30 µg ATP/0.5 ml. Despite the fact that the hydrated enzyme was kept on ice at all times, its activity did decay measurably during the 3-4 hours required to complete a run. To compensate for this, a standard curve was run at the beginning and at the end of the analysis and the regression line was calculated from both sets of values (Figure 2.12). Quality of the enzyme also varied considerably from batch to batch. Corrected C.P.M. for 0.2 µg/0.5 ml ATP standards using fresh enzyme preparations ranged from 31,500 cpm to 102,000 cpm. Although it was considered that the quality of ATP primary standards might be at fault, the reproducibility of the experimental results ruled out this possibility. ATP activity was also found to decay rapidly at room temperature. ATP samples were therefore thawed in an ice bath, as were primary standards; secondary standards were made up in Tris Buffer taken directly from the refrigerator.

Figure 2.12 ATP standard curve

µg ATP/0.5 ml vs counts minute x 10^{-4}


5
4
3
2
1
0
CPM x 10⁴
0
0.05
0.10
0.15
0.20
0.25
0.30
μg ATP/0.5 ml
Begining
End

2.8.9 Data Reduction

Values for intracellular chlorophyll a, carbohydrate, protein, lipid, ATP, and total particulate carbon from the experimental continuous cultures all showed a marked diurnal periodicity. When data for replicate runs were pooled, the periodic responses were still apparent, but scatterance was high and it would have been meaningless to define the periodicity in terms of some non-linear function (Ross, personal communication). Instead, each periodic response was reduced by linear regression analysis to a set of intersecting straight lines, one with positive slope relative to the left-hand axis representing the increasing portion of the periodic response, and one with positive slope relative to the right-hand axis representing the decreasing portion of the periodic response.

The resulting "sawtooths" each described by two linear equations lend themselves to simple geometric or integral calculations of baseline values, daily peak concentrations, daily and part-day means, and apparent (and in some cases real) rates of synthesis.

3 RESULTS

3.1 Residual Inorganic Nitrogen and Phosphate

Based on the nutrient content of Hudson Canyon Sea Water, the dilution of this water with distilled water in making the 20°/00 growth medium, and the nitrogen added to enrich the growth medium, ammonia medium would have an initial inorganic nitrogen composition of 180 µeq/l of ammonia, 0.456 µeq/l of nitrate, and 0.054 µeq/l of nitrite. Nitrate medium would have an initial inorganic nitrogen composition of 180 µeq/l of nitrate, 0.144 µeq/l of ammonia, and 0.054 µeq/l of nitrite. Thus the ammonia medium contained 0.28% of inorganic nitrogen other than ammonia, and the nitrate medium contained 0.11% of inorganic nitrogen other than nitrate.

Mean residual inorganic nitrogen values for ammonia and nitrate in the medium from steady state continuous cultures under both light regimes ranged from 0.52-1.48 µeq/l of nitrate, 0.194-0.308 µeq/l of nitrite, and 4.74-6.42 µeq/l of ammonia (Table 3-a). No pattern was discernable in the "nitrogen" composition of the steady state medium. The ammonia and nitrate media under both light regimes contained similar amounts of nitrate, nitrite and ammonia. In all cases, the contaminating nitrogen sources (nitrate and nitrite for the ammonia medium, ammonia and nitrite for the

TABLE 3-a

MEAN COMPOSITION OF RESIDUAL NITROGEN IN STEADY STATE CONTINUOUS CULTURES

chemostat	μeq NO_3/l	μeq NO_2/l	μeq NH_3/l	μeq total N/l	mean % removal
hi light ammonia 1	0.52	0.194	6.42	7.1	96.0
hi light ammonia 2	0.95	0.306	5.88	7.1	96.0
hi light nitrate 1	0.56	0.247	6.00	6.8	96.2
hi light nitrate 2	0.89	0.279	5.70	6.9	96.2
medium light ammonia 1	1.48	0.308	4.74	6.5	96.4
medium light ammonia 2	0.96	0.213	4.92	6.1	96.6
medium light nitrate 1	0.866	0.275	5.04	6.2	96.5
medium light nitrate 2	0.96	0.281	5.0	6.2	96.5

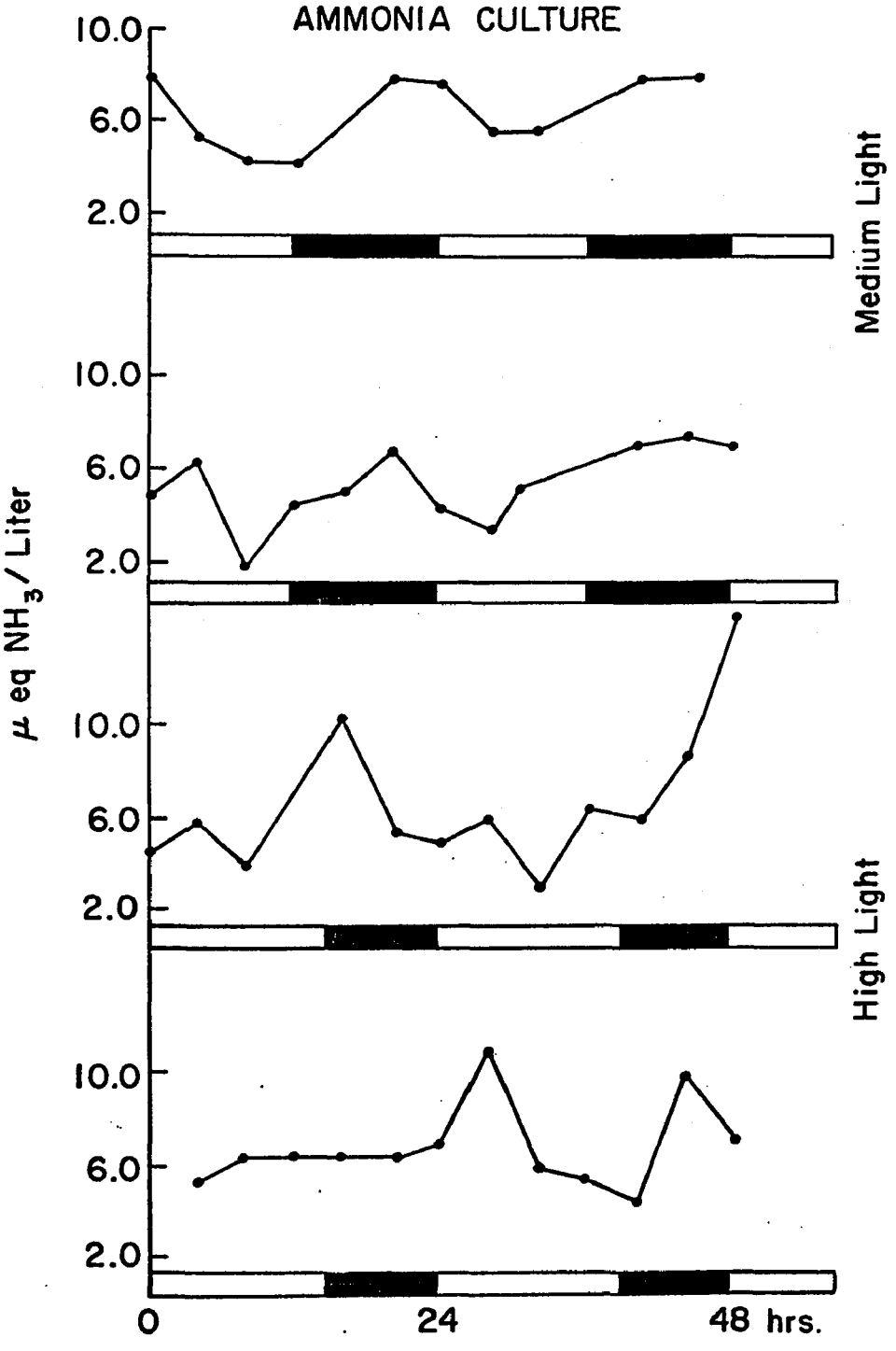
nitrate medium) were higher in the used medium than in the freshly made medium.

Mean total residual inorganic nitrogen values ranged from 6.1-7.1 μeq/l. Thus mean percent nitrogen removal ranged from 96.0-96.6%.

Individual values for residual inorganic nitrate, nitrite and ammonia for the continuous culture medium under steady state growth conditions are presented in figures 3.1, 3.2, and 3.3. Although no periodicity in medium inorganic "nitrogen" content was noted, there were frequent peaks in the "nitrogen" values. These were most prevalent in the medium light cultures. The peaks were most probably a result of the technical limitations encountered in maintaining a constant and continuous flow through the cultures. Although flow rates were reproducible when measured on a 24-hour basis, short term measurements of flow rates revealed a great deal of variability. For example, at a turnover rate of 102%/day, two consecutive 24-hour flow measurements differed by only 18 mls or 0.43%. However, individual flow readings taken at 15-minute intervals within the 24-hour periods varied by as much as 9 mls or 20.5%. In addition, when the cultures were being sampled, the periodic removal of a large volume of culture for analysis resulted in interruptions of outflow for periods of time that ranged from 2.3-3.5 hours. This obviously interrupted continuous flow and steady state. Nevertheless, it is doubtful that these fluctuations masked any light dependent periodicity in "nitrogen" uptake since maximum residual

Figure 3.1 Residual ammonia in steady state continuous culture medium

$\mu eq\ NH_3\ liter^{-1}$ vs time (hours)


AMMONIA CULTURE
10.0
6.0
2.0
Medium Light
10.0
6.0
2.0
μ eq NH3 / Liter
10.0
6.0
2.0
High Light
10.0
6.0
2.0
0
24
48 hrs.

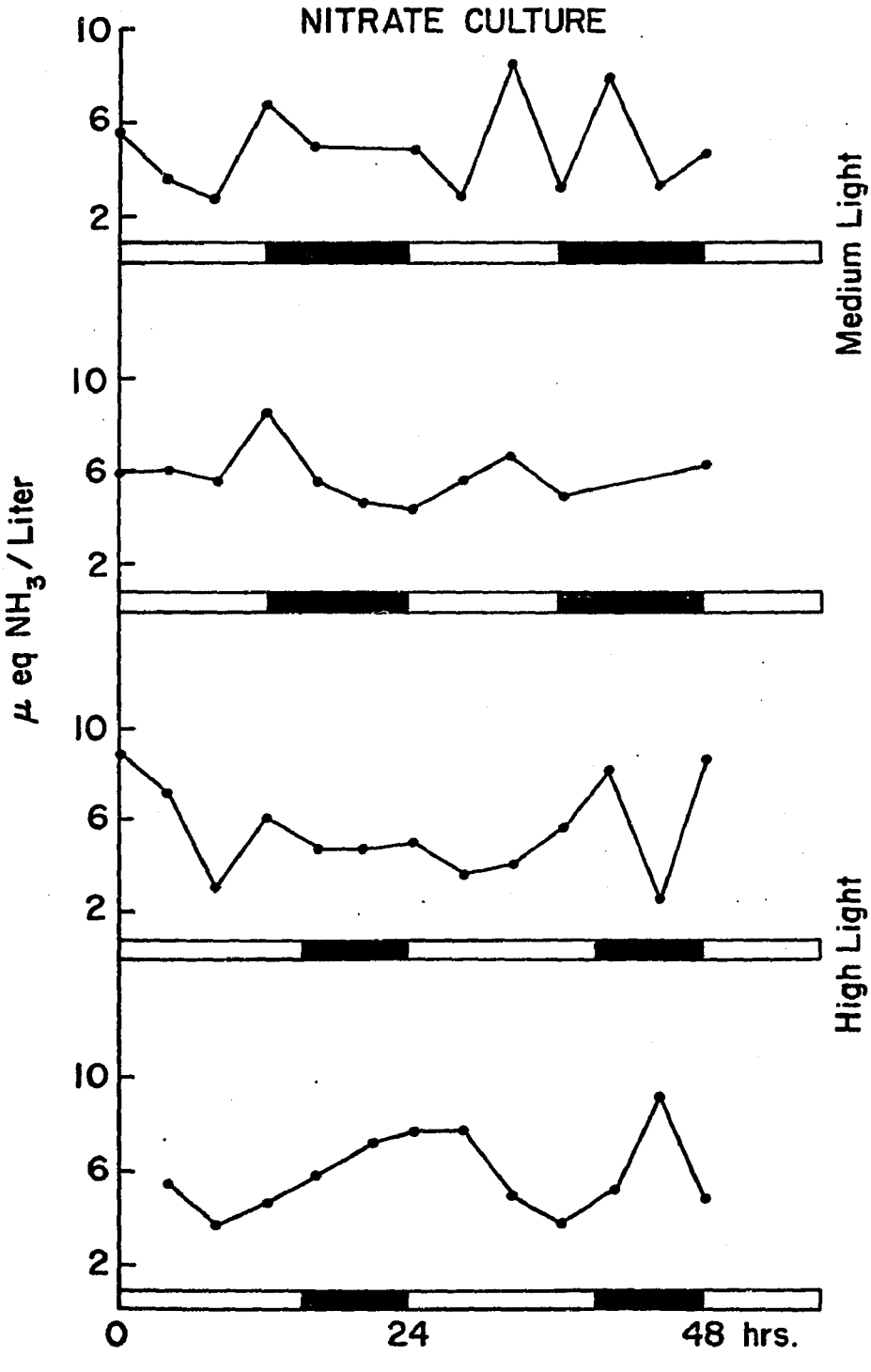
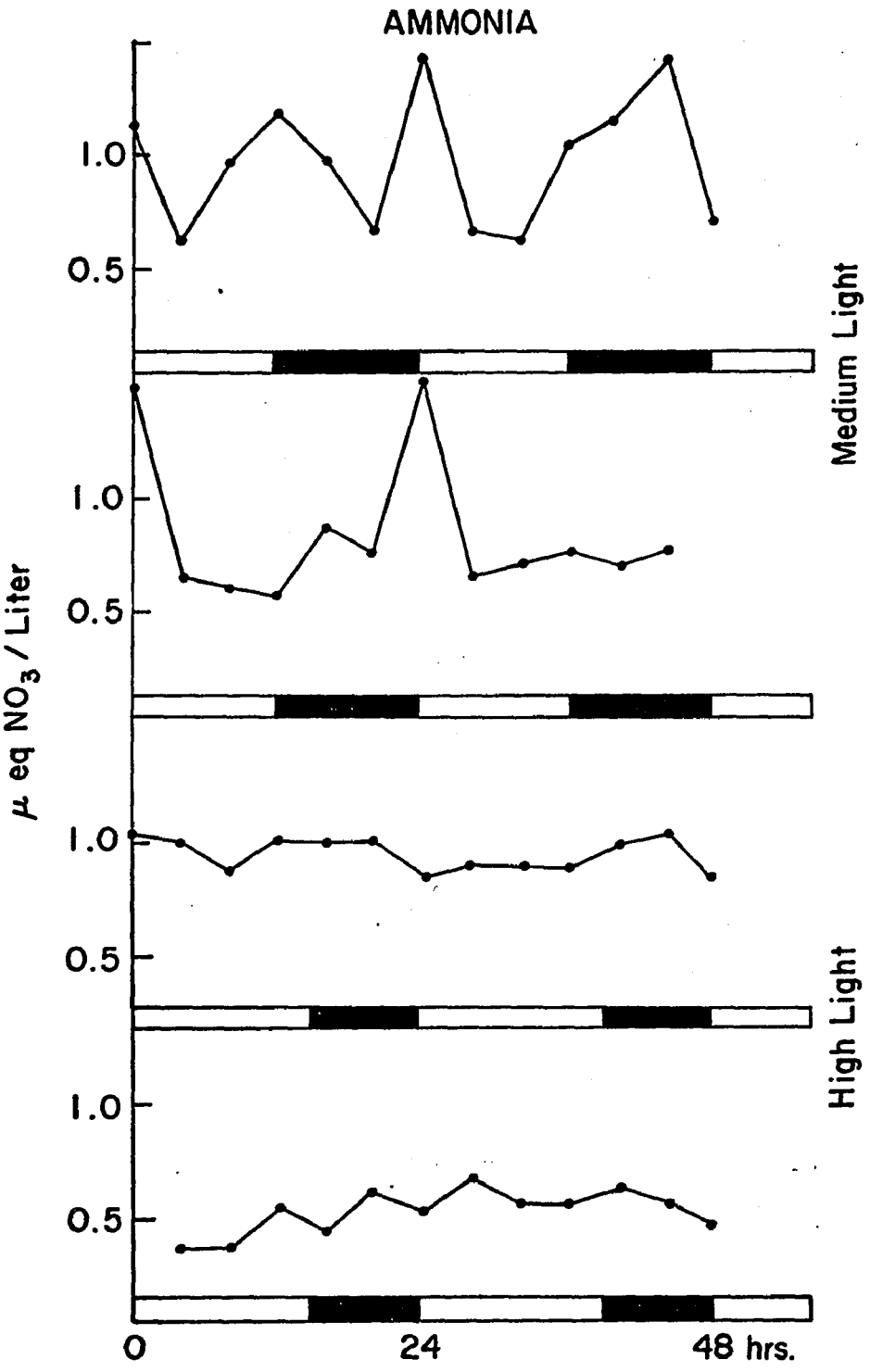

NITRATE CULTURE
10
6
2
Medium Light
10
6
2
μ eq NH3 / Liter
10
6
2
High Light
10
6
2
0
24
48 hrs.

Figure 3.2 Residual nitrate in steady state continuous culture medium

$\mu eq\ NO_3\ liter^{-1}$ vs time (hours)


AMMONIA
1.0
0.5
Medium Light
1.0
0.5
μ eq NO_3 / Liter
1.0
0.5
High Light
1.0
0.5
0
24
48 hrs.

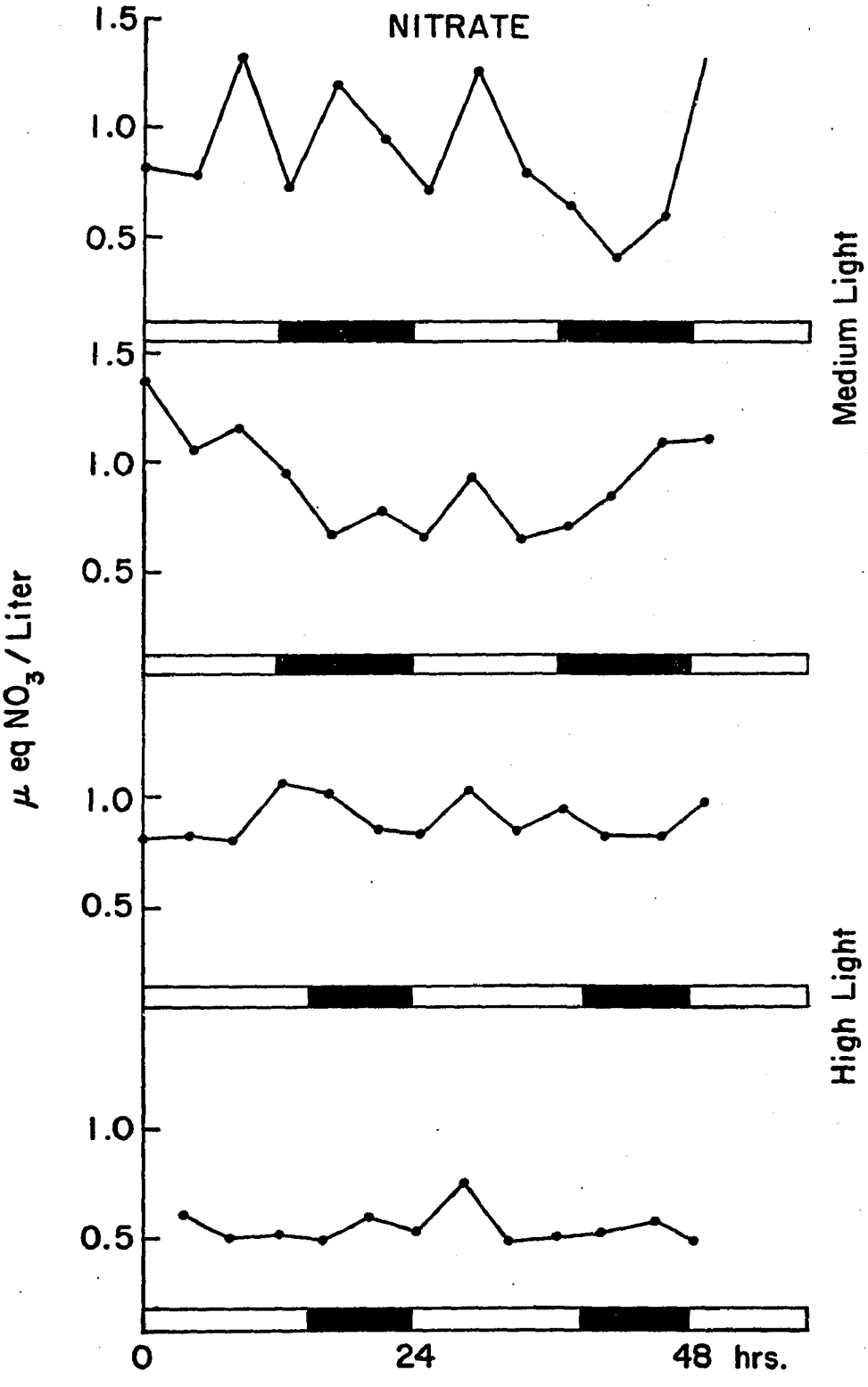
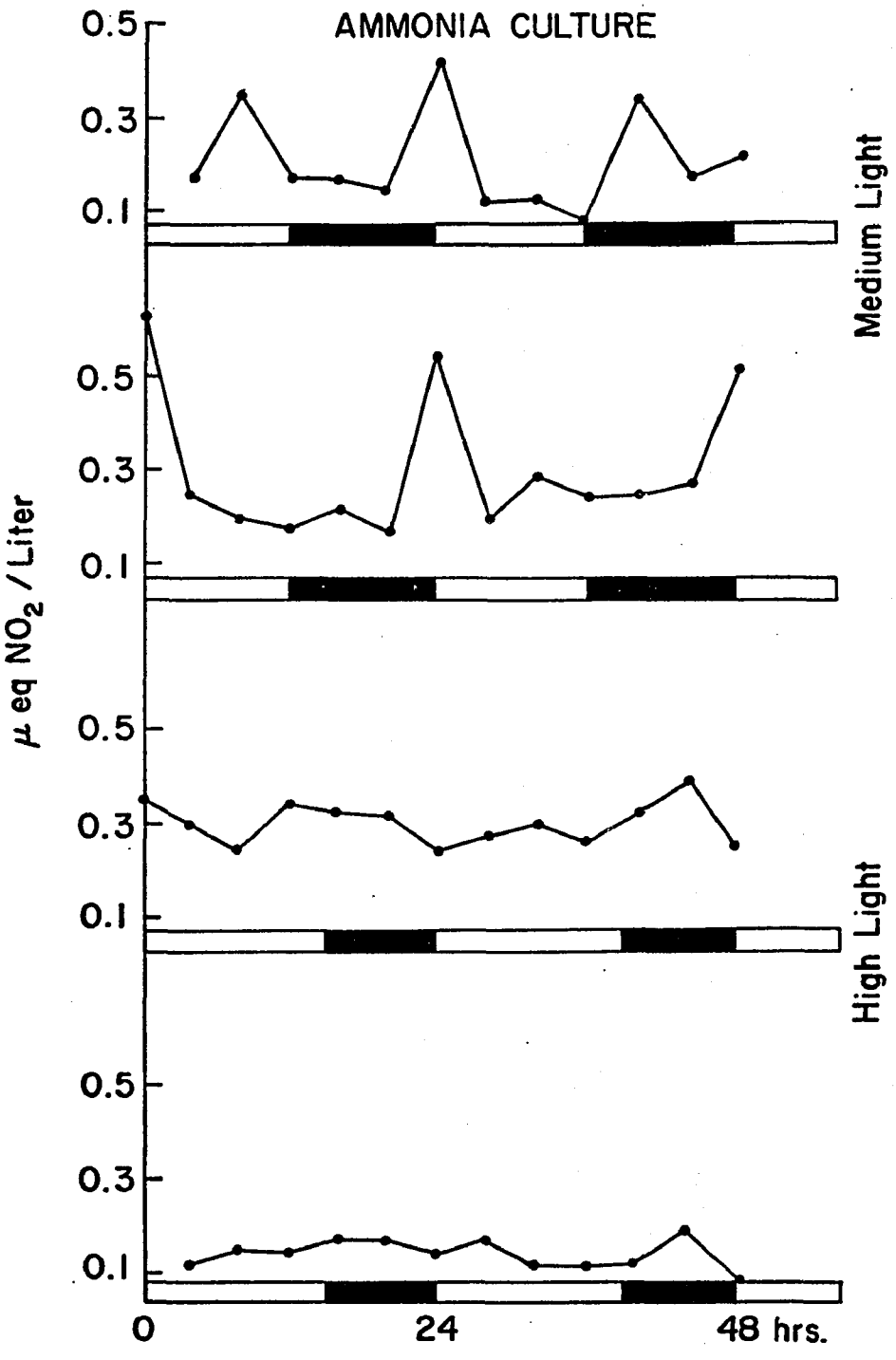
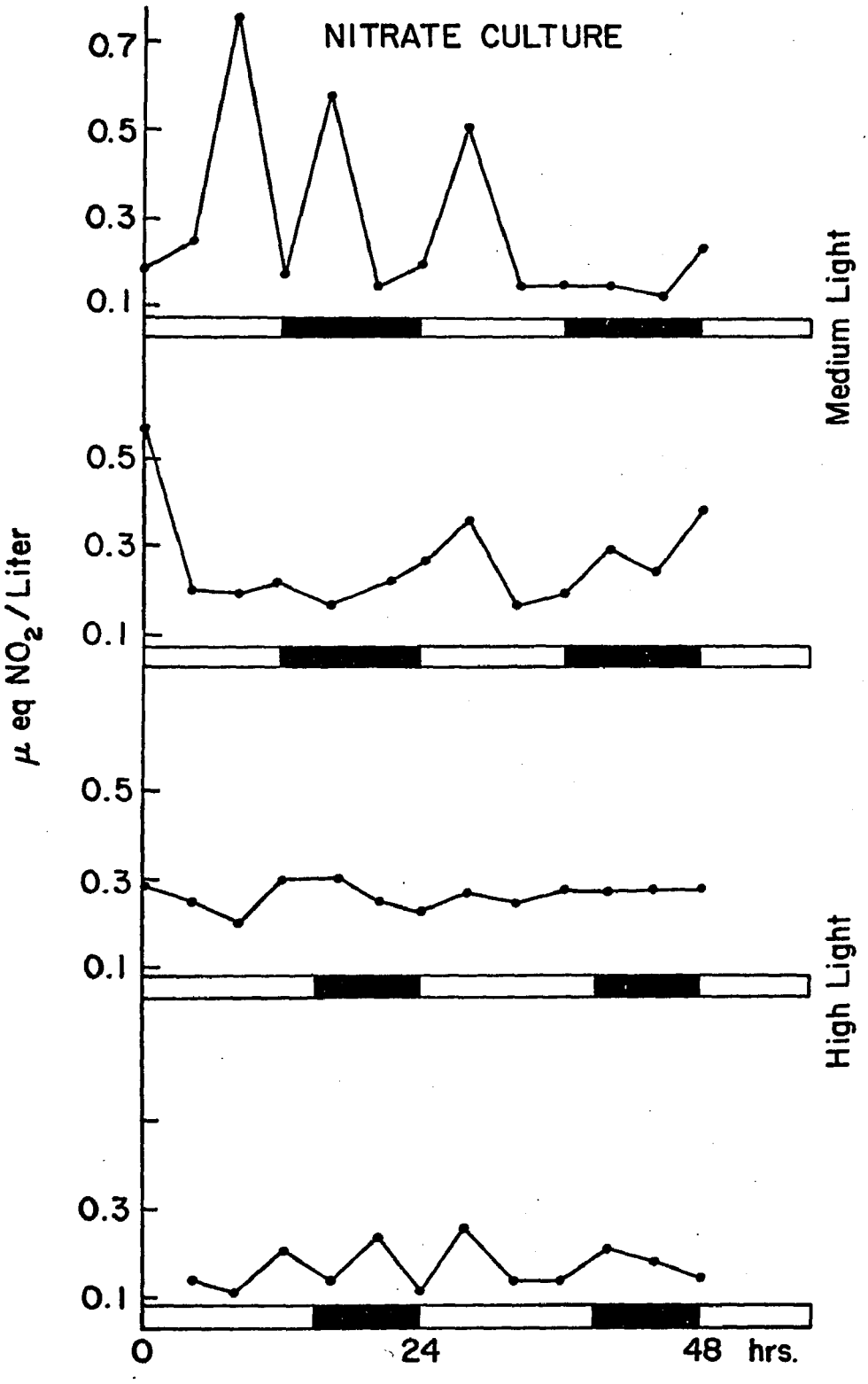

NITRATE
1.5
1.0
0.5
Medium Light
1.5
1.0
0.5
μ eq NO3 / Liter
1.0
0.5
High Light
1.0
0.5
0
24
48 hrs.

Figure 3.3 Residual nitrite in steady state continuous culture medium

$\mu eq\ NO_2\ liter^{-1}$ vs time (hours)


AMMONIA CULTURE
0.5
0.3
0.1
Medium Light
0.5
0.3
0.1
μ eq NO2 / Liter
0.5
0.3
0.1
High Light
0.5
0.3
0.1
0
24
48 hrs.


NITRATE CULTURE
0.7
0.5
0.3
0.1
Medium Light
0.5
0.3
0.1
μ eq NO2 / Liter
0.5
0.3
0.1
High Light
0.3
0.1
0
24
48 hrs.

nitrate and ammonia values were of the order of 10 µeq/l while it can be calculated that if uptake were to decrease appreciably in the dark, given the concentration of input "nitrogen", and the turnover rates used in this study, peak residual nitrogen concentrations should be 4-5 times higher. In fact, calculations of uptake rates for the experimental cultures showed a maximum dark:light difference only 0.91% for both nitrate- and ammonia-grown cells. Similarly, although values for percent removal of "nitrogen" reflected the peaks in culture "nitrogen" concentration, stripping never fell below 95%, suggesting that uptake of both substrates proceeded at a rapid rate independent of light:dark periodicity. This has been confirmed for *Dunaliella tertiolecta* by Grant (1967).

A poorly defined diurnal periodicity in medium orthophosphate content was noted in the continuous cultures (Figure 3.4). It is probable that the same flow rate and sampling limitations which affected the medium "nitrogen" data obscured the clarity of this periodicity.

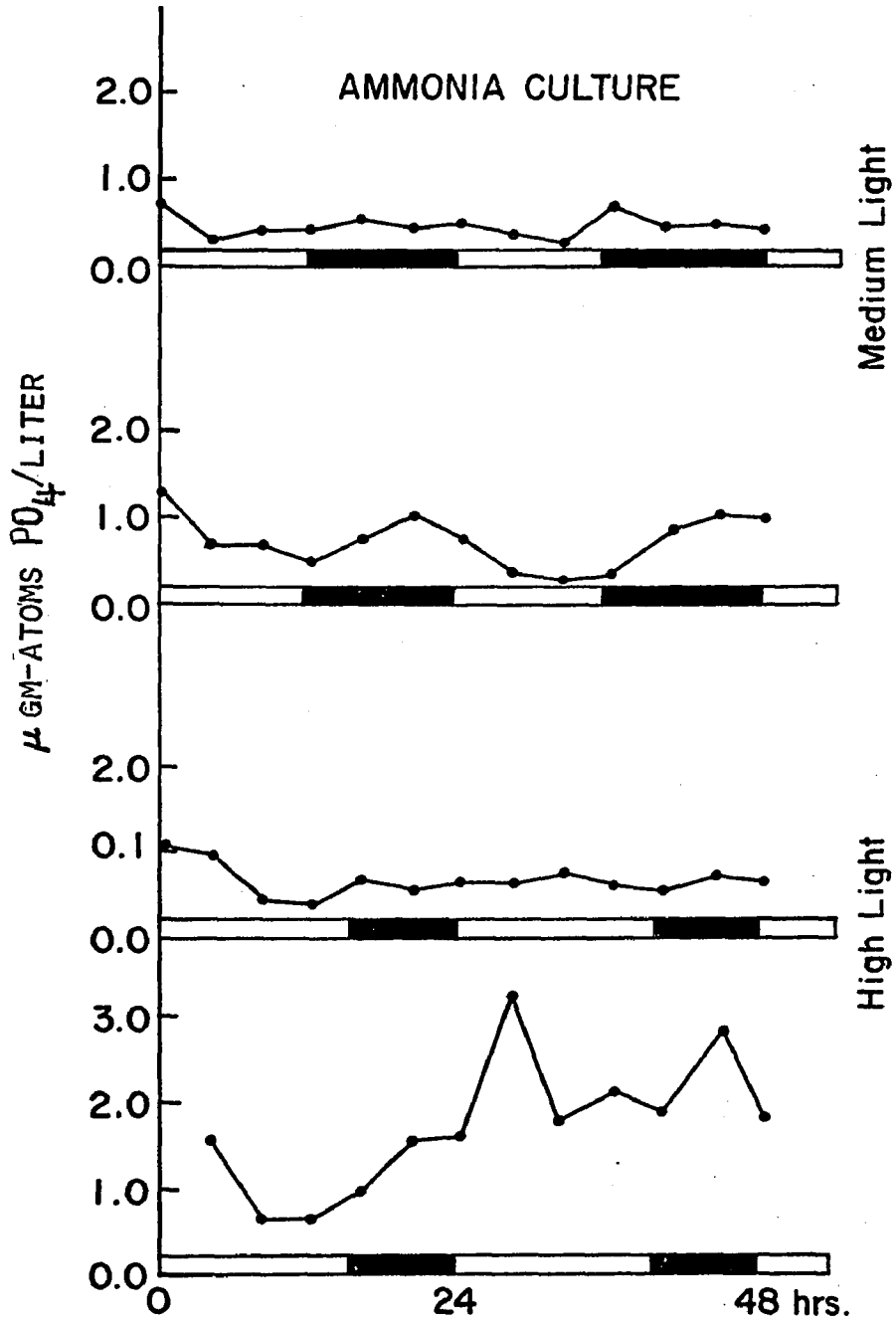
Mean orthophosphate values, and mean residual N:P ratios, are presented in table 3-b.

3.2 Maximum Attainable Growth Rate

Maximum attainable growth rates for ammonia and nitrate continuous cultures under the three light regimes are presented in table 3-c. Growth rates, which are directly related to the maximum attainable percent turnover, were highest in high light cultures, and lowest in low light cultures for each nutrient substrate. At all light intensity - photoperiod combinations, ammonia gave better growth (in terms of

Figure 3.4 Residual orthophosphate in steady state continuous culture medium

μg-atoms PO_4 $liter^{-1}$ vs time (hours)


AMMONIA CULTURE
2.0
1.0
0.0
Medium Light
2.0
1.0
0.0
μ GM-ATOMS PO4/LITER
2.0
0.1
0.0
High Light
3.0
2.0
1.0
0.0
0
24
48 hrs.

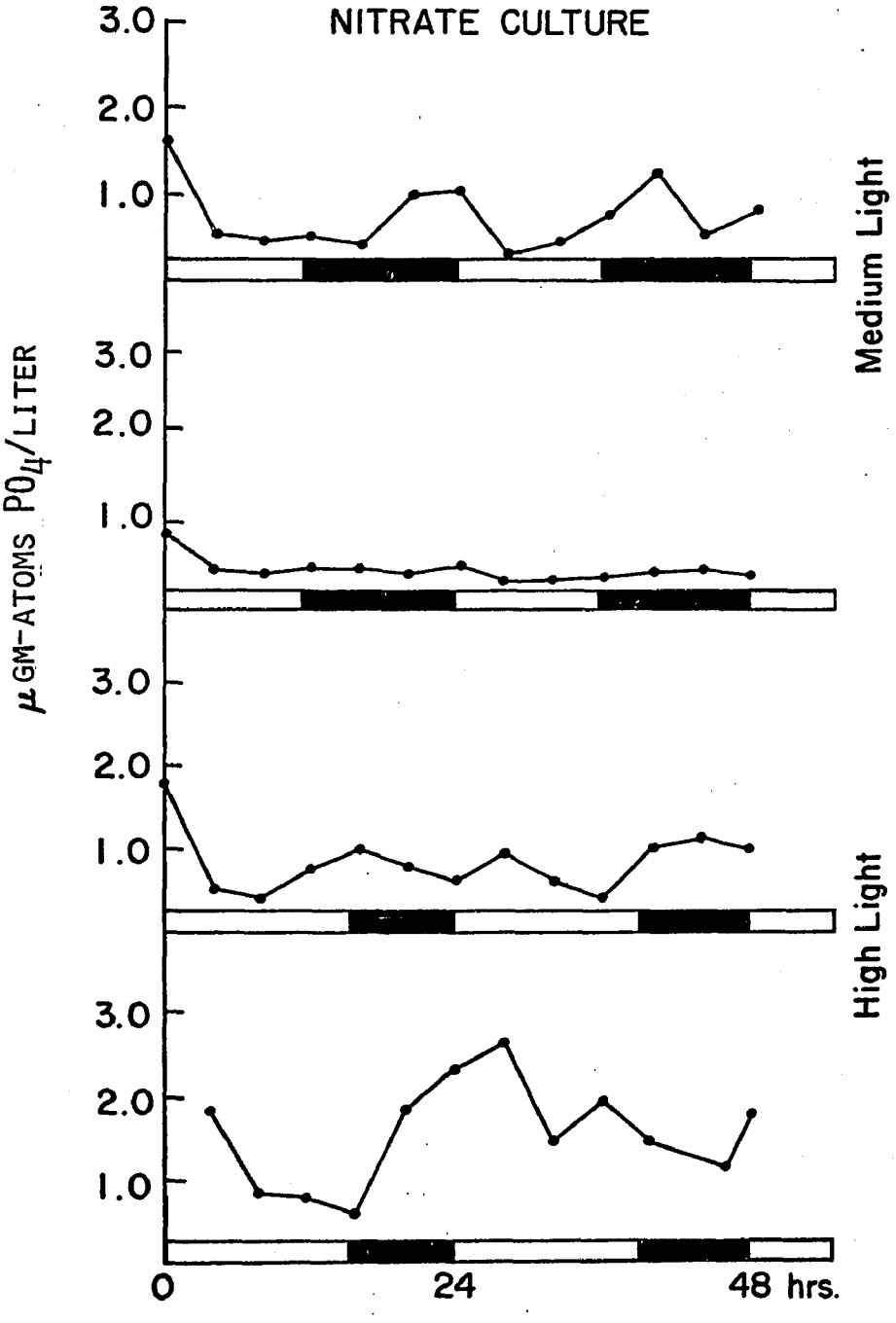

NITRATE CULTURE
3.0
2.0
1.0
Medium Light
3.0
2.0
1.0
3.0
2.0
1.0
High Light
3.0
2.0
1.0
μGM-ATOMS PO4/LITER
0
24
48 hrs.

TABLE 3-b

MEAN ORTHOPHOSPHATE AND MOLAR N:P RATIOS

Culture condition		mean µg-atom PO_4/l	mean µg-atom total N/l	N/P ratio
Hi Lt Ammonia	1	1.73	7.1	4.10
Hi Lt Ammonia	2	0.73	7.1	9.7
Hi Lt Nitrate	1	1.52	6.8	4.47
Hi Lt Nitrate	2	0.88	6.9	7.84
Med Lt Ammonia	1	0.72	6.5	9.02
Med Lt Ammonia	2	0.47	6.1	12.9
Med Lt Nitrate	1	0.52	6.2	11.9
Med Lt Nitrate	2	0.73	6.2	8.5

TABLE 3-c

MAXIMUM ATTAINABLE GROWTH RATES-NITRATE AND AMMONIA CULTURE UNDER 3 LIGHT INTENSITY:PHOTOPERIOD COMBINATIONS

chemostat	mean div/day continuous culture	div/day exponential growth
hi lt ammonia 1	0.835	1.04
hi lt ammonia 2		1.00
hi lt nitrate 1	0.687	0.72
hi lt nitrate 2		0.50
medium lt ammonia 1	0.664	0.71
medium lt ammonia 2		0.67
medium lt nitrate 1	0.484	0.55
medium lt nitrate 2		0.52
low lt ammonia 1	0.470	0.52
low lt ammonia 2		
low lt nitrate 1	0.178	0.24
low lt nitrate 2		

number of divisions/day) than nitrate. The same effect was noted during the exponential-growth, scale-up phase of the continuous cultures. Division rates for this phase of growth were calculated by the following formula (Hobson 1974):

$$\mu = \frac{\log_2 C_2 - \log_2 C_1}{t_2 - t_1}$$

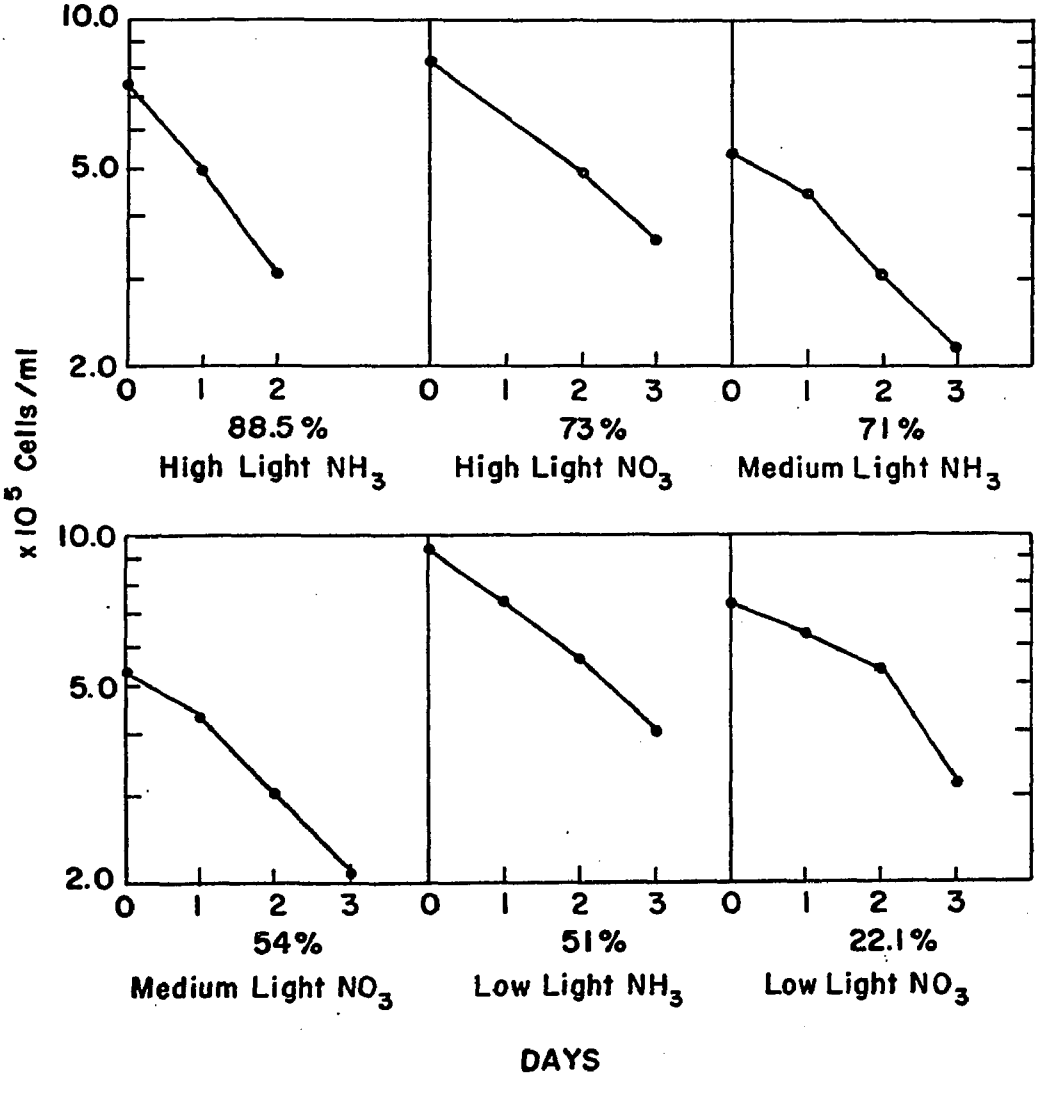
where μ is divisions per day and C_2 and C_1 are cells/ml at times t_1 and t_2 in days. Division rates during exponential growth were relatively similar to the growth rates maximally attainable in continuous culture at the same levels of total daily irradiance, although there was a tendency for exponential growth rates to be slightly higher than continuous culture growth rates (Table 3-c). This is understandable in view of the low cell densities and greater culture transparency characteristic of the early stages of exponential growth.

As a partial check on the validity of the observed values for maximum specific growth rate, the continuous cultures were run at flow rates exceeding presumed μ max by 5% for periods of three days, to see whether a consistent trend in cell density could be established (Figure 3.5). In all cases, cell densities decreased by greater than 50% over the three-day period. While it has been suggested that the observed decreases were merely lag responses to the changes in flow rate, the magnitude of the responses would seem to argue against such an interpretation.

No conclusive evidence can be offered to establish that the observed growth rates were indeed those maximally attain-

Figure 3.5 Effect of increasing continuous culture flow rates by 5% after the establishment of "steady state"

Total cells ml x 10^{-5} vs time (days)


10.0
5.0
2.0
x 10^5 Cells/ml
0 1 2
88.5%
High Light NH3
0 1 2 3
73%
High Light NO3
0 1 2 3
71%
Medium Light NH3
10.0
5.0
2.0
0 1 2 3
54%
Medium Light NO3
0 1 2 3
51%
Low Light NH3
0 1 2 3
22.1%
Low Light NO3
DAYS

able under the experimental conditions. However, the close correspondence between exponential and continuous culture growth rates, and the behavior of the continuous cultures when flow rate was increased by 5%, would indicate that the observed growth rates were probably close to μ_{max}.

3.3 Number of Dividing Cells per ml

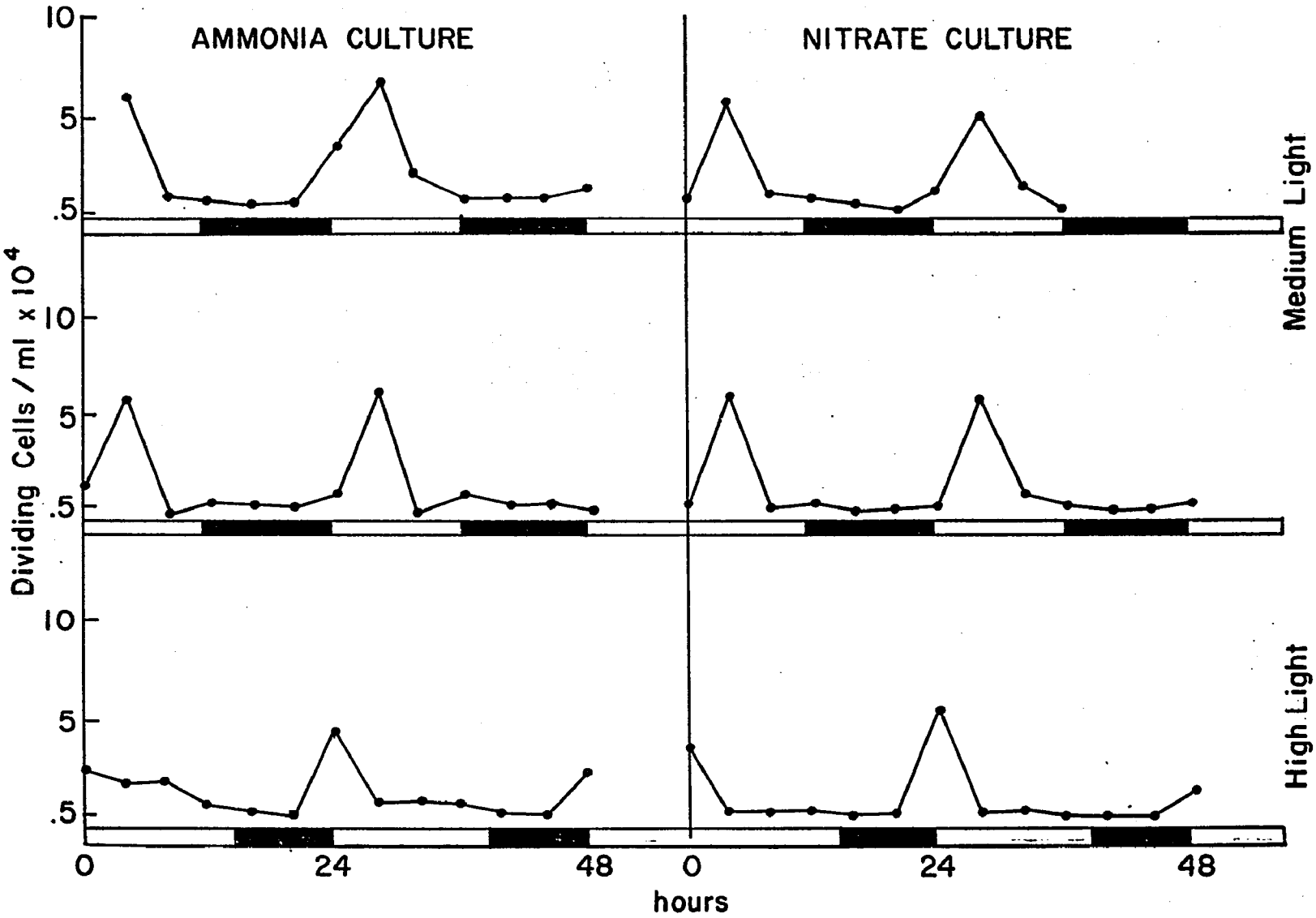
The effects of light intensity and photoperiod on continuous culture growth rates are reflected in the patterns of cell division observed in the cultures (Figure 3.6). Under high light growth conditions, both ammonia and nitrate cultures showed some synchrony, with peak numbers of dividing cells appearing in the 06:00 hours samples, immediately after the beginning of the light phase. Under conditions of medium light, medium photoperiod, both ammonia and nitrate cultures still showed synchrony. However, the period of peak cell division had shifted four hours into the light phase and occurred at 10:00 hours.

One consistent difference in patterns of cell division between ammonia and nitrate cultures was noted. Although both ammonia and nitrate cultures showed synchrony of cell division, synchrony was more pronounced in the nitrate cultures. Under high light conditions, nitrate cultures showed no dividing cells[1] between the 06:00 hour periods of peak

[1] No dividing cells < $1x10^3$ cells/ml. The minimum countable density with a Spiers Levy Eosinophil Slide. Dividing Cells = Late anaphase or telophase cells. See Materials and Methods.

Figure 3.6 Synchrony in continuous culture cell division

Telophase cells ml x 10^{-4} vs time (hours)


AMMONIA CULTURE
NITRATE CULTURE
Medium Light
High Light
Dividing Cells / ml x 10^4
10
5
.5
0
24
48
hours

cell division. Under conditions of medium light, nitrate cultures showed no dividing cells for from four to twelve hours during the dark phase. Ammonia cultures under conditions of high light showed dividing cells in all samples. Under conditions of medium light, ammonia cultures showed dividing cells in all samples with the exception of the 02:00-hour sample. Thus the general trend is for ammonia cultures to show one synchronous daily peak in cell division and to continue dividing at low levels over the entire 24-hour light:dark cycle. In contrast, nitrate cultures show the same synchronous daily peak, but are restricted to a shorter daily period of continuing low level division. Since the magnitudes of the division peaks under each light regime are the same for ammonia and nitrate cultures, the faster rates of growth in ammonia cultures may be dependent on the difference in the low level component of cell division.

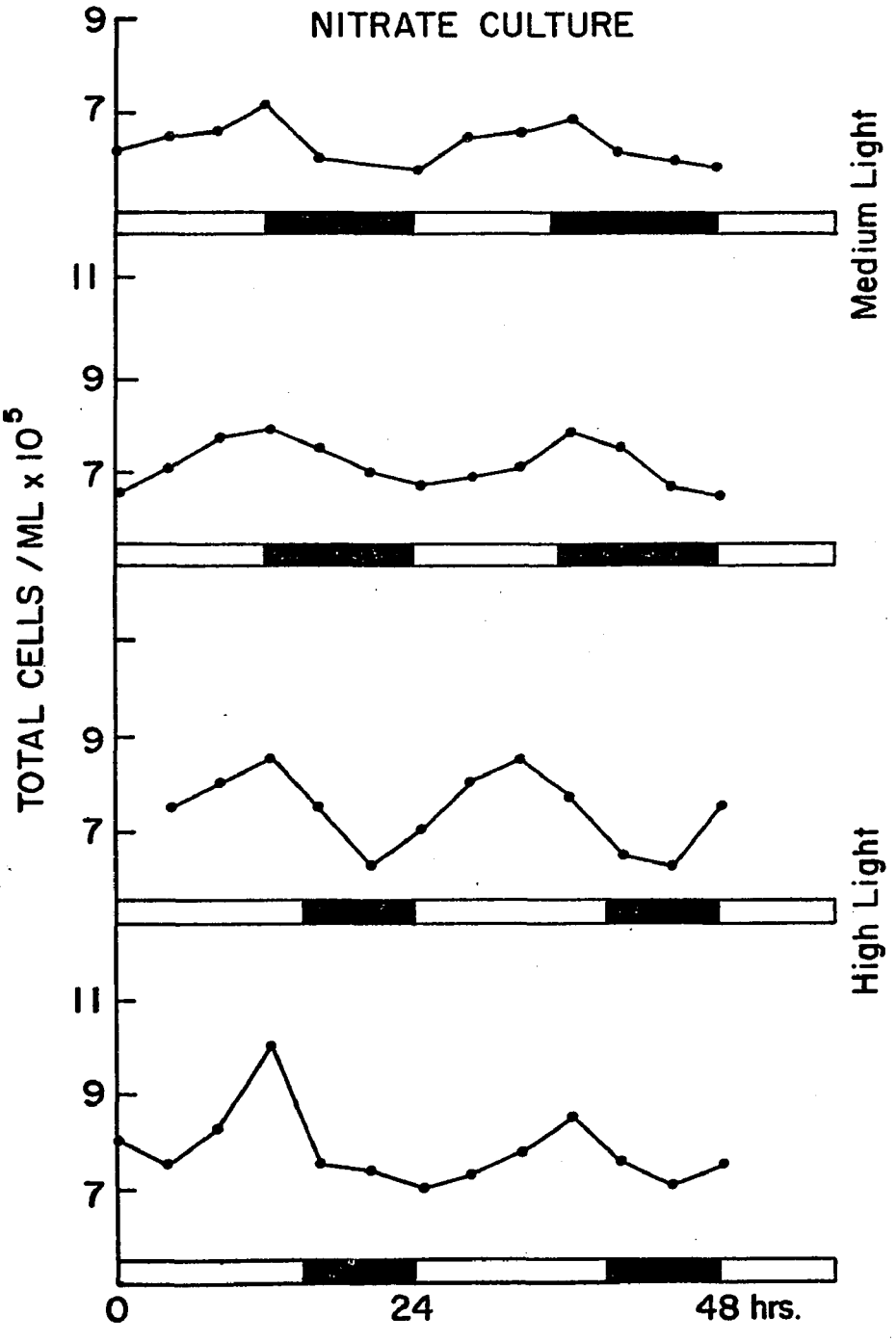
Dividing cells/ml could not be quantitatively compared to total culture growth rate or maximum attainable turnover rate since some cells probably entered late anaphase, and completed telophase between any two sampling periods.

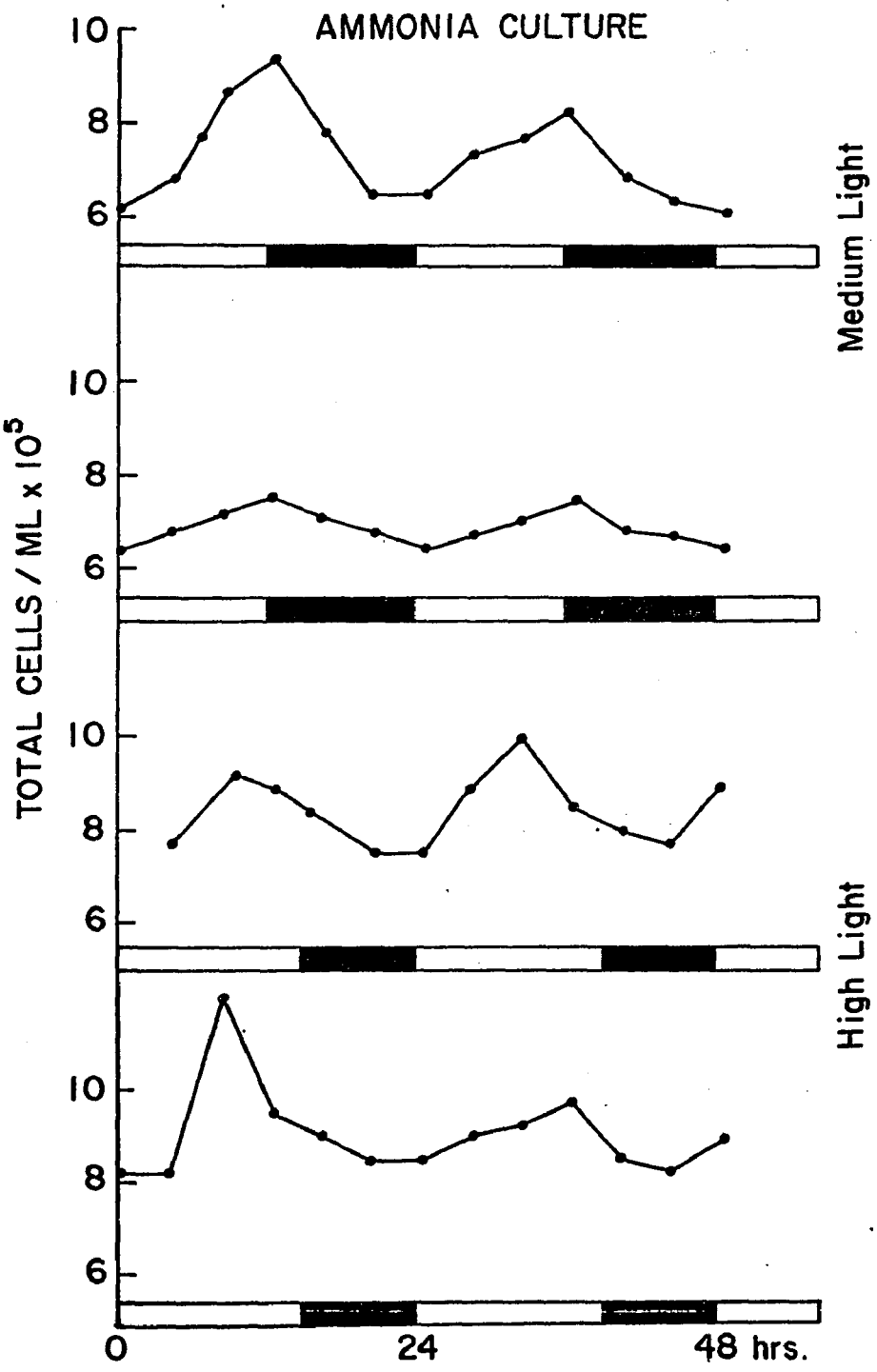
3.4 Total Cells/ml

Cell densities in steady state continuous cultures under high light, and medium light conditions in both nitrate and ammonia medium showed a diurnal periodicity. The basic pattern of this periodicity is a gradual increase in cell densities during the light phase, and a gradual decrease during the dark phase (Figure 3.7). Under conditions of

Figure 3.7 Diurnal periodicity in continuous culture cell density

Total cells ml x 10^{-5} vs time (hours)


NITRATE CULTURE
9
7
11
9
7
9
7
11
9
7
TOTAL CELLS / ML x 10^5
Medium Light
High Light
0
24
48 hrs.


AMMONIA CULTURE
TOTAL CELLS / ML x 10^5
10
8
6
Medium Light
High Light
0
24
48 hrs.

high light in both ammonia and nitrate medium, cell densities began to increase at 02:00 hours in six out of eight cases. In all cases, cell densities peaked, and began to decline between 14:00 and 18:00 hours. Thus, cell densities began to increase during the dark phase, and began to decline four to eight hours before the onset of the dark phase. In contrast, under conditions of medium light, in both ammonia and nitrate medium, cell densities did not begin to increase until 06:00 hours, after the onset of the light phase, and four hours later than the onset of cell density increase in most high light cultures. Similarly, under medium light conditions, all cultures did not peak and begin to decline until 18:00 hours, four hours later than the onset of decline in several of the high light cultures.

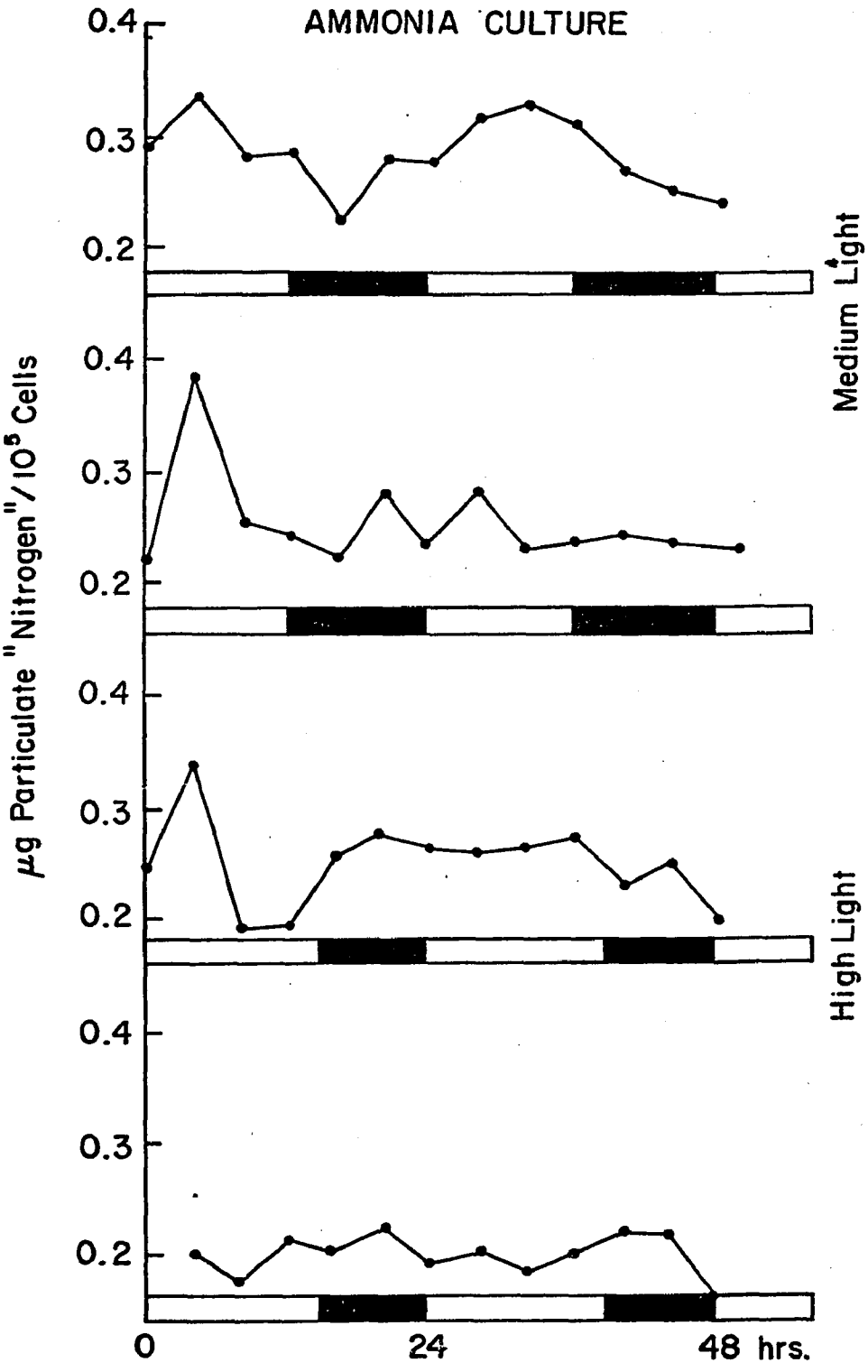
Average daily cell densities in the continuous cultures varied from approximately 7×10^5 to 9×10^5 cells/ml. Using the equations presented on pages 153 and 154, it can be calculated that these differences in cell density resulted in a maximum variation in light penetration in the experimental culture vessels of only 4.7%, and therefore would have been relatively insignificant in terms of overall culture kinetics.

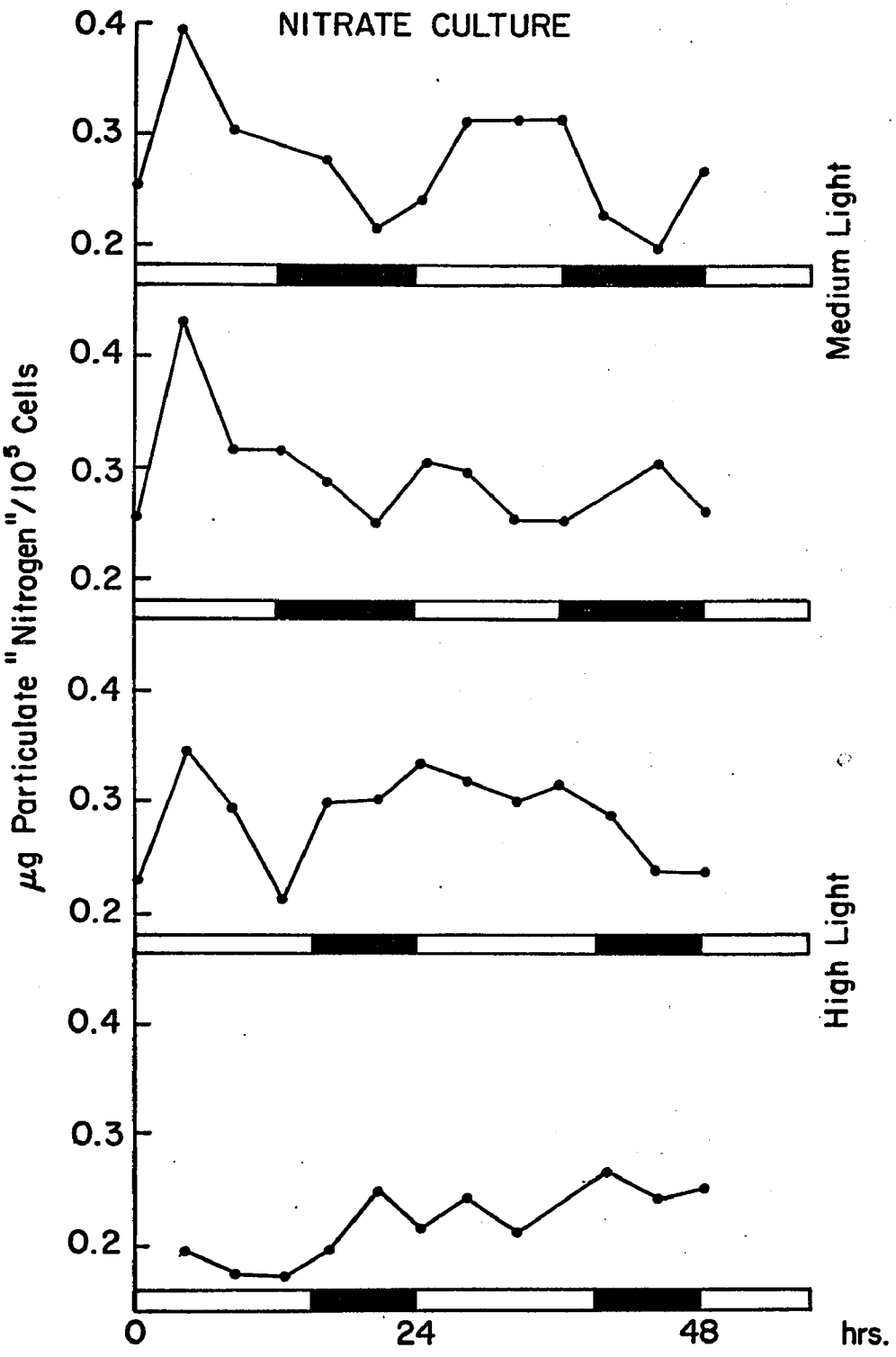
3.5 Total Particulate "Nitrogen"

Individual values for total particulate "nitrogen" on a per cell basis are presented in figure 3.8. Values for total particulate "nitrogen" were characterized by non-periodic fluctuations with a maximum amplitude of 0.175 µg total N/10^5 cells.

Figure 3.8 Particulate "nitrogen" content of *Dunaliella tertiolecta*

µg particulate "nitrogen" cells x 10^{-5} vs time (hours)


AMMONIA CULTURE
0.4
0.3
0.2
Medium Light
High Light
μg Particulate "Nitrogen"/10⁵ Cells
0
24
48 hrs.


NITRATE CULTURE
0.4
0.3
0.2
Medium Light
High Light
μg Particulate "Nitrogen"/10⁵ Cells
0
24
48
hrs.

3.6 Total Particulate "Carbon"

Total particulate "carbon" showed diurnal periodicity under both light regimes in ammonia and nitrate culture (Figure 3.9).

3.7 Chlorophyll a and Lipid

Intracellular chlorophyll a and lipid responded similarly to light. Both showed a marked diurnal periodicity in response to the light:dark cycle and a doubling in baseline concentration in response to a halving in incident light (Figures 3.10 and 3.11). Under both light regimes, there was little quantitative difference in intracellular chlorophyll a and lipid content between ammonia and nitrate cultured cells.

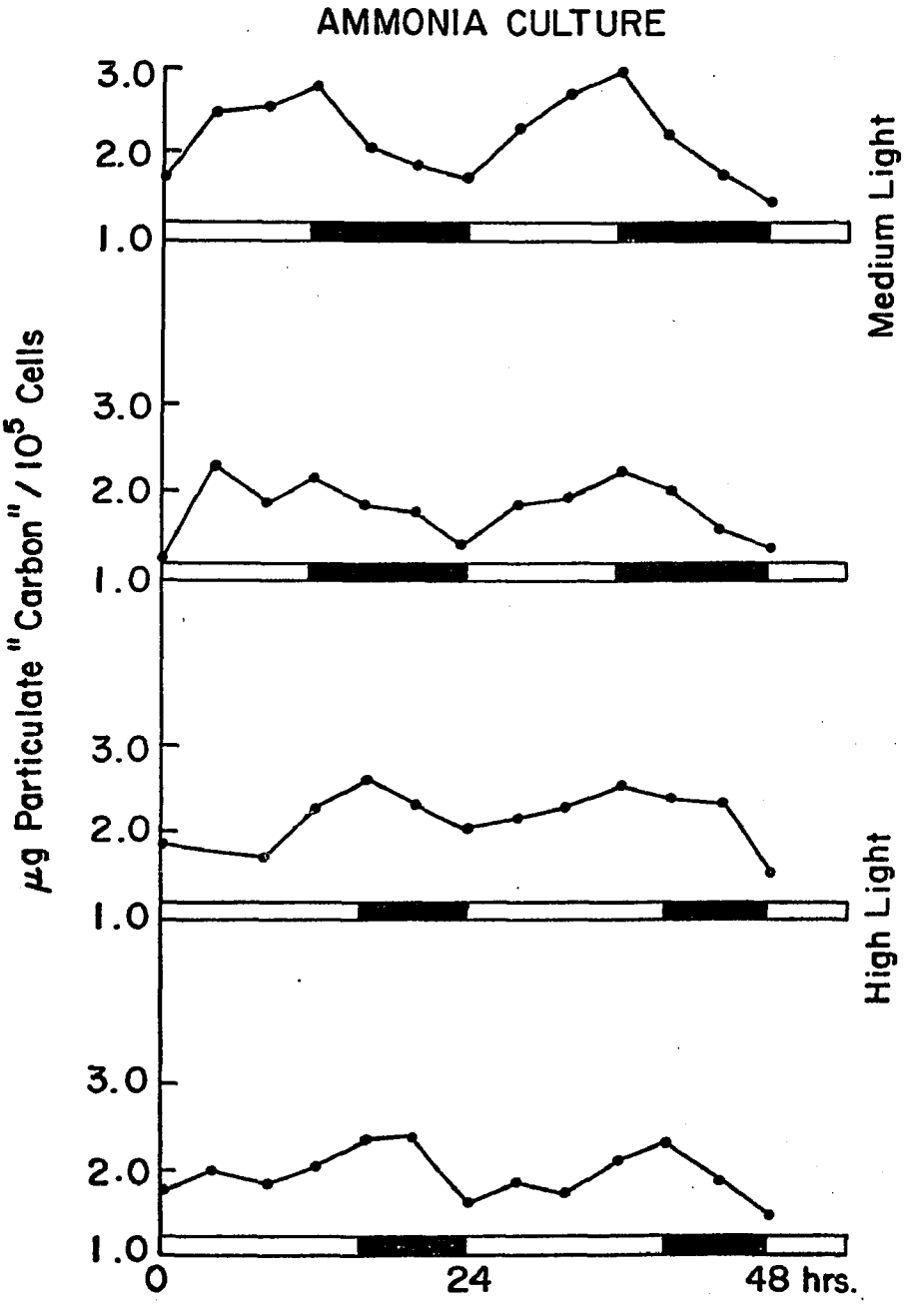
Mean values of chlorophyll a per cell for *Dunaliella tertiolecta* in the steady state continuous cultures were of the same order of magnitude as values reported for *Phaeodactylum tricornutum* by Griffiths (1973), but about one-half as concentrated as the mixed phytoplankton cultures studied by Eppley et al. (1971). Constantopoulos and Block (1967), working with photoauxotrophic cultures of *Euglena gracilis* found that under constant illumination at varying light intensities, the same relationship between chlorophyll a and light existed.

3.8 ATP

Cellular ATP content in both ammonia and nitrate cultures showed similar patterns of periodicity, depending on the light regime used. Unlike the other intracellular parameters measured, the periodicity shown by cellular ATP was markedly out

Figure 3.9 Particulate "carbon" content of *Dunaliella tertiolecta*

µg particulate carbon cells x 10^{-5}
vs time (hours)


AMMONIA CULTURE
3.0
2.0
1.0
Medium Light
3.0
2.0
1.0
μg Particulate "Carbon" / 10^5 Cells
3.0
2.0
1.0
High Light
3.0
2.0
1.0
0
24
48 hrs.

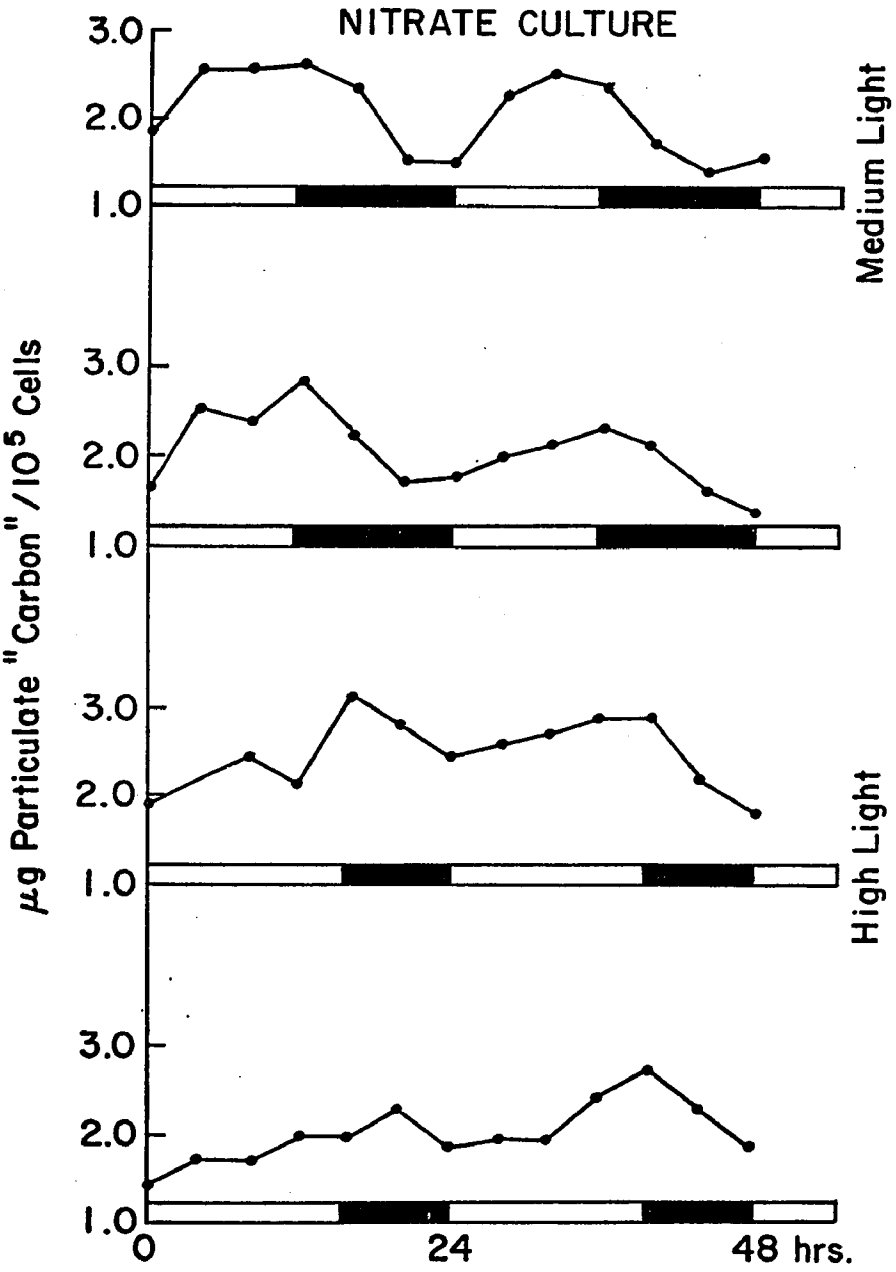
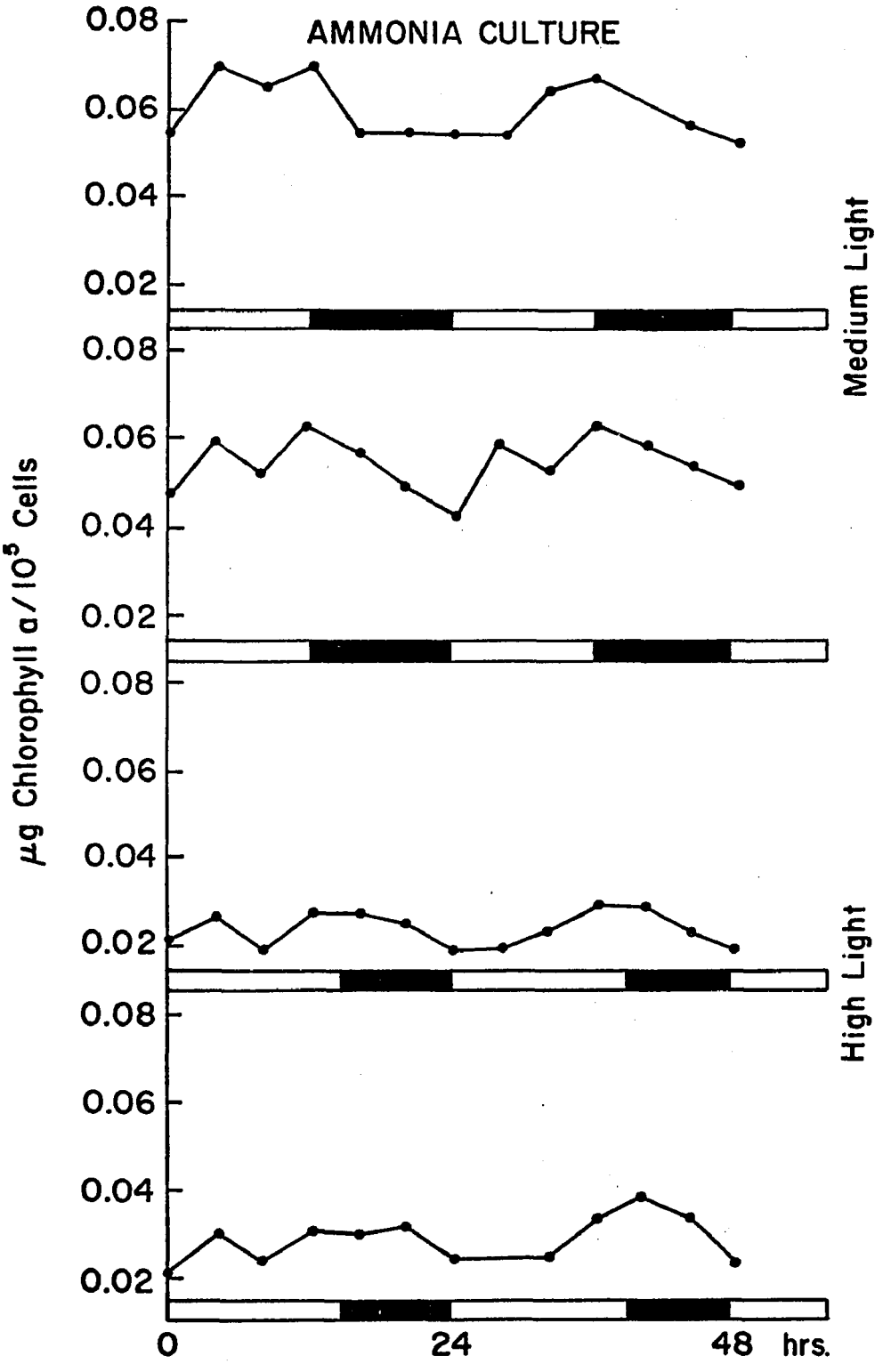

NITRATE CULTURE
3.0
2.0
1.0
Medium Light
3.0
2.0
1.0
3.0
2.0
1.0
High Light
3.0
2.0
1.0
0
24
48 hrs.
μg Particulate "Carbon" / 10^5 Cells

Figure 3.10 Chlorophyll a content of *Dunaliella tertiolecta*

µg chlorophyll a cells x 10^{-5}
vs time (hours)


AMMONIA CULTURE
0.08
0.06
0.04
0.02
Medium Light
High Light
μg Chlorophyll a / 10⁵ Cells
0
24
48
hrs.

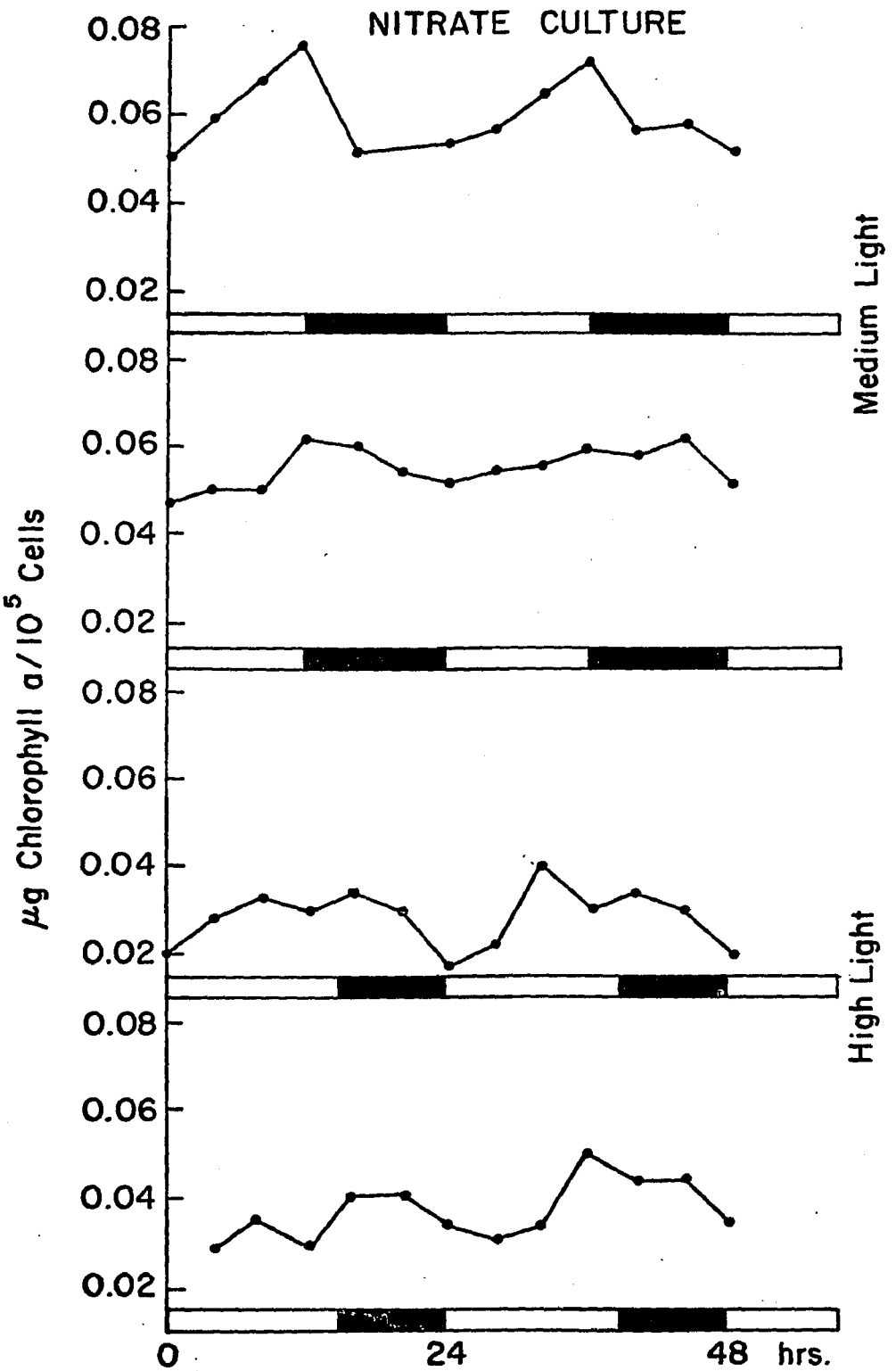
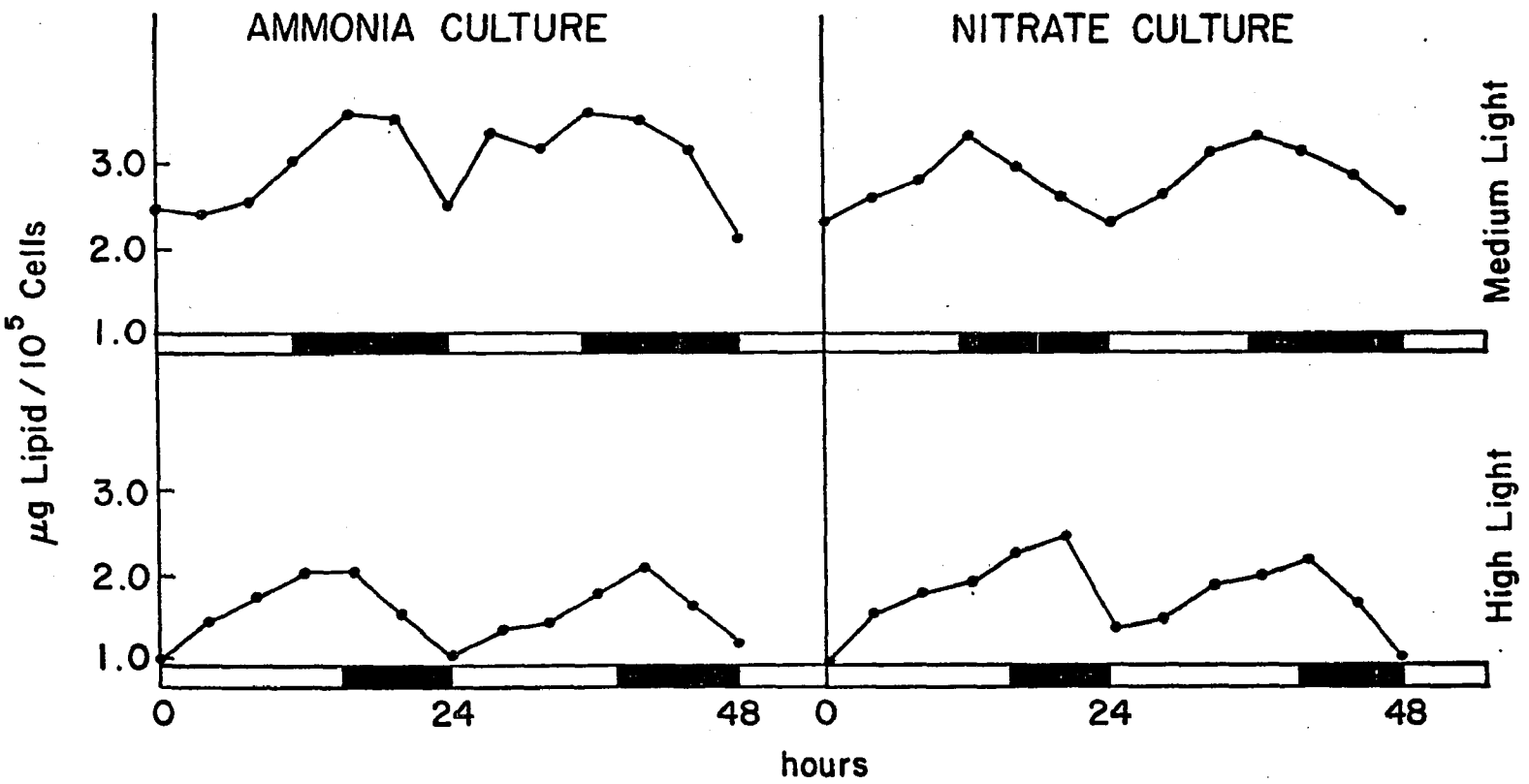

NITRATE CULTURE
0.08
0.06
0.04
0.02
Medium Light
High Light
μg Chlorophyll a/10^5 Cells
0
24
48 hrs.

Figure 3.11 Lipid content of *Dunaliella tertiolecta*

µg lipid cells x 10^{-5} vs time (hours)


AMMONIA CULTURE
NITRATE CULTURE
Medium Light
High Light
μg Lipid / 10^5 Cells
3.0
2.0
1.0
0
24
48
hours

of phase with the light:dark cycle (Figure 3.12).

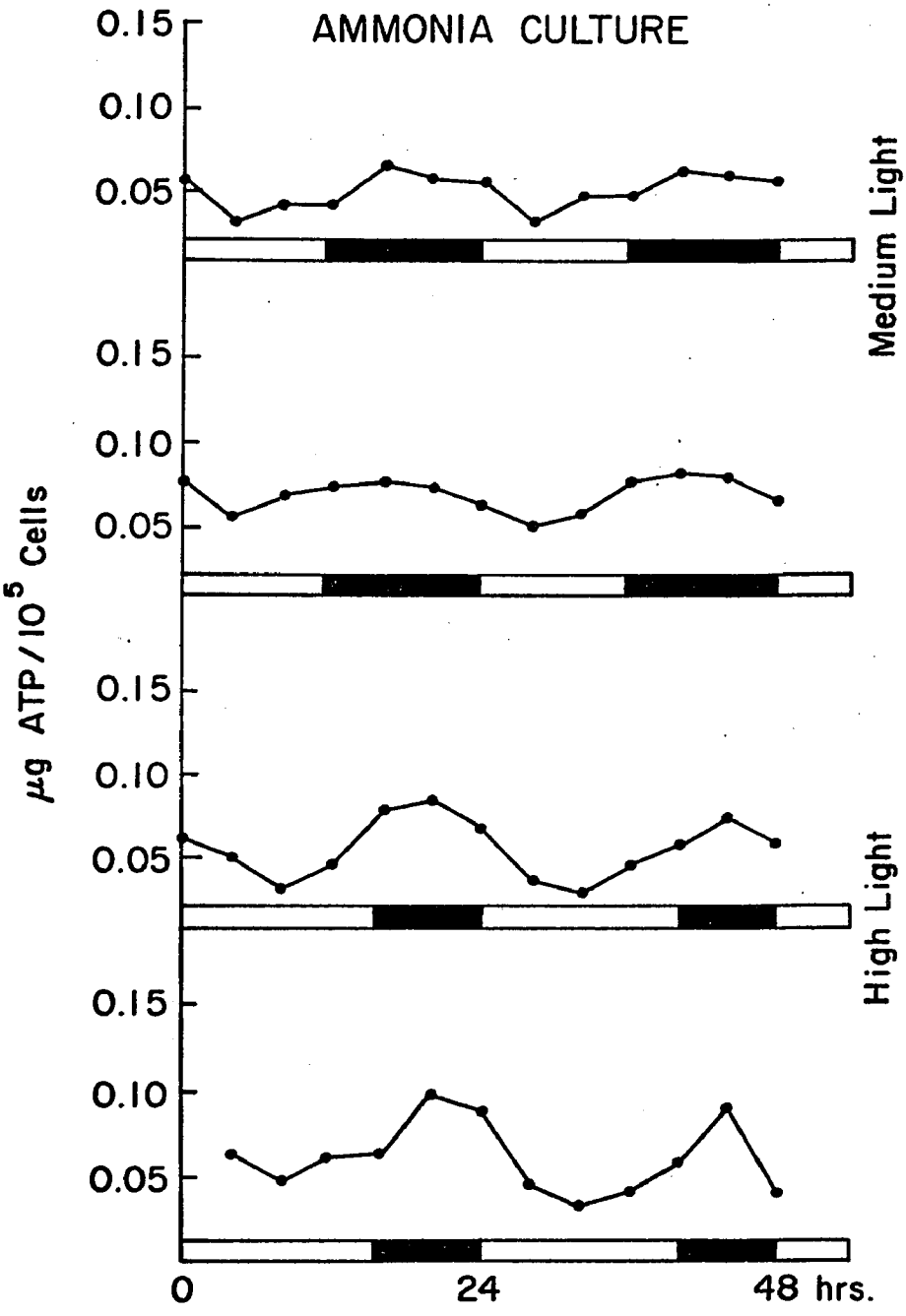
Under high light conditions in ammonia culture, cellular ATP reached minimum values, and began the daily increase at 14:00 hours, eight hours after the onset of the light cycle. Peak ATP values, and the beginning of the daily decline occurred at 02:00 hours, four hours after the onset of the dark phase. Under high light conditions in nitrate cultures, a four-hour shift in maximum and minimum ATP values was noted. Minimum ATP values occurred at 10:00 hours, four hours after the onset of the light cycle. Maximum ATP values occurred between 22:00 and 02:00 hours, 0-4 hours after the onset of the dark phase. Under medium light conditions, in both ammonia and nitrate medium, minimum ATP values and the beginning of the daily increase occurred at 10:00 hours, four hours after the onset of the light phase. Maximum ATP values occurred between 18:00 and 22:00 hours, 0-4 hours after the onset of the dark phase.

3.9 Carbohydrate and Protein

Intracellular carbohydrate and protein showed a marked diurnal periodicity closely attuned to the light:dark cycle under which the cells were cultured. Both carbohydrate and protein content decreased in response to the decrease in total daily irradiance. Under both light regimes, ammonia grown cells had lower mean protein concentrations than nitrate grown cells (Figures 3.13 and 3.14).

A summary of the patterns of diurnal periodicity shown by

Figure 3.12 ATP content of *Dunaliella tertiolecta*
μg ATP cells x 10^{-5} vs time (hours)


AMMONIA CULTURE
0.15
0.10
0.05
Medium Light
High Light
μg ATP / 10^5 Cells
0
24
48 hrs.

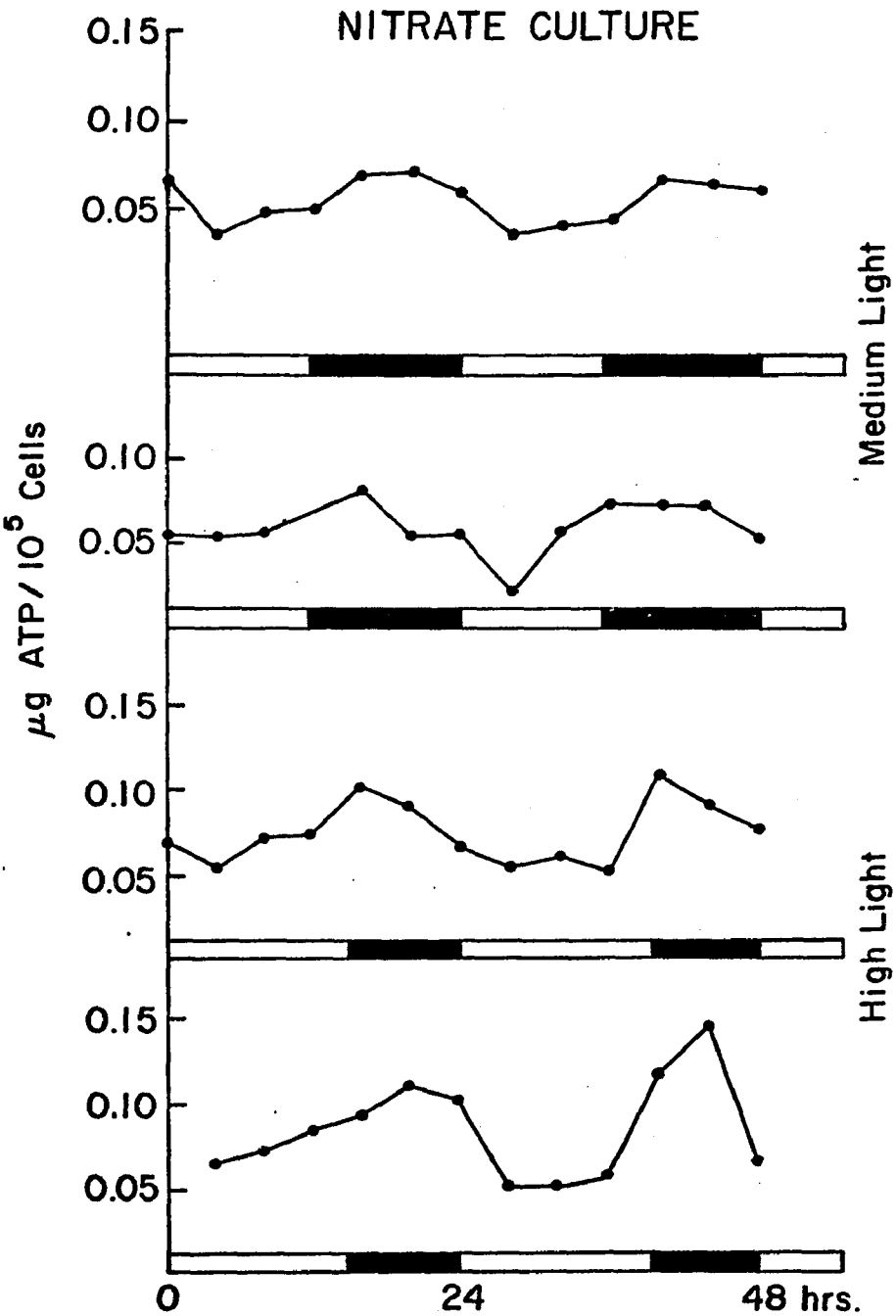
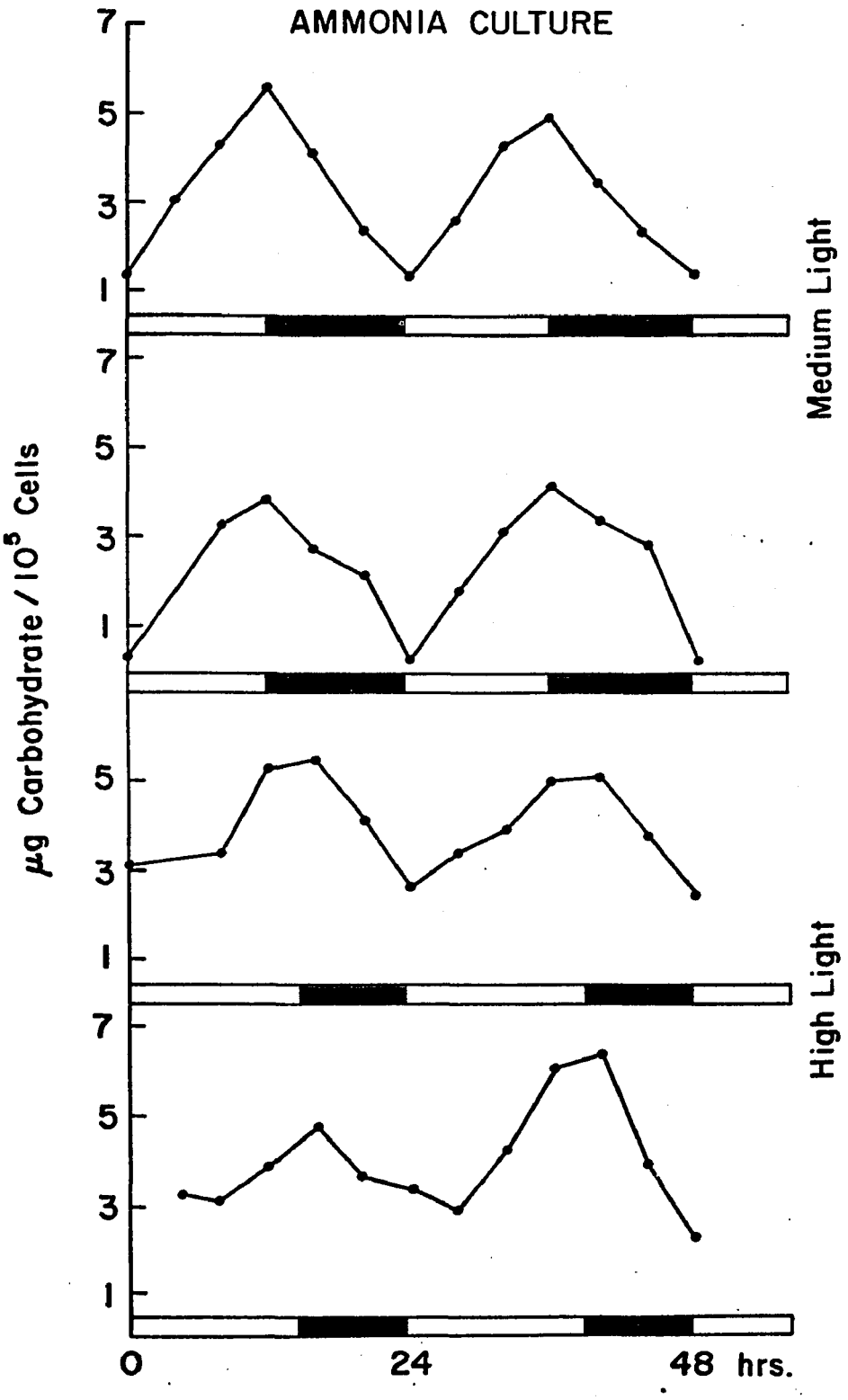

NITRATE CULTURE
0.15
0.10
0.05
Medium Light
0.10
0.05
μg ATP / 10^5 Cells
0.15
0.10
0.05
High Light
0.15
0.10
0.05
0
24
48 hrs.

Figure 3.13 Carbohydrate content of *Dunaliella tertiolecta*

µg carbohydrate cells x 10^{-5}
vs time (hours)


AMMONIA CULTURE
μg Carbohydrate / 10⁵ Cells
7
5
3
1
Medium Light
High Light
0
24
48 hrs.

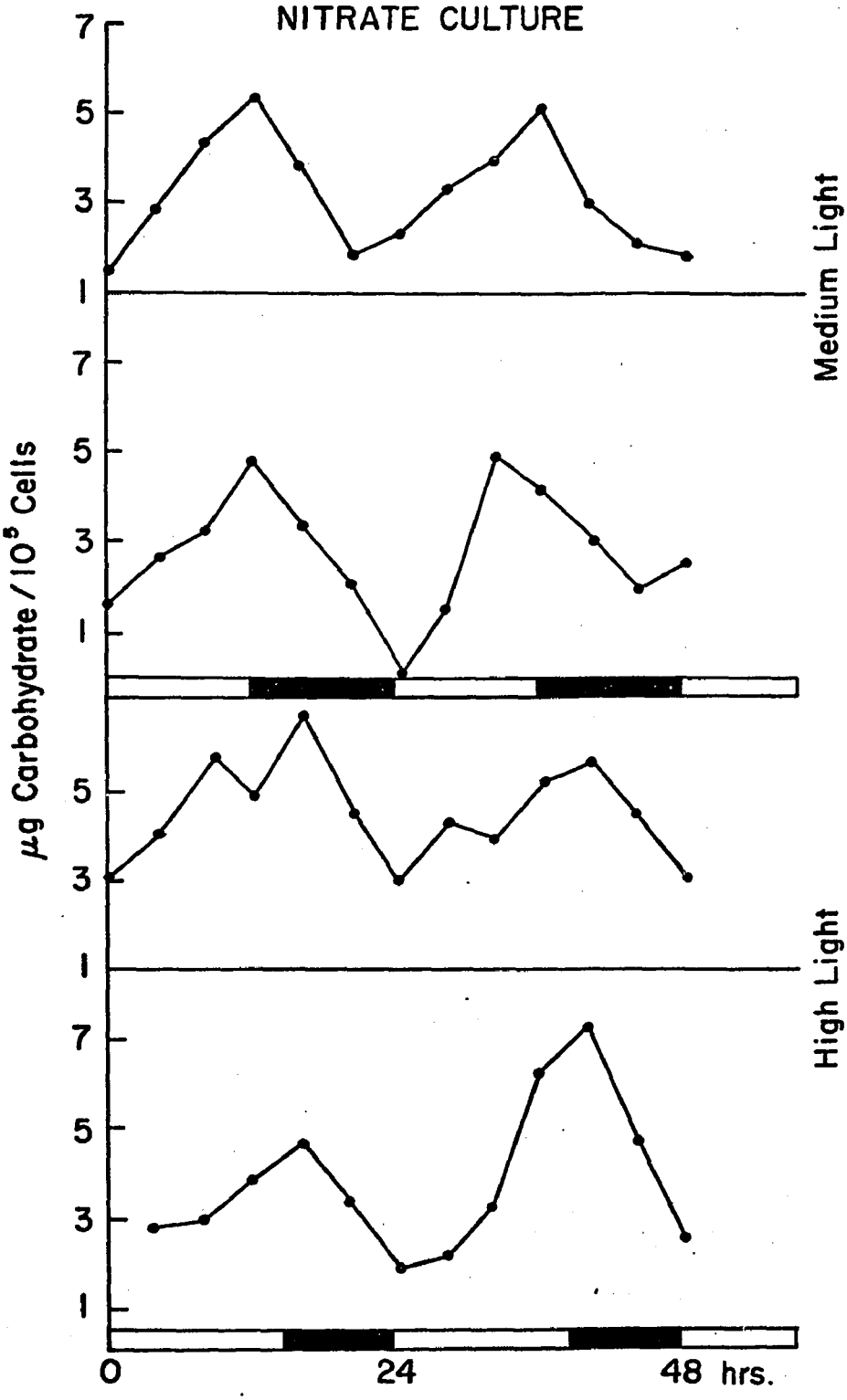
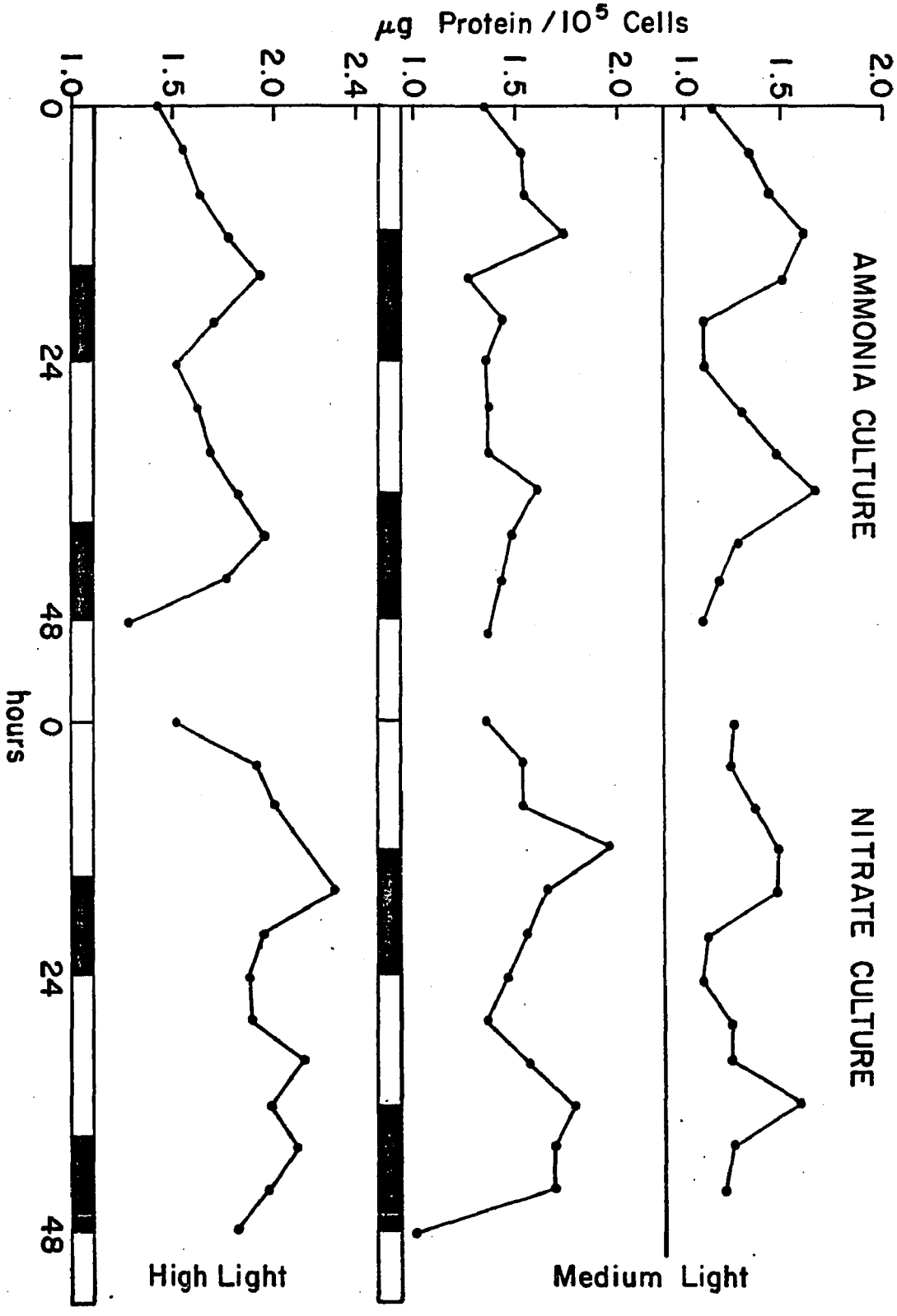

NITRATE CULTURE
7
5
3
1
Medium Light
High Light
μg Carbohydrate / 10⁵ Cells
0
24
48 hrs.

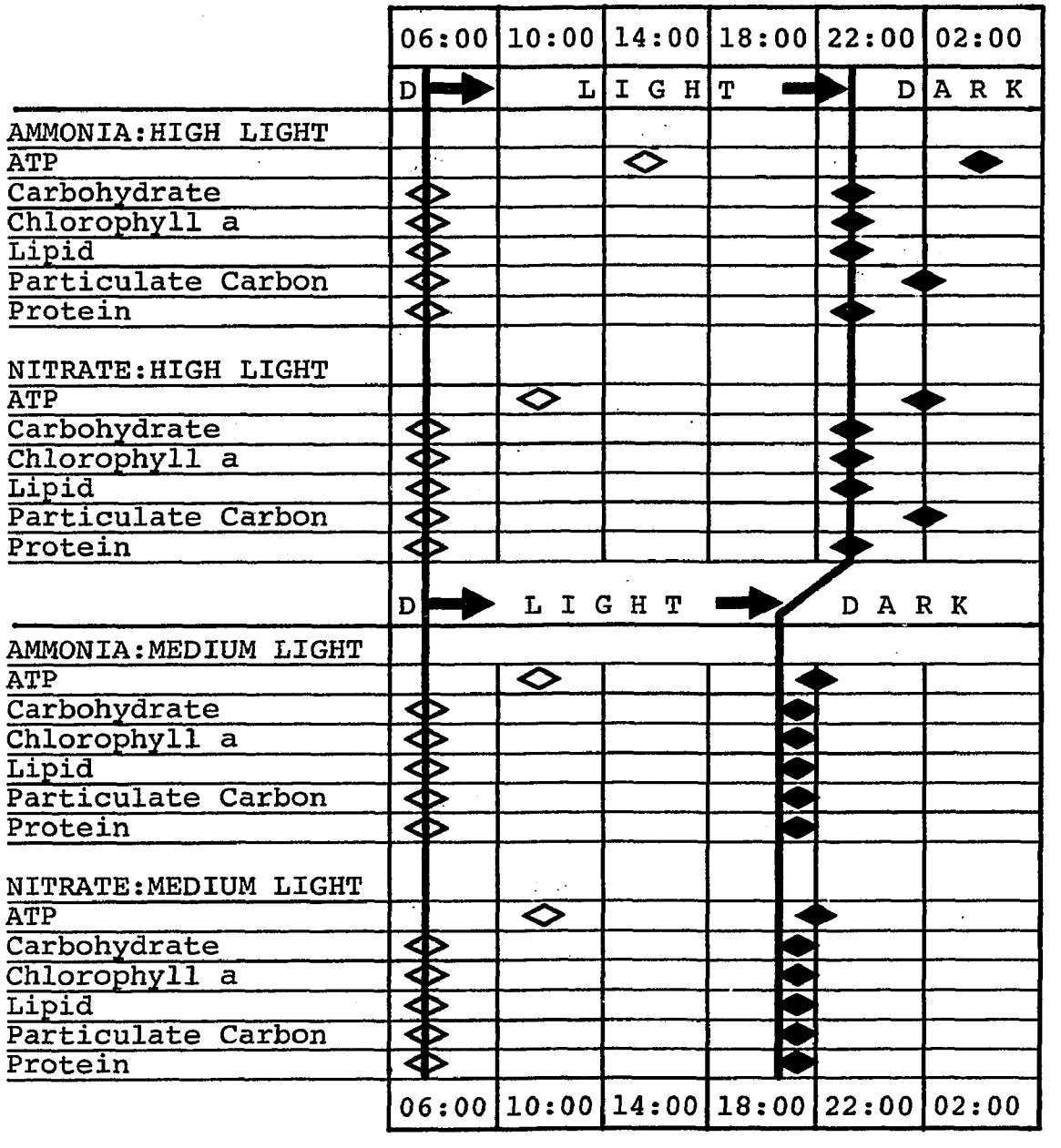
Figure 3.14 Protein content of *Dunaliella tertiolecta*

µg protein cells x 10^{-5} vs time (hours)


μg Protein / 10⁵ Cells
AMMONIA CULTURE
NITRATE CULTURE
hours
High Light
Medium Light

intracellular carbohydrate, protein, lipid, chlorophyl *a*, ATP and particulate "carbon", and their responses to ammonia and nitrate substrates and changing light regimes are presented in table 3-d.

TABLE 3-d



SUMMARY OF THE PERIODICITIES SHOWN BY VARIOUS CELLULAR COMPONENTS OF *DUNALIELLA TERTIOLECTA*

4 DISCUSSION

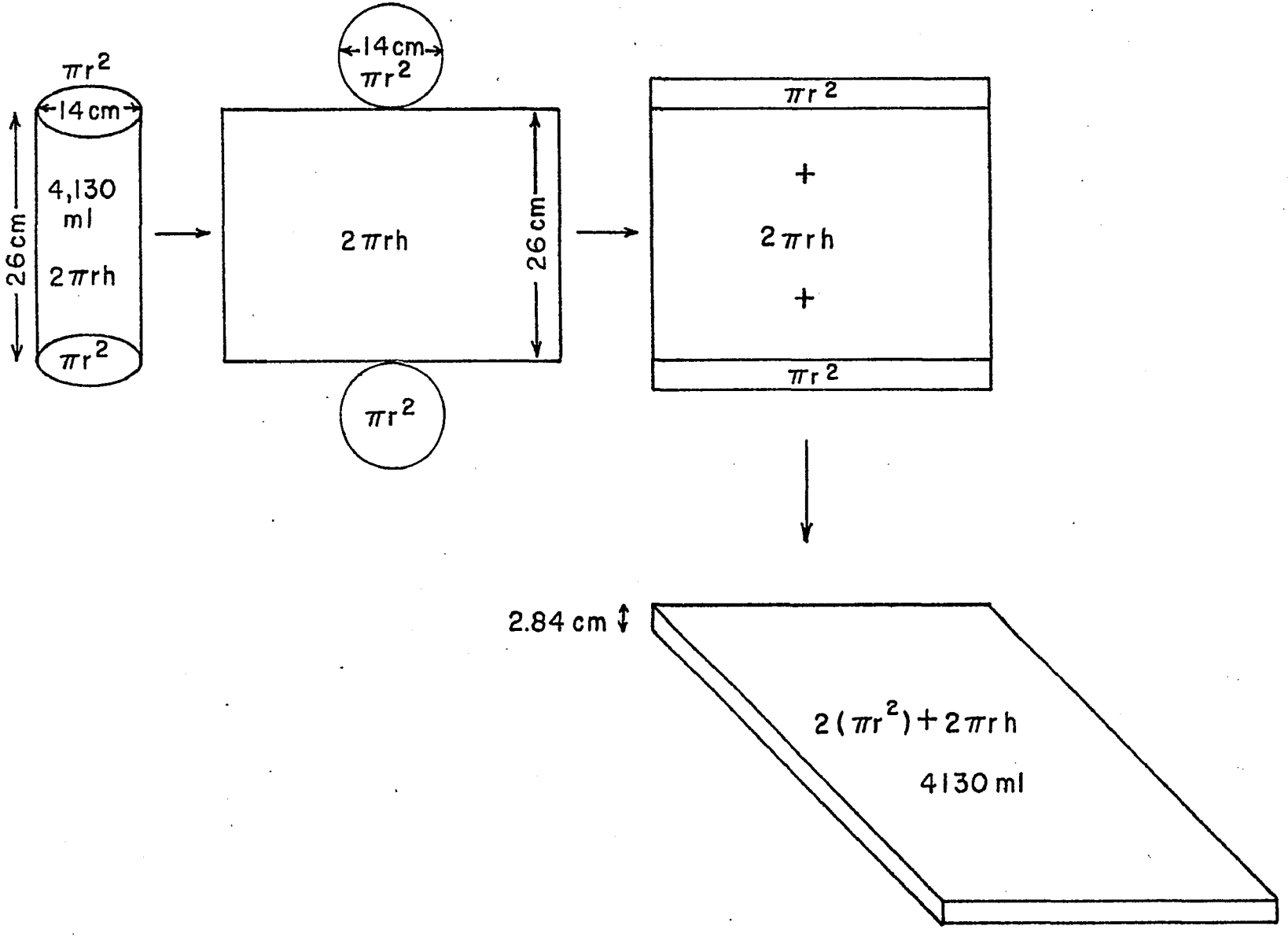
4.1 Light and Growth

Most light attenuation equations assume unidirectional illumination in a plane perpendicular to the depth gradient. These equations are therefore not directly applicable to the experimental culture vessels, which were cylindrical in shape and illuminated from all sides. For comparative purposes, the depth of culture in the experimental vessel was taken to be the depth occupied by 4130 ml (the volume of the culture vessel) in a rectangular tank with a surface area equal to the surface area of the experimental vessel (Figure 4.1). Based on this, the depth of culture in the experimental vessels was 2.84 cm (less than the radius of the culture).

The parameter of major importance in a study of the effects of light on growth and cellular composition is the total quantity of light absorbed by each cell in the culture. The average light absorbed in a 1 cm^2 column of culture can be obtained from the expression: (Jerlov 1968)

$$I_{D_1} = I_0 e^{-KD_1} \text{ and } I_0 - I_D = \text{gm cal abs. under a } cm^2 \text{ area of depth } D_1$$

Figure 4.1 Conversion of culture vessel dimensions to approximate conditions of unidirectional illumination


πr²
14 cm
4,130 ml
2πrh
26 cm
πr²
14 cm
πr²
2πrh
26 cm
πr²
πr²
+
2πrh
+
πr²
2.84 cm
2(πr²)+2πrh
4130 ml

Where: I_O = light incident on the culture surface (corrected for reflectance).

I_D = light remaining at depth D.

D_1 = depth in cm.

K = the attenuation (extinction) coefficient.

The attenuation coefficient K is composed of three additive parameters, absorbance by water, absorbance by cells, and scatterance by the cells. In a dense cell suspension, the effects of scatterance can be ignored since "scattering within a cell suspension (which tends to > absorption/unit pathlength) is approximately compensated for by the packaging effect (which causes the absorption of a cell suspension to be less than that of the dissolved pigments)." (Bannister 1974). Absorbance by water is a constant, equal to .004 (Riley 1956), and should be omitted from the total "K" if absorbance by the cells alone is to be calculated. Absorbance by the cells is dependent on cell density, the chlorophyll content of the cells, and the coefficient of absorption of light/unit plant pigment (generally taken to be per unit chlorophyll a). Bannister (1974) expressed this relationship as KcC, where Kc is the coefficient of absorption/unit chlorophyll a, and C is the chlorophyll concentration/unit volume of culture. Adopting Bannister's notations, the light energy absorbed by the cells in a cm^2 column of culture of given depth is:

$$I_{D_1} = I_O e^{-(kcC)D_1} \text{ and } I_O - I_D = \frac{gm}{cm^2} \text{ cal abs. under a area of depth } D_1$$

C is the product of: (µg chlorophyll *a*/10^5 cells) x $\frac{\text{cells/ml}}{10^5}$, and since both these parameters were measured in the experimental high and medium light cultures, C could be calculated directly. Chlorophyll concentration in the low light cultures was not measured, but since chlorophyll *a* concentration shows an inverse hyperbolic response to changing light intensity, low light chlorophyll concentration could be approximated from a modified Michaelis Menten type curve constructed to fit the experimental data.

To calculate Kc, it was assumed that absorption/unit chlorophyll is constant at any light intensity within the maximum response portion of the chlorophyll:light curve (Bannister 1974). The absorption spectrum of a medium light adapted culture of *Dunaliella* (0.0589 µg chlorophyll *a*/10^5 cells) of known cell density was measured over the range 400-700 nm. Kc was calculated as follows based on the mean percent absorbance over this range:

$$Kc = (\text{abs/cm culture}) - (\text{abs/cm medium}) \div \left(\frac{\text{cell density}}{10^5}\right)(\text{chl } a/10^5 \text{ cls})$$

Based on this, Kc for the experimental cultures was 0.33. Table 4-a presents the resultant values per gm cal abs/min/cm^2 of culture (2.84 cm deep) and gm cal abs/day.

From the values shown in this table, the energy absorbed/cell/minute or per day can be calculated as follows (Table 4-b).

TABLE 4-a. LIGHT ABSORBED BY THE EXPERIMENTAL CULTURES PER cm^2 CELLS

Light Regime	gm cal abs/cm^2x2.84/min	gm cal abs/cm^2x2.84/day
high light	.0046	4.14
medium light	.00453	3.26
low light	.00380	2.052

TABLE 4-b. LIGHT ABSORBED BY THE EXPERIMENTAL CULTURES PER 10^5 CELLS

Light Regime	gm cal abs/10^5cells/min	gm cal abs/10^5cells/day
high light	0.00021	0.189
medium light	0.00020	0.144
low light	0.00018	0.097

$$\text{gm cal abs}/10^5 \text{ cells/min} = \frac{(\text{gm cal abs}/2.84)}{(\text{cell density}/10^5)}$$

$$\text{gm cal abs}/10^5 \text{ cells/day} = (\text{gm cal abs}/10^5 \text{ cells/min}) \cdot (60) \cdot (h)$$

In the maximal response portion of the chlorophyll: light curve, a decrease in incident light results in a proportional increase in chlorophyll a content, so that the energy absorbed per cell remains relatively constant. Thus, cells of *Dunaliella tertiolecta* grown under high and medium light fix relatively similar amounts of carbon/day, while the carbon fixed:chlorophyll a ratio decreases by roughly one half (Table 4-c).

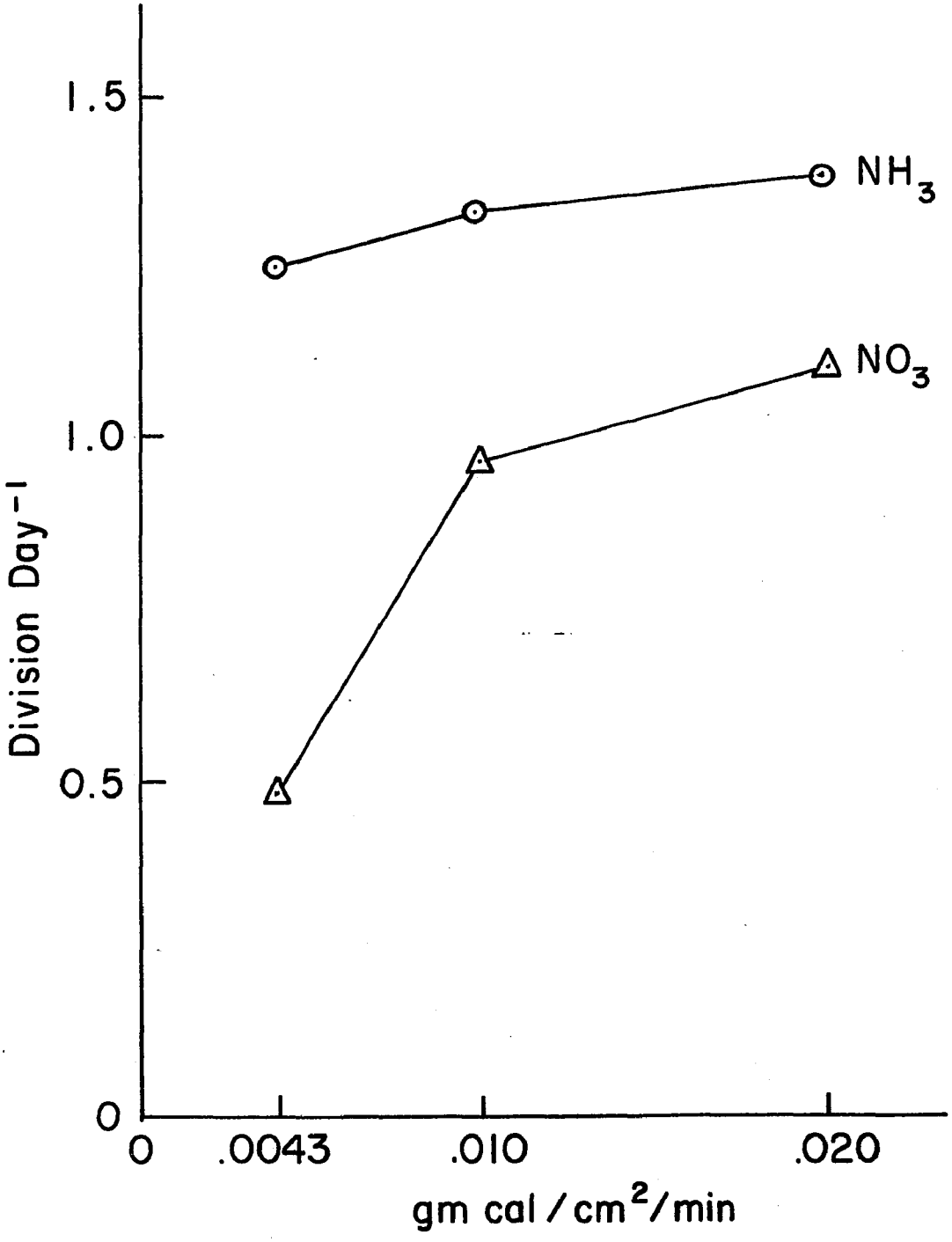
As a result, observed daily growth rates at these low intensities might be expected to become primarily dependent on the length of the photoperiod (i.e., total calories of irradiance/cm^2/day). In fact, this is the case for ammonia grown cells (Figure 4.2). When 24-hour growth rates for ammonia grown cells are calculated on the basis of divisions hour illumination^{-1} x 24, the resultant plot is a very shallow hyperbola, indicating that the increase in chlorophyll a content (shade adaptation) is almost completely compensating for the decrease in incident light. However, when this same calculation is applied to nitrate grown cells, the resultant plot is a much steeper hyperbola, suggesting (in view of the essentially similar chlorophyll a content of ammonia and nitrate grown cells) that another light dependent mechanism is operating to limit growth in nitrate culture.

Grant (1968) reported that the light-dependent component

TABLE 4-c. CARBON FIXATION AND CELLULAR CHLOROPHYLL *a*

Light Regime	µg C fixed/10^5 cells/day	µg C fixed:µg Chl *a*
high light ammonia	10.42	388
high light nitrate	12.32	382
med light ammonia	9.48	165
med light nitrate	15.24	252

Figure 4.2 Division rates and incident light
Division day^{-1} vs gm $cal/cm^2/min$


1.5
1.0
0.5
0
Division Day⁻¹
NH3
NO3
0
.0043
.010
.020
gm cal / cm² / min

of nitrate assimilation in *Dunaliella tertiolecta* becomes saturated at intensities of approximately 340 foot candles (0.018 ly/min)*. Thus it seems that for nitrate grown cells, light limited growth at low intensities may be mediated by nitrate assimilation ability rather than by carbon reducing ability.

In developing a model for light limited growth of *Dunaliella tertiolecta*, the relationship between light intensity, chlorophyll a concentration, efficiency of light absorption, and energy transfer into growth must be established. For nitrate grown cells, a light limited growth model may also have to include the effects of light on nitrate assimilation at low intensities.

The response of Chlorophyll a to changing light intensity is inversely hyperbolic and can be described by the following modification of the Michaelis Menten equation:

$$Vm - \frac{VmS}{Ks + S} = V$$

where: Vm = maximum chlorophyll concentration µg chlorophyll a/10^5 cells

Ks = S where V = Vm/2

S = Light intensity

Such a curve was calculated for *Dunaliella* by a modification of the linear transformation of the Michaelis Menten equation given in Eppley, Rogers and McCarthy (1969), figure 4.3.

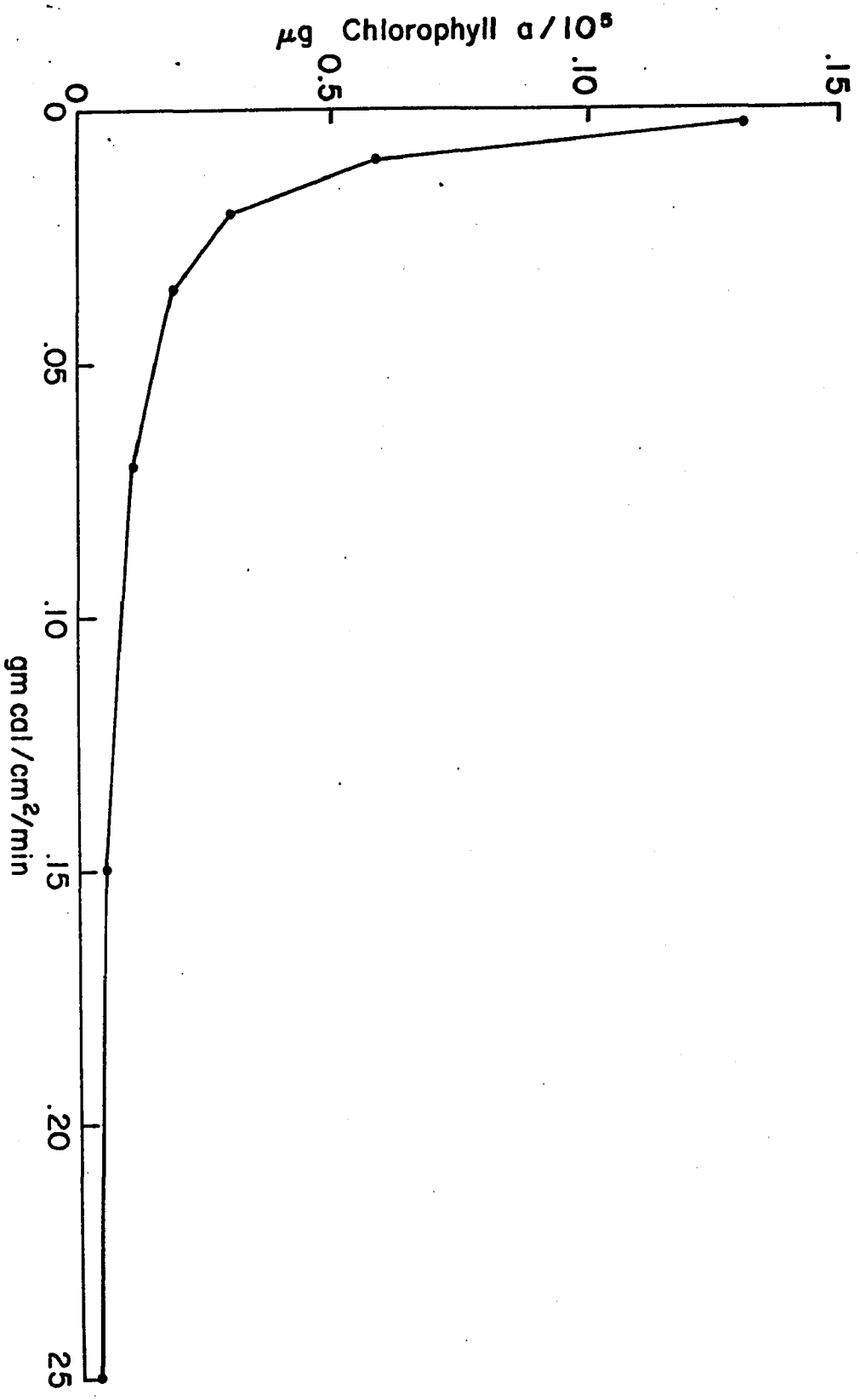
Implicit in Bannister's (1974) Kc is the idea that a constant proportion of I is absorbed/unit chlorophyll a, at

---

* By a conversion factor from Parsons and Takahashi (1974).

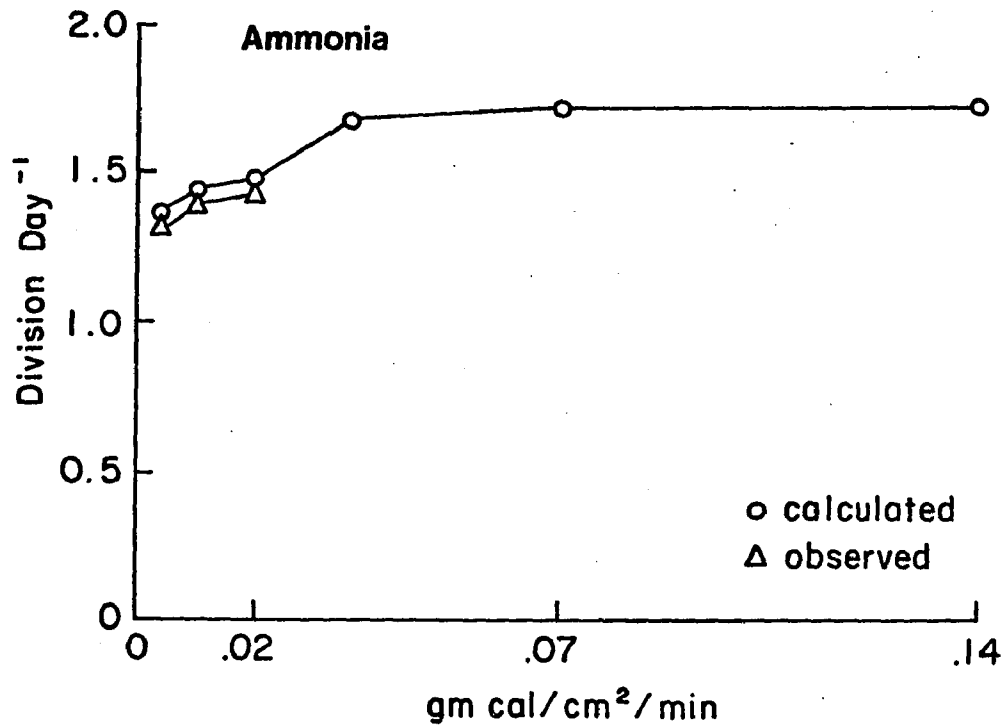
Figure 4.3 Response of cellular chlorophyll *a* to changing light intensity

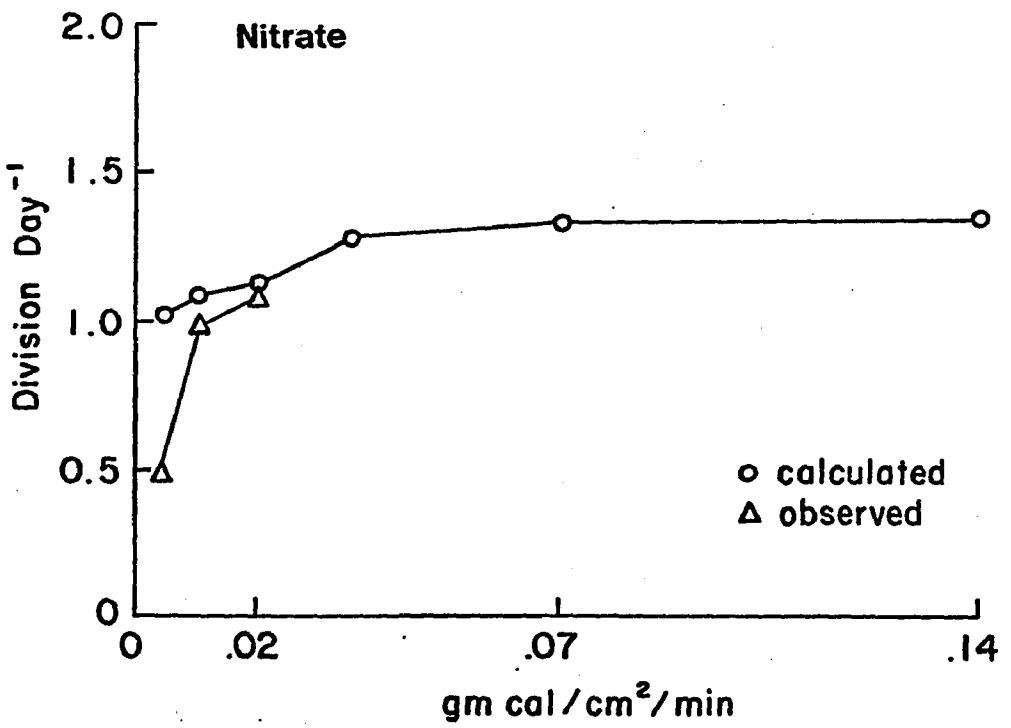
µg chl *a*/10^{-5} cells vs gm cal/cm^2/min


μg Chlorophyll a / 10⁵
0
0.5
.10
.15
0
.05
.10
.15
.20
25
gm cal / cm²/min

Figures 4.4 and 4.5 Observed and calculated growth rates for *Dunaliella tertiolecta* in ammonia and nitrate medium

Divisions day^{-1} vs gm $cal/cm^2/min$


Ammonia
2.0
1.5
1.0
0.5
0
Division Day⁻¹
o calculated
Δ observed
0
.02
.07
.14
gm cal/cm²/min


Nitrate
2.0
1.5
1.0
0.5
0
Division Day⁻¹
o calculated
Δ observed
0
.02
.07
.14
gm cal/cm²/min

least within the maximum response portion of the chlorophyll: light curve. This proportion was calculated to be 0.33 for *Dunaliella*. Therefore:

$$(0.33 \times I)\ (\mu g\ chl\ \underline{a}/10^5\ cells) = gm\ cal\ abs/10^5\ cells/min$$

where: 0.33 = proportion of I absorbed/μg chlorophyll a equivalent to Kc.

and

$$(gm\ cal\ abs/10^5\ cells/min)\ (60)\ (h) = gm\ cal\ abs/10^5\ cells/day$$

where h = photoperiod hours.

Based on the experimental data, at an intensity of 0.02 ly/min 10^5 ammonia grown cells of *Dunaliella* must absorb 0.2 gm calories to accomplish a division, while 10^5 nitrate grown cells require 0.26 calories. Eppley and Sloan (1966) have shown that growth rate is proportional to the product of light intensity x time over a wide range of intensities and levels of total daily irradiance. Thus:

$$\frac{gm\ cal\ abs/10^5\ cells/day}{gm\ cal\ required/10^5\ cells/division} = divisions/day$$

where: gm cal req/10^5 cells/division = 0.20 for ammonia
0.26 for nitrate

Figures 4.4 and 4.5 present a comparison of observed growth rates, and growth rates calculated by the following series for *Dunaliella tertiolecta* in nitrate and ammonia medium:

1) $C = Vm - \frac{VmS}{Ks + S}$ where: C = μg chl/10^5 cells

2) $(0.33 \times I)\ (C)\ 60 \times h = gm\ cal\ abs/10^5\ cells/day$

3) $\frac{\text{gm cal abs } 10^5 \text{ cells/day}}{\text{calories req}/10^5 \text{ cells/division}}$ = divisions/day

The close correspondence between the observed and calculated growth rates for *Dunaliella* in ammonia medium suggest that the model is an adequate description of growth kinetics at low light intensities in ammonia medium. However, the calculated growth rates for *Dunaliella* in nitrate medium gave values of 15% and 100% higher at 0.01 and 0.0043 ly/min, respectively, than those actually observed, although correspondence was good at 0.02 ly/min. Grant's (1967) data for the light dependent component of nitrate assimilation in *Dunaliella tertiolecta* indicates that the N assimilatory mechanism would be at 50% of saturation at 0.0043 ly/min, 80% of saturation at 0.01 ly/min, and 100% of saturation at 0.02 ly/min. These values agree well with the differences in calculated and observed growth rates, and in view of the absence of light: dark differences in nitrate uptake would seem to confirm the role of N assimilation kinetics in light limited growth at low light intensities, in nitrate medium.

If the model is to be generalized for both the nitrate and the ammonia medium, an additional term (N) must be included for percent saturation of the nitrogen assimilating mechanism: Thus, equation 3 becomes:

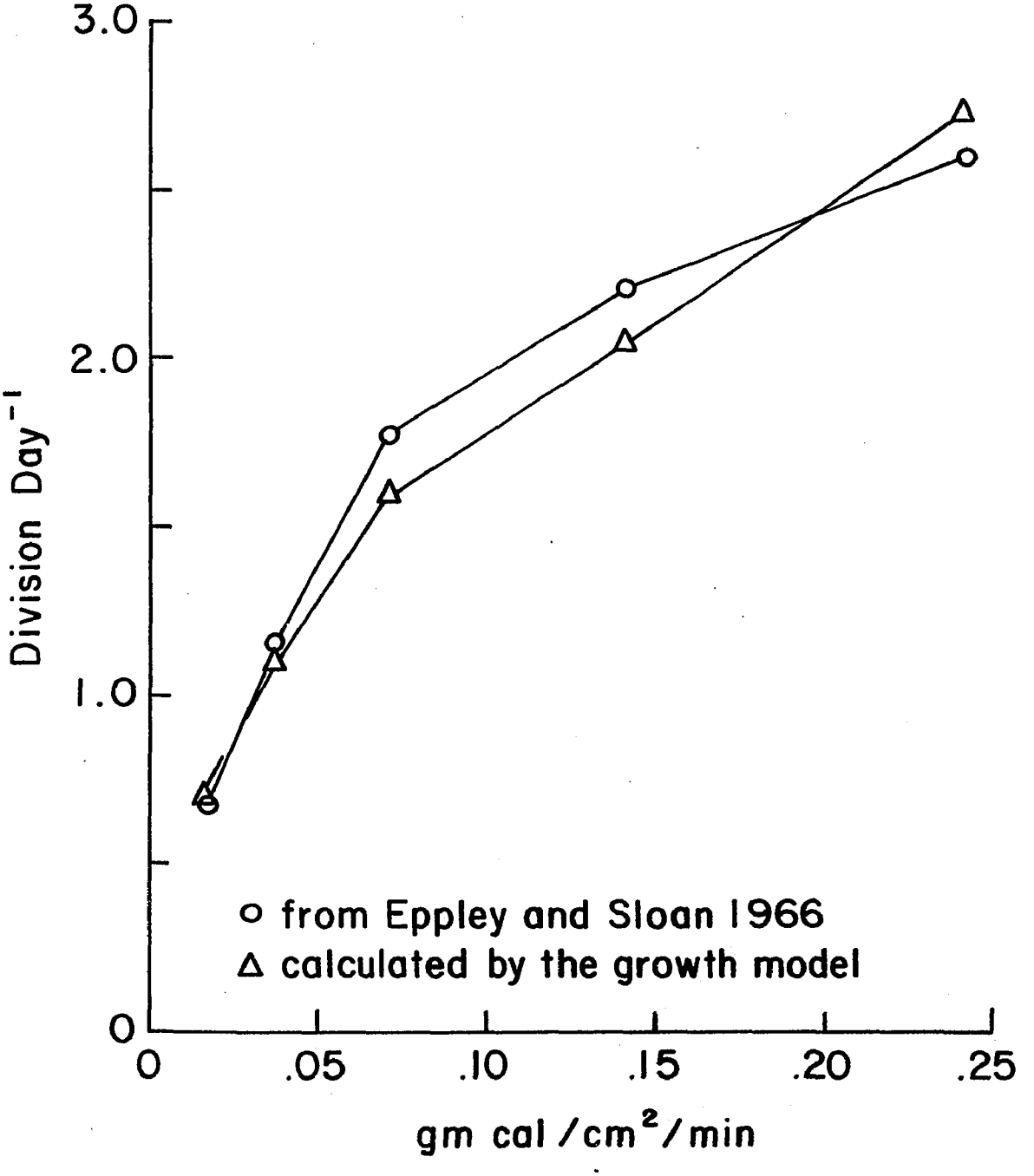
4) $\frac{\text{gm cal abs}/10^5 \text{ cells/min} \times 60 \times h}{\text{gm cal req}/10^5 \text{ cells/div}}$ (N) = div/day

where: N = a correction factor for percent saturation of the NO_3 assimilating mechanism. (N = 1 for ammonia grown cells at all light intensities studied).

To test the predictive validity of the model at higher light intensities, Eppley and Sloan's (1966) light, chlorophyll concentration, and growth rate data for *Dunaliella tertiolecta* were used. Eppley and Sloan's chlorophyll a data were substituted directly into equation 2 of the model. Figure 4.6 presents a comparison of Eppley's growth rate data and a theoretical curve calculated from the model. Correspondence between the two curves is generally good, although there is a tendency for the theoretical curve to overestimate growth at total daily irradiances higher than 274 gm cal/cm^2. It seems probable that this is due to a decrease in the coefficient of absorption/μg chlorophyll at high intensities, possibly related to a contraction or migration of chloroplasts resulting in increased self shading, similar to the behavior noted in *Lauderia borealis* by Kiefer (1973).

Finally, attention should be called to the shapes of the nitrate and ammonia, light limited growth curves generated by the model. As predicted by Eppley and Sloan (1966) and Eppley and Coatsworth (1968), the growth response of *Dunaliella tertiolecta* in nitrate medium is essentially hyperbolic. However, the response is hyperbolic not only because of the relationship between light absorbed and growth rate, but probably because of the interaction of the light-dependent component of nitrate assimilation at low intensities, and the relationship between light and chlorophyll a at higher intensities. In contrast, in ammonia medium, where growth rate is solely

Figure 4.6 Comparison of calculated growth rates for *Dunaliella tertiolecta* with those reported by Eppley and Sloan (1966)


3.0
2.0
1.0
0
Division Day⁻¹
o from Eppley and Sloan 1966
Δ calculated by the growth model
0
.05
.10
.15
.20
.25
gm cal /cm²/min

dependent on the light:chlorophyll relationship, growth rate shows a non-hyperbolic response to changing light due to a secondary plateau occurring at 0.01-0.02 ly/min, and resulting from the nearly linear inverse response of chlorophyll a to changing light in the maximal response portion of the light vs. chlorophyll curve.

4.2 "Nitrogen" Uptake and Assimilation

When "nitrogen" uptake is calculated based on the measured depletion of inorganic N from the culture medium, nitrate and ammonia grown cells of *Dunaliella tertiolecta* show relatively similar values, and a similar trend toward the accumulation of more N/cell at lower turnover rates.

When the "expected" concentrations of intracellular "nitrogen" based on uptake data are compared to the observed concentrations of Particulate "Nitrogen" measured in the cells, ammonia grown cultures show good correspondence, 100-108% of the NH_3 removed from the medium being accounted for as Particulate "Nitrogen". However, when the same comparison is made for nitrate grown cells, only 74-75% of the NO_3 removed from the medium can be accounted for as intracellular "N". The balance of the inorganic "N" removed from the medium must be presumed either to have been excreted by the cells in some organic form, or to have been part of the intracellular inorganic nitrate pool which was lost during the preparation of the cells for Particulate "Nitrogen" analysis.

When intracellular protein content measured by the Lowry technique is compared with protein content calculated from

Particulate "Nitrogen" data by the factor 6.25, nitrate grown cells show good correspondence between the two values. When the same comparison is made for ammonia grown cells, only 62-74% of the measured Particulate "Nitrogen" content can be accounted for as protein. Ammonia grown cells of *Dunaliella* thus appear to contain a large pool of non-protein organic nitrogen similar to the intracellular pool of soluble amide and amino nitrogen found in ammonia grown cells of the diatom *Biddulphia aurita* (Lui and Roels 1972). Since high concentrations of ammonia are toxic to the cell, it seems probable that this intracellular amino and amide nitrogen pool may represent an alternative method of storing cell nitrogen against later utilization in protein synthesis (Tabel 4-d).

TABLE 4-d

KINETICS OF NITROGEN METABOLISM IN *DUNALIELLA TERTIOLECTA*

	μeq N/10^5 cells			Protein μg/10^5 cells			
Light regime	as P.N.	N. depletion	N as cell N	Lowry	P.N. x 6.25	% P.N.	% non protein P.N.
Hi lt. NH_3	0.025	0.023	108%	1.63	2.21	74%	26%
Med lt. NH_3	0.026	0.026	100%	1.38	2.24	62%	38%
Hi lt. NO_3	0.0179	0.024	74.5%	1.65	1.56	105%	0%
Med lt. NO_3	0.020	0.027	74%	1.62	1.77	92%	8%

4.3 Cellular Components

4.3.1 Chlorophyll a and Lipid

As noted previously, chlorophyll a content showed a diurnal periodicity in response to length of photoperiod and an inverse relationship between mean daily chlorophyll content and light intensity, a characteristic "sun" and "shade" adapting response (Jorgensen and Steeman-Nielsen 1965; Ryther and Menzel 1959; Steeman-Nielsen and Jorgensen 1968).

Based on the data presented in table 4-e, it seems that the amplitude of the daily change in cell chlorophyll and the overall response of cellular chlorophyll a content to light are relatively independent of each other, and may be under the control of separate aspects of daily irradiance. The majority of the adaptive increases or decreases in chlorophyll a content in response to changing intensity appears in the daily minimum (baseline) values. Thus, cells of *Dunaliella*, which had been exposed to the experimental light intensities for up to two weeks before sampling was started, had developed a specific chlorophyll content which was maintained throughout the daily light-dark cycle. In contrast, the changes in cellular chlorophyll a content which result in the observed daily amplitude are relatively constant, although they represent different percentages of total cell chlorophyll in the high light and medium light cells.

The observed daily amplitude of changes in cellular chlorophyll a seems to be unrelated to the light regime.

TABLE 4-e

COMPONENTS OF THE PERIODIC RESPONSE OF CELLULAR CHLOROPHYLL A

Light Regime	Mean Chl a $\mu g/10^5$ cells	Daily min. value	Amplitude Chl day -1 (Daily max-daily min)
Hi lt. NH_3	.0268	.020	.011
Med lt. NH_3	.0575	.0505	.014
Hi lt. NO_3	.0321	.0260	.013
Med lt. NO_3	.0603	.0510	.014

The similarity between the behavior of intracellular lipid and chlorophyll a in *Dunaliella tertiolecta*, coupled with the fact that lipid is not a typical storage product of the *Chlorophyceae* (Fritsch 1935), suggests that most of the measured lipid may be associated with the lamellar glycolipid fraction of chlorophyll. Like cellular chlorophyll a, daily minimum (baseline) lipid concentrations doubled in response to a halving of the incident light intensity, while the daily changes in cellular lipid which resulted in the observed amplitude of the periodic response remained relatively constant regardless of the light regime under which the cells were cultured (Table 4-f).

However, when the lipid:chlorophyll a ratio is compared for daily minimum values, and for the extent of the observed daily increase (Table 4-g), a constant ratio of 45/1 is obtained for daily minimum values, while the daily Δ lipid: daily Δ chlorophyll ratios vary from 100/1 to 64/1 and 80/1 to 69/1 in high light and medium light ammonia and nitrate cultures, respectively. Perhaps the difference in the lipid: chlorophyll ratios represents a portion of the daily lipid synthesis which is put into short term storage and ultimately oxidized as an energy source.

4.3.2 ATP

Since the half-life of a molecule of ATP is extremely brief, the observed periodicity in cellular ATP represents a nearly instantaneous measurement of the cell's metabolic activity at the time of sampling rather than a storage phenomenon.

TABLE 4-f

COMPONENTS OF THE PERIODIC RESPONSE OF CELLULAR LIPID

Light Regime	Mean µg lipid/ 10^5 cells	Daily min. µg lipid/10^5 cells	Daily Δ lipid daily max-daily min.
Hi lt. NH_3	1.46	0.9	1.10
Med lt. NH_3	2.86	2.45	0.90
Hi lt. NO_3	1.57	1.075	1.03
Med lt. NO_3	2.70	2.20	0.97

TABLE 4-g. LIPID:CHLOROPHYLL A RATIOS

Light Regime	Daily min. lipid: Chl a	Daily Δ lipid: daily Δ Chl a
Hi lt. NH_3	45	100
Hi lt. NO_3	42	80
Med lt. NH_3	48	64
Med lt. NO_3	43	69

A consideration of table 4-h demonstrates that *Dunaliella tertiolecta* roughly doubles its respiration rate over the course of a day.

A similar elevation in cellular ATP was noted by Bornefeld and Simonis (1974) when the blue-green alga *Anacystis nidulans* was exposed to light. By use of various inhibitors, it was established that this increase was attributable to cyclical photosynthetic phosphorylation. While it is probable that some portion of the observed daily increase in ATP activity in *Dunaliella* is attributable to the same mechanism, the overall magnitude of the response must be partially due to an increase in mitochondrial activity as well since peak metabolic activity always occurred two to four hours after the onset of the dark phase, when the photosynthetic phosphorylating mechanism was no longer operating. Some portion of the overall periodicity shown by cellular ATP activity may also be related to the observed synchrony in cell division. In all but the high light ammonia cultures, minimum ATP concentrations occur synchronously with the observed peaks in telophase configuration cells (10:00 hours), while maximum ATP concentrations occur 8-12 hours prior to the observed telophase configuration peaks. The relationship between the two cycles seems reasonable in view of the fact that just prior to the onset of division, and in the earliest stages of division, cellular activity is high while in the later stages metabolic activity is minimal.

TABLE 4-h

CELLULAR ATP, DAILY MINIMUM AND DAILY MAXIMUM VALUES

	μg ATP/10^5 cells	
Light Regime	Daily Minimum	Daily Maximum
Hi lt. NH_3	.035	.088
Med lt. NH_3	.043	.076
Hi lt. NO_3	.0575	.110
Med lt. NO_3	.038	.076

4.3.3 Carbohydrate and Protein

When daily maximum, minimum, and mean values of intracellular carbohydrate and protein are expressed per dividing cell, a number of nitrogen substrate specific, and light specific trends become apparent.

Intracellular protein content shows a relatively constant nitrate-substrate-specific level that seemingly must be attained before division occurs, regardless of the light regime. This level is 40-42% higher in nitrate-grown cells than in ammonia-grown cells. In contrast, intracellular carbohydrate content shows a double trend, decreasing in response to decreasing light, but always being approximately 40% higher in nitrate-grown cells. However, under each light regime, the protein:carbohydrate ratio remains constant, regardless of the nitrogen substrate used to culture the cells (Table 4-i).

The fact that nitrate-grown cells contain 40% more protein and carbohydrate than ammonia-grown cells at each light regime suggests that nitrate-grown cells are 40% larger than ammonia-grown cells or that they must synthesize 40% more protein, and fix 40% more carbohydrate before division can occur. Undoubledly, this difference in critical cell size or critical cell synthesis contributes to the observed differences in maximum specific growth rate between nitrate and ammonia cultures.

TABLE 4-i

RELATIONSHIP BETWEEN CELLULAR PROTEIN, CARBOHYDRATE, AND CELL DIVISION

	μg Protein/10^5 dividing cells			μg Carb./10^5 dividing cells			Protein:Carb. ratio		
Light Regime	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean
Hi lt. NH_3	1.73	2.33	1.95	2.81	6.22	4.76	1.62	2.66	2.44
Hi lt. NO_3	2.60	3.26	2.82	3.76	8.69	6.24	1.45	2.66	2.21
Med lt. NH_3	1.83	2.43	2.05	1.49	7.16	4.23	0.81	2.94	2.06
Med lt. NO_3	2.55	3.46	3.29	2.95	9.89	6.26	1.15	2.85	1.91

4.3.4 Cell Energetics

At 0.0043 and 0.010 gm cal/cm^2/min the differences noted in μ_m of nitrate-and ammonia-grown cells may not be directly related to fundamental differences in the energy requirement of growth on oxidized and reduced nitrogen substrates, since, at these intensities, light-limited growth in ammonia-grown cells is mediated through the chlorophyll-light relationship while growth in nitrate medium is being mediated by the light-nitrate assimilation relationship and the chlorophyll-light relationship. However, at 0.02 gm cal/cm^2 min the observed nitrogen substrate specific differences in growth may be related to a fundamental difference in energy requirements since at this intensity, the light dependent nitrate assimilation mechanism, and the chlorophyll concentration-light mechanism are at saturation, and growth is coming under control of the carbon reducing and phosphorylating reactions of photosynthesis. Therefore, the energy saved in growth on a reduced nitrogen substrate is equivalent to the energy necessary to accomplish 0.23-0.24 division/day, assuming that all of the energy saved is channeled into growth. Table 4-j presents the ratio of calories absorbed/division for NH_3 and NO_3 cells grown under high and medium light conditions.

Thus nitrate-grown cells require 30 and 38% more energy to accomplish a division than ammonia-grown cells. Energy in this case is simply input energy, and the differences shown make no assumptions about the energy requirements of

TABLE 4-j

CALORIES ABSORBED/DIVISION FOR AMMONIA AND NITRATE CULTURED CELLS OF *DUNALIELLA TERTIOLECTA*

Light Regime	Cal Abs/Div	% Difference
Hi lt. NH_3	0.20	30%
Hi lt. NO_3	0.26	
Med. lt. NH_3	0.21	38%
Med. lt. HO_3	0.29	

the various cellular processes in NH_3 and NO_3 grown cells, nor about the way in which energy is partitioned into respiration, division, and synthesis.

The primary sources of cellular energy are photosynthetic phosphorylation, and mitochondrial synthesis of ATP with energy supplied by the oxidation of photosynthetically reduced carbon. Although the contribution of photosynthetic phosphorylation to the total energy budget of the cell can not be determined from the experimental data, an estimate of the energy contribution from reduced carbon can be made if it is assumed that the daily decline in intracellular carbohydrate represents respiration without any additional synthesis. Respiration per division can then be obtained by the expression,

$$\text{respiration/div} = ((\text{daily decline} \div \text{hrs darkness})24)\left(\frac{1}{\text{div/day}}\right)$$

and can be expressed on a caloric basis by adopting Lehninger's (1961) value of 345 Kcal/mole of glucose converted to ATP by mitochondrial synthesis (Table 4-k).

When the energy production (consumption) of carbohydrate/division is compared with the light absorbed/division, an overall photosynthetic efficiency of 12-13% is obtained. This is in line with other published values (Rabinovitch and Govindjee 1968) and lends support to the general validity of this interpretation of the data.

Nitrate-grown cells are thus consuming at least 30% more energy than ammonia-grown cells to accomplish a division under high light conditions. However, the most germane question is

TABLE 4-k

CALORIES OF MITOCHONDRIAL ATP EXPENDED/DIVISION

High Light	Calories Mitochondrial ATP expended/Division
NH_3	.024
NO_3	.035

what this additional energy is being utilized for. Syrett (1958) showed a requirement for reducing energy of 10.4 Kcal/mole of ammonia and 84 Kcal/mole of nitrate converted to amino nitrogen. Superficially, this seems like a large difference. However, when total calories as mitochondrial ATP expended/division is compared to the quantity of Particulate "Nitrogen" assimilated/division for ammonia- and nitrate-grown cells of *Dunaliella*, the resultant values are 800 Kcal/mole of ammonia assimilated and 1,300 Kcal/mole of nitrate assimilated. Thus, energy directed towards the reduction of inorganic nitrogen represents only 1.25% and 6.4% of total respiration for ammonia- and nitrate-grown cells, respectively. Reducing energy alone would seem to compose too small a percentage of total cell energy to account for the observed differences in growth and respiration in ammonia and nitrate medium. There remains the 40% difference in protein synthesized/division noted previously, and it seems likely that the additional energy required by nitrate-grown cells to complete a division is largely directed towards this protein synthesis. What this additional protein synthesis represents cannot be determined from the experimental data, but since the synthesis must in some way be related to the different nitrogen substrates, a reasonable assumption might be that this protein is involved with the synthetic machinery necessary to the production of the various enzymes involved in the uptake and assimilation of inorganic nitrate.

4.4 Effluent Mariculture

4.4.1 Growth, Area, Depth and Volume Throughput in an Effluent Mariculture System

Any consideration of a photosynthetically powered effluent mariculture system must begin with an evaluation of the light parameters that will be of greatest significance for the phytoplankton grown in the system. Of primary importance in such an evaluation are the following three characteristics of the phytoplankton phase of an effluent mariculture system:

1) Tanks or ponds will be relatively shallow.
2) Tanks or ponds will be thoroughly and rapidly mixed.
3) Phytoplankton standing crops will be considerably higher than those encountered in natural waters, under bloom conditions.

As a result, cells will be moving rapidly through a comparatively steep light gradient, a situation atypical of most natural plankton populations. How cells respond to such a situation in terms of their chlorophyll a concentration will ultimately determine the growth response of the culture since growth is dependent on the energy absorbed by the cells which is in turn a function of cell chlorophyll concentration.

Eppley and Dyer (1965) suggested that cell pigment concentration was a function of the highest intensity to which the cell was exposed, even if the exposure was extremely brief. However, in view of Kiefer (1973), Takahashi and Shimura (1971) and Harris and Lott's (1973) studies on the time course of photo-adaptive and photo-inhibitive responses, cited below, such a response seems highly unlikely. Harris and Lott (1973) found that natural phytoplankton populations required approximately two hours for photo-adaptation to occur. Takahashi and Shimura (1971) measured the time course of photo-inhibition of photosynthesis in natural phytoplankton populations at 70 Klux. They noted the first measurable changes in photosynthetic rate after an incubation of 30 minutes. Kiefer (1973) noted a decay in fluorescence activity of chlorophyll associated with chloroplast contraction and migration in cells of *Lauderia borealis* exposed to inhibitory light intensities. However, even this physical change required two minutes for the contraction response to occur.

Alternatively, in a rapidly mixed culture, cells may be responding to the average intensity incident on each cell as suggested by Rabe and Benoit (1962). Average incident intensity can be calculated by an

integration of the light attenuation equation:

$$I_D = I_0 e^{-KD}$$

Thus:

$$I_{av} = \frac{1}{D_1} \int_0^{D_1} I_0 e^{-(KD_1)}$$

$$I_{av} = \frac{I_0}{KD_1} - \frac{I_0}{KD_1} e^{-KD_1}$$

where: I_{av} = average intensity incident on each cell under a cm^2 area of depth D_1.

However, the above integration ignores the feedback loop that exists between intensity, chlorophyll a concentration, and culture transparency. Therefore, to obtain I_{av}, the method of approach by successive approximation must be used with the following three equations to obtain the cellular chlorophyll concentration and I_{av} for a given tank depth (cm), cell density, and I_o:

$$I_{av} = \left(\frac{I_o}{K_{cm}D_1}\right) - \left(\frac{I_o}{K_{cm}D_1}\right) e^{-KD_1}$$

$$Kcm = 0.004 + 0.0088c + 0.054c\ 2/3 \quad \text{(Riley)}$$

$$C_{ml} = \left(V_m - \frac{VmS}{K_s+S}\right) \left(\frac{\text{cells/ml}}{10^5}\right)$$

where: I_{av} = average light incident (gm cal/min) under a cm^2 area of depth D_1

Kcm = attenuation co-efficient/cm increase in depth

C_{ml} = µg chlorophyll/ml

$S = I_{av}$

V_m = maximum chlorophyll concentration/10^5 cells

k_s = S at which C = 1/2 V_m

The resultant values of I_{av} and $C_{ml} \div \frac{\text{cells/ml}}{10^5}$ can then be substituted into the growth rate model presented previously.

Finally, the maximum total volume (m^3) that can be treated in a given area per unit time (days) is the product:

$A \times D \times \mu_m$ = total m^3 through/day

where: A = area m^2

D = depth m

μ_m = growth rate division/day

If it is assumed that under the light-limited conditions characteristic of effluent mariculture the total quantity of nitrogen that can be assimilated by the system per unit time is a function of the nitrogen content of the cells, the rate of cell division, and the density of the standing crop, then the portion of the total daily flow that can be composed of

secondary sewage effluent, if adequate nitrogen removal at μm is to be attained equals:

$$\frac{\text{total volume through } m^3}{\left(\frac{\mu\text{eq N/liter } 2^{o} \text{ effluent}}{(\mu\text{eq N/cell})(\text{cells/ml})(1000)}\right)}$$

where: μeq N/cell = 2.3×10^{-7} - NH_3 culture

3.2×10^{-7} - NO_3 culture

Figure 4.7 presents a comparison of maximum volume throughput for a 1 m^2 area at I_o = 0.146 ly/min (the average summer value measured at the Tallman Island Pollution Control Plant), 13 hours light, and a standing crop density of 8×10^5 cells/ml for nitrate and ammonia rich medium, and tank depths of 0.5 - 10.5 meters. Figure 4.8 presents a comparison of the portion of this total daily flow that can be composed of secondary sewage effluent if adequate nitrogen removal at μ_m is to be attained at standing crops of 6×10^5, 8×10^5, and 1.2×10^6 cells/ml.

In terms of maximizing total volume throughput for a given area, an ammonia rich effluent is definitely advantageous. Although the advantage is relatively small in shallow tanks where I_{av} is high, and both ammonia- and nitrate-grown cells are in the minimum response portion of the chlorophyll: light curve, at greater depth, where the growth response in nitrate medium becomes limited by the light dependent component of nitrate-assimilation and the growth response in ammonia medium is being mediated by the maximum response portion of the chlorophyll:light curve, the advantage of an ammonia

Figure 4.7 Maximum volume throughput in a homogenous, rapidly mixed, effluent mariculture system

$m^3\ day^{-1}$ vs depth (meters)

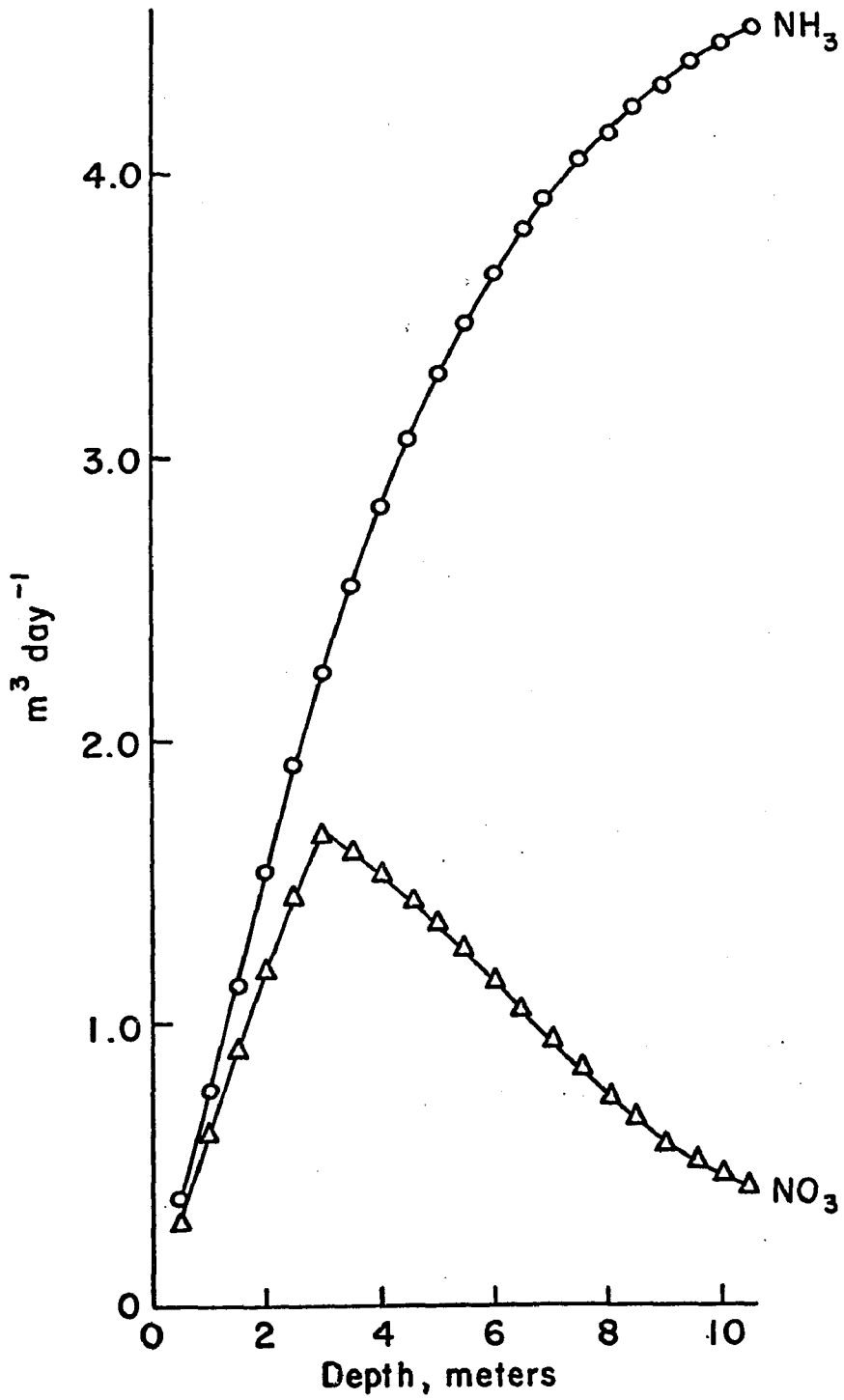
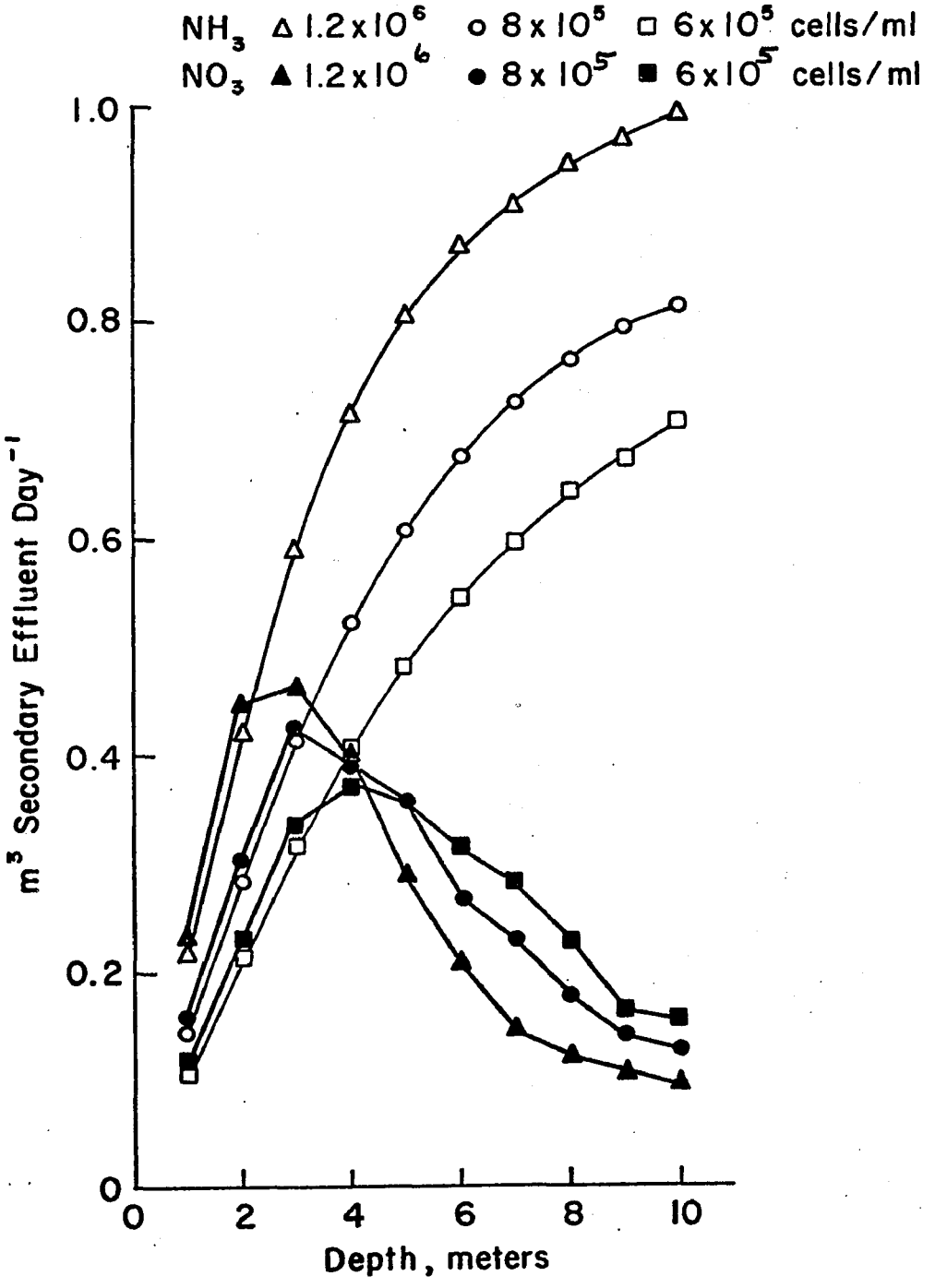

NH3
NO3
4.0
3.0
2.0
1.0
0
0
2
4
6
8
10
m3 day-1
Depth, meters

Figure 4.8 Volume secondary effluent treatable day^{-1} in a homogenous, rapidly mixed, effluent mariculture system

m^3 secondary effluent day^{-1} vs depth (meters)


NH3 Δ 1.2x10^6 ○ 8x10^5 □ 6x10^5 cells/ml
NO3 ▲ 1.2x10^6 ● 8x10^5 ■ 6x10^5 cells/ml
1.0
0.8
0.6
0.4
0.2
0
m³ Secondary Effluent Day⁻¹
0
2
4
6
8
10
Depth, meters

rich medium becomes increasingly more pronounced. At tank depths of less than two to three meters, nitrate-grown cells compare favorably with ammonia-grown cells in terms of nitrogen stripping ability, since their higher nitrogen content compensates for their slower growth rate. However, at the low levels of I_{av} characteristic of tanks of greater depth, the advantages of an ammonia rich medium again become increasingly apparent. In addition, it should be noted that due to the compensatory nature of the chlorophyll:light response in ammonia-grown cells, an increase in standing crop density results in a proportional increase in "N" stripping ability, since the resultant decreases in culture transparency and I_{av} do not have a marked effect on μ_m.

Since one of the major constraints on effluent mariculture will be the availability of space for the phytoplankton culturing phase of the system, the following would seem to be important strategies to consider in the development of such a system:

1) Avoidance of secondary sewage treatment processes that result in the production of highly nitrified effluents.
2) Use of phytoplankton tanks or pools that are as deep as possible within the limits of engineering constraints.
3) Development of the highest standing crops possible under the culture conditions used.

4.4.2 Cellular Composition and Shellfish Growth

Despite the limited range of light intensities and photoperiods over which the cellular composition of *Dunaliella tertiolecta* was studied, a number of general inferences can be made about the potential impact of the observed changes in intracellular carbohydrate, protein, and lipid for the shellfish phase of an effluent mariculture system.

Assuming that a deep-tank approach is adopted, the low I_{av} characteristic of such a system will result in the production of relatively lipid rich cells. In addition, if the response of intracellular lipid to changing light intensity does indeed parallel the response of intracellular chlorophyll *a*, small changes in I_{av} will results in large changes in intracellular lipid content and in the carbohydrate:lipid ratio. For example, over the range 0.01-0.02 ly/min, the carbohydrate:lipid ratio decreases from 2.73:1 to 0.99:1. In terms of total daily phytoplankton calories produced/day, the high lipid content of low I_{av} grown cells may represent an additional advantage of a deep-tank approach, since on an equal weight basis the caloric yield of lipid is twice that of carbohydrate or protein (Albanese 1970). However, recent work by Castell and Trider (1974) and DeZwann and Zandee (1971) suggest that the marked changes in intracellular lipid in response to changing light intensity may have a major physiological effect on shellfish, in an effluent mariculture system, and possibly in the estuarine environment as well.

Castell and Trider (1974) found that in *Crassostrea virginica* an increase in the carbohydrate component of the diet resulted in shellfish glycogen production, while an increase in the lipid component of the diet induced the development of gonadial tissue. Thus, it would appear that the seasonal cycle of shellfish glycogen production and spawning activity are at least partially dependent on the nutritional composition of the phytoplankton food source.

DeZwann and Zandee (1971) report that the annual glycogen cycle of *Mytilus edulis* consists of a rapid increase in spring and summer followed by a decline in autumn and early winter as gonad formation begins. Thus, *Mytilus* accumulates glycogen during a period of the year when it is feeding on phytoplankton grown at high light intensities (low lipid content), while gonad formation and the related decrease in stored glycogen occurs when the animals are feeding on phytoplankton grown at low light intensities (high lipid content).

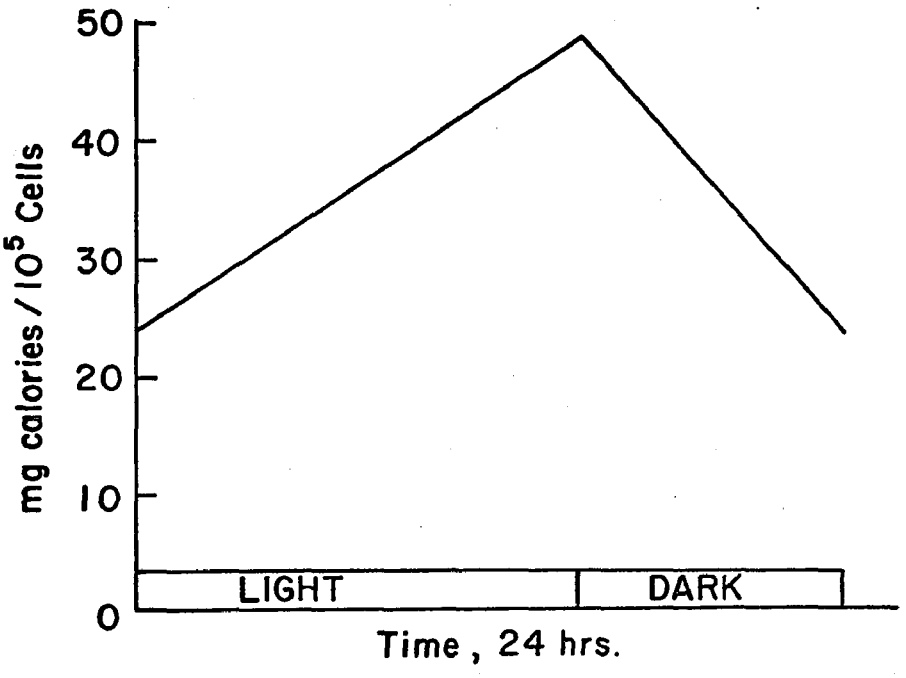
The strong diurnal periodicity in cellular carbohydrate, protein, and lipid content shown by *Dunaliella tertiolecta* cultured under alternating light:dark cycles (Figure 4.9) suggests that shellfish growth might be enhanced if the daily production of phytoplankton was to be retained and fed during a restricted period of the day, bracketing the time of maximum intracellular content. Figure 4.10 presents the average caloric content of cells fed in a continuous fashion, and in discontinuous intervals of 16, 8, and 4 hours equally bracketing the time of peak cell caloric content. The average daily

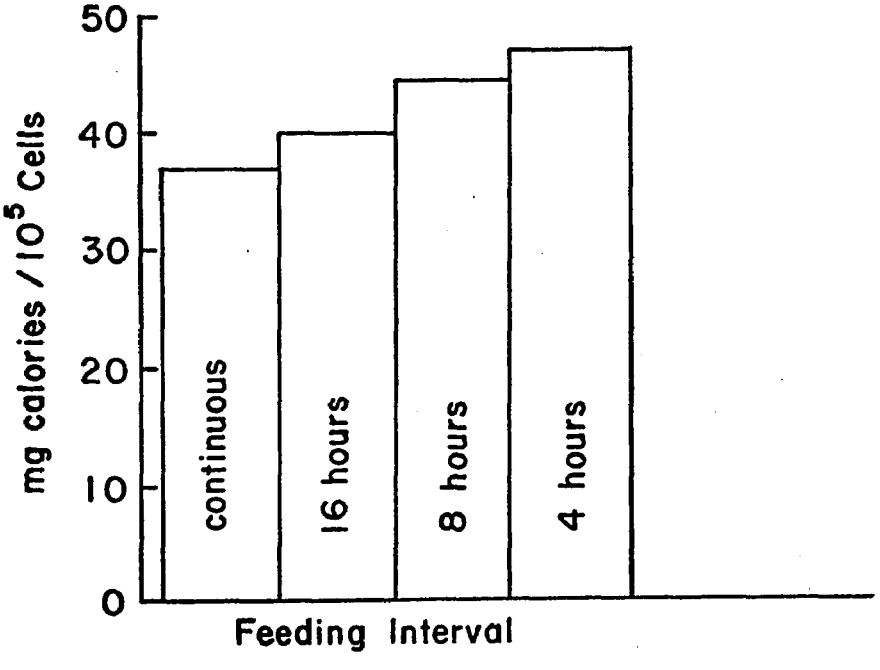
Figure 4.9 Diurnal periodicity in the total caloric content of *Dunaliella tertiolecta*

mg calories/10^5 cells vs time (days)

Figure 4.10 Continuous vs discontinuous feeding

Averaging calories/10^5 cells vs length of feeding interval


50
40
30
20
10
0
mg calories / 10^5 Cells
LIGHT
DARK
Time , 24 hrs.


50
40
30
20
10
0
mg calories / 10^5 Cells
continuous
16 hours
8 hours
4 hours
Feeding Interval

caloric content, when cells are fed continuously is 37 mg cal/10^5 cells, while when the feeding interval is restricted to two hours on either side of the time of peak cell caloric value, the average daily caloric content increases to 46-47 mg calories/10^5 cells.

Ultimately, the advantage of a discontinuous feeding regime rests on the kinetics of the continuous inflow-discontinuous outflow method of phytoplankton culturing required to implement such an approach, and on the effects of introducing a period of passive (non-feeding) shellfish metabolism into the system. Unfortunately, there are no inputs available on the effects of the former constraint. However, it seems probable that the introduction of a non-feeding interval should have no detrimental effects, since the passive metabolic rate of *Mytilus edulis* is less than 1% of the active metabolic rate (Thompson and Bayne 1972) while the caloric value of the phytoplankton food source might be increased by as much as 26% through such an approach.

5 CONCLUSIONS

The maximum growth rates attainable by *Dunaliella tertiolecta* grown in light-limited continuous cultures at three levels of total daily irradiance in nitrate and ammonia medium were measured. Daily changes in cellular ATP, Particulate "Carbon", Particulate "Nitrogen", Chlorophyll a, protein, carbohydrate and lipid were monitored at four-hour intervals at the two highest levels of total daily irradiance.

Cells cultured in ammonia medium had consistently higher hourly growth rates at all levels of light intensity, the difference increasing from 22% at the highest intensity to 62% at the lowest intensity due to a rapid decrease in growth in nitrate medium at the lowest intensity and a much smaller decrease in ammonia medium. Growth in ammonia medium was shown to be dependent on I abs/unit chlorophyll, the response of cellular chlorophyll a to changing light intensity and the energy demands of division. At the highest intensity studied, nitrate-grown cells showed a similar response to light, while at lower intensities, light-limited growth was primarily mediated by the light dependent component of nitrate assimilation. As a result, nitrate-grown cells show a hyperbolic response to changing light, while ammonia-grown cells show a non-hyperbolic response caused by a secondary plateau in growth rate at low intensities where the chlorophyll:light

curve is in its maximal response portion. A theoretical model of light-limited growth was developed based on these results.

Nitrate- and ammonia-grown cells showed similar levels of intracellular ATP, chlorophyll *a* and lipid, suggesting that the cellular concentration of these components is a function of illumination rather than of the nature of the available "nitrogen" substrate.

Cellular ATP showed a diurnal periodicity which was out of phase with the light:dark cycle. This periodicity was probably a result of the combined effects of photosynthetic phosphorylation, and the partial synchrony in cell division observed in the experimental cultures.

Cellular chlorophyll *a* and cellular lipid showed a rapid increase in response to decreasing light intensity, related to the shade adapting chlorophyll:light response. These changes appear in the minimum daily (baseline) values for both parameters. Both parameters, but especially chlorophyll *a*, showed a relatively constant daily increase in concentration regardless of the baseline values, and a possible mechanism of light absorption dependent decay and replacement of chlorophyll *a* was suggested to explain this constancy.

Nitrate-grown cells showed a tendency to synthesize 40% more protein/division than ammonia-grown cells. It was suggested that it is this additional synthesis, rather than the energy differences in uptake and assimilation of nitrate and ammonia, which account for the observed differences in growth

rate at light intensities of 0.018 ly/min, where the light dependent component of nitrate assimilation is saturated. Further, it was suggested that this additional protein synthesis might be related to the synthetic machinery and enzymes involved in the reduction of nitrate to ammonia by the cell.

A consideration of potential strategies for the operation of an effluent mariculture system was developed based on the experimental results, and the following three conclusions were generated:

1) The use of a homogeneous, rapidly mixed, approach to maximize volume treatable in a given area.
2) The advantage of using non-nitrified secondary effluents as the primary nutrient source in effluent mariculture.
3) The potential advantages to shellfish growth of using a discontinuous feeding pattern which takes advantage of the observed diurnal changes in cellular carbohydrate, protein, and lipid content.

6 LITERATURE CITED

Albanese, A. A. 1970. Newer Methods of nutritional biochemistry, Vol. 1 and 2. New York: Wiley Interscience.

Arnon, D. 1960. The role of light in photosynthesis. Scientific American Nov. 1960.

Bannister, T. T. 1974. A general theory of steady state phytoplankton growth in a nutrient saturated mixed layer. Limnol. Oceanogr. 19(1):13-30.

Batt, T. and D. H. Brown. 1974a. The influence of inorganic nitrogen supply on amination and related reactions in the blue green alga, *Anabaena cylindrica*. Planta 116:27-37.

_____. 1974b. The influence of inorganic N supply on carbohydrate and related metabolism in the blue-green alga *Anabaena cylindrica*. Planta 116:197-206.

Bongers, L. H. J. 1956. Aspects of nitrogen assimilation by cultures of green algae. Mededel Landbouwhoogesch Wageningen 56(15):1-52.

Bornefled, T. and W. Simonis. 1974. Effects of light, temperature, pH and inhibitors on the ATP level of the blue-green algae *Anacystis nidulans*. Planta 115:309-318.

Boyle, W. C. and P. M. Berthouex. 1974. Biological wastewater treatment model building fits and misfits. Biotech. Bioeng. 16:1139-1159.

Caperon, J. and J. Meyer. 1972a. Nitrogen limited growth of marine phytoplankton I. Changes in population characteristics with steady state growth. Deep-Sea Res. 19: 601-618.

_____. 1972b. Nitrogen limited growth of marine phytoplankton II. Uptake kinetics and their role in nutrient limited growth of phytoplankton. Deep-Sea Res. 19:619-632.

Castell, J. D. and P. J. Trider. 1974. Preliminary feeding trials using artificial diets to study the nutritional requirements of *Crassostrea virginica*. J. Fish. Res. Bd. Can. 31(1):95-99.

Colowick, S. P. and N. O. Kaplan. 1957. Methods in Enzymology, Vol. 3, pp. 79-80. New York: Academic Press.

Constantopoulos, G. and K. Block. 1967. Effect of light intensity on the lipid composition of *Euglena gracillis*. J. Biol. Chem. 15(242):3538-3542.

De Zwaan, A. and D. I. Zandee. 1972. Body distribution and seasonal changes in the glycogen content of the common sea mussel *Mytilus edulis*. Comp. Biochem. Physiol. 43(A): 53-58.

Eppley, R. W. and J. L. Coatsworth. 1968. Uptake of nitrate and nitrite by *Ditylum brightwellii*-kinetics and mechanisms. J. Phycol. 4:151-156.

_____ and D. L. Dyer. 1965. Predicting production in light limited continuous cultures of algae. Appl. Microbiol. 3:833-837.

_____, Rogers and McCarthy. 1969. Half saturation constants for uptake of nitrate and ammonium by marine phytoplankton. Limnol. Oceanogr. 14:912-920.

_____ and Sloan. 1966. Growth rates of marine phytoplankton: correlation with light absorption by cell chlorophyll a. Physiol. Planta 19:47-59.

Fritsch, F. E. 1935. The structure and reproduction of algae, Vol. 1, pp. 5-9. New York: Cambridge Univ. Press.

Grant, B. R. 1967. The action of light on nitrate and nitrite assimilation by the marine chlorophyte *Dunaliella tertiolecta* (Butcher). J. Gen. Microbiol. 48:379-389.

_____. 1968. The effect of carbon dioxide concentration and buffer system on nitrate and nitrite assimilation by *Dunaliella tertiolecta*. J. Gen. Microbiol. 54: 327-336.

_____ and Turner. 1969. Light stimulated nitrate and nitrite assimilation in several species of algae. Comp. Biochem. Physiol. 29:995-1004.

Griffiths, D. J. 1973. Factors affecting the photosynthetic capacity of laboratory cultures of the diatom *Phaeodactylum tricornutum*. Mar. Biol. 21:91-97.

Haines, K. C. 1972. Personal communication.

Harris, G. P. and J. N. A. Lott. 1973. Light intensity and photosynthetic rates in phytoplankton. J. Fish. Res. Bd. Can. 30:1771-1778.

Hobson, L. A. 1974. Effects of interactions of irradiance, daylength, and temperature on division rates of three sp. of marine unicellular algae. J. Fish. Res. Bd. Can. 1974:391-395.

Holm-Hansen, Osmund and Charles R. Booth. 1966. The measurement of adenosine triphosphate in the ocean and its ecological significance. Limnol. Oceanogr. II:510-519.

JRB Company. Manual for the operation of the JRB ATP Photometer.

Jerlov, N. G. 1968. Optical Oceanography. Amsterdam: Elsevier Publ. Co.

Jorgensen, E. G. and E. Steemann-Nielsen. 1965. Adaptation in plankton algae in primary productivity in aquatic environments. Ed. Cr. Goldman Mem. Inst. Ital. Idrobiol. pp. 40-46.

Kiefer, D. A. 1973. Chlorophyll a fluorescence in marine centric diatoms: responses of chloroplasts to light and nutrient stress. Mar. Biol. 23:39-46.

Lehninger, A. 1961. How cells transform energy. Reprinted from Sci. Amer. Sept. 1961.

Lowry, O. H., J. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurements with the folin phenol reagent. J. Biol. Chem. 193:265-275.

Lui, N. S. T. and O. A. Roels. 1972. Nitrogen metabolism of aquatic organisms II the assimilation of nitrate, nitrite and ammonia by *Biddulphia aurita*. J. Phycol. 8:259-264.

Morris and Syrett. 1963. The development of nitrate reductase in chlorella and its repression by ammonia. Arch. Mikrobiol. 47:32-41.

Paasche, E. 1971. Effect of ammonia and nitrate on growth, photosynthesis and ribulosediphosphate carboxylase content of *Dunaliella tertiolecta*. Phys. Planta. 25: 294-299.

Parsons and Takahashi. 1974. Biological oceanographic processes. New York: Pergamon Press.

Rabe and Benoit. 1962. Mean light intensity: a useful concept for correlating growth rates of dense cultures of microalgae. Biotech. Bioeng. IV:377-319.

Rabinovitch and Govindjee. 1965. "The role of chlorophyll in photosynthesis" in The Molecular Basis of Life, pp. 307-315. San Francisco: W. H. Freeman Co.

Ross, George. 1974. Personal communication.

Ryther, J. H. and R. W. Menzel. 1959. Light adaptation by marine phytoplankton. Limnol. Oceanogr. 4:492-497.

Sawyer, C. N. and P. L. McCarty. 1967. Chemistry for sanitary engineers. New York: McGraw Hill.

Sharfstein, B., O. A. Roels, C. G. Garside, S. Williams, and N. Kostyk. 1972. "Sewage and the New York Estuary. Nutrient levels in the effluent from the Tallman Island Pollution Control Plant and in the nearby East River."

Steele and Menzel. 1962. Conditions for maximum primary productivity in the mixed layer. Deep-Sea Res. 9:39-49.

Steeman-Nielsen, E. and E. Jørgensen. 1968. The adaptation of plankton algae. Physiol. Planta. 21:401-413.

Strickland, J. D. H. and T. R. Parsons. 1968. A practical handbook of sea water analysis. Bull. 167, Fish. Res. Bd. Can., Ottawa.

Syrett, P. J. 1958. Respiration rate and internal ATP concentration in *Chlorella*. Arch. Biochem. Biophys. 75:117-129.

Takahashi, Shimura, Yamaguchi, Fujita. 1971. Photo-inhibition of photoplankton photosynthesis as a function of exposure time. J. Oceanogr. Soc. Japan 27:43-50.

Thompson, R. J. and B. L. Bayne. 1972. Active metabolism associated with feeding in the mussel *Mytilus edulis*. J. Exp. Mar. Biol. Ecol. 9:111-124.

Williams, F. M. 1971. "Dynamics of microbial populations", Vol. 1, pp. 197-267. In Systems analysis and simulation in ecology. B. C. Patten, ed. New York: Academic Press.

Winkenbach, F., B. R. Grant, and R. G. S. Bidwell. 1972. The effects of nitrate, nitrite and ammonia on photosynthetic carbon metabolism of *Acetabularia* chloroplast preparation compared with spinach chloroplasts and whole cells of *Acetabularia* and *Dunaliella*. Can. J. Bot. 50:2545-2551.