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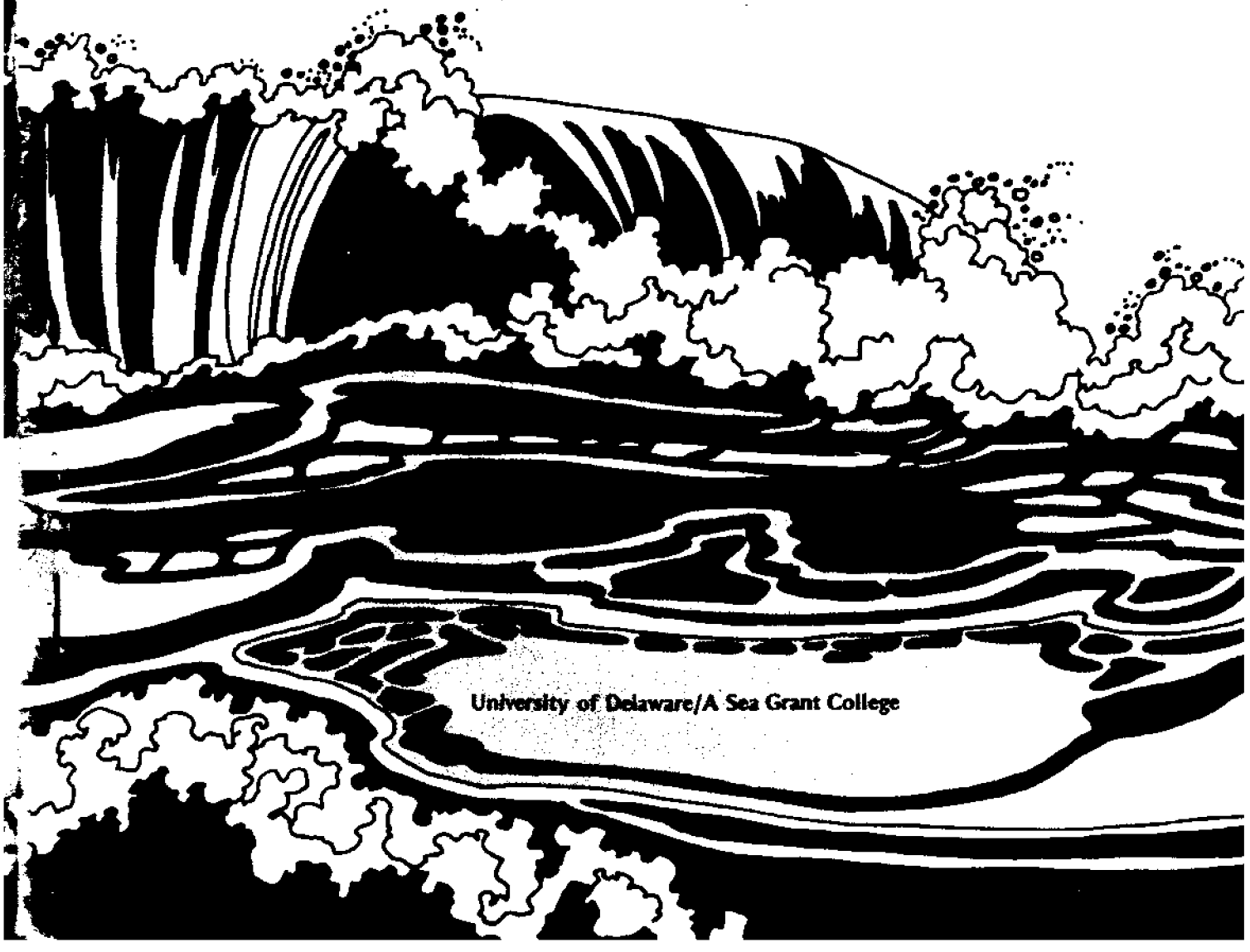
EFFECT OF pH, CARBON DIOXIDE, OXYGEN AND LIGHT ON THE GROWTH OF THALASSIOSIRA PSEUDONANA (HUSTEDT) HASLE AND HEIMDAL CLONE 3H, AN IMPORTANT FOOD FOR BIVALVE MOLLUSCAN MARICULTURE

$3.00



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$3.00

by

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The best part of every mind is not that
which he knows, but that which hovers
in gleams, suggestions, tantalizing, unpossessed,
before him. His firm recorded knowledge soon
loses all interest for him. But this dancing chorus
of thoughts and hopes is the quarry of his future,
is his possibility, and teaches him that his
man's life is of a ridiculous brevity and meanness.

On Man and God by Ralph Waldo Emerson

He had been Eight Years upon a Project for extracting
Sun - Beams out of Cucumbers, which were to be
put in Vials hermetically sealed, and let out to warm
the Air in raw inclement Summers. He told me, he
did not doubt in Eight Years more, that he should be
able to supply the Governors Gardens with Sunshine
at a reasonable Rate; but he complained that his
Stock was low, and entreated me to give something as
an Encouragement to Ingenuity, especially since
this had been a very dear Season for Cucumbers.

Gulliver's Travels by Jonathan Swift

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ACKNOWLEDGMENTS

I express my appreciation to my advisory committee, composed of biologists Dr. Ellis T. Bolton and Melbourne R. Carriker and engineers Drs. Frederick A. Costello, Richard I. Dick, William S. Gaither, and George Tchobanoglous. Their encouragement, advice, and constructive criticism is acknowledged.

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ABSTRACT

The effect of pH, carbon dioxide, oxygen, and light on the growth of Thalassiosira pseudonana (Hustedt) Hasle and Heimdal clone 3H was studied. Algal cultures were grown in an enriched artificial-seawater medium under constant illumination in 500-ml gas wash bottles. Population density, particulate carbon, and particulate nitrogen were measured periodically during exponential growth. Growth rates are reported as the coefficient k from the expression $Y = a\ e^{kt}$ computed by regression analysis for 43 treatment combinations of pH, carbon dioxide, oxygen, and light levels.

Overall, cell division proceeded somewhat faster than either carbon or nitrogen fixation. Growth rate did not increase at concentrations of CO_2 above 16.0 µmole/L. Freshly inoculated algal cultures did not grow in an inorganic-carbon-free medium bubbled with CO_2-free air. The addition of HCO_3^- to the various cultures initially supported rapid growth of algae. The growth rate decreased if the pH of the medium rose above 8.8. Carbon- and nitrogen-fixation rates were inhibited at high concentration of oxygen, while cell-division rate was not inhibited. The differential inhibition aggravated nonsteady-state conditions. Saturation light intensity was about 1500 $\mu w/cm^2$ (5000 lux) and there was no inhibition at 3500 $\mu w/cm^2$ (11,500 lux). The growth rate was unaffected by pH over a range from

6.8 to 8.4. Precipitation of inorganic carbon occurred at elevated $CO_3^=$ concentrations (4000 μmole/L). Ratios of carbon to chlorophyll ranged from 20:1 to 125:1 and were affected by both light intensity and pH.

In some experiments, the growth of 3H was limited by pH levels and CO_2 concentrations caused by dehydration and direct hydroxyl reactions of HCO_3^-. A model was developed relating rates of CO_2 generation from HCO_3^- to pH, temperature, and minimum CO_2 concentrations. Under certain conditions, CO_2 generation from HCO_3^- can limit growth of algae. Methods of introducing inorganic carbon to the medium, which is a critical consideration in the mass cultivation of algae, are discussed.

The mass cultivation of algae for bivalve molluscan mariculture may be an integral part of a controlled-environment system. In that instance, special consideration must be given to maintaining conditions compatible with the algae and the molluscs. Operating limits for CO_2, pH, O_2, and light intensity are suggested.

Chapter I

INTRODUCTION

Controlled-environment molluscan mariculture on a commercial scale requires an abundant and reliable supply of food. The literature is devoid of prepared diets that would sustain molluscs through their life cycle and allow rapid growth to a marketable size. Certain marine algae have long been known to serve as adequate food sources, but their consistent culture in sufficient quantities for commercial production of molluscs has been an elusive goal.

Thalassiosira pseudonana (Hustedt) Hasle and Heimdal clone 3H, hereafter referred to as 3H, is a marine diatom that is being considered as a food for molluscan mariculture (Pruder et al., 1976, 1977). It grows rapidly over the range of temperatures from 15°C to 25°C and over the range of salinities from 0.5 to 32 parts/1000 (Guillard and Ryther, 1962). 3H is known for its nutritive value for the Eastern oyster, Crassostrea virginica (Gmelin), and for the hard clam Mercenaria mercenaria Linne (Matthieson and Toner, 1966; Epifanio et al., 1976). Commercial shellfish hatcheries use 3H as food for larvae (Hargraves and Guillard, 1974), but it has not been produced consistently in quantities large enough for sustained growth and development of shellfish in succeeding life stages. Mass culture of algae remains a major stumbling block to the large-scale development of a shellfish mariculture industry (Goldman and Stanley, 1974).

A number of investigators have successfully cultured 3H in the laboratory (Guillard and Ryther, 1962; Guillard and Cassie, 1963; Matthieson and Toner, 1966; Fuhs, 1969; Guillard et al., 1973; Haines and Guillard, 1974; Goldman and Stanley, 1974; Larson, 1975; Ferguson et al., 1976; and Srna, 1976). These investigators have studied the growth of 3H as a function of temperature, salinity, light intensity, nitrogen source and concentration, and concentrations of vitamin B_{12}, silicon, and phosphorous.

Guillard and Ryther (1962) identified 3H as a chief component of algal blooms in the productive shellfish waters of Moriches Bay, Long Island, New York. Ryther (1954) suggested that phytoplankton that became dominant in these waters flourished because of their ability to use uric acid and other products of the degradation of organic nitrogenous matter.

Environmental conditions in outdoor mass algal cultures do not support the growth of 3H, whereas conditions maintained in laboratory cultures--and present in at least some estuaries--sustain its growth. Certain algal species thrive particularly well under mass cultivation. Chlorella pyrenoidosa, a freshwater coccus, has been cultured on a large scale worldwide for a number of years (Badour et al., 1972). Phaeodactylum tricornutum, a marine diatom, has been grown well and maintained with relative ease in large outdoor cultivation experiments (Ansell et al., 1963, 1964; Goldman and Stanley, 1974). P. tricornutum usually is not found in large numbers in natural marine waters, which further suggests selective differences in environmental conditions.

Pruder et al. (1977) considered the conditions that characterize outdoor pond cultures and reported some progress in the culture of 3H in 9000-L pools in a greenhouse. High-yield cultures (carbon fixation of 14 $g/m^2 \cdot day$ were maintained by controlling oxygen, carbon dioxide, and pH. Experience with such cultures suggested the involvement of photorespiration in the failure of some algae to grow well in partially controlled outdoor cultures. Photorespiration is the loss of carbon dioxide following oxygen uptake that occurs in many photosynthetic tissues exposed to light and can reduce net carbon fixation from a few to nearly 100 percent (Tolbert, 1974). With the objective of increasing photosynthetic efficiency and plant yield, many investigators are continuing to study the phenomenon of photorespiration (Zelitch, 1971, 1975; Dodd and Bidwell, 1971; Andrews et al., 1973, 1975; Tolbert and Zill, 1956; Tolbert, 1974; Morris and Beardall, 1975; Black et al., 1976; Lloyd et al., 1977; Bassham, 1977; Burris, 1977; and Ku and Edwards, 1977a and 1977b). These investigators have shown that oxygen concentration, carbon-dioxide concentration, pH, and light intensity affect the degree of photorespiratory loss.

The purpose of the present investigation was to determine the effect of carbon-dioxide concentration, oxygen concentration, pH, and light intensity on the growth of 3H. This understanding will be used to control the interplay of these factors for optimal production in mariculture. The scope of the investigation was limited to consideration of these variables at 20 parts/1000 salinity, 23°C to 25°C, and one seawater nutrient-enrichment recipe.

The present investigation showed that carbon-dioxide concentration and pH play a crucial role in high-yield cultures of 3H, and that high oxygen concentrations aggravate nonsteady-state conditions, which can lead to failure of 3H cultures. These findings are fundamental to the establishment of an abundant and reliable food supply for bivalve molluscan mariculture. The findings of this investigation, along with the knowledge of nutrients already in hand, should lead to increased success in the mass culture of selected marine plants.

Chapter II

METHODS AND MATERIALS*

Experimental Organism

Thalassiosira pseudonana clone 3H is a marine, centric diatom, 4 to 9 µm in diameter, isolated in 1958 from Moriches Bay, Long Island, New York, by Guillard and Ryther (1962). It was mistakenly assigned the name Cyclotella nana by Hustedt, a name by which it was known from 1962 to 1970. Hasle and Heimdel (1970) corrected the name assignment and discussed the organism's morphology and its geographic distribution. Dr. R. R. L. Guillard has maintained bacteria-free cultures of 3H and kindly supplied a number of cultures from his laboratories at the Woods Hole Oceanographic Institution. Figure 1 shows a scanning electron micrograph of 3H from Guillard's culture. The algae were prepared using the procedures of Underhill (1976).

In the laboratories of the Center for Mariculture Research, University of Delaware, Lewes, Delaware, 15-ml bacteria-free stock cultures are maintained in screw-top test tubes at 16°C under constant illumination by cool-white fluorescent lamps. Intermediate 25-ml cultures started every 30 days became the seed cultures for this investigation.

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*See Appendix A for equipment models and manufacturers.

Fig. 1. A scanning-electron micrograph of Thalassiosira pseudonana Hasle and Heimdal clone 3H (7500X). Micrograph taken by Peter Underhill and included through the courtesy of Melbourne Carriker.

Artificial Seawater

Artificial seawater prepared in a single large batch from "Instant Ocean" synthetic sea salts was used throughout this investigation. A chemical analysis of the sea-salt solution is given in Table 1. Tap water from the city of Lewes, Delaware, was treated with charcoal, de-ionized, and distilled. This was accomplished using a glass still with quartz heating elements, an adsorber column, and an ion-exchange column.

The de-ionized, distilled tap water was blended with a single 3.63-kg package of synthetic sea salts in sufficient quantity to yield seawater with a final salinity of 20 parts/1000. The liquid trace-metal mixture supplied with the package was added. Despite vigorous and lengthy mixing, a small amount of the sea salts did not go into solution; the undissolved sea salts were not identified.

The artificial seawater was acidified to a pH of 3.7 by adding HCl to drive off inorganic carbon as CO_2. The acidified seawater was vacuum filtered at about 50 cm of mercury through 12.5-cm-diameter AH glass-fiber filters. The seawater was transferred to 18-L glass carboys and autoclaved for either 3 hours at 15 psi, or for 8 hours at 6 psi. Following autoclaving, the seawater was clear, with no indication of precipitation. While the carboys were still warm, their cotton plugs were replaced with silicone-rubber stoppers. The carboys, seven in all, were stored in nine dark coldboxes at or below 6°C until needed.

Enrichment Media

Stock nutrient solutions were prepared according to the

Table 1

COMPOSITION OF "INSTANT OCEAN" SYNTHETIC SEA SALTS[a]

Material	Concentration	
	ppm	mole/L
Cl	18400	0.52×10^{0}
Na	10220	0.44×10^{0}
SO_4	2518	26.2×10^{-3}
Mg	1238	50.9×10^{-3}
Ca	390	9.7×10^{-3}
K	370	9.5×10^{-3}
HCO_3	142	2.3×10^{-3}
Br	60	0.75×10^{-3}
H_3BO_3	25	0.40×10^{-3}
Sr	6	0.07×10^{-3}
SiO_3	3	0.04×10^{-3}
PO_4	1.3	13.6×10^{-6}
F	1.0	52.6×10^{-6}
MoO_4	0.6	3.7×10^{-6}
S_2O_3	0.3	2.7×10^{-6}
Li	0.2	29.2×10^{-6}
Rb	0.1	1.2×10^{-6}
I	0.07	0.6×10^{-6}
EDTA	0.06	
Al	0.04	1.5×10^{-6}
Mn	0.02	0.4×10^{-6}
Zn	0.02	0.3×10^{-6}
V	0.02	0.4×10^{-6}
Co	0.01	0.2×10^{-6}
Fe	0.01	0.2×10^{-6}
Cu	0.003	0.05×10^{-6}

[a] In a solution adjusted to specific gravity of 1.025 and a temperature of 15°C.

concentrations and procedures recommended by Guillard (1973 and 1975). Separate solutions were prepared for vitamin B_{12}, nitrate, phosphate, silicate, borate, and trace metals. The flasks containing the solutions were fitted with cotton plugs, sterilized, and stored along with the seawater. The flasks were removed from the coldbox only long enough to enrich seawater for a given experiment then were returned immediately to storage. One set of stock nutrient solutions was used throughout the course of this investigation.

Seed Cultures

Seed cultures started with algal inoculant from the intermediate cultures described earlier provided the inoculant for the batch cultures examined in this investigation. To prepare the growth medium, artificial seawater was taken from storage and enriched with the stock nutrient solutions to yield what Guillard (1973) calls the "f/2" medium, less thiamin and biotin, which 3H does not require. (Appendix B lists the composition of Guillard's f/2 enriched algal medium.) Sodium bicarbonate was added to the enriched medium to yield a preinoculation pH of 7.8. The seed cultures were grown in 500-ml Erlenmeyer flasks at 15°C to 16°C under constant illumination by cool-white fluorescent lamps at an intensity of about 300 $\mu w/cm^2$ (990 lux) at the culture surface. When the seed cultures reached a density of 1×10^6 to 2×10^6 cells/ml, they were used to inoculate the experimental batch cultures.

Immediately before their use as inocula, cells in each seed culture were counted and examined under an optical microscope at

400X using a hemocytometer. Cells in the seed cultures were well-shaped and pigmented, many in the state of dividing, and there was no indication of cell clumping or of contaminating algae. Bacterial populations were inconsequential, never exceeding 1×10^4 cells/ml.

Culture Type

Batch-culture techniques were employed in this investigation because of their simplicity and reliability. To provide sufficient samples for measurement of particulate carbon (PC), particulate nitrogen (PN), and chlorophyll, duplicate or triplicate cultures were blended together. The experimental cultures were inoculated to initial concentrations of about 1×10^6 cells/ml to avoid nutrient limitation of algal growth. (Laboratory cultures enriched according to the f/2 composition routinely grow to concentrations of 3×10^6 cells/ml.) At the maximum concentration of 1×10^6 cells/ml, light intensity on the side of the culture vessel facing away from the light was never less than 80 percent of the light intensity on the side of the culture vessel facing toward the light. Commercially available custom gas mixtures were bubbled through the algal cultures at a rate sufficient to prevent algal cells from settling. To maximize gas transport in and out of the algal culture medium, 500-ml gas wash bottles with 20-mm-diameter, coarse-porosity fritted discs were employed as culture vessels. Measured levels of oxygen concentration did not vary as cell concentration increased. The maximum pH change during the growth of the algae was 0.3 pH units, except in those experiments where CO_2-free gas was used.

Custom Gas Mixtures

Cylinders of 15 different custom gas mixtures were obtained from a commercial supplier; partial pressures and equilibrium concentrations of CO_2 and O_2 in these mixtures are reported in Table 2. The O_2 to CO_2 ratio varied from zero to 89. The gas mixtures were metered into individual cultures through calibrated flow meters. The gas flow to each culture was controlled between 0.12 and 0.15 std. L/min. (1 atmosphere and 21°C). At this flow rate, algal cells did not settle, and pH and O_2 concentration were held constant. The amount of CO_2 delivered to the cultures was far in excess of that needed for photosynthesis: from 200 to 800 mg/L·day. The efficiency of CO_2 utilization ranged from 4 to 15 percent.

Light-Energy Source

Experimental cultures were grown under "cool white" fluorescent lamps. Fluorescent lamps with the "cool white" spectral properties are commonly used in routine production and much experimental work with algae (Guillard, 1975). Some consideration was given to using special-purpose lamps such as "Grolux" and "Vita-lite"; there is evidence that the makeup of the spectral emission of the lamps under which plants are grown affects the growth and chemical composition of the plants (Bickford and Dunn, 1972). However, there is no clear evidence that special-purpose lamps are better for growing marine algae, most of which--like 3H--are golden brown in color and well adapted to absorbing the bluish-green light that has maximum penetration in clear seawater (Guillard, 1975).

Table 2

COMPOSITION OF CUSTOM-MIXED GASES

P_{CO_2}	$[CO_2]^a$	P_{O_2}	$[O_2]^b$	$[O_2]/[CO_2]$
0.000442	14.0	0.999558	1250	89/1
0.001000	31.8	0.999000	1250	39/1
0.002543	80.8	0.997457	1246	15/1
0.005000	159.0	0.995000	1244	8/1
0.010000	318.0	0.990000	1238	4/1
0.000459[c]	14.6	-	-	-
0.001100[c]	35.0	-	-	-
0.002500[c]	80.0	-	-	-
0.005000[c]	159.0	-	-	-
0.010000[c]	318.0	-	-	-
0.00510	16.2	0.2095	262	16/1
0.00980	31.2	0.2095	262	8/1
0.002500	80.0	0.2095	262	3.3/1
0.005000	159.0	0.2096	262	1.6/1
0.010000	318.0	0.2095	262	0.8/1

[a] CO_2 solubility 318 x 10^{-4} moles/L/atmos.

[b] O_2 solubility 1.25 x 10^{-3} moles/L/atmos.

[c] CO_2-enriched nitrogen.

For light intensities of 350, 750, and 1500 $\mu w/cm^2$, neutral-density polyethylene screens were interposed between the cultures and Westinghouse "cool white" fluorescent lamps; for a light intensity of 3500 $\mu w/cm^2$, Sylvania "cool white" fluorescent lamps were trained directly on the cultures without neutral-density screens. Comparison of the spectral energy-distribution curves for both these lamps yielded no differences (Westinghouse Electric, Inc., catalog no. A-8850A, 1974; Bickford and Dunn, 1972). With a radiometer, light intensities were measured between the wavelengths of 0.375 and 0.740 μm. Illumination was estimated using 1 $\mu w/cm^2$ = 3.3 lux (from Westinghouse Research Lamp Division). A typical experimental setup is shown in Figure 2.

Experimental Procedures

This investigation included eighteen separate experiments. Each experiment examined three to nine treatment combinations and involved nine to eighteen individual cultures. The basic procedures for all experiments were essentially the same:

1. Artificial seawater was transferred from storage carboys to one or more 4-L Fernbach flasks that were placed in a water bath to raise the temperature to 23° to 25°C. For each six cultures to be grown, 3.5 ml of seawater was prepared.

2. Sufficient nutrient solution was added to achieve concentrations the same as Guillard's "f/2" enriched medium. The addition of nutrients, principally silicates, raised the pH of the seawater from 3.7 to 6.7.

3. One custom gas mixture was used for each experiment. The appropriate gas mixture was bubbled through the enriched medium. Because the bubbled gas lowered the pH, sodium bicarbonate in solution

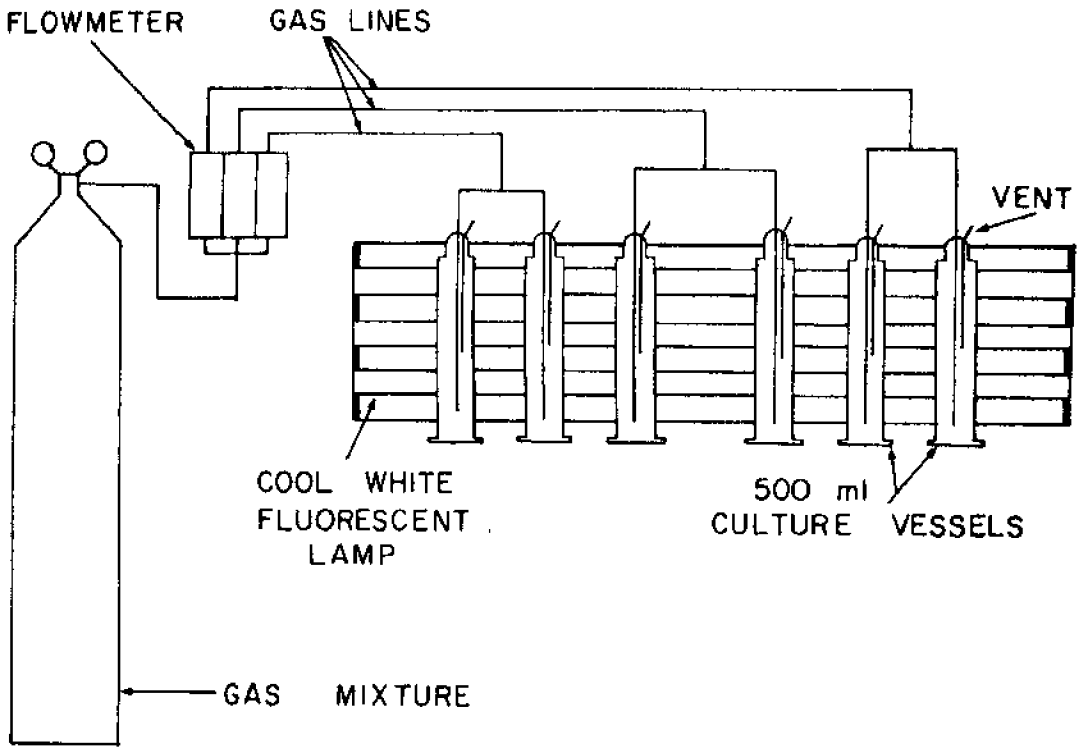


Figure 2. Diagram of apparatus for culturing algae.

(0.5 molar) was added to the seawater slowly until the pH stabilized at 6.5.

4. A portion of seed culture was counted and examined. Then sufficient volume of the seed culture was added to the enriched seawater to yield a starting concentration of 5×10^4 cells/ml.

5. Additional inorganic carbon, principally as $NaHCO_3$, was added to the inoculated seawater until the desired pH was reached (i.e., 6.8, 7.6, 8.4). Sodium bicarbonate was added to achieve higher pH levels. The solubility of alkali earth carbonates placed limits on the combinations of pH and CO_2 that could be used effectively.

6. After equilibrium was reached at the desired pH, as evidenced by constant pH for at least 30 minutes, the inoculated medium was transferred to individual culture bottles. When the transfer was complete and proper gas flow established, experiment time-zero was noted.

7. The remaining, untransferred, inoculated seawater was sampled for cell concentration, PC, and PN.

8. The nominal experimental period was 20 to 27 hours. The rapid-growing cultures reached cell concentrations in excess of 1×10^6 cells/ml. Approximately every four hours, 5 ml of medium from each culture bottle was withdrawn for measurement of cell concentration and pH. After 8 to 10 hours, 100 ml of each culture was withdrawn for measurements of PC, PN, and chlorophyll. These measurements were repeated at the end of the experiment using the remaining culture volume.

A typical culture bottle is shown in Figure 3. Gas was introduced through a fritted disc on one side and was vented through a tube

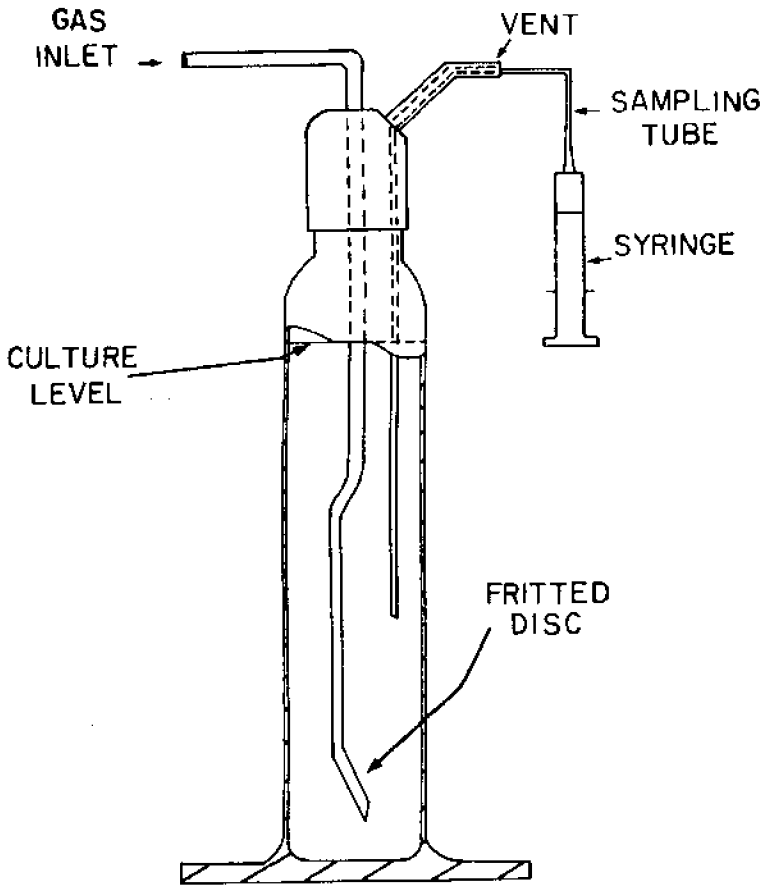


Figure 3. Culture vessel (500-ml gas wash bottle).

on the opposite side. To sample the culture, a small-diameter plastic tubing was inserted into the ventilation tube down to the approximate center of the culture where vigorous mixing was taking place. A hypodermic syringe was fitted to the other end of the plastic tubing to withdraw a sample for analysis. The plastic tubing and syringe were removed and the sample was transferred to an appropriate container for subsequent analysis.

Experimental Variables and Constants

The experimental variables considered in this investigation were the concentration of CO_2 as dissolved gas, the concentration of O_2 as dissolved gas, the pH of the algal medium, and the light intensity at the culture surface. These variables had the following values:

CO_2: 0, 16, 32, 159, and 318 μmoles/L

O_2: 15, 262, and 1250 μmole/L

pH: 6.8, 7.3 to 7.7, 8.4, and 9

light: 350, 750, 1500, and 3500 $\mu w/cm^2$

These ranges were selected to encompass levels above and below anticipated optimum levels.

Experimental conditions from trial to trial throughout the experiment included temperature--23° to 25°C; salinity--20 parts/1000; light source--cool-white fluorescent lamps; artificial seawater media with f/2 enrichment; culture vessel--gas wash bottle; culture type--batch; initial cell concentration--5×10^4/ml; trial duration--20 to 27 hours; preparation and sampling procedures as discussed.

Experimental Response

The objective of this investigation was to determine the effect of carbon-dioxide concentration, oxygen concentration, pH, and light intensity on the growth of 3H. Cell concentration, particulate-carbon concentration (PC), and particulate-nitrogen concentration (PN) were measured at time intervals during the growth of the algae in batch culture. The growth rate is a first-order reaction and may be expressed as

$$\frac{dY}{dt} = k\ Y$$

where Y equals cell concentration, PC, or PN at time t and k equals specific growth rate. This equation can be integrated as

$$Y = Y_o\ e^{kt}$$

where Y = cell concentration, PC, or PN at time t

Y_o = cell concentration, PC, or PN at time t = 0

k = specific growth-rate coefficient hr^{-1}

t = time, hr

The measured values of cell concentration, PC, and PN at time t were fitted to the exponential function $Y = Y_o\ e^{kt}$ by the least-squares fit according to the linear equation $\ln Y = \ln Y_o + kt$. Growth coefficients for change in cell concentration were reported as k_{cc}, for change in particulate carbon as k_{pc}, and for change in particulate nitrogen as k_{pn}.

For comparison purposes, a growth-rate coefficient of 0.087 is equivalent to three doublings per day, a growth-rate coefficient of 0.116 is equivalent to four doublings per day, and a growth-rate coefficient of 0.145 is equivalent to five doublings per day.

Measurement Techniques and Instruments

The experimental algal cultures were sampled periodically during the 24-hour growth period. Cell concentrations were measured by counting cells in a known volume using a hemocytometer. Particulate carbon and particulate nitrogen were measured using a CHN analyzer. Chlorophyll was measured using a DU spectrophotometer for acetone-extracted samples. Bacteria were counted through a microscope with ultraviolet epi-illumination.

Cell Concentration. The cell concentration was determined by counting cells on a hemocytometer. The basic source of variability of the count using this procedure arises from the random settling of cells in a designated area of the counting chamber. This error was called "field error" by Berkson et al. (1940), who confirmed the results of earlier work (Student, 1907) that the number of cells falling within an area of designated size in the hemocytometer chamber follows the Poisson distribution and accordingly, the standard deviation is expressed relatively in the coefficient of variation V_f as a percentage of the mean, $V_f = 100/m^{1/2}$. If more than one field is counted, the coefficient of variation becomes $V_f = 100/n^{1/2}$, where n is the total number of cells counted. The field error is not dependent upon the number of hemocytometer squares counted, but on the total number of cells counted.

During the early part of an experiment, while cell concentrations were low, ten fields were counted. A typical cell count of 80 yielded a coefficient of variation of 11 percent. At higher cell concentrations, only five fields were counted. At typical counts of 500 cells, the coefficient of variation was about 5 percent. This error is fundamental with the hemocytometer method and is unavoidable if the hemocytometer is used. The same hemocytometer chamber and coverslip were used throughout the experiment by only one operator and no dilution was required. It is likely that field error was the only significant counting error.

The Coulter counter was considered and thoroughly tested as an alternative counting method. Difficulties were encountered in obtaining reproducible results despite attempts to standardize techniques and confidence was not established for using the process to count algal cells.

Particulate Carbon and Particulate Nitrogen. Particulate-carbon and particulate-nitrogen concentrations were determined using an elemental analyzer. Samples of the algal cultures were filtered through 1.0-μm, 47-mm-diameter silver filters using 25 cm vacuum; the filters had been washed in acetone and precombusted for 2 hours at 400°C. Silver filters were selected over glass filters because of the ease of handling and low retention of seawater (Gordon and Sutcliffe, 1974). Immediately following filtration, individual filters were frozen and stored in individual petri dishes. Several blank filters were prepared during each trial. The sample volume per filter ranged from 30 to 100 ml, depending on the algal culture density. The

samples withdrawn from replicate cultures were blended and filtered on duplicate filters. On the day of analysis, never more than two weeks following the experiment, filters were removed from the freezer and dried at 40°C for one hour, rolled tightly, and stored in a dessicator until analyzed.

The elemental analyzer was operated with a combustion-tube temperature of 860°C and a reduction-column temperature of 680°C. Four ladle blanks and five standards were run each day as preparation and calibration for analysis of algal-culture samples.

Forty blank filters were analyzed using the procedures outlined. The mean and standard deviation for carbon blanks were 17.75 µg ± 7.1 µg. The mean and standard deviation for the nitrogen blanks were 0.65 µg ± 1.0 µg. The standard deviations are within the range reported by Gordon and Sutcliffe (1974) using the same type machine and the same type filters.

High carbon intercepts were encountered by Gordon and Sutcliffe (1973), who investigated the phenomenon without isolating the cause. In this investigation, blank values of 18 µg/ml carbon and 0 µg/ml nitrogen were subtracted from machine readings to obtain sample concentration.

Ten replicate samples were analyzed using algal samples on filter pads processed according to the procedures described. The mean and standard deviation values were . . .

Particulate carbon	130 µg/ml ± 5.9 µg/ml
Particulate nitrogen	18 µg/ml ± 0.6 µg/ml

The coefficients of variability expressed as a percentage of the mean

at the two sigma levels were 9 percent for carbon and 6 percent for nitrogen.

Silver filters with a pore size of 1.0 μm were used throughout this experiment. This pore size was selected for convenience. Duplicate filters were analyzed for each of five pore sizes (0.2, 0.45, 0.8, 1.2, and 3.0 μm) with equal volumes for the same algal culture. For reasons that are not clear, the highest carbon concentration was found on the 1.2-μm filters (120 and 118 μg/ml) and the lowest carbon concentration was reported from the 0.2-μm filters (107 and 113 μg/ml).

Chlorophyll "a". Chlorophyll "a" concentration was determined by the method of Strickland and Parsons (1972) except that algal-culture samples were collected on 0.45-μm, 47-mm-diameter filters. The filters were dissolved immediately in 5 ml of a 90-percent solution of acetone in double-distilled water. Extinction coefficients were measured at the appropriate wavelengths in 1-cm pathlength cells on a spectrophotometer.

Bacterial Count. Samples for bacterial counts were drawn directly from the culture bottles into 10-ml sterile syringes. Then 7 ml of each sample was transferred into sterile, rubber-stoppered tubes to which 0.36 ml of formalin was added. The tubes were then shaken well and stored in the refrigerator until the cells were counted, usually within 24 hours. The bacterial cells were counted on a microscope with ultraviolet epi-illumininations by the method of Hobbie et al. (1977).

Chapter III

RESULTS

A total of 123 individual experimental algal cultures were grown under 43 treatment combinations. A treatment combination comprises one set of CO_2, O_2, pH, and light-intensity levels. Additional algal cultures were grown under conditions where the pH and concentrations of CO_2 were allowed to vary during the growth period.

The algae were not acclimated to individual treatment combination conditions before each experiment. Significant differences were found between growth-rate coefficients k_{cc}, k_{pc}, and k_{pn} calculated for individual cultures. This is an indication of nonsteady-state conditions where cell division, carbon fixation, and nitrogen fixation proceed at different rates. The examination of nonsteady-state growth is relevant to the growth of algae under changing CO_2, O_2, pH, and light intensities that are common to mass outdoor algal cultures.

Cell concentrations were measured and a growth-rate coefficient k_{cc} was calculated for each individual algal culture by the least-squares fit of the measurements to the expression $Y = Y_o e^{kt}$. The growth-rate coefficients k_{cc}, presented in Table 3, are mean values from duplicate and triplicate algal cultures grown at given treatment combinations. Representative plots of the growth of 3H are presented in Appendix C. To obtain sufficient sample volumes for measuring concentrations of particulate carbon (PC) and particulate

Table 3

CELL-CONCENTRATION GROWTH-RATE COEFFICIENT k_{cc} OF 3H FOR THE 43 TREATMENT COMBINATIONS OF CO_2, O_2, pH, AND LIGHT

O_2 (μmole/L)	pH	Light ($\mu w/cm^2$)	CO_2 (μmole/L) 318			159			32			16		
			n	$\bar{x}$	σ	n	$\bar{x}$	σ	n	$\bar{x}$	σ	n	$\bar{x}$	σ
15	7.5	350	3	0.114	0.001	6	0.110	0.003	3	0.090	0.005	6	0.111	0.010
15	7.5	750	3	0.138	0.002	6	0.131	0.004	3	0.124	0.002	6	0.134	0.016
15	7.5	1500	3	0.143	0.002	3	0.137	0.008	3	0.121	0.004	6	0.139	0.010
1250	7.5	350				3	0.105	0.002	2	0.100	0.019	3	0.111	0.005
1250	7.5	750				3	0.133	0.006	1	0.118	-	3	0.144	0.002
1250	7.5	1500				3	0.130	0.006	3	0.140	0.008	3	0.150	0.006
1250	8.4	350										3	0.117	0.008
1250	8.4	750										3	0.125	0.002
1250	8.4	1500										3	0.136	0.007
1250	8.4	3500										3	0.128	0.005
262	6.8	350				2	0.113	0.008				2	0.117	0.001
262	6.8	1500				2	0.127	0.004				2	0.151	0.003
262	6.8	3500				2	0.126	0.004				2	0.144	0.006
262	7.6	350				2	0.114	0.000				2	0.115	0.002
262	7.6	1500				2	0.128	0.003				2	0.153	0.000
262	7.6	3500				2	0.134	0.006				2	0.149	0.016
262	8.4	350				2	0.073	0.019 [a]				2	0.117	0.018
262	8.4	1500				2	0.096	0.001				2	0.146	0.002
262	8.4	3500				2	0.088	0.006				2	0.142	0.001

[a] Carbonate precipitation; abnormal growth-rate coefficients.

nitrogen (PN), it was necessary to combine samples from duplicate or triplicate cultures. The growth-rate coefficients k_{pc} and k_{pn} were calculated for each treatment combination by the least-squares fit of PC and PN measurements to the expression $Y = Y_o e^{kt}$. The k_{pc} and k_{pn} growth-rate coefficients are presented in Tables 4 and 5, respectively. These data were grouped and evaluated statistically to express the growth of 3H in terms of elevated CO_2 concentrations, different pH and light-intensity levels, and different O_2 concentrations. Differences between growth-rate coefficients were examined at different O_2 concentrations and light intensities. Algal growth in carbon-free medium and with HCO_3^- as the carbon source was observed in a separate set of experiments.

Algal Growth at Elevated CO_2 Concentrations

The growth rate of 3H as mean k_{cc} was examined at CO_2 concentrations of 16, 32, 159, and 318 µmole/L under four light intensities and three O_2 concentrations. The data were grouped and analyzed at CO_2 concentrations of 16 and 159 µmole/L at each of four light intensities as shown in Table 6. Note that the k_{cc} values for different oxygen concentrations were combined within the light-intensity groups. This grouping was supported by an analysis of variance using the methods of Lapin (1973), comparing variation within and between sampled means of k_{cc} at different O_2 concentrations. No significant change in mean k_{cc} was attributable to change in O_2 concentration. Sample calculations are presented in Appendix D.

Table 4

PARTICULATE-CARBON GROWTH-RATE COEFFICIENT k_{pc} OF 3H
FOR THE 43 TREATMENT COMBINATIONS OF CO_2, O_2, pH, AND LIGHT

Oxygen (µmole/L)	pH	Light ($\mu w/cm^2$)	Carbon Dioxide (µmole/L)			
			318	159	32	16
15	7.5	350	0.096	0.090, 0.091	0.084	0.094, 0.102
15	7.5	750	0.120	0.118, 0.117	0.117	0.218, 0.130
15	7.5	1500	0.144	0.130,	0.131	0.130, 0.138
1250	7.5	350		0.082	0.070	0.080
1250	7.5	750		0.099	0.098	0.108
1250	7.5	1500		0.104	0.124	0.120
1260	8.4	350				0.073
1260	8.4	750				0.092
1260	8.4	1500				0.104
1260	8.4	3500				0.105
262	6.8	350		0.093		0.095
262	6.8	1500		0.109		0.129
262	6.8	3500		0.117		0.124
262	7.6	350		0.072		0.089
262	7.6	1500		0.111		0.125
262	7.6	3500		0.123		0.128
262	8.4	350		0.061 [a]		0.093
262	8.4	1500		0.084		0.121
262	8.4	3500		0.078		0.126

[a] Carbonate precipitation; abnormal growth-rate coefficients.

Table 5

PARTICULATE-NITROGEN GROWTH-RATE COEFFICIENT k_{pn} OF 3H FOR THE 43 TREATMENT COMBINATIONS OF CO_2, O_2, pH, AND LIGHT

O_2 (μmole/L)	pH	Light ($\mu w/cm^2$)	CO_2 (μmole/L) 318	159	32	16
15	7.5	350	0.096	0.094, 0.088	0.077	0.088, 0.094
15	7.5	750	0.011	0.109, 0.116	0.114	0.124, 0.125
15	7.5	1500	0.129	0.124	0.096	0.121, 0.130
1250	7.5	350		0.083	0.071	0.082
1250	7.5	750		0.097	0.089	0.107
1250	7.5	1500		0.101	0.105	0.118
1250	8.4	350				0.078
1250	8.4	750				0.096
1250	8.4	1500				0.107
1250	8.4	3500				0.102
262	6.8	350		0.098		0.094
262	6.8	1500		0.114		0.126
262	6.8	3500		0.120		0.122
262	7.6	350		0.101		0.089
262	7.6	1500		0.115		0.124
262	7.6	3500		0.126		0.124
262	8.4	350		0.053[a]		0.091
262	8.4	1500		0.077		0.119
262	8.4	3500		0.070		0.124

[a]Carbonate precipitation; abnormal growth-rate coefficients.

Table 6

CELL-CONCENTRATION GROWTH-RATE COEFFICIENT k_{cc} OF 3H AT ELEVATED CO_2 CONCENTRATIONS[a]

Grouped Treatment Combinations[b]	CO_2 (µmole/L)					
	16			159		
	n	$\bar{x}$	σ	n	$\bar{x}$	σ
L_1 (O_1,O_2, O_3)	13	0.113	0.007	13	0.110	0.005
L_2 (O_1, O_3)	9	0.137	0.013	9	0.132	0.005
L_3 (O_1,O_2,O_3)	13	0.145[c]	0.101	10	0.131[c]	0.007
L_4 (O_2)	4	0.146[c]	0.011	4	0.130[c]	0.006

[a]This table includes all data from Table 3 for CO_2 concentrations of 16 and 159 µmole/L, except at pH 8.4.

[b]Treatment levels:

Light ($\mu w/cm^2$)		Oxygen (µmole/L)	
L_1 =	350	O_1 =	15
L_2 =	750	O_2 =	262
L_3 =	1500	O_3 =	1250
L_4 =	3500		

[c]Differences between CO_2 concentrations of 16 and 159 µmole/L are significant at the 95% confidence level.

Between CO_2 concentrations of 16 and 159 µmole/L, no significant difference was found for mean k_{cc} at light intensities of 350 and 750 $\mu w/cm^2$, but significant difference was found at light intensities of 1500 and 3500 $\mu w/cm^2$. The difference between means was tested using the t-test and methods of Volk (1970), and was found significant at the 95-percent confidence level. The growth-rate coefficient k_{cc} was lower at the higher CO_2 concentration. CO_2 concentration above 16 µmole/L did not increase the growth rate of 3H.

Algal Growth at Different pH Levels

The growth rate of 3H as k_{cc} was not significantly different for pH levels of 6.8, 7.6, and 8.4 at a CO_2 concentration of 16 µmole/L. At a higher CO_2 concentration, 159 µmole/L, a significant decrease in growth rate was found at pH 8.4. (See Table 7.) The k_{cc} values were analyzed in matched pairs and mean pair differences were tested for significance using the t-test. The growth rates at various pH and CO_2 levels are plotted in Figure 4 in relative units of percent rate. Nine algal cultures were grown at pH 9.0 and above. Precipitation occurred and the results were difficult to interpret. As a best estimate, the growth rate of 3H at pH 9.0 is about 20 percent of that at lower pH levels. The data from these nine cultures are not included in Tables 3, 4, or 5. The decrease in growth rate at pH 8.4 at elevated CO_2 concentrations involved carbonate precipitation as described in the following section.

Carbonate Precipitation at High pH and CO_2 Concentrations

The growth rate of 3H was depressed at pH 8.4 with a CO_2 concentration of 159 µmole/L. In comparison, there was no depression at

Table 7

CELL-CONCENTRATION GROWTH-RATE COEFFICIENT k_{cc} OF 3H AT DIFFERENT pH LEVELS[a]

Grouped Treatment Combinations[b]	pH		
	6.8	7.6	8.4
$O_2L_1C_2$	0.108	0.114[c]	0.060[c]
	0.119	0.114[c]	0.087[c]
$O_2L_1C_5$	0.118	0.114	0.105
	0.116	0.117	0.130
$O_2L_3C_2$	0.128	0.130[c]	0.095[c]
	0.122	0.126[c]	0.096[c]
$O_2L_3C_5$	0.153	0.153	0.148
	0.145	0.153	0.145
$O_2L_4C_2$	0.123	0.138[c]	0.092[c]
	0.129	0.130[c]	0.084[c]
$O_2L_4C_5$	0.148	0.161	0.143
	0.139	0.138	0.142

[a]Actual calculated values of k_{cc} for individual algal cultures are shown as means in Table 3 at oxygen concentration of 262 µmole/L.

[b]Treatment levels:

Oxygen (µmole/L)		Light ($\mu w/cm^2$)		Carbon Dioxide (µmole/L)
O_2 = 262	L_1 =	350	C_2 =	159
	L_3 =	1500	C_5 =	16
	L_4 =	3500		

[c]Differences between pH 7.6 and 8.4 are significant at the 95% confidence level.

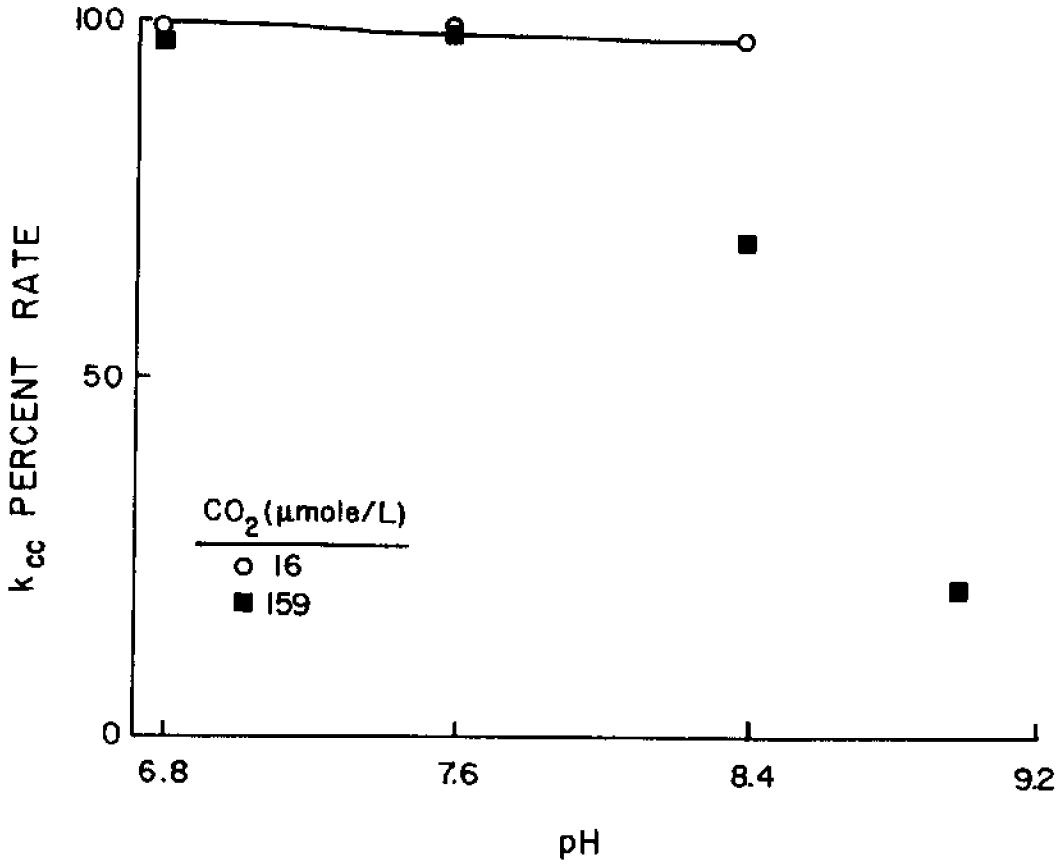


Figure 4. Growth rate of 3H at different pH levels.

pH 8.4 with a CO_2 concentration of 16 µmole/L. It is instructive to compare the change in PC and PN exhibited by algal cultures at the two levels of CO_2. Figure 5 shows that PC increased more rapidly in the culture grown at high CO_2. However, the increase is equivalent to a k_{pc} of 0.206, which is over seven doublings per day. The highly abnormal rate was accompanied by a carbon-to-nitrogen ratio of 29:1. This indicates that the nitrogen did not increase at a comparable rate. Precipitation of $CO_3^=$ was suspected. At a pH of 8.4 with a CO_2 concentration of 159 µmole/L, the equilibrium concentration of $CO_3^=$ is 4000/µmole/L. Pytkowicz (1965) reported precipitation of carbonate at this concentration required a nucleation time of about 10 hours.

Algal Growth at Different Light Intensities

The growth rate of 3H as k_{cc} increased with light intensity to 1500 µw/cm^2 (5000 lux) and was not inhibited at 3500 µw/cm^2 (11,500 lux) with a CO_2 concentration of 16 µmole/L. At a CO_2 concentration of 159 µmole/L, the maximum k_{cc} occurred at 750 µw/cm^2. The data are plotted in Figure 6. Note that k_{cc} is unaffected by O_2 concentration. The treatment combination grouping in Table 6 is appropriate for statistical analysis of the difference between mean k_{cc} values at each light intensity. Using the methods of Volk (1970) it was found that differences between k_{cc} at 350, 750, and 1500 µw/cm^2 were significant at the 95-percent confidence level at 16 µmole/L CO_2 concentration. At 159 µmole/L CO_2 concentration, the difference between k_{cc} at 350 and 750 µw/cm^2 was significant at the 95-percent confidence level.

The growth rate of 3H as k_{cc} was sensitive to elevated CO_2 concentrations and insensitive to changing O_2 concentrations. In

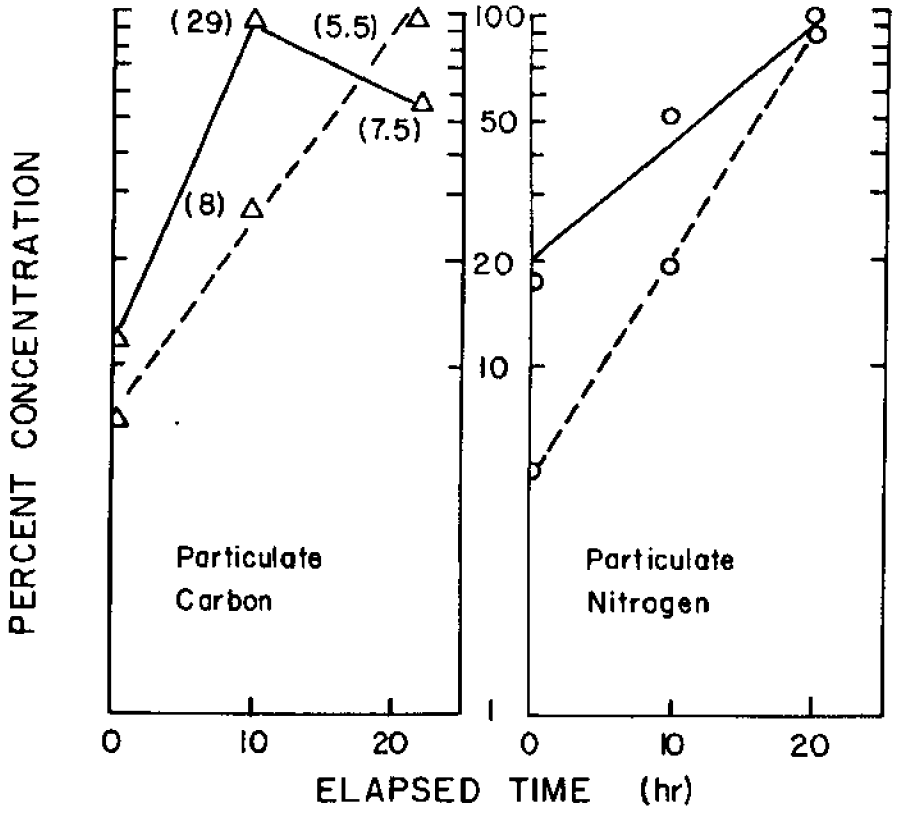


Figure 5. Growth characteristics of 3H at 16 (---) and 159 (——) µmole/L CO_2. The numbers in parentheses is the carbon-to-nitrogen ratio.

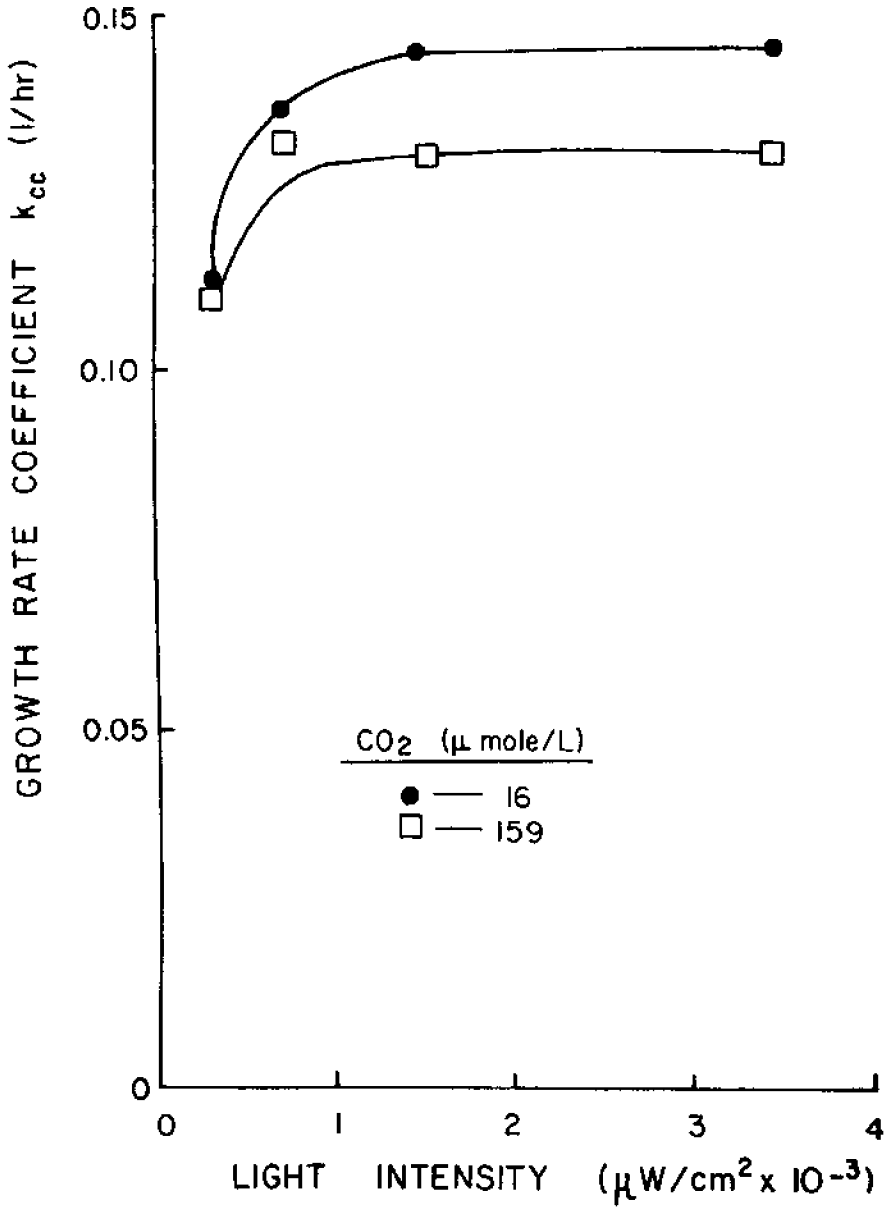


Figure 6. Growth rate of 3H as k_{cc} at different light intensities.

contrast, the growth rate of 3H as k_{pc} was unaffected by CO_2 concentrations but decreased significantly at increased O_2 concentration. The mean k_{pc} is plotted as a function of light intensity at two oxygen levels in Figure 7.

Algal Growth as k_{cc} and k_{pc} at Different O_2 Concentrations

The growth of 3H as k_{cc} was not affected by O_2 concentration. An analysis of variance using the methods of Lapin (1973) to compare variation within and between mean values for k_{cc} at different O_2 concentrations showed no significant relationship. Sample calculations are included in Appendix D. The growth rate of 3H as k_{pc} was significantly affected by O_2 concentration. The mean k_{pc} decreased as O_2 increased, as shown in Table 8 and plotted in Figure 7. The decrease in k_{pc} was significant, between 15 and 1250 µmole/L at 350, 750, and 1500 $\mu w/cm^2$ using the procedures of Volk (1970). The inhibition of k_{pc} by O_2 concentration at each light intensity is given in Table 9.

Differences Between Growth-Rate Coefficients

Significant differences were found between growth-rate coefficients k_{cc}, k_{pc}, and k_{pn}, as shown in Table 10. At low O_2 concentrations, the growth rate of 3H as k_{cc} was higher than the growth rate as k_{pc}, which itself was higher than the growth rate expressed as k_{pn}. At high O_2 concentration, the k_{cc} was unaffected, while both the k_{pc} and k_{pn} decreased. The decrease in k_{pc} was greater than the decrease in k_{pn} such that the difference between k_{pc} and k_{pn} became insignificant. The algal cultures were growing in nonsteady-state conditions.

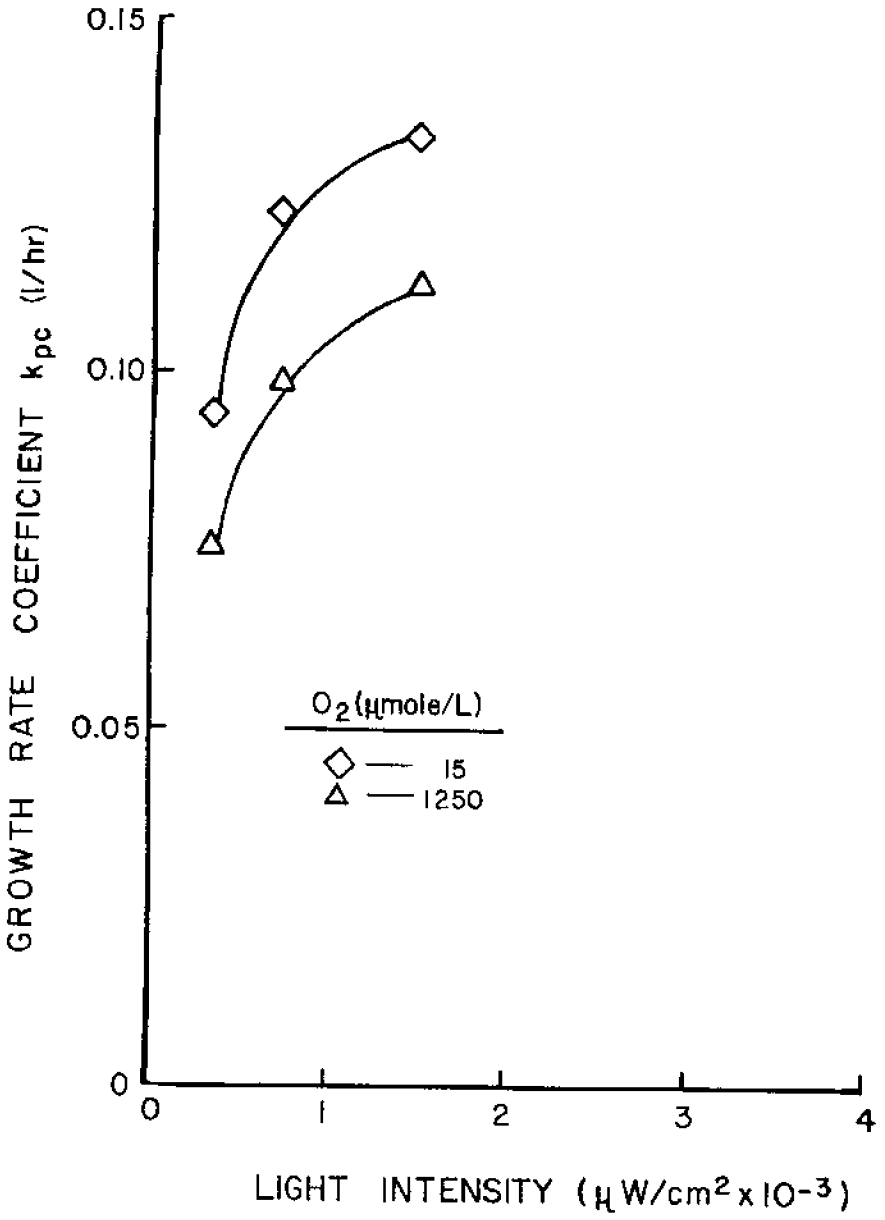


Figure 7. Growth rate of 3H as k_{pc} at different light intensities.

Table 8

PARTICULATE-CARBON SPECIFIC GROWTH-RATE COEFFICIENT k_{pc} OF 3H FOR DIFFERENT LIGHT INTENSITIES AT THREE O_2 CONCENTRATIONS[a]

Grouped Treatment Combinations[b]	O_2 (µmole/L)[c]								
	15			262			1250		
	n	$\bar{x}$	σ	n	$\bar{x}$	σ	n	$\bar{x}$	σ
L_1 (C_2, C_5)	4	0.094	0.005	4	0.087	0.010	3	0.078	0.005
L_2 (C_2, C_5)	4	0.123	0.007				3	0.100	0.008
L_3 (C_2, C_5)	3	0.133	0.005	4	0.118	0.010	3	0.109	0.009

[a]This table includes all data from Table 4 at carbon-dioxide concentrations of 16 and 159 µmole/L, except at pH 8.4.

[b]Treatment levels:

	Light[d] ($\mu w/cm^2$)		Carbon Dioxide (µmole/L)
L_1 =	350	C_2 =	159
L_2 =	750	C_5 =	16
L_3 =	1500		

[c]Differences in k_{pc} between oxygen concentrations of 15 and 1250 µmole/L are significant at the 95% confidence level at all light intensities.

[d]Differences in k_{pc} between L_1, L_2, and L_3 are significant at the 95% confidence level at all oxygen concentrations.

Table 9

INHIBITION OF THE GROWTH RATE OF 3H AS k_{pc} AT HIGH OXYGEN CONCENTRATIONS[a]

Light$_2$ ($\mu w/cm^2$)	Oxygen Inhibition[b] (%)
350	29
750	24
1250	18

[a]Data for k_{pc} from Table 8 at O_1 and O_3.

[b]Oxygen inhibition $= 100 \dfrac{k_{pc}\ (O_1) - k_{pc}\ (O_3)}{k_{pc}\ (O_3)}$

	Oxygen (μmole/L)
O_1 =	15
O_3 =	1250

Table 10

DIFFERENCE BETWEEN SPECIFIC-GROWTH-RATE COEFFICIENTS AT TWO OXYGEN CONCENTRATIONS[a]

Grouped Treatment Combinations[b]		$k_{cc} - k_{pc}$[c]			$k_{cc} - k_{pn}$[d]			$k_{pc} - k_{pn}$[e]		
		n	$\bar{x}$	σ	n	$\bar{x}$	σ	n	$\bar{x}$	σ
O_1 $\begin{pmatrix} C_1, C_2 \\ C_4, C_5 \end{pmatrix}$	L_1	6	0.015	0.008	6	0.018	0.006	6	0.003	0.005
	L_2	6	0.011	0.006	6	0.016	0.007	6	0.005	0.003
	L_3	6	0.001	0.008	5	0.015	0.006	5	0.015	0.012
O_3 $\begin{pmatrix} C_2, C_4 \\ C_5 \end{pmatrix}$	L_1	3	0.028	0.004	3	0.027	0.004	3	0.002	0.001
	L_2	3	0.030	0.005	3	0.034	0.004	3	0.004	0.004
	L_3	3	0.024	0.007	3	0.029	0.004	3	0.008	0.005

[a]Data for specific growth rates taken from Table 3, 4, and 5 at pH 7.5.

[b]Treatment levels:

Oxygen (µmole/L)		Carbon Dioxide (µmole/L)		Light ($\mu w/cm^2$)	
O_1 =	15	C_1 =	318	L_1 =	350
O_3 =	1250	C_2 =	159	L_2 =	750
		C_4 =	32	L_3 =	1500
		C_5 =	16		

[c]Difference significant at the 95% confidence level except at O_1L_1.

[d]Difference significant at the 95% confidence level.

[e]Difference significant at the 95% confidence level except at O_1L_2 and O_1L_3.

The light intensity and the O_2 concentration affected the difference between growth-rate coefficients, as shown in Figure 8.

Algal Growth in Carbon-Free Medium

In a separate experiment involving six individual algal cultures, the growth of 3H in a carbon-free medium was compared to its growth with HCO_3^- as the carbon source. The observations are shown in Figure 9. The open symbols represent cell-concentration measurements from three individual cultures grown in carbon-free media. The artificial seawater prepared for this investigation was treated to remove inorganic carbon by acidification. This seawater with nutrients added was bubbled with CO_2-free air. No appreciable growth occurred following inoculation in these three cultures (open symbols in Figure 9). The pH of the carbon-free medium was adjusted to between 7.8 and 8.0 with sodium hydroxide prior to inoculation.

At the same time, three cultures were prepared similarly, except that HCO_3^- was added and the medium brought to equilibrium with atmospheric air. Following inoculation, the atmospheric air was removed and replaced with CO_2-free air. The growth of the algae and the purging of CO_2-free air caused the pH of the algal medium to rise. The growth rate of 3H, as k_{cc}, during the initial 20 hours, was 0.140, which is comparable to growth in cultures with CO_2-enriched air. It appears that the rate of CO_2 generation from HCO_3^- was sufficient to support the growth of the algae. At a pH of about 8.8, the growth rate decreased and all three cultures appeared to be entering a stationary phase. In Figure 9, these cultures are represented by solid

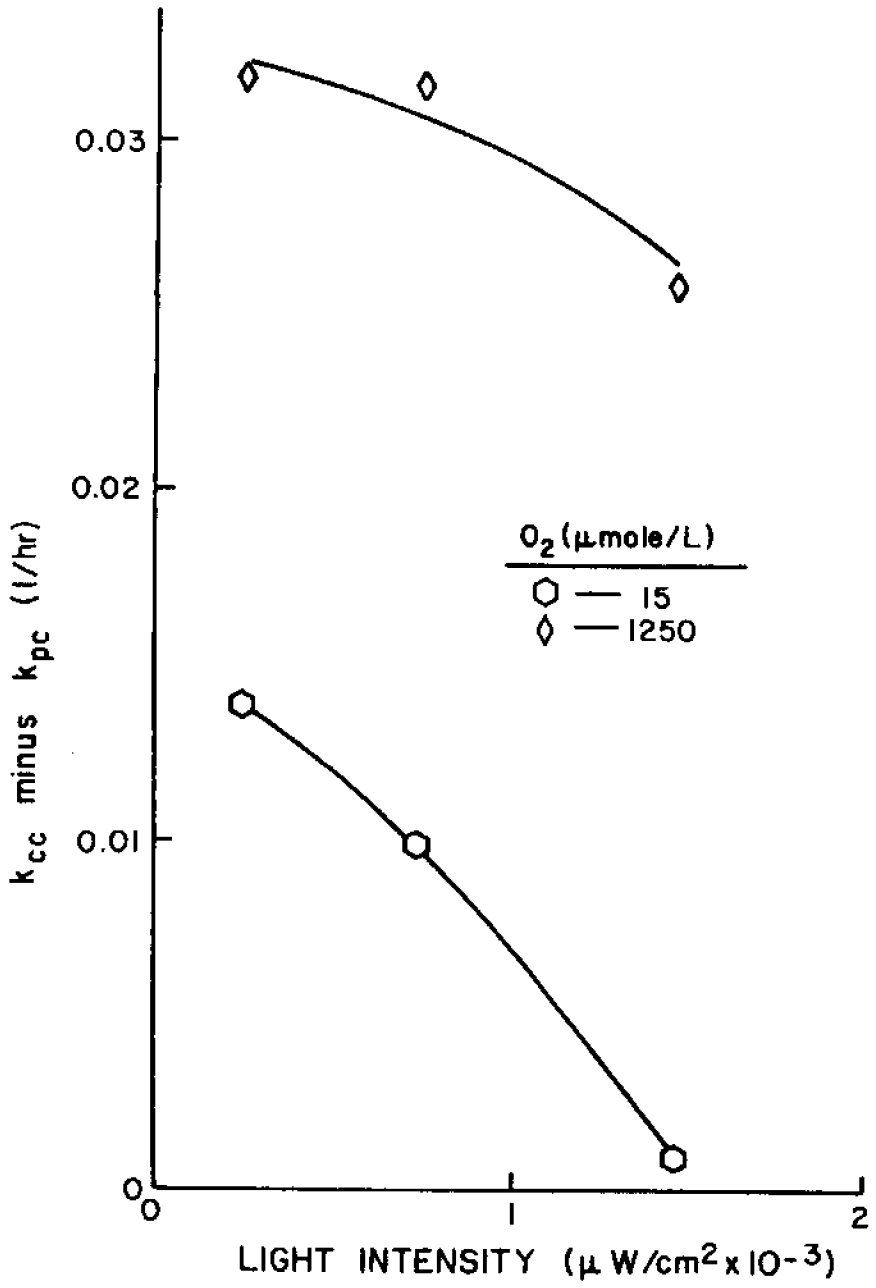


Figure 8. Difference between growth-rate coefficients.

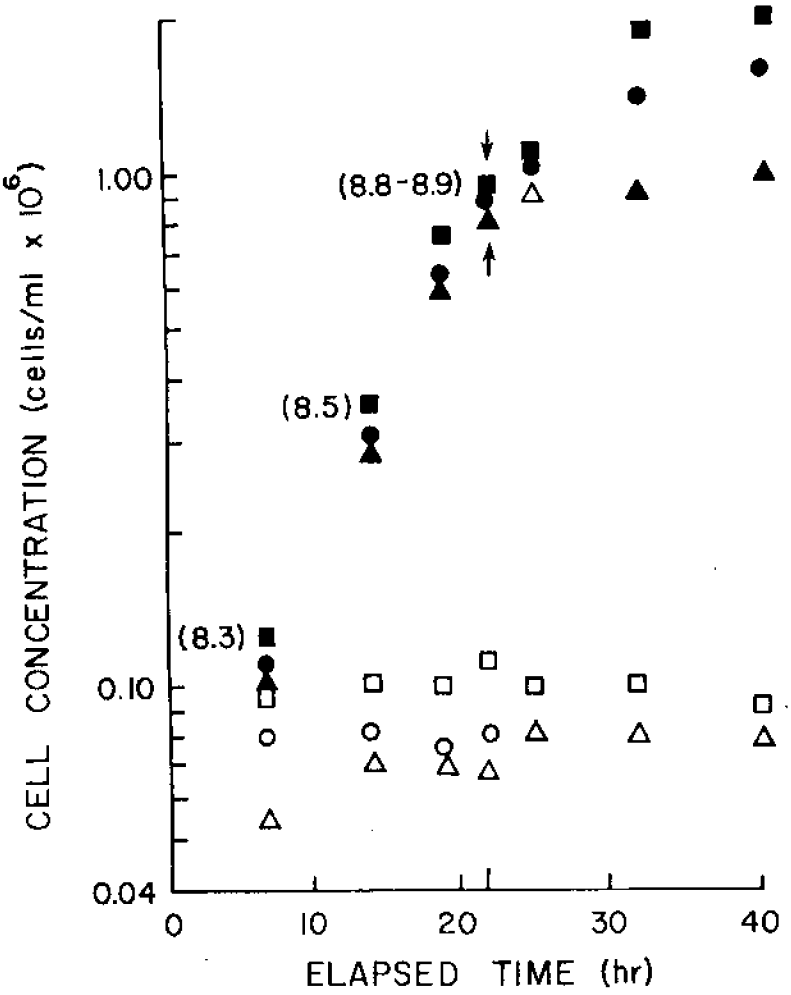


Figure 9. Growth of 3H in CO_2-free medium (open symbols) and with HCO_3^- added (closed symbols). Acid added to (■) and HCO_3^- added to (●) at 22 hours. pH is in parentheses.

symbols. At about 22 hours, hydrochloric acid was added to one of these three cultures and the growth rate increased (solid squares in Figure 9). Additional HCO_3^- was added to another of these cultures and modest increased growth resulted (solid circles in Figure 9). The remaining culture was not modified and entered a stationary growth phase at about 1 x 10^6 cells/ml (solid triangles, Figure 9).

Carbon Fixation in Relation to Chlorophyll "a" Content

Table 11 presents the growth rate of typical cultures of 3H from this experiment in terms of μmole C/mg chlorophyll·hr. In Figure 10, the carbon-to-chlorophyll ratios are presented for cultures at three light intensities and three pH levels. Note that this ratio was affected by both light intensity and pH level.

Table 11

GROWTH RATE OF 3H[a] IN µMOLE C/MG CHLOROPHYLL "a"·HR

pH	Light Inten. ($\mu w/cm^2$)	k_{pc}	Carbon Fixation (µmole/L·hr)	Chloro-phyll "a" (mg/L)	(µmole C/ mg chlor.)	Carbon Conc. (mg/L)	Carbon-to-Chlorophyll Ratio
6.8	350	0.095	30	0.193	158	4.0	20.7
6.8	1500	0.129	84	0.159	528	8.3	52.2
6.8	3500	0.124	59	0.094	628	6.1	64.9
7.6	350	0.089	23	0.103	225	3.3	32.0
7.6	1500	0.125	78	0.086	909	8.0	93.0
7.6	3500	0.128	72	0.056	1276	7.1	126.8
8.4	350	0.093	27	0.097	278	3.6	37.1
8.4	1500	0.121	64	0.087	740	6.8	78.2
8.4	3500	0.126	71	0.059	1208	7.2	127.1

[a]At 16 µmole CO_2/L and 262 µmole O_2/L, included in Table 4.

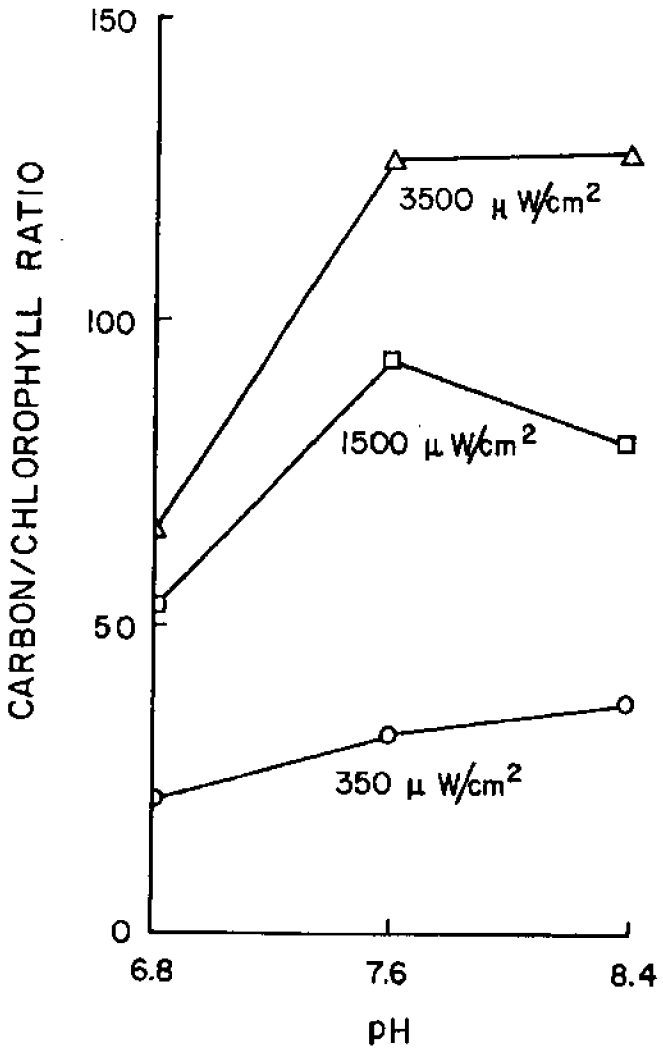


Figure 10. Carbon-to-chlorophyll ratios for cultures of 3H.

Chapter IV

DISCUSSION

The kinetics of inorganic carbon are viewed from the standpoint of chemical-reaction rates including molecular transport of CO_2 from the gas phase, generation of CO_2 from HCO_3^- by dehydration and direct hydroxyl reactions, and CO_2 uptake by the algae. Particular attention is paid to conditions where CO_2 uptake by the algae exceeds CO_2 transport from the gas phase resulting in the generation of CO_2 from HCO_3^-. Oxygen inhibition of photosynthesis is discussed in light of the decrease in net carbon fixation and potential aggravation of nonsteady-state conditions. Engineering aspects include the development of a model for estimating CO_2 generation rate from HCO_3^-, the best operating conditions for algal growth, and special considerations for bivalve molluscan mariculture.

Inorganic Carbon

Seawater at a salinity of 20 parts/1000 and 24°C at equilibrium with the atmosphere has a "free" or molecular carbon-dioxide concentration (hereafter denoted as CO_2) of 10.5 μmoles/L. Increasing CO_2 to 16 or 159 μmoles/L did not increase the growth rate of Thalassiosira pseudonana clone 3H as presented in Table 6. Yet, it was demonstrated (Pruder, et al., 1977) with the same clone that CO_2 enrichment of an

aerating gas markedly increased the yield and useful life of 9,000-L pool cultures of 3H in a greenhouse. These findings are not incompatible, nor are they new.

Warburg (1919) found that ordinary atmospheric air (0.033 percent CO_2) would not support the growth of certain cultures of Chlorella and resorted to a mixture of air and 5 percent CO_2. Subsequently, Warburg (1952) suggested that a high concentration of CO_2 was essential for optimum photosynthesis in Chlorella. This suggestion was disputed by Nielson (1966), who reported that Chlorella was able to photosynthesize and grow optimally at 0.033 percent CO_2, and even less.

The role of inorganic carbon in limiting the growth of algae in natural waters is a subject of controversy. On one side Lange (1970), Kuentzel (1969), and Kerr (1970) discount atmospheric CO_2 and bicarbonate alkalinity as major sources of inorganic carbon for algal growth. In contrast, Goldman (1974) supports the position that the availability of inorganic carbon from bicarbonate alkalinity is sufficient to ensure some other nutrient in algal-growth limiting. Persuasive experimental evidence suggests substantial benefit from CO_2 enrichment. Based upon the data presented in this dissertation, the value of increasing CO_2 above 16 µmoles/L is questionable. Yet, some factor associated with inorganic carbon limits the growth of 3H in mass cultures.

It has been demonstrated experimentally in this investigation that diffusion and chemical conversion of HCO_3^- were the only sources of inorganic carbon available for photosynthesis. The growth of 3H was poor in the prepared algal media bubbled with CO_2-free air (Figure 9)

The addition to the media of HCO_3^- as an $NaHCO_3$ solution supported excellent growth of 3H, which is also shown in Figure 9. The growth-rate coefficient k_{cc} was 0.140/hr, which is not significantly different from the k_{cc} levels in CO_2-enriched cultures reported in Table 3. The rate of generation of CO_2 from the HCO_3^- did not restrict the growth rate of 3H at moderate pH levels. The growth rate did begin to decline as the pH approached 8.8. The direct or indirect involvement of pH was indicated when growth increased markedly following the addition of hydrochloric acid.

The growth characteristics of the experimental cultures shown in Figure 9 are representative of batch-culture problems with 3H. It is therefore instructive to analyze the kinetics of inorganic-carbon mobilization, including concentrations, reaction rates, and CO_2 transport, generation, and uptake. In the discussion that follows, CO_2 transport refers to gas transfer by molecular diffusion, CO_2 generation refers to dehydration or direct hydroxyl reactions of HCO_3^-, and CO_2 uptake refers to CO_2 removed from the medium by algae. In this analysis, it is assumed that CO_2 is the species taken up by the algae and that catalytic agents (for example, the enzyme carbonic anhydrase) do not play a significant role. Further a simplified carbon-dioxide system will be considered; ion pairing and complexing with organic components will not be considered.

Miller et al. (1971) suggested a differential equation to express $d/dt\ (CO_2)$ in terms of the kinds of reactions, rate constants, and equilibrium and dissociation constants involved. Expanding that differential equation to include the CO_2-uptake activities of algae in

the system yields

$$\frac{d}{dt}(CO_2) = -k_1(CO_2) + \frac{k_1}{K_h}(H_2CO_3) - k_2(OH^-)(CO_2) + \frac{k_2K_w}{K_1'}(HCO_3^-) - k_3(CO_2) + \frac{k_3}{K_p}(CO_2)_g - k_4(CO_2) + k_4'(PC) \quad (1)$$

where d/dt (CO_2) = change in CO_2 concentration in the algal medium per unit time

k_1 = rate constant $\quad CO_2 + H_2O \xrightarrow{k_1} H_2CO_3$

k_2 = rate constant $\quad CO_2 + OH^- \xrightarrow{k_2} HCO_3^-$

k_3 = rate constant $\quad CO_2\ (solution) \xrightarrow{k_3} CO_2\ gas$

k_4 = rate constant $\quad CO_2 \xleftarrow[photosynthesis]{k_4} PC$

k_4' = rate constant $\quad CO_2 \xrightarrow[respiration]{k_4'} PC$

K_h = equilibrium constant $\quad \frac{(H_2CO_3)}{(CO_2)}$

K_p = equilibrium constant $\quad \frac{(CO_2)gas}{(CO_2)}$

K_w = ion product of water $\quad (OH^-)(H^+)$

K_1' = first dissociation constant of carbonic acid $\quad \frac{(H^+)(HCO_3^-)}{(CO_2)}$

K_2' = second dissociation constant of carbonic acid $\quad \frac{(H^+)(CO_3^=)}{(HCO_3^-)}$

The particular culture conditions chosen for detailed analysis at 24°C included the algal medium previously described. The values of constants and other quantities essential and appropriate to this analysis are

$k_1 = 3.2 \times 10^{-2}$ (Miller et al., 1971)

$k_2 = 3.2 \times 10^{4}$ (Miller et al., 1971)

k_3 = see discussion of net CO_2 transport

k_4 and k_4' = see discussion of net CO_2 uptake rate estimates

$K_h = 2.0 \times 10^{-3}$ (Miller et al., 1971)

$K_1' = 0.851 \times 10^{-6}$ (Lyman, 1956)

$K_2' = 0.501 \times 10^{-9}$ (Lyman, 1956)

CO_2 Solubility = 31,800 μmole/l·atm
(Murray and Riley, 1971)

Total inorganic carbon = 1370 μmole/L

The system described by the differential equation (1) presented earlier is shown schematically in Figure 11.

Net CO_2 Uptake by Algae

$$\frac{d}{dt}(CO_2) = [-k_4 (CO_2) + k_4' (PC)]$$

The net carbon-uptake rate (net carbon-fixation rate) by algae varies considerably, depending upon the organism, the environment, and the stage of growth. Typical experimental algal cultures grown as part of this investigation fixed carbon at 84 μmole/L·hr maximally

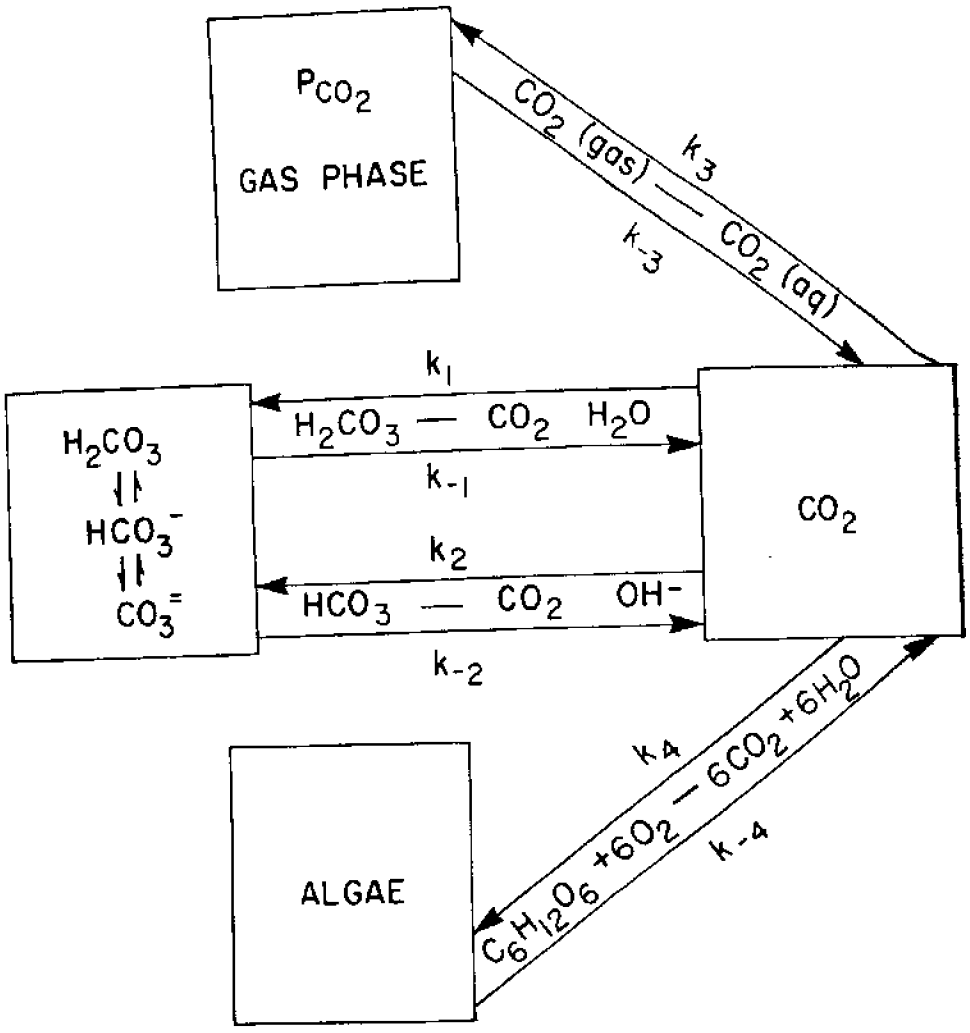


Figure 11. Schematic diagram of the relationship between reactions involving CO_2.

(Table 11). This rate is 50 to 100 times faster than some less dense, slower growing laboratory cultures. However, it is considerably slower than the 600 μmole/L·hr fixation rate we have achieved in 400-L tubes using CO_2 and pH control (unpublished). Bassham (1977) reports maximum yields for Chlorella of 28 g/m^2·day, total dry matter. Assuming a 50 percent carbon content and a 12-hour growing period, the average carbon fixation rate is 0.1 mole C/m^2·hr. The depth of the culture determines the fixation rate per unit volume. For example at a 1-meter depth, the fixation rate is 100 μmole/L·hr; and at a 10-cm depth, the rate is 1000 μmole/L·hr. For the purposes of this discussion, net CO_2 uptake rates of up to 1000 μmole/L·hr were considered.

Net CO_2 Transport by Molecular Diffusion

$$\frac{d}{dt}(CO_2) = [-k_3 (CO_2) + \frac{k_3}{K_p} (CO_2)g]$$

The diffusion pathway, considering only ordinary air, is generally discounted as significant as a source of inorganic carbon to support algal blooms. The pathway is considered here to demonstrate its viability for high-yield algal cultures if higher CO_2 partial pressures (P_{CO_2}) and/or higher gas-liquid interface areas are used.

The transport of gaseous CO_2 into the algal medium is initiated by dissolution into an outer layer of liquid at the gas-liquid interface. The CO_2 concentration in this layer is equal to the product of the gas phase P_{CO_2} and the solubility coefficient in the medium. The CO_2 concentration in the medium exists at the inner layer of the

diffusion barrier. The differential concentration provides the driving force to transport CO_2 from the gas to the liquid phase.

Kanwisher (1963) utilized a simple film model in discussing the diffusion of CO_2 from the atmosphere into seawater. The mathematical model and his values for diffusivity and apparent diffusion barrier thickness are

$$\text{Flux} = \frac{CO_2 \text{ concentration x diffusivity}}{\text{apparent barrier thickness}} \quad (2)$$

where

Flux = μmole/cm^2·sec

CO_2 = μmoles/L

Diffusivity = 2×10^{-5} cm^2/sec

Thickness = 47 μm under strong stirring

127 μm under weak stirring

Danckwerts (1970) considers this model unrealistic, but reports that predictions based upon its use are usually remarkably similar to those based upon more sophisticated models.

The maximum flux of CO_2 from the atmospheric air into "weakly stirred" media, assuming zero CO_2 concentration, is 590 μmole/m^2·hr. This is less than 1 percent of the carbon-uptake rate by algae in high-yield cultures. The flux can be increased by CO_2 enrichment of the aerating gas and through media agitation. Increased gas-liquid interface area, by the introduction of small bubbles, will increase the CO_2 transport rate.

Net CO_2 Generation by Dehydration and Hydroxyl Reactions of HCO_3^-

$$\frac{d}{dt}(CO_2) = \left| -k_1(CO_2) + \frac{k_1}{K_h}(H_2CO_3) - k_2(OH^-)(CO_2) + \frac{k_2 K_w}{K_1'}(HCO_3^-) \right|$$

To calculate the maximum generation rate of CO_2 from HCO_3 it is necessary to estimate the concentration of CO_2, HCO_3, and OH in the algal medium. Ionization proceeds faster than hydration, justifying the following substitution:

$$(H_2CO_3) = \frac{K_h}{K_1'}(a_H)(HCO_3^-) \qquad (3)$$

Under equilibrium conditions the net CO_2 generation rate from HCO_3^- is zero and the equilibrium expressions are

$$\text{dehydration } k_1(CO_2) \rightleftharpoons \frac{k_1}{K_1}(a_H)(HCO_3^-) \qquad (4)$$

$$\text{hydroxyl reaction } k_2(OH^-)(CO_2) \rightleftharpoons \frac{k_2 K_2}{K_1}(HCO_3^-) \qquad (5)$$

It can be seen that if the concentration of CO_2 in the medium is zero, maximum rate of CO_2 generation will occur in both reactions. The maximum CO_2 generation rates for the algal medium are 1200 μmole/L·hr by dehydration and 1660 μmole/L·hr by direction hydroxyl reaction. The combined maximum generation rate exceeds the requirements of high-yield cultures by a factor of almost three.

However, it is important to recognize that an algal culture using bicarbonate does so because the CO_2 concentration in the medium has dropped below equilibrium as the result of CO_2 uptake by the algae. At equilibrium conditions the net CO_2 generation rate is zero and the CO_2 concentration is 10.5 μmole/L in equilibrium with the atmosphere and other carbon species in the medium. If the CO_2 concentration in the medium is assumed to be zero, we have a 10.5 μmole/L differential CO_2 concentration. It can be shown that the pH increases with algal uptake of CO_2 and the equilibrium concentration of CO_2 decreases, lowering the maximum differential CO_2 concentration and the maximum CO_2 generation rate.

Artificial seawater at 20 parts/1,000 salinity has a total inorganic carbon content of 1370 μmole/L, listed in Table 1 as HCO_3^-. The CO_2 concentration, assuming equilibrium with air and a solubility coefficient of 31,800 μmole/L·atm, is 10.5 μmoles/L. Using the classical treatment of pH and carbonate alkalinity of Buch (1930) where

$$P_{CO_2} = \frac{CA\ a_h}{K'\ \alpha_s[1 + 2K'_2/a_H]} \tag{6}$$

and

$$C_T = CA \left| \frac{1 + K_2/a_H + a_H/K'}{1 + 2K'_2/a_H} - \right| \tag{7}$$

initial pH and carbonate alkalinity CA can be calculated. This was

accomplished using the computer in a program to find the root to the equation $f(a_H) = 0$. The calculated initial (a_H) was 0.703×10^{-8} or a pH of 8.15. The CA was then calculated to be 1390 μmole/L.

The equilibrium concentrations of HCO_3^-, $CO_3^=$, and CO_2 can be calculated using the expressions

$$c_{HCO_3^-} = \frac{CA}{1 + 2K'_2/a_H} \tag{8}$$

$$c_{CO_3^=} = \frac{CA\ (K_2')}{[a_H + 2K_2']} \tag{9}$$

$$c_{CO_2} = \frac{CA\ a_H}{K_1'[1 + 2K_2'/a_H]} \tag{10}$$

The algae remove CO_2 from the medium and assuming isolation from the atmosphere, C_T (total inorganic carbon) decreases and pH increases. Using the equations above, equilibrium concentrations were calculated for incremental increases in pH. The concentration of various carbon forms are presented in Table 12 and plotted in Figure 12. Substituting the a_H and HCO_3^- concentrations into equations 4 and 5 the maximum CO_2 generation rate can be calculated for a growing algal culture. The CO_2 generation from HCO_3^- by dehydration and direct hydroxyl reactions are presented in Table 13 and plotted in Figure 13. It is assumed in this treatment that CA remains constant during the algal growth and that the a_H-CA relationship is properly expressed by equations 8, 9, and 10. This is useful as a first approximation. It is recognized that total alkalinity does change during algal growth

Table 12

CALCULATED EQUILIBRIUM CONCENTRATIONS OF VARIOUS CARBON FORMS AND pH AS CO_2 IS REMOVED FROM AN ALGAL MEDIUM ISOLATED FROM THE ATMOSPHERE

	Carbon Form (μmole/L)			
pH	Total Carbon	CO_2	HCO_3^-	$CO_3^=$
8.14	1370	10.5	1230	80
8.30	1290	6.9	1170	120
8.50	1240	3.9	1060	170
8.70	1170	2.2	930	240
8.90	1090	1.1	780	310
9.10	1010	0.6	620	390
9.30	930	0.3	470	460

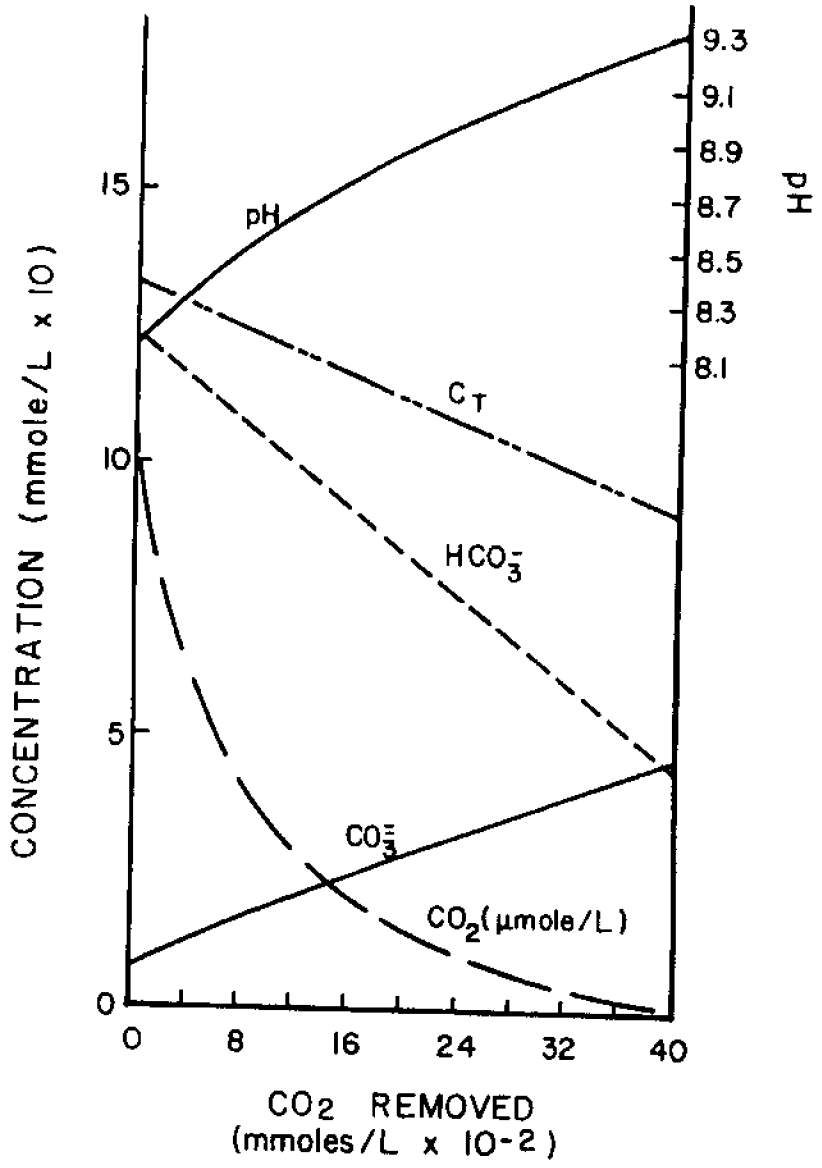


Figure 12. Calculated pH and equilibrium concentrations as CO_2 is removed from an algal medium isolated from the atmosphere.

Table 13

CALCULATED MAXIMUM GENERATION RATE OF CO_2 FROM HCO_3^-
BY DEHYDRATION AND DIRECT HYDROXYL REACTION
AS CO_2 IS REMOVED FROM AN ALGAL MEDIUM
ISOLATED FROM THE ATMOSPHERE

HCO_3^- (μmole/L)	pH	CO_2 Generation Rate (μmole/L·hr)	
		Dehyrdation	Hydroxyl
1230	8.14	1200	1660
1170	8.30	790	1570
1060	8.50	450	1430
930	8.70	260	1260
780	8.90	130	1050
620	9.10	70	840
470	9.30	30	630

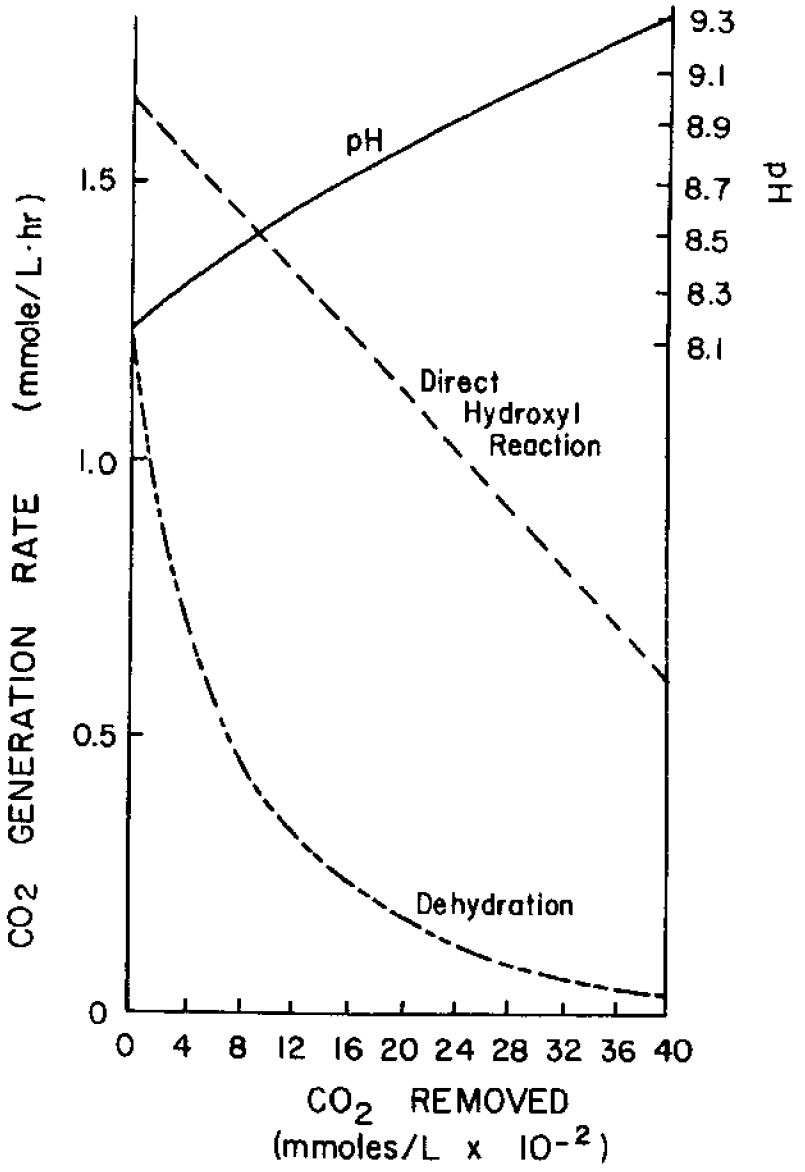


Figure 13. Calculated pH and maximum CO_2-generation rate from HCO_3^- as CO_2 is removed from an algal medium isolated from the atmosphere.

(Brewer and Goldman, 1976) and that at high pH levels borate alkalinity becomes appreciable at about 30 percent of the carbonate alkalinity level.

Inorganic-Carbon-Induced Growth Failure of 3H Cultures

From the results of this investigation, it can be concluded that increasing the concentration of CO_2 above 16 µmole/L did not enhance the growth of 3H (Table 6). Further, it has been shown that CO_2 generation from HCO_3^- at moderate pH levels did not limit the growth of 3H. The growth on HCO_3^- was not appreciably different from that obtained under the conditions of CO_2 enrichment. Yet, as shown in Figure 9, algal cultures utilizing HCO_3^- could not sustain the high growth rate as the pH increased. As the pH approached 8.8, the growth rate started to slacken and cultures were virtually stationary by pH 9. It is enlightening to examine the concentration of carbon forms and the maximum CO_2 generation rate available at pH 8.8. The total carbon, HCO_3^-, and $CO_3^=$ concentrations are clearly above levels needed to support very rapid growth of 3H under different pH and CO_2 concentration as clearly established by the growth achieved at pH 6.8 with 16 µmole/L CO_2 (Table 7). Examination of Figure 11 shows that the maximum CO_2^- generation rate at pH 8.8 is more than 1300 µmole/L·hr. The calculated uptake by the cultures in Figure 9 is less than 50 µmoles/L·hr or less than 4 percent of the available generation rate. It becomes increasingly obvious that 3H will not grow well at pH 8.8 and a CO_2 concentration of about 2 µmoles/L for reasons not related to generation rate per se of CO_2 or to the availability of HCO_3^-. It

may be an effect of the CO_2 concentration, or the pH, but nonetheless the combination does not support the rapid growth of 3H. As the pH rises further, reaching 9, the CO_2 concentration drops to less than 1 1 µmole/L and 3H essentially stops growing.

Attempts to separate the effects of pH and CO_2 concentration have involved the utilization of special buffering materials with unknown biological affect. Swift (1966) used high concentrations of inorganic carbon to increase the CO_2 concentrations at higher pH levels with CO_2-enriched gas. The results of this thesis indicate that algal growth may be inhibited in super-saturated carbonate solutions. However, it was also found in the present work that growth of 3H may not be inhibited until CO_2 concentrations decrease to 2 µmole/L or lower. It may be possible to separate the pH and CO_2 effects by capitalizing on nonequilibrium conditions where different CO_2 concentrations can exist at a given pH, depending on the uptake rate of CO_2 by the algae.

Should it be determined that the low CO_2 is the limiting factor, then little argument will be found for the suggestions that algae able to use HCO_3^- directly, or even having a functional carbonic anhydrase, would enjoy advantages over 3H in an environment characterized by CO_2 concentrations below 2 µmole/L. Similarly, if high pH is eventually found to be the cause of reduced growth rate, it would appear reasonable to consider that an algal species able to grow well at high pH would have an advantage over 3H.

A principal difficulty in the successful mass culture of 3H lies in failure to maintain control of the critical parameters CO_2 and pH. This assumes that proper supplies of nutrients, vitamins, and silicates

have been provided. The addition of fertilizers common to semicontrolled mass cultures of algae accelerates the growth of the species present. The increased growth increases the demand on the system for CO_2, which is readily supplied by the carbonate system, but at a price. The price involves the consumption of H^+ and the production of OH^-, leading to an environment unsuitable for 3H. There are, of course, algal species that are less inhibited at the high pH and low CO_2 concentrations that characterize semicontrolled mass algal cultures. It would appear that Phaeodactylum tricornutum, the algae that dominates and renders ineffective many mass algal culture ponds, comes into its own because of the very conditions that inhibit the growth of 3H. It also suggests a rationale for why P. tricornutum seldom dominates in natural blooms.

It is not the concentration of inorganic carbon nor its reaction kinetics that are central to its role in growth failure of some algae. It is the consequence of its utilization in raising pH, especially in highly productive waters, that selects which particular algae will dominate. If photosynthetic activity is encouraged by the addition of fertilizers and the algae of choice has a CO_2 concentration-pH limit, then one must attend to the carbon system.

Oxygen Inhibition of Photosynthesis (Photorespiration)

High O_2 concentrations, 1200 µmole/L, were shown to inhibit the growth rate of 3H over a range of CO_2 concentrations and light intensities. The inhibition affected the rate of carbon and nitrogen fixation, but not the rate of cell division. This is illustrated by differences

between growth rate coefficients k_{cc}, k_{pc}, and k_{pn}, shown in Table 10 and plotted in Figure 8.

Inhibition of the rate of photosynthesis by high O_2 concentrations is considered evidence of photorespiration in the pentose cycle (C_3) plants (Downes and Hesketh, 1968; Jackson and Volk, 1970). Other investigators have reported the presence of photorespiration in micro and macro marine algae (Morris and Beardall, 1976; Black et al., 1976; and Burris, 1977). Black et al. (1976) concluded their paper by suggesting that photorespiration was present in the marine plants they studied at activities comparable to terrestrial pentose cycle plants. Photorespiratory loss can vary from a few percent to nearly 100 percent of the total CO_2 fixation (Tolbert, 1974).

The 18- to 29-percent photorespiratory loss of 3H under these experimental conditions is higher than the 12- to 18-percent inhibition reported for the marine diatom Phaeodactylum tricornutum by Morris and Beardall (1976). In contrast, Black et al. reported 19- to 83-percent inhibition and Burris (1977) reported a 67-percent inhibition of Thalassiosira pseudonana* growing in high oxygen concentrations.

The wide disparity in the magnitude of photorespiratory loss may be due in part to the organism, in part to the experimental conditions, and in part to the methods of measure and reporting. In the present study the percent inhibition was calculated from differences in given growth-rate coefficients derived for cultures growing in CO_2-enriched nitrogen and CO_2-enriched oxygen. The algae were grown at pH 7.3 to

*Obtained from the Food Chain Research Group Collection, Scripps Institution of Oceanography.

7.5 at 24°C over 24 hours. The growth-rate coefficients were calculated from actual measurements of cell concentration, PC, and PN. Black et al. (1976) and Burris (1977) utilized $NaH^{14}CO_3$ in 5- to 10-ml tubes bubbled with 100 percent oxygen and 100 percent nitrogen or helium. The experimental period was from 20 to 30 minutes and the photosynthetic rates were reported as μmole C/mg chlorophyll·hr.

A comparison of photosynthetic rates can be made using the results given in Table 11. The photosynthetic rates in the present work ranged from 158 to 1276 μmole C/mg chlorophyll·hr. Both Black et al. (1976) and Burris (1977) reported photosynthetic rates between 24 and 168 μmole C/mg chlorophyll·hr. It is suggested that algal growth in the tracer experiments was poor even for the cultures grown in nitrogen.

The photorespiratory loss found here and that reported by Black (1976) and Burris (1977) may reflect the true activities of the organism in different environments. The problems described by Pruder et al. (1977) could well have included a very high respiratory loss which was aggravated by poor algal growth at high pH and low CO_2 levels.

The inhibition of carbon- and nitrogen-fixation rates but not cell-division rates causes an additional problem. The presence of high oxygen concentrations may be detrimental in aggravating nonsteady-state conditions. The large differences between k_{cc} and k_{pc} and between k_{cc} and k_{pn} at high O_2 concentrations will result in a more rapid decrease in cell size or the amount of carbon and nitrogen per cell. Algal cultures enter a stationary phase if the cells decrease below a minimum size. The presence of high O_2 concentrations may

contribute to both a decrease in carbon-fixation rate and also to the premature failure of the culture itself.

Engineering Considerations

Within the framework of bivalve molluscan mariculture, successful mass cultivation of algae requires the efficient utilization of light to grow certain desirable algal species reliably at high concentrations and at minimum cost. Findings of the present research can provide useful guidelines for selecting operating parameters for the mass cultivation of Thalassiosira pseudonana clone 3H.

1. The amount of inorganic carbon in seawater, about 2300 µmole/L as HCO_3^- at a salinity of 34 parts/1,000, is insufficient to support the growth of high-yield algal cultures with net CO_2 uptake rates of 1000 µmole/L·hr.

2. CO_2 concentration above 16 µmole/L did not increase the growth rate. No decrease in growth rate occurred until a pH of 8.8 was reached, where the calculated equilibrium concentration of CO_2 drops below 2 µmole/L.

3. Growth rate was not affected over a pH range from 6.8 to 8.4 when carbonate alkalinity was 1390 µmole/L. Growth rate decreased at pH 8.8 and above.

4. Growth rate was decreased at pH 8.4 when carbonate alkalinity was 14,000 µmole/L and carbonate precipitation was implicated.

5. Growth rate did not increase at light intensities above 1500 $\mu w/cm^2$ (5000 lux) nor did it decrease at 3500 $\mu w/cm^2$ (11,500 lux).

6. High O_2 concentration decreased the rate of carbon and

nitrogen fixation 18 to 29 percent but had no effect on the rate of cell division.

7. Cell-division rate was consistently higher than carbon- and nitrogen-fixation rates. The carbon and nitrogen content per cell decreased as cell division continued under these experimental conditions.

Inorganic Carbon

Inorganic carbon must be added to the algal medium to sustain the growth of high-yield cultures. This, of course, is no different in principle from the long-recognized need to add nitrogen, phosphorus, silicates, vitamins, and trace metals. The desirability of automatic-control feeder systems is clear if a net CO_2 uptake by the algae of 1000 μmole/L·hr is considered. Addition of carbon as well as other nutrients might be required hourly or even continuously. A balanced additive solution could satisfy algal requirements from a single sensor-controller combination. Inorganic carbon may be added as gaseous CO_2 or in carbonate or bicarbonate forms if a concomitant supply of hydrogen ions is provided.

Carbon dioxide from the air in natural systems has been generally discounted because of slow diffusion rates. It was shown on page 53 ff. that increasing the air-liquid interface area could provide sufficient CO_2 transport for high-yield algal cultures. In Metcalf and Eddy (1972) aeration in the biological treatment of waste water was discussed and efficiencies for commercial-size surface aerators of 2 to 4 pounds O_2/hr·hp were reported. It is possible to estimate the efficiency and cost of CO_2 transport for algae using similar devices. Since both CO_2

and O_2 are slightly soluble gases with similar diffusivities (2.0 x 10^{-5} and 2.4 x 10^{-5} cm^2/s respectively), their respective fluxes are dependent upon differential concentrations of each gas across the diffusion barrier. Equilibrium concentrations for CO_2 and O_2 are 10.5 and 262 μmole/L respectively. As a first approximation, a surface aerator could be expected to transport about 4 percent as much CO_2 as O_2 for a given expenditure of energy. The costs related to CO_2 transport from the atmosphere are not grossly different from the costs of purchasing and introducing pure CO_2. Air can be considered as a serious candidate for CO_2 supply. There are two important considerations: (1) The algae of choice must be evaluated under any particular intended mixing conditions, since mechanical shear and spreading on surfaces are generally poorly tolerated; (2) generation of foam is to be avoided in algal cultures since it blocks light and interferes with photosynthesis.

There are applications where the hydrated form of inorganic carbon may be used to advantage. Large, outdoor ponds, open to the air, may present formidable problems to the efficient transport of CO_2 into the medium. In these cases, $NaHCO_2$ and acid additions should be considered. It was clearly shown that CO_2-generation rate from HCO_3^- was not rate limiting in the laboratory cultures (page 40 ff.). Maximum CO_2-generation rates were calculated assuming a minimum CO_2 concentration of zero and the data were plotted in Figure 13. The zero-CO_2-concentration assumption may not be realistic at least for some algal species. The findings here indicate that a CO_2 concentration of 2 μmoles/L or less may depress the growth of 3H. It is instructive to

consider CO_2 generation rates over a range of minimum CO_2 concentrations. It is also instructive to consider the effect of temperature on the CO_2 generation rates. It can be shown that CO_2 generation rates from HCO_3^- under certain combinations of pH, temperature, and carbon-fixation rates can limit culture growth for at least some algal species. To illustrate the point consider the mathematical model, a revised form of equation (1):

$$\frac{d}{dt}(CO_2) = -k_1(CO_2) + \frac{k_1}{K_1'}(a_H)(HCO_3^-) - \frac{k_2K_w}{(a_H)}(CO_2) + \frac{k_2K_w}{K_1}(HCO_3^-) \tag{11}$$

Express the rate constants and the equilibrium constants in the form

$$k = \text{constant } e^{E/RT} \text{ and } K = \text{constant } e^{H/RT}$$

Using the temperature-rate constant data from Miller et al. (1971)

$$k_1 = 8.04 \times 10^{11}\ e^{-9.22 \times 10^3/T(K^\circ)} \tag{12}$$

$$k_2K_w = 3.20 \times 10^{9}\ e^{-1.31 \times 10^4/T(K^\circ)} \tag{13}$$

Using the temperature-equilibrium constant data from Lyman (1956)

$$K'_1 = 3.64 \times 10^{-5}\ e^{-1.13 \times 10^3/T(K^\circ)} \tag{14}$$

$$K'_2 = 1.71 \times 10^{-6}\ e^{-2.4 \times 10^3/T(K^\circ)} \tag{15}$$

Expressing the (CO_2) and (HCO_3^-) in terms of carbonate alkalinity as

shown on page 57 and assuming a carbonate alkalinity of 1390 μmole/L, the inorganic carbon system is in equilibrium at pH 8.1 and CO_2 generation is zero.

In a rapidly growing culture, CO_2 is removed from the algal medium at a rate higher than it is supplied from air. This results in a drop of CO_2 concentration in the medium, driving the carbon system out of equilibrium and resulting in the generation of CO_2 from HCO_3^-. This generation of CO_2 from HCO_3^- is accompanied by a compensatory shift of HCO_3^- to $CO_3^=$. The result is a change in carbon species composition and pH. Once again the culture medium is considered to be isolated from the air for convenience. If the medium were exposed to the air, slight CO_2 transport would occur, complicating the analysis but having no significance in short-term, high-yield cultures.

The model was bound to a contour-plot program and computer trials were carried out calculating CO_2 transport rates in μmole/L·hr. A unit conversion from mole/L·s required multiplication by 3.6×10^6. The temperature range considered was 283°K to 303°K in 2.5-degree increments. The pH range was 8.1 to 9.3 in 0.1 unit increments. The CO_2 generation rates were calculated for combinations of temperature and pH for five levels of minimum CO_2 concentration, 0, 1, 2, 3, and 4 μmole/L (Figures 14, 15, 16, 17, and 18). The data are presented as contour plots representing intersections of a three-dimensional data surface (X = pH, Y = temperature, and Z = CO_2 transport rate) with planes parallel to the X-Y axis at the following selected CO_2-generation rates: 0, 200, 300, 400, 800, 1000, 1500, 2000, 2500, 3000, 3500, 4000, and 4500 μmole/L·hr. It appears obvious that the higher the free CO_2 concentration required by algae the narrower the acceptable

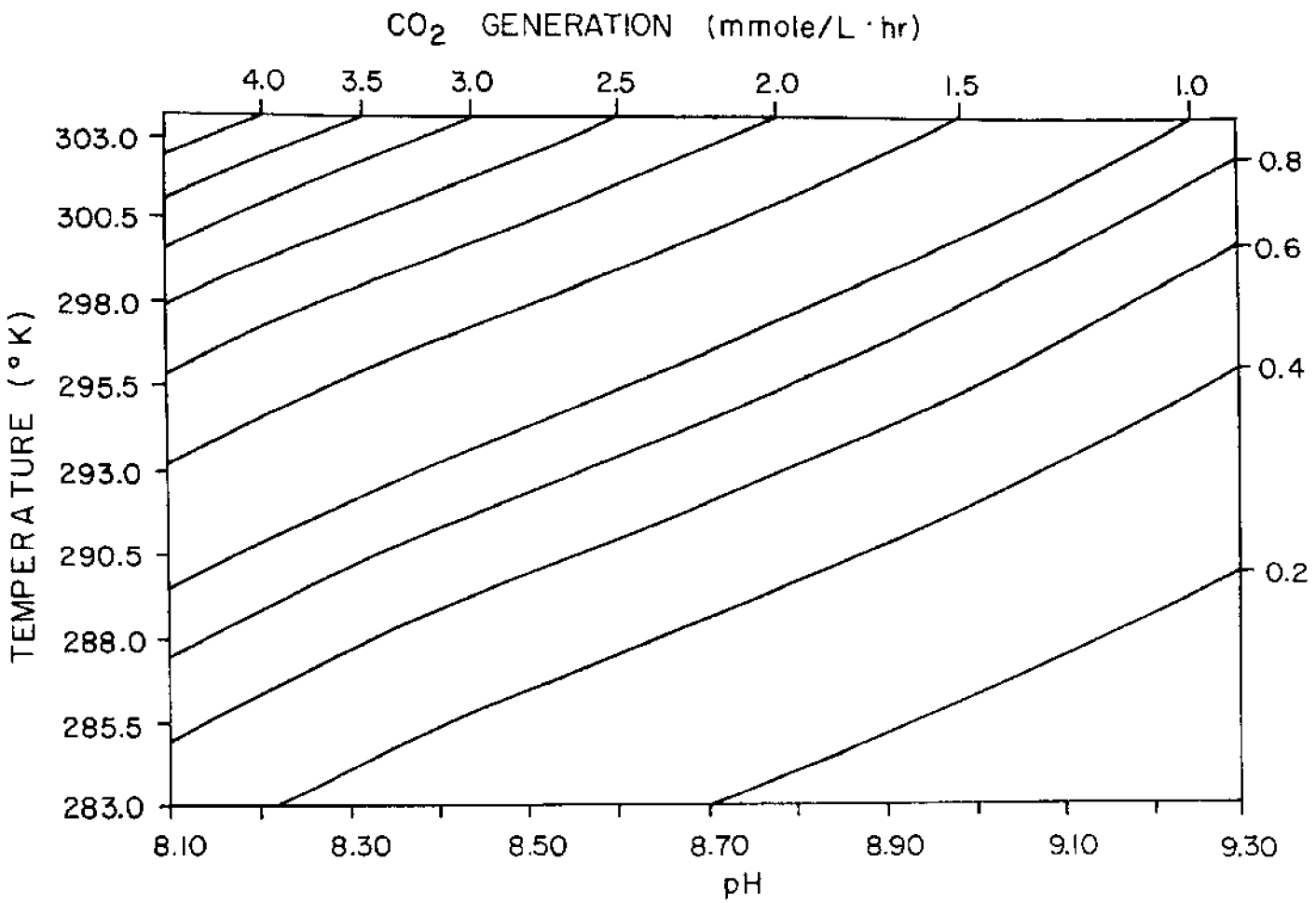


Figure 14. Maximum CO_2-generation rate from HCO_3^- as CO_2 is removed from an algal medium isolated from the atmosphere when minimum CO_2 concentration is zero.

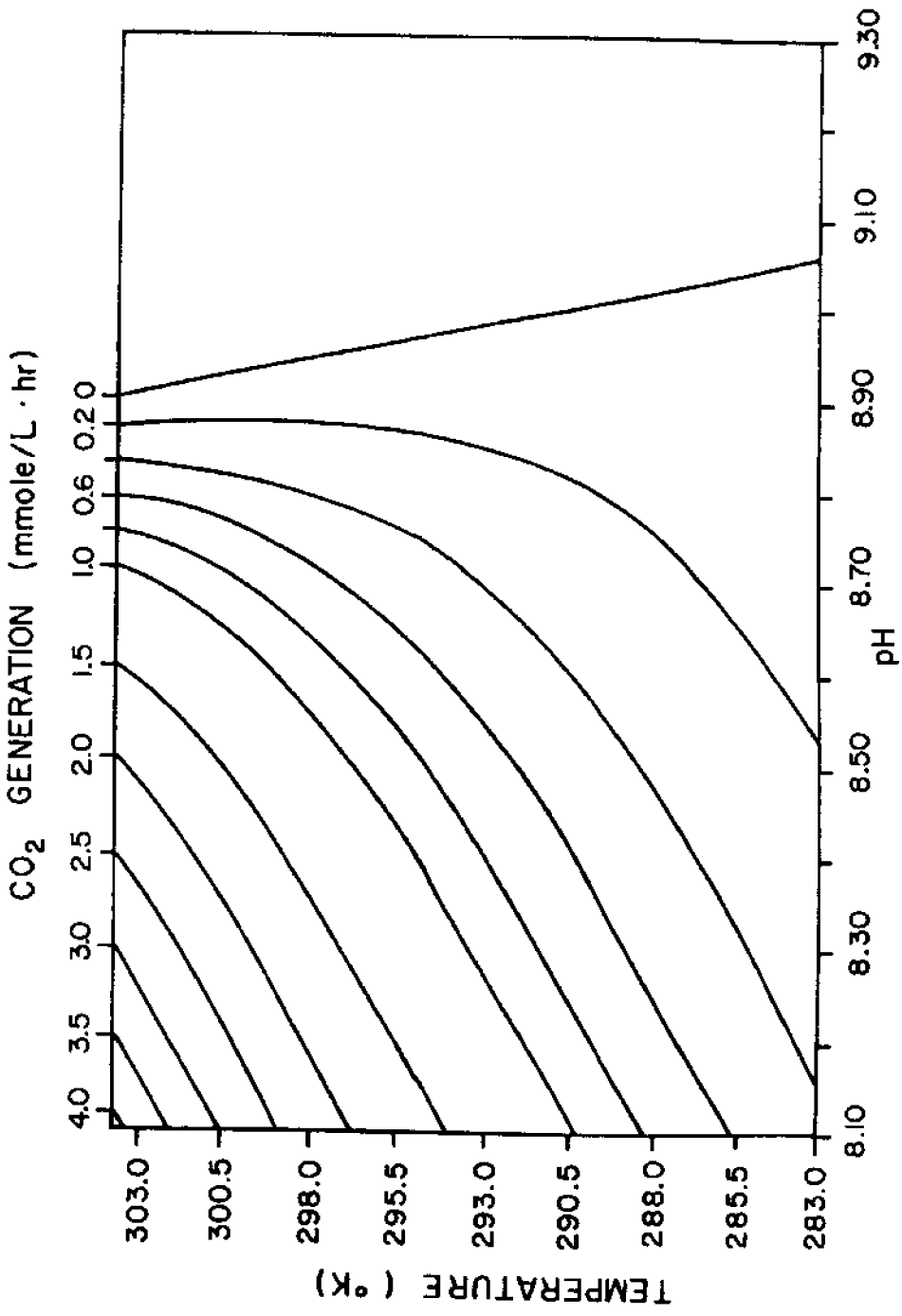


Figure 15. Maximum CO_2-generation rate from HCO_3^- as CO_2 is removed from an algal medium isolated from the atmosphere when minimum CO_2 concentration is 1 µmole/L.

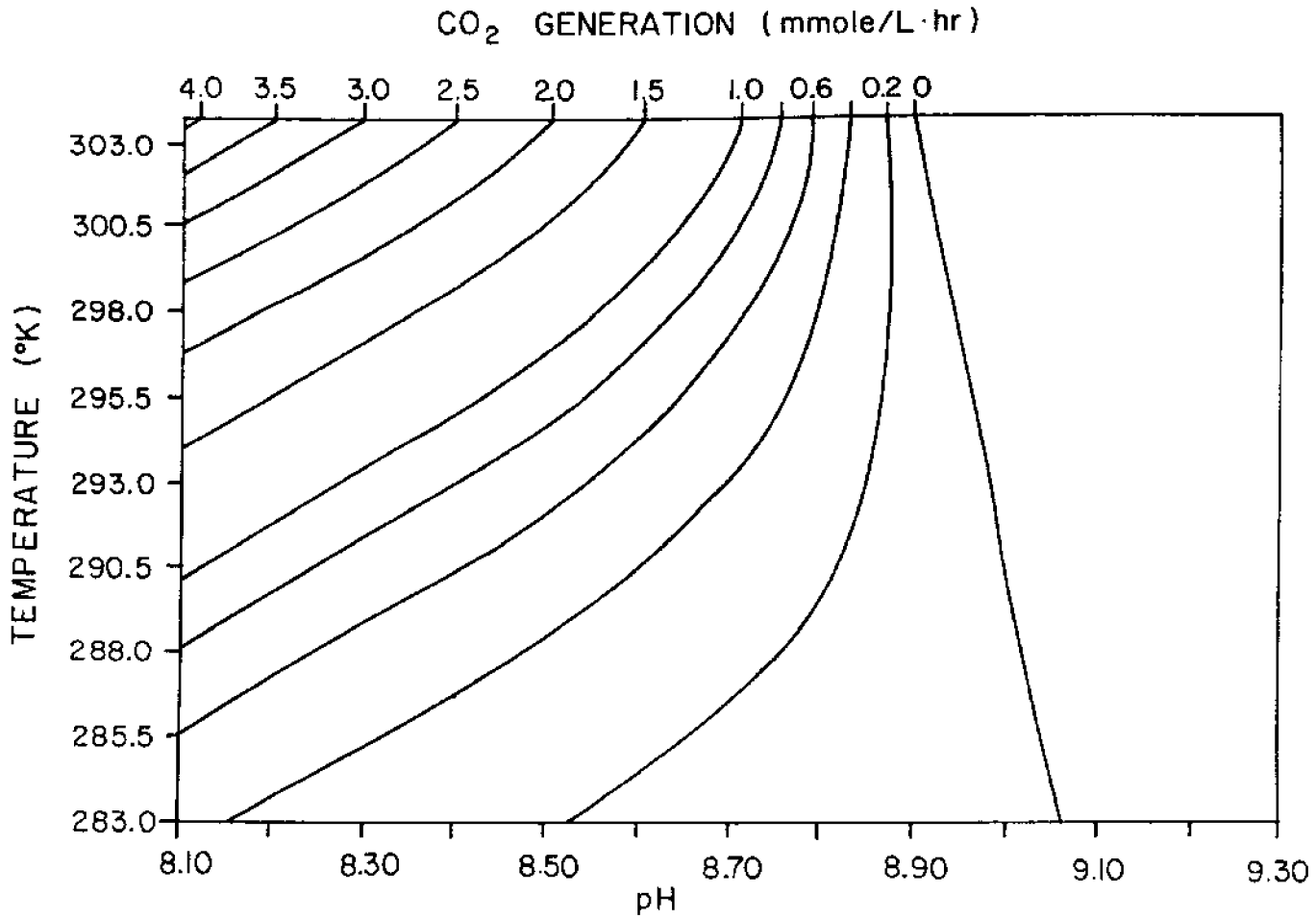


Figure 16. Maximum CO_2-generation rate from HCO_3^- as CO_2 is removed from an algal medium isolated from the atmosphere when minimum CO_2 concentration is 2 μmole/L.

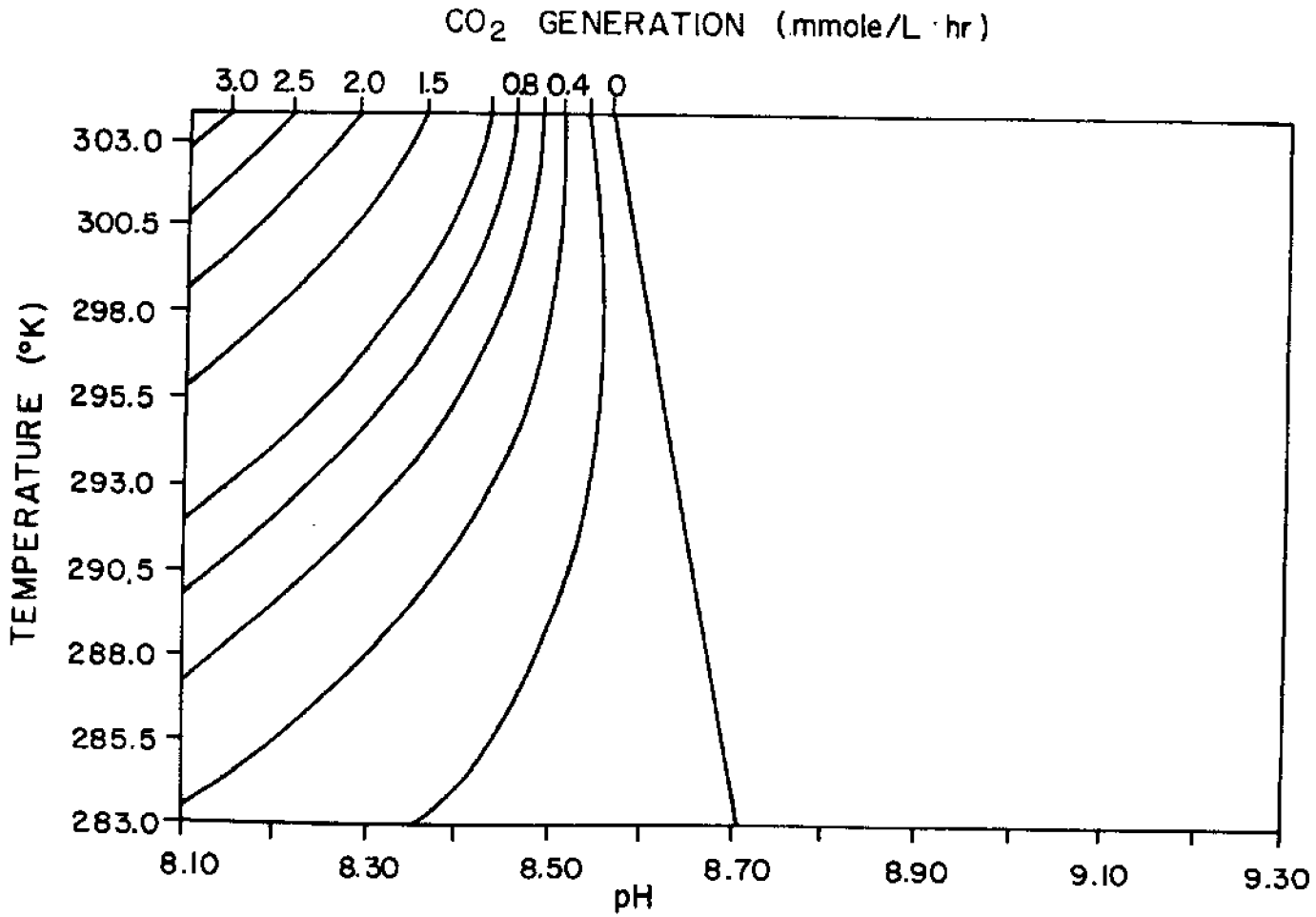


Figure 17. Maximum CO_2-generation rate from HCO_3^- as CO_2 is removed from an algal medium isolated from the atmosphere when minimum CO_2 concentration is 3 μmole/L.

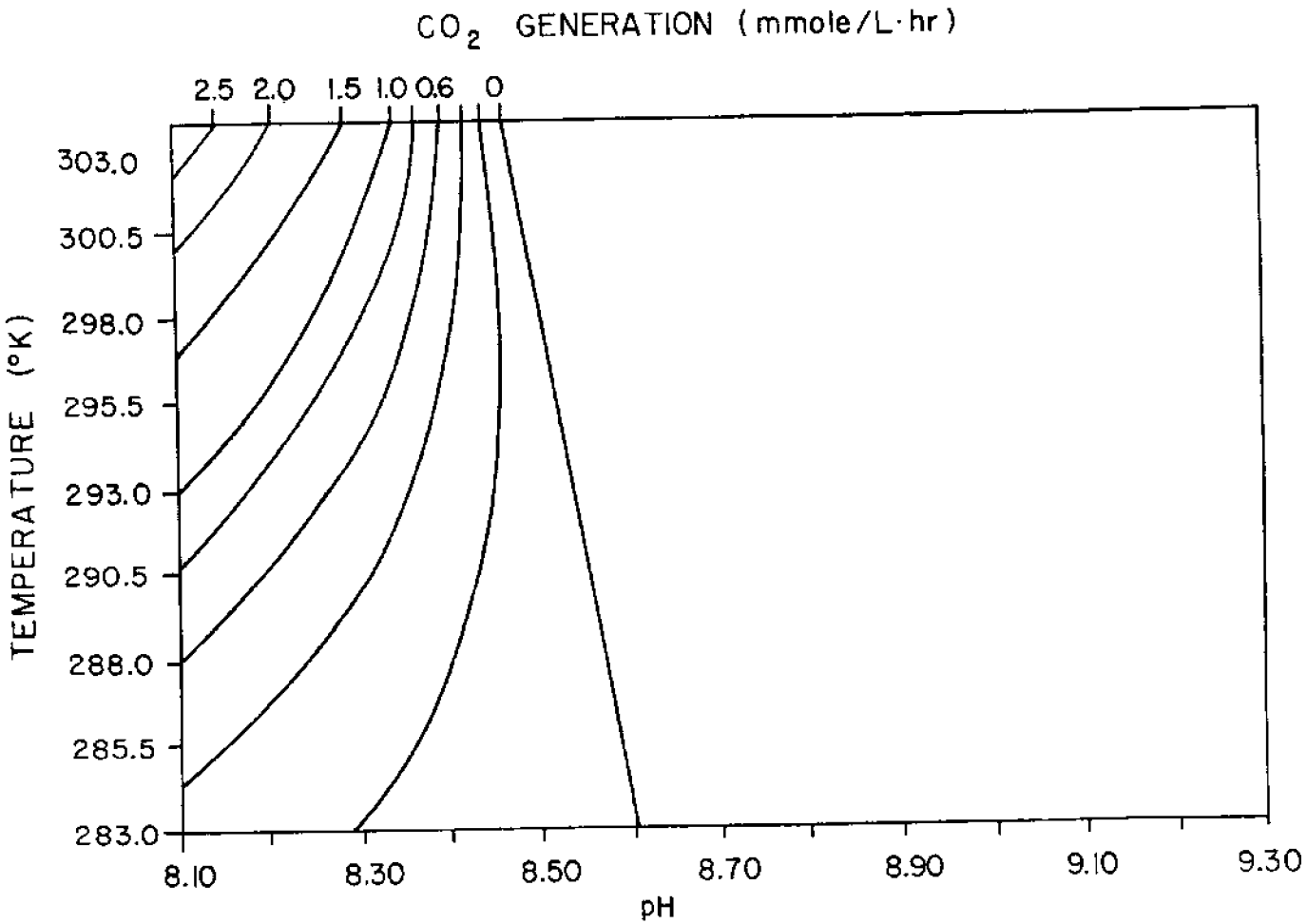


Figure 18. Maximum CO_2-generation rate from HCO_3^- as CO_2 is removed from an algal medium isolated from the atmosphere when minimum CO_2 concentration is 4 μmole/L.

range of temperature and pH at any given carbon-fixation rate. This is interpreted as limiting algal growth by the dehydration or direct hydroxyl reaction of HCO_3^- for at least some species in mass culture and perhaps in natural systems during algal blooms. In the case of 3H, an algal bloom will cease and the culture will die as a direct result of excessive carbon uptake without sufficient control of pH and CO_2. Further, it appears obvious that algal species that could use HCO_3^- directly or that have an active carbonic anhydrase are at an ecological advantage over algal species requiring CO_2 at higher concentrations. These findings substantiate the position of King (1970) and Wetzel (1972), while questioning the position of Goldman et al. (1974) and Hough (1974).

In controlled-environment, mass algal culture, the dehydration of the HCO_3^- pathway can be exploited to meet the demanding requirements of high-yield cultures by maintaining proper pH and CO_2 concentrations. Note that the temperature affects the CO_2-generation rate and should be considered in selecting maximum operating pH. The contour plot in Figure 16 should be used in conjunction with the culture specifications for 3H.

pH Level

Algal growth was unaffected by pH from 6.8 to 8.4 but was depressed at pH 8.8 and above. Particular care should be taken to avoid supersaturated carbonate solutions, which may cause precipitation and interfere with algal growth. All else being equal, it is suggested that the algal cultures be maintained between pH 7.8 and

and 8.2, which is compatible with bivalve mollusc culture.

Light Intensity

Algal growth does not increase further as the light intensity reaches 1500 $\mu w/cm^2$. There was no inhibition at a light intensity of 3500 $\mu w/cm^2$. These light intensities are low when compared to direct sunlight, which has a maximum of 42,000 $\mu w/cm^2$. However, direct sunlight does not inhibit the growth rate of 3H (Pruder, et al., 1977).

O_2 Concentration

High O_2 concentration reduced the carbon and nitrogen fixation rate 18 to 29 percent while not affecting the cell-division rate. This selective effect aggravated nonsteady-state growing conditions, as evidenced by increasing difference between k_{cc} and k_{pc}. This difference in rates could limit the number of cell divisions possible for an algal culture before it enters stationary phase. There are possible difficulties with feeding bivalves in a medium that is supersaturated with O_2. O_2 concentration should be limited to saturation levels or below.

Appendix A

EXPERIMENTAL EQUIPMENT AND MATERIALS

Descriptions of the equipment and materials used in this investigation, along with model numbers and manufacturers, are given in Table 14.

Table 14

EXPERIMENTAL EQUIPMENT AND MATERIALS

Description	Model Number	Trade Name or Type	Manufacturer
ANALYZER, elemental	240		Perkin-Elmer Corp. Norwalk, CT
FILTERS			
Glass Fiber: 12.5-cm dia		AH	Reeve Angel Whatman, Inc. Clifton, NJ
Silver: 1.0 μm, 47-mm dia			Selas Flotronics Spring House, PA
0.45 μm, 47-mm dia	GA-6	Metricel	Gelman Instrument Co. Ann Arbor, MI
GAS (custom mixtures)			Linde Division Union Carbide Corp. Keasbey, NJ
GLASSWARE			
Bottle, gas wash: 500 ml	31760		Corning Glass Works Corning, NY
Column, adsorber	5062-333333		Bellco Glass, Inc. Vineland, NJ
Column, ion exchange	5062-222222		"
Still (with quartz heating elements)	5046		"
HEMOCYTOMETER		Bright-Line	American Optical Corp. Buffalo, NY
METER, flow (gas)		Rotameter	Brooks Instrument Div. Emerson Electric Co. Hatfield, PA
MICROSCOPE, optical (with UV epi-illumin)		Vanox	Olympus Corp. of America New Hyde Park, NY
RADIOMETER	PR-1000	Spectra	Photo Research, Inc. Burbank, CA

Continued

Table 14

(Continued)

Description	Model Number	Trade Name or Type	Manufacturer
SALTS, sea (synthetic)		Instant Ocean	Aquarium Systems, Inc. East Lake, OH
SPECTROPHOTOMETER		DU	Beckman Instruments, Inc. Fullerton, CA
"			Carl Zeiss New York, NY

Appendix B

COMPOSITION OF "f/2" ENRICHED ALGAL MEDIUM

Table 15 presents the composition of Guillard's (1973 and 1975) "f/2" enriched algal medium.

Table 15

COMPOSITION OF "f/2" ENRICHED ALGAL MEDIUM

Material[a]	Quantity (mg)	Primary Element		
		Element	Quantity (µg)	Approx. Molar Equivalent (µmole)
Major Nutrients				
$NaNO_3$	75	-	-	883
$NaH_2PO_4 \cdot H_2O$	5	-	-	36.3
$NaSiO_3 \cdot 9H_2O$	30	Si	3000	107
Trace Metals				
$Na_2 \cdot EDTA^+$	4.36	-	-	11.7
$FeCl_3 \cdot 6H_2O^+$	3.15	Fe	650	11.7
$CuSO_4 \cdot 5H_2O$	0.01	Cu	2.5	0.04
$ZnSO_4 \cdot 7H_2O$	0.022	Zn	5	0.08
$CoCl_2 \cdot 6H_2O$	0.01	Co	2.5	0.05
$MnCl_2 \cdot 4H_2O$	0.18	Mn	50	0.9
$Na_2MoO_4 \cdot 2H_2O$	0.006	Mo	2.5	0.03
Vitamins				
Thiamin·HCl[b]	0.1	-	-	-
Biotin[b]	0.0005	-	-	-
B_{12}	0.0005	-	-	-

[a]Materials added to 1 liter of seawater.

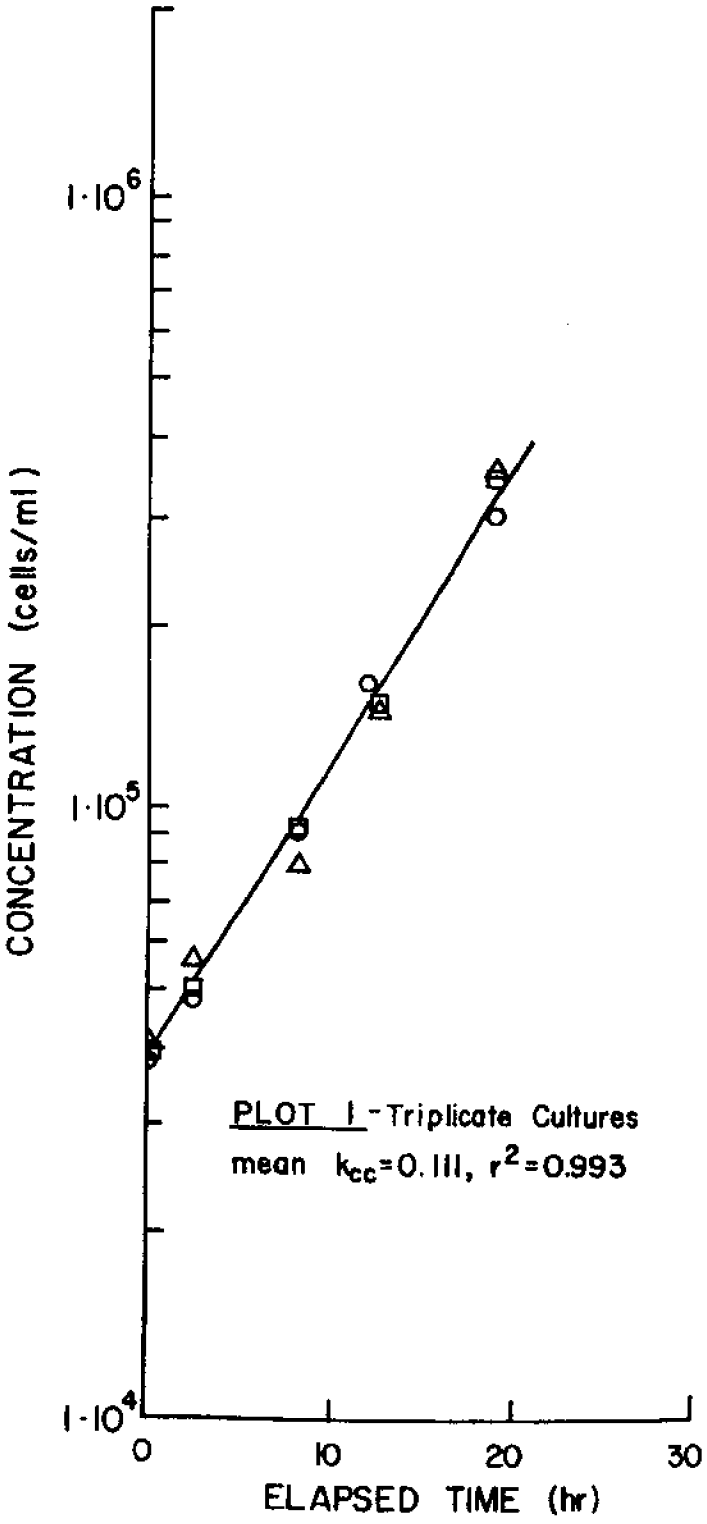
[b]Not required for 3H.

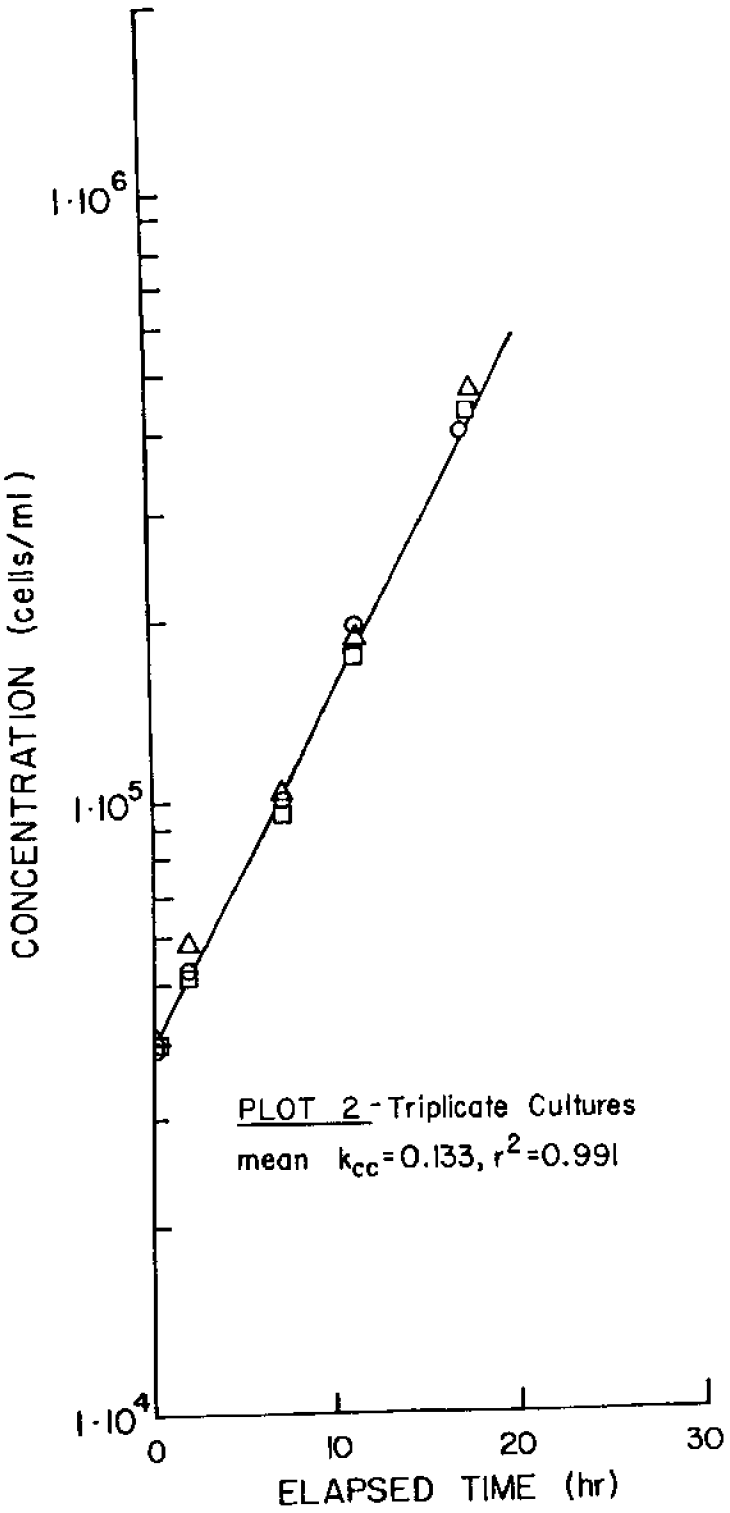
Appendix C

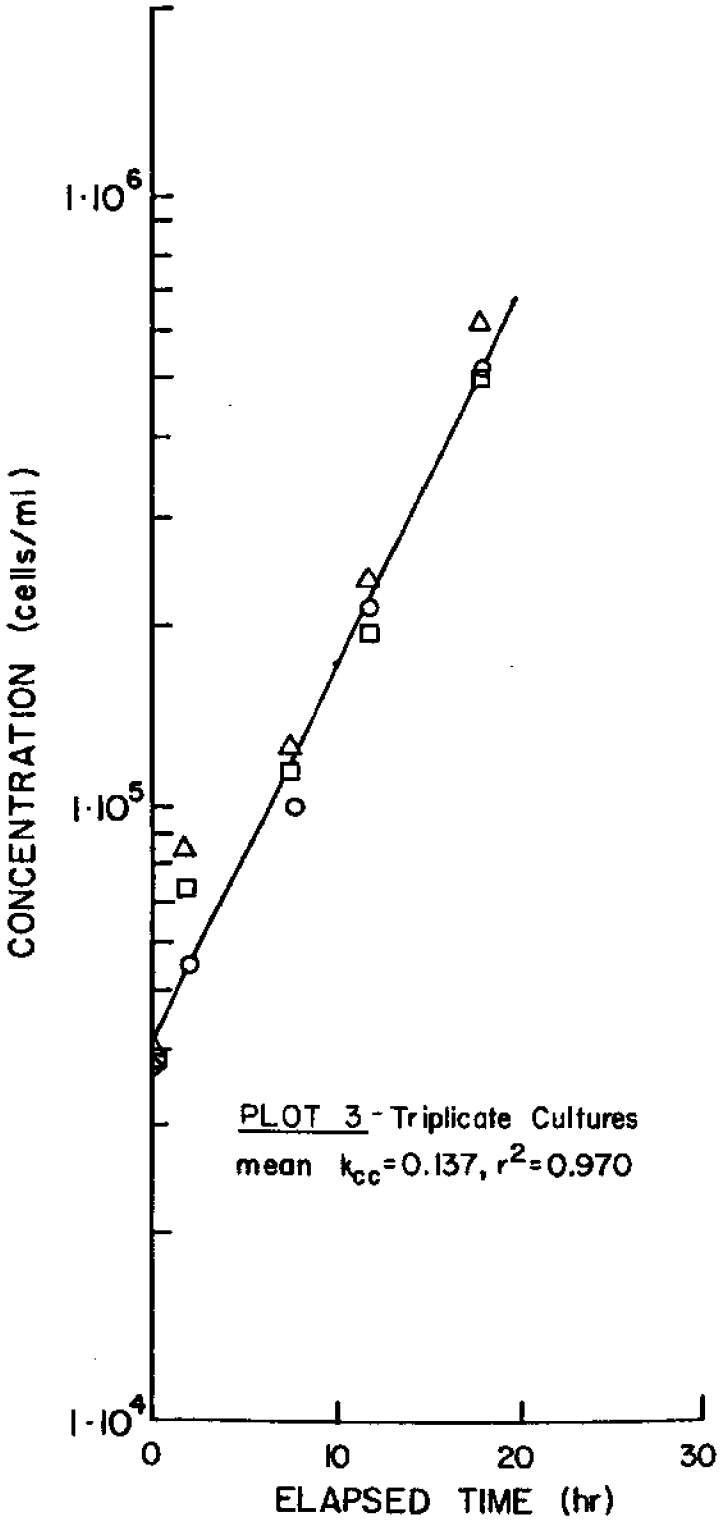
REPRESENTATIVE GROWTH PLOTS OF 3H

The following three plots illustrate the growth rate of 3H at various experimental conditions:

Plot	Light ($\mu w/cm^2$)	Carbon Dioxide (μmole/L)	Oxygen (μmole/L)	pH
1	350	159	15	7.5
2	750	159	15	7.5
3	1500	159	15	7.5


1·10^6
1·10^5
1·10^4
CONCENTRATION (cells/ml)
0
10
20
30
ELAPSED TIME (hr)
PLOT 1 - Triplicate Cultures
mean k_{cc}=0.111, r^2=0.993


1·10^6
1·10^5
1·10^4
CONCENTRATION (cells/ml)
0
10
20
30
ELAPSED TIME (hr)
PLOT 2 - Triplicate Cultures
mean k_{cc}=0.133, r^2=0.991


1·10^6
1·10^5
1·10^4
CONCENTRATION (cells/ml)
0
10
20
30
ELAPSED TIME (hr)
PLOT 3 - Triplicate Cultures
mean k_{cc}=0.137, r^2=0.970

Appendix D

SAMPLE CALCULATIONS

Comparing Several Means: Analysis of Variance (Lapin, 1973)

Compare population means of growth-rate coefficient k_{cc} for three oxygen concentrations at a carbon-dioxide concentration of 16 μmole/L and a light intensity of 350 $\mu w/cm^2$.

	Oxygen (μmole/L)		
Sample	15	262	1250
1	0.116	0.118	0.108
2	0.129	0.116	0.117
3	0.103	0.114	0.108
4	0.102	0.117	-
5	0.107	-	-
6	0.110	-	-
	0.667	0.465	0.333
Mean = $\bar{x}$ =	0.111	0.116	0.111

$$\text{Grand Mean} = \bar{\bar{x}} = \frac{(0.667 + 0.465 + 0.333)}{(6 + 4 + 3)} = 0.113$$

	$(x_1 - \bar{x}_1)^2$	$(x_2 - \bar{x}_2)^2$	$(x_3 - \bar{x}_3)^2$
1	0.000025	0.000004	0.000009
2	0.000324	0.000000	0.000036
3	0.000064	0.000004	0.000009
4	0.000081	0.000001	-
5	0.000016	-	-
6	0.000001	-	-
	0.000511	0.000009	0.000054

$$SS_w = \Sigma(x_1 - \bar{x}_1)^2 + \Sigma(x_2 - \bar{x}_2)^2 + \Sigma(x_3 - \bar{x}_3)^2$$

$$= 0.000511 + 0.000009 + 0.000054 = 0.000574$$

$$SS_b = n_1(\bar{x}_1 - \bar{\bar{x}})^2 + n_2(\bar{x}_2 - \bar{\bar{x}})^2 + n_3(\bar{x}_3 - \bar{\bar{x}})^2$$

$$= 0.000024 + 0.000036 + 0.000012 = 0.000072$$

$$MS_w = \frac{SS_w}{n - m} = \frac{0.000574}{13 - 3} = 0.000057$$

$$MS_b = \frac{SS_b}{3 - 1} = \frac{0.000072}{2} = 0.000036$$

$$F = \frac{MS_b}{MS_w} = \frac{0.000036}{0.000057} = 0.631$$

F distribution table for 0.05 is 4.1; therefore, differences between populations are not significant at the 95% confidence level.

Difference Between Two Means: The "t" Test (Volk, 1969)

Compare mean k_{cc} for two carbon-dioxide concentrations at various grouped treatment combinations of oxygen concentration and light intensity.

Grouped Treatment Combinations	Carbon Dioxide (μmole/L)	
	159	16
O_1L_3	0.144	0.146
	0.128	0.124
	0.139	0.150
		0.140
		0.145
		0.129
O_3L_3	0.128	0.143
	0.137	0.153
	0.125	0.152
O_2L_3	0.128	0.153
	0.122	0.145
	0.130	0.153
	0.126	0.153
Mean =	0.131	0.145
Std. Dev. =	0.007	0.0095
Σx^2 =	0.00044	0.00109
n =	10	13

$\bar{S}_x$ = pooled estimate of standard deviation

$$= \left[\frac{\Sigma x_1^2 + \Sigma x_2^2}{n_1 + n_2 - 2}\right]^{1/2} = 0.0085$$

$$t = \frac{|\bar{x}_1 - \bar{x}_2|}{\bar{S}_x\left[1/n_1 + 1/n_2\right]^{1/2}} = \frac{0.014}{0.0036} = 3.89 \qquad t \text{ table } 0.05 \quad 1.721$$

Means are significantly different at the 95% confidence level.

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