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The Interrelation of Carbon and Phosphorous in Regulating Heterotrophic and Autotrophic Populations in Aquatic Ecosystems

Department of the Interior • Federal Water Quality Administration

THE INTERRELATION OF CARBON & PHOSPHORUS IN REGULATING HETEROTROPHIC AND AUTOTROPHIC POPULATIONS IN AQUATIC ECOSYSTEMS

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Preface

The research findings presented in this report were presented at the Purdue Industrial Wastes Conference on May 6, 1970. These results, along with other publicity concerning the research at FWQA's Southeast Water Laboratory, have been utilized by some individuals and groups outside FWQA to support a contention that carbon rather than phosphorus or nitrogen is the limiting nutrient in the control of excessive algal blooms, a distortion of the conclusions of this research. Interestingly, most of these arguments were raised well before the research data and results became available at Purdue.

The research reported here does not alter FWQA's position with regard to the importance of reducing phosphate discharges to the environment. The research findings assist in elucidating the highly complex interrelationships of the various algal and bacterial nutrients and, if any single action conclusion were to be drawn, it would be that even more stringent controls should be placed on carbonaceous (BOD) discharges than at present. The findings in no way suggest that any less attention should be given to phosphates and, in fact, additional evidence is presented that phosphorus plays an important part in stimulating bacterial growth in water in addition to its role as an algal nutrient. The FWQA advocates the highest degree of nutrient control necessary for each receiving water so as to achieve the desired ecological balance.

Research at the Southeast Water Laboratory pertains to the "fate" of various materials in the aquatic environment and involves, among other things, the mechanisms of interaction between decomposing organic matter and the stimulating effect of this decomposition on algae in the overlying water. The symbiotic relationship between algae and bacteria is a phenomenon recognized by biologists for many years.

The findings reported are preliminary in that they result from limited laboratory and field experiments. At this time we cannot say, scientifically, how typical or atypical were the situations studied but it is unwarranted to extrapolate these results to waters other than those studied. The research on the role of carbonaceous materials in aquatic ecosystems is being continued in coordination with the work of FWQA's National Eutrophication Research Program.

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ABSTRACT

Laboratory and field investigations were conducted on the fate and cycling of carbon and phosphorus in selected aquatic ecosystems. Inorganic carbon, as CO_2, supplied by both bacterial cultures and cylinder gases, stimulated the growth of the blue-green alga Anacystis nidulans. The carbon requirement (10^{-5} ug CO_2 per cell) for this alga was determined for a single set of experimental conditions. The addition of CO_2 to natural water low in phosphorus (5 ug P) and nitrogen (5 ug N) in the laboratory stimulated the growth of indigenous algal populations. The limiting and luxury cellular concentrations of phosphorus for starved Anacystis nidulans were found to be 0.3×10^{-8} ug P and 3.0×10^{-8} ug P per cell, respectively.

Diel studies of a stream which received biologically-treated sewage demonstrated that the dissolved CO_2 and HCO_3 continually produced in the system were essentially depleted by the autotrophic organisms during daylight hours, while the concentration of phosphorus (1.3-2.2 mg/1 P) remained unchanged.

Addition of organic carbon and inorganic nitrogen and phosphorus alone and in combination to the waters studied directly stimulated the oxidative metabolism of the heterotrophic population, which resulted in increased dissolved CO_2 and HCO_3^-. This increased availability of inorganic carbon, rather than the direct metabolic removal of dissolved phosphorus by the algae, appeared to be directly responsible for the growth of the algal populations in the waters studied.

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INTRODUCTION

The increasing demands to control water pollution, to curb eutrophication, and to develop rational predictive models for water quality management require a fuller quantitative and qualitative understanding of the fundamental biological, chemical, and physical reactions and interactions occurring in natural waters.

The research described in this paper is a portion of the work being done by the National Pollutants Fate Research Program at the Southeast Water Laboratory designed to study the cycling of carbon, phosphorus, and nitrogen through aquatic ecosystems.

Algal blooms frequently accompany cultural enrichment of waters. Increased amounts of available nitrogen and phosphorus have been suggested as the cause of these blooms. Although the importance of carbon in regulating algal growth has long been known, it received little attention until recently (Kuentzel, 1969). Increased supplies of carbon (as well as nitrogen and phosphorus) had to be available to support the increased algal growth associated with enriched waters.

The relative chemical composition of living organisms demonstrates the importance of carbon as a nutrient. Carbon accounts for 50-77% of the dry weight of algae and 45-55% of the dry weight of bacteria.

In aquatic ecosystems, autotrophs fix and transform inorganic forms (CO_2 and HCO_3^-) of carbon from the environment into organic compounds, and through oxidation, heterotrophs return portions of this carbon to the environment as CO_2. Essential to the functioning of this cycle are phosphorus, nitrogen, sulfur, and other nutrients which are required by both the autotrophic and heterotrophic organisms.

The growth of individual species of organisms depends on the availability of specific utilizable forms of carbon in the system. Bacteria take up and utilize most forms of organic carbon. While most algae can take up and transform organic carbon, they *grow* (divide) only on CO_2 and HCO_3^-. Although the literature concerning algae growth on organic substrates is conflicting, Allen (1952), Hoare and Moore (1965), Pearce and Carr (1967), and others report uptake but no increased cell division (when compared to an inorganic control) in axenic cultures of algae grown on organic media. Also, there is no evidence that algae can utilize their intracellular carbon compounds to support growth.

In general, the availability of any ionic species of carbon in an aquatic ecosystem is dependent upon: 1) direct additions and losses resulting from flow of the water mass; 2) exchange between sediments and the water and atmosphere; 3) chemical reactivity in the water; 4) removal or release by biological activity on soluble compounds, sediments, or other organisms; 5) photolysis of compounds and autolysis of organisms in the water; and 6) secretion, excretion, and exchange phenomena within the biota. The

availability of carbon or other growth nutrients depends also upon physical parameters such as temperature, light, and pH.

Foremost, and least understood, among all factors affecting the carbon cycle are the rates of supply and demand. The literature concerning the rates of chemical equilibria between the carbonates, water, and atmospheric CO_2 is devoid of absolute rate coefficients, with most researchers resorting to adjectives such as fast, relatively fast, or slow. Also, the rates of replenishment of the required ionic species to the enzymatic sites of the organisms, by diffusion or mass transport, are poorly understood and ill defined. However, many of the field studies reported in the literature demonstrate that the rates of supply of carbon were insufficient to meet the demands of the autotrophic populations studied.

The quantity of CO_2 exchanged at the air-water interface depends not only upon the transfer rate but also upon the concentration of CO_2 in air and water. The saturation concentration in water in equilibrium with normal air ranges from 0.4 to 1.0 mg CO_2/liter water, depending upon the pH and buffering capacity.

For terrestrial plants, Quinn and Jones (1936), Brix (1968), Johnson and Kelley (1969), and others have shown that CO_2 is the primary factor limiting their potential growth, even though there is a relatively constant source of CO_2 in the atmosphere (0.6 mg CO_2/liter air).

In the aquatic system, Birge and Juday (1911) measured the complete removal of CO_2 and severe depletion of HCO_3^- and $CO_3^=$ from soft, medium, and hard water lakes of Wisconsin in 1904-1906. They also stressed the importance of bacterial oxidation of organic material as a source of CO_2. Pearsall (1932) found that in the English Lake District, richer populations of planktonic diatoms occurred in water containing more $CaCO_3$. Chu (1942) reported that growth of *Pediastrum boryanum* increased with increasing carbonate content up to 22 ppm. The more soluble K_2CO_3 was preferable to $CaCO_3$ as a source of carbon.

Some of the most definitive work is that of Myers (1944), who studied the effect of CO_2 and pH on the growth of *Chlorella pyrenoidosa*. He found CO_2 to be growth-limiting even at the low light intensity of 100 foot candles and stated:

> "The culture obtaining its carbon dioxide by diffusion through the cotton plug was clearly limited in its rate of growth. A still greater relative limitation might be expected at higher light intensities."

Myers further stated:

> "The only carbon dioxide pressure of significance is that existing within the liquid medium and available to the cells. During photosynthesis, this carbon dioxide pressure at the surface of the algal cell can never be as great as that in equilibrium with the gas phase since a diffusion gradient must always exist.... It is clear that the carbon dioxide pressure within the medium

must be a complex function of the partial pressure in the gas phase, the area of the liquid-gas interface, the rate of aeration, and the rate of uptake of carbon dioxide by the algae. Even in any one culture in which the first three of the above factors are held constant, the multiplication of the algae must result in increasing rates of uptake of carbon dioxide, thus continuously decreasing the pressure of carbon dioxide within the media. The effective carbon dioxide concentration must fall off continuously during the growth curve."

Myers (1947) and Kratz and Myers (1955) reported other data stressing the importance of CO_2 in regulating the growth of algae.

Allen (1955) showed the algae in sewage oxidation ponds were utilizing CO_2 rather than organic carbon for growth and that this CO_2 was produced by heterotrophic activity.

Wright and Mills (1967) and Wright (1969) reported a downstream reduction in both primary productivity and macrophyte standing crop on the Madison River. The downstream decline in productivity was correlated with successively lower concentrations of free CO_2. The decline in free CO_2 was due to photosynthetic removal during the day and to outward diffusion of CO_2 during both day and night.

In an estuarine environment, Kelley (pers. comm., 1970) has measured changes in pCO_2, in a dense eel grass *(Zostera)* lagoon near Cold Bay, Alaska, during the summer and fall. At low tide during the summer the water was hyposaturated, and at high tide the water was nearly saturated with CO_2. In the fall, when the eel grass was reaching normal dormancy, the water was supersaturated with CO_2. The pH of the water varied between 6.5 and 11.5 in summer and 7.8 and 8.3 in the fall.

Evidence in the literature also relates qualitative as well as quantitative changes in aquatic plant communities to CO_2. Wright (1969) postulated that the relative amounts of CO_2 and HCO_3^- are of importance in control of species distribution of higher plants as well as the production of biomass. Bristow's (1969) data indicated that the concentration of CO_2 is important not only in regulating the production of biomass but also in controlling morphogenic response of the heterophyllous amphibious species, *Ranunculus flabellaris* and *Myriophyllum brasilense*. Neither of these species is capable of utilizing HCO_3^-. Bristow concluded that a concentration of CO_2 higher than air saturation may be essential for the normal growth and development of submerged amphibious plants. Martin *et al.* (1969) showed that HCO_3^- content of Pickwick Reservoir was important in regulating the growth of a rooted aquatic weed, *Najas sp.*

Data from several studies of artificial fertilization of ponds and lakes indicate the important role of carbon in the growth of algae. Thomaston and Zeller (1961) reported that the addition of inorganic nitrogen and phosphorus fertilizer to fish ponds did not always result in increased phytoplankton populations. They found plankton growth controlled by the availability of CO_2.

Walker (1949) added lime and cottonseed meal to ponds receiving swamp drainage (pH 5.2) which contained no carbonates. In the pond receiving the lime and cottonseed meal, a very thick green bloom was produced, while in the unlimed ponds, which also received cottonseed meal, only a very light bloom was produced.

Neess (1946), reviewing the literature from Central Europe, emphasized the importance of an environment suitable for heterotrophic activity which would allow rapid cycling of nutrients in fish ponds. He reported that the water quality of moor ponds (low pH, methane, H_2S) inhibited bacterial activity, resulting in peat accumulations and reduced productivity. Additions of lime to previously unfertilized and very unproductive moor ponds with muck bottoms resulted in immediate and phenomenal increases in yield of phytoplankton.

Neess also reported literature stressing the importance of the bottom soil and the additions of organic materials to fish ponds in regulating fish production, and he stated:

> "Plant debris is of particular importance in ponds where reliance is placed only on inorganic mineral fertilizers."

Neess stated further:

> "Cottonseed or soybean meals (C/N=40) have been found to be unusually effective fertilizers (Embody and Sadler, 1934; Hogan, 1933; unpublished observations of Neess and Hasler at Madison, Wisconsin) applied either alone or in conjunction with phosphorus."

Waters (1957) found that additions of lime to bog lakes in Northern Michigan resulted in higher HCO_3^- concentrations, thus increasing CO_2 utilization by the nanoplankton and producing immediate increases in phytoplankton yield.

Swingle (1947) reported that CO_2 is a limiting factor in fish production. He was able to increase fish yield by the addition of organic material with and without the addition of inorganic fertilizer. He stated that addition of stable manure increased fish production 43 lbs/acre per ton of manure. Addition of 200 lbs of superphosphate to the manure increased production 4.5 lbs above that for manure alone. He reported the work of Burr and others demonstrating that algae attain their maximum efficiency at much higher CO_2 tensions than normally occur in natural waters.

Swingle emphasized the importance of buffering capacity in regulating availability of CO_2. Addition of lime with inorganic fertilizers or with small amounts of organic material can inhibit fish production. The author attributed this inhibition to competition for CO_2 between the lime and the phytoplankton. Addition of lime with large amounts of organic material resulted in the growth of extensive phytoplankton populations; in this case, heterotrophic production of CO_2 was sufficient to overcome the reaction with calcium, allowing more CO_2 and HCO_3^- to be available to the algae.

Swingle discussed the importance of bacteria in nutrient cycling and stated that the bacterial populations were capable of completely utilizing the nitrogen and phosphorus available in the water under certain conditions.

The phosphorus content of ponds and lakes has long been of interest because phosphorus has been considered a limiting factor in the production of algae and, hence, of fish in many natural waters. One of the early studies of the phosphorus and nitrogen content of natural waters was made by Juday and Birge (1931) who, in the 1920's, determined the nitrogen and phosphorus content of 479 lakes in Wisconsin. The arithmetic mean of the entire set of phosphorus determinations was 0.023 mg of phosphorus/liter; the average soluble phosphate content was found to be 0.003 mg of phosphorus/liter; and the average organic phosphorus content was 0.020 mg of phosphorus/liter. In another study of the phosphorus content of a pond, Hutchinson (1941) reported the overall mean content of Linsley Pond as 0.021 mg phosphorus/liter.

Mackereth (1953) stated that measurements of dissolved phosphate in Lake Windermere have shown the concentration of phosphate phosphorus to vary from below 1.0 mg phosphorus/liter to a maximum of 2 mg phosphorus/liter However, Mackereth said it bore no relationship to the size of the population of *Asterionella formosa*, the dominant algal species in this lake.

Rigler (1956), from his observations of the phosphorus cycle in the waters of Toussaint Lake, estimated that 62-65% of the phosphorus is found as particulate organic bound and only 15-30% as soluble organic. He also found that fractions previously described as soluble may actually be associated with particles between 0.1 u and 0.45 u.

Rigler used ^{32}P tracer to demonstrate that both attached and suspended bacteria added to autoclaved lake water utilized inorganic phosphorus for growth. Rigler concluded that the growth of large bacterial populations must be an important factor in the removal of phosphate from solution.

Hayes and Phillips (1958) showed that uptake of phosphorus by bacteria and higher aquatic plants prevented the elements from being incorporated into lake sediments. They concluded that bacteria are more efficient than higher plants in removing phosphorus from water and actually keep the phosphorus available in the water column under both anaerobic and aerobic conditions When the bacteria were killed by antibiotic treatment, only 10% of the ^{32}P was found in the water after one week; while in the control, 66% of the ^{32}P remained in the water. Earlier work reported by Hutchinson and Bowen (1950) on Linsley Pond showed the same general phenomenon.

Rigler (1956) stated that bacteria and algae take up significant amounts of phosphate from solution. Using ^{32}P as a tracer, he found that in 20 minutes an equilibrium had been attained, with approximately 93% of the ^{32}P taken up by plankton. The plankton were separated into bacterial and algal fractions by filtering the culture through Nos. 10 and 20 bolting cloth, No. 2 Whatman paper and a Millipore filter. He found that 98% of the algae had been removed by the Whatman paper, and they contained 21.3% of the ^{32}P. The Millipore filter removed the bacteria and the other 2% of the algae and contained 68.4% of the ^{32}P. Since the total phosphate concentration in solution remained 0.8 ug/liter throughout the experiment, he concluded that the

incorporation of ^{32}P represented turnover rather than uptake. He reported turnover times of 3.6 and 5.4 minutes for two similar experiments. From these experiments, Rigler concluded that the bacteria are largely responsible for the turnover of inorganic phosphate and thus, "might be competing with the algae for the inorganic phosphate".

Bacteria are reported to contain a larger percentage of phosphorus on a dry weight basis than algae. McKinney (1962) stated that bacteria contain 2.5% phosphorus and Taylor (1946) reported 2.72% phosphorus for B strain *E. coli*. Sanders (pers. comm.) found the phosphorus content of suspended stream bacteria ranged from 1.07 to 2.02%; that of attached bacteria ranged from 1.5 to 3.1% phosphorus.

Bacteria cells not only contain small amounts of phosphorus, but also apparently do not increase their uptake of this element when the phosphorus concentration in the medium is increased.

Levin (1963), using 30% raw sewage, 1.5mM sodium succinate/liter, 0.5mM glucose/liter, and varying concentrations of KH_2PO_4, found that regardless of initial concentration, the absolute amount of dissolved orthophosphate taken up by the bacteria was remarkably similar. Within the concentration range of approximately 5 - 33 mg of dissolved orthophosphate/liter the rate and amount of uptake were independent of concentration.

Porcella (1969) reported approximately 10^{-10} mg of phosphorus utilized per cell of *Selenastrum capricornutum*. Al Kholy (1956) reported a maximum phosphorus content of 1.50×10^{-6} ug and a minimum content of 0.10×10^{-6} ug phosphorus per cell of *Chlorella*. He found no growth below this minimum concentration. Mackereth (1953) found that 1 ug of phosphorus supported the growth of 16×10^6 cells of *Asterionella formosa* and that the limiting amount was 0.06 ug phosphorus per 10^6cells.

Both Al Kholy (1956) and Mackereth (1953) demonstrated phospnorus uptake and storage by algae from external concentrations of 1 ug/liter or less. This accumulated phosphorus was used to support growth and cell division in the absence of an external supply of the element.

Batterton and van Baalen (1968) reported the normal level of pnosphorus in *Anacystis nidulans* was approximately 3.7 ug Pi/mm^3 cells. This value fell to 0.5 ug Pi/mm^3 cells under prolonged phosphorus starvation. Even at low cellular phosphate levels, cells were viable and continued to divide slowly.

Goldberg, Walker, and Whisenand (1951) demonstrated that the size of the population of *Asterionella japonica* (a marine diatom) was the same, when the cells were grown in 2.7, 4.2, 8.4, or 12.8 ug-atoms phosphorus/liter. This alga also exhibited luxury consumption of phosphorus and was capable of dividing in the absence of an external supply. These data indicated that the final phosphate content of the diatoms and the maximum cell population were essentially independent of the initial inoculum and the initial phosphate level.

Ketchum (1939), Chu (1942); Lund (1950), Humenik and Hanna (1969); and others provide data indicating comparably small amounts of phosphorus required for the growth of algae.

Plants require large amounts of carbon and are incapable of growing on their cellular carbon compounds. Conversely, plants require small amounts of nitrogen and phosphorus and possess the capability of growing on cellular nitrogen and phosphorus compounds.

Our literature review indicates:

(1) Removal of all the CO_2 and severe depletion of the HCO_3^- and $CO_3^=$ in soft and hard water lakes has been attributed to green plants.

(2) Additions of nitrogen and phosphorus to *some* waters do not stimulate algal growth. These waters have been shown to contain little available CO_2 and HCO_3^-.

(3) Bacterial oxidation of organic carbon is a principal source of CO_2 in aquatic ecosystems.

Since many industrial and domestic wastes include organic material, increased organic carbon as well as nitrogen and phosphorus has been added to the receiving water. Addition of organic carbon should stimulate the heterotrophic population, thus resulting in the production of more CO_2 which could be utilized by the aquatic plants. Because of their generally faster metabolic rates, growing populations of heterotrophic microorganisms may need more nitrogen and phosphorus than autotrophic organisms. If this assumption is correct, addition of nitrogen and/or phosphorus to some waters should stimulate the heterotrophic oxidation of organic carbon.

This report includes some of our research findings to date on the relationships of carbon, nitrogen, and phosphorus in regulating the growth of heterotrophic and autotrophic populations in aquatic ecosystems.

Emphasis has been on the role of carbon and/or nitrogen plus phosphorus in regulating the growth of an axenic culture of the Blue-Green alga, *Anacystis nidulans*, of isolated heterotrophic populations in sterile media, and of mixed natural populations of heterotrophs and autotrophs in non-sterile media.

For clarity and ease of presentation in this report, the work has been divided into eight phases:

Phase I: Stimulation of Algal Growth by Bacteria.

Phase II: CO_2 Requirement of *Anacystis nidulans*.

Phase III: Stimulation of Algal Growth by CO_2.

Phase IV: Phosphorus Requirements of *Escherichia coli* and *Anacystis nidulans*.

Phase V: Regulation of the Growth of *Anacystis nidulans* by addition of Carbon and/or Nitrogen plus Phosphorus to Sterilized, Infertile Pond Water.

Phase VI: Regulation of Growth of Natural Heterotrophic Organisms by Addition of Carbon and/or Nitrogen plus Phosphorus to Sterilized, Infertile Pond Water.

Phase VII: Regulation of the Growth of Natural Populations of Heterotrophs and Autotrophs by the Addition of Carbon and/or Nitrogen plus Phosphorus to Nonsterile, Infertile Pond Water.

Phase VIII: Growth of Heterotrophic and Autotrophic Populations and Nutrient Cycling in an Enriched Lake.

MATERIALS & METHODS

Organisms

Axenic cultures of the Blue-Green alga, *Anacystis nidulans* (Starr Culture #625) were obtained from the University of Indiana Algae Collection. Natural populations of algae were obtained from Lake Hartwell, South Carolina, and Shriner's Pond near Athens, Georgia.

Escherichia coli was obtained from W. J. Payne, University of Georgia Natural populations of heterotrophs were isolated from the water of Shriner's Pond, Oconee River, and Sycamore Valley Lake, Athens, Georgia.

Growth Media

The standard algae medium, pH 6.8, used in the laboratory was that of Allen and Arnon (1955) containing Arnon A_5 micronutrients. Unless otherwise indicated *E. coli* were cultured in the basal salts medium of Payne and Feisal (1963), with either 0.25% or 1% glucose as the carbon source.

Isotopes and Reagents

Carrier-free $^{32}PO_4$ was obtained from Mallinckrodt/Nuclear.* Specific activity was calculated by dividing the disintegrations/minute/liter by the ug phosphorus/liter.

All chemicals were reagent grade except the commercial fertilizer. Commercial fertilizer components were obtained from the Royster Fertilizer Company, Athens, Georgia, and mixed according to the formulation for 20-20-5 fish pond fertilizer. This mixture was used as a source of nitrogen (N) and phosphorus (P) at the same concentrations (0.8 mg N/liter, 0.6 mg P/liter, and 0.33 mg KCl/liter) recommended by the Georgia Extension Service for fertilizing fish ponds.

Gases

CO_2 and air mixtures (5% CO_2, 1% CO_2, and compressed air) were obtained in commercial cylinders. Zero grade helium was used as the carrier gas for the gas chromatograph and for purging air from the gas collection and reaction flasks.

Glassware

All glassware used in the phosphorus experiments was acid washed and rinsed in double distilled water just prior to use.

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* Mention of commercial products does not constitute endorsement by the Federal Water Quality Administration.

Growth Measurements

The growth of algae in the laboratory and in the field was measured by direct cell counts. Algae were fixed in 5% phenol containing 0.5% crystal violet and enumerated by direct microscopic counts at 1500X with a Petroff-Hausser Bacteria Counter.

Bacteria in all field studies and in all laboratory experiments, unless otherwise indicated, were enumerated by the Millipore filter technique (Millipore Corp. 1969), using 47 mm, 0.45 u filters and Trypticase Glucose Yeast Broth Medium, at pH 7.0, and incubated at 27°C for 48 hours.

Contamination

Extreme caution was exercised in maintaining the algae cultures free from bacterial contamination. Microscopic examination at 1500X and growth in various liquid media in shake cultures at 23°C were employed before, during, and after individual experiments. Cultures found to be contaminated were discarded.

Sampling

Aliquants taken in the laboratory for biological and chemical analyses were withdrawn from the culture flasks with sterile 6" stainless steel needles and syringes.

Field samples were taken with a 2-liter Kemmerer bottle at desired locations and depths. All biological and chemical analyses were performed on aliquants from the same water sample.

Sediments for bacterial analysis were also removed with the Kemmerer bottle and immediately placed in sterile bottles. All bacterial samples were stored in ice and returned immediately to the laboratory (travel time approximately two hours) where they were cultured.

Water samples from Shriner's Pond used in the laboratory experiments were collected and returned to the laboratory (1 hr) where they were filtered through 47mm, 0.45 u Millipore filters, autoclaved, and stored until used. Chemical analyses were performed on the autoclaved water by the Analytical Chemistry Section, Technical Services Program, Southeast Water Laboratory.

Analytical Procedures

Quantitative chemical determinations were made in dilution triplicate. Analytical tests were checked for chemical interferences. Substances have been found in samples which interfered with all three phosphate methods used.

N and P determinations were made on samples filtered through 47mm, 0.45 u Millipore filters. Inorganic carbon determinations were made on samples filtered through 13mm, 0.22 u Millipore filters.

Total inorganic carbon (CO_2, HCO_3^-, and $CO_3^=$) was determined on aliquants stored in 2-ml gas-tight bottles. For analysis, 1 ml was removed with a syringe and injected into a 130-ml helium-filled, tared, reaction flask with side arms, stop cocks, and rubber septum. The flask was re-weighed and sufficient H_2SO_4 injected into the sample to reduce the pH to 4. At this pH all inorganic carbon should be released as CO_2 with little or no oxidation of the soluble organic fraction. After shaking for 5 minutes, 200-ul samples of the head gas in the flask were injected into a Model 1532 Varian Aerograph Gas Chromatograph. From the total CO_2 in the aliquant and the pH of the original samples, calculations were made of the amounts of CO_2, HCO_3^-, and $CO_3^=$ that could have existed in the original water sample.

CO_2 determinations were performed on gas samples by flushing the gas stream directly through the helium-filled, 130-ml reaction flasks (2 minutes), and injecting 50 ul of this sample into the gas chromatograph.

Total organic carbon was determined with the Beckman Total Carbon Analyzer Model 915 by the Analytical Chemistry Section, Technical Services Program, Southeast Water Laboratory.

Phosphate-P concentrations were determined by the Fiske-Subbarow procedure (Fiske and Subbarow, 1925), the Lowry modification of the Fiske-Subbarow procedure (Lowry *et al.*, 1954) and the Lowry-Lopez method (Lowry and Lopez, 1946).

Since the absolute amounts of inorganic and total P in all initial samples were identical when the first two methods were used, it was suspected that organic P was being hydrolyzed to PO_4 by the acid in the reagents. Examination of the procedures showed that hydrolysis was apparently occurring. Rigler (1968) suspected this hydrolysis to be a source of error. Since the Lowry-Lopez procedure is done in a buffered solution, hydrolysis is reduced, if not eliminated.

Nitrate-N concentrations were determined by the Brucine method (Standard Methods, 1965).

Ammonia and total Kjeldahl-N were determined by the Kjeldahl procedure (Standard Methods, 1965) by the Analytical Chemistry Section, Technical Services Program, Southeast Water Laboratory.

Dissolved oxygen was determined by the Azide modification of the Winkler method (Standard Methods, 1965).

Radiophosphorus disintegrations were counted in a low background Nuclear-Chicago planchet counter.

The pH determinations were made with either a battery-operated Beckman Model N pH meter (field) or a Zeromatic model pH meter (lab). In all cases the meters were standardized with two buffer solutions just prior to use.

Light measurements in the field were made with a submersible Weston photometer at all depths each daylight sampling time. Light intensities in

the laboratory were measured with a Weston Model 756 Illumination Meter. Light quality measurements were also made in the laboratory with an ISCO spectroradiometer at the surface of the water bath.

Three white light sources are available in the laboratory:

(1) 200 ft-c fluorescent source

(2) 2000 ft-c fluorescent - incandescent source

(3) 4000 ft-c fluorescent - incandescent source

EXPERIMENTAL RESULTS

Phase I: Stimulation of Algae Growth by Bacteria.

Initial growth experiments were performed to verify the reports of Kratz and Myers (1955), Smith *Et al.* (1967), Hoare *et al.* (1967), and Pearce and Carr (1967) concerning the obligate photoautotrophic nature of the Blue-Green alga, *A. nidulans*. The studies were performed with axenic cultures of *A. nidulans* in shake flasks at 22°C under the 200 ft-c light source. Specific uptake studies using ^{14}C-labeled acetate, glycine, serine, and glucose, respectively, according to standard isotope techniques, were conducted at various pH's for each compound. Results indicate that acetate and serine were removed from the media, but glycine and glucose were not removed from the media. The results also indicate that growth in the algae medium containing these substrates was no greater than growth in the standard algae medium alone.

Figure 1 shows the comparative uptake of glucose and acetate for a 12-hour period at pH 6.8 and 4.7, respectively. The acetate curve at pH 4.7 was chosen because that is the pK and represents maximum uptake.

A second series of experiments was performed to demonstrate the growth stimulatory effect of the presence of a bacterial culture on the growth of

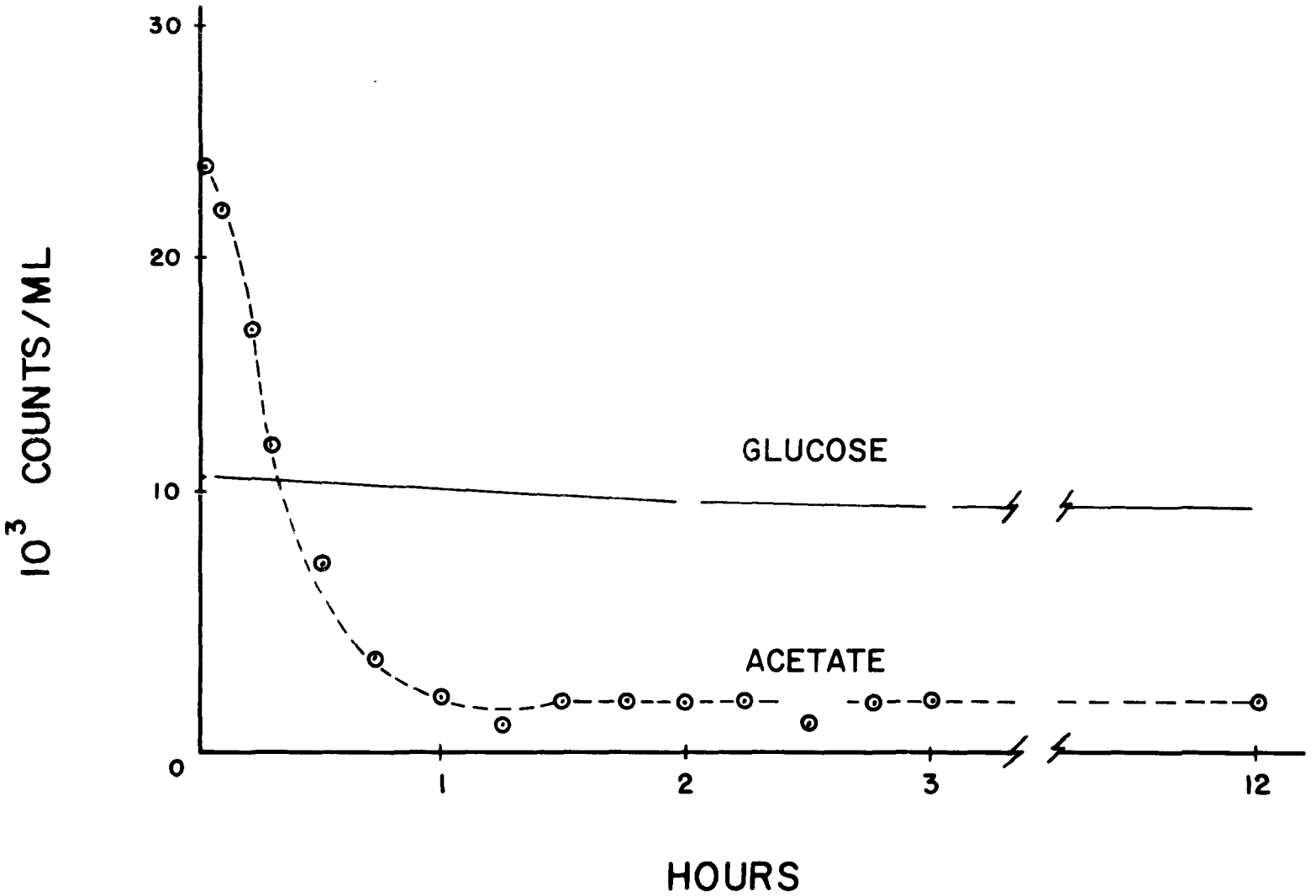
Axenic cultures of *A. nidulans* were grown in 500 ml of the standard culture medium, modified by adding 0.5 g NH_4Cl, 0.5 g $(NH_4)_2SO_4$, and 0.25 g glucose per 100 ml, in 1-liter Erlenmeyer flasks on a rotary shaker and were maintained at 22°C under the 200 ft-c light source. A 3" long dialysis bag was suspended in each flask. Twenty milliliters of the modified medium were added to the bag in each control flask while 15 ml of medium plus 5 ml of a heavy inoculum (33% transmittance with a 420 mm filter) of *E. coli* were added to the bag in each test flask. Appropriate dialysis bags were added to all flasks every eight hours. Since *A. nidulans* does not take up glucose, this sugar was used as the carbon source for the bacteria. All cultures were stoppered with cotton plugs to allow gas exchange with the atmosphere.

The growth rate of *A. nidulans* was doubled when grown in the presence of the cultures of *E. coli* (Fig. 2). After 43 hours the algal cultures with bacteria contained an average of 6.5×10^7 algae/ml and the control cultures contained an average of 3.7×10^7 algae/ml.

Since there is much evidence that *A. nidulans* is an obligate photoautotroph, it was suspected that CO_2 produced by the bacteria stimulated the growth of this algae.

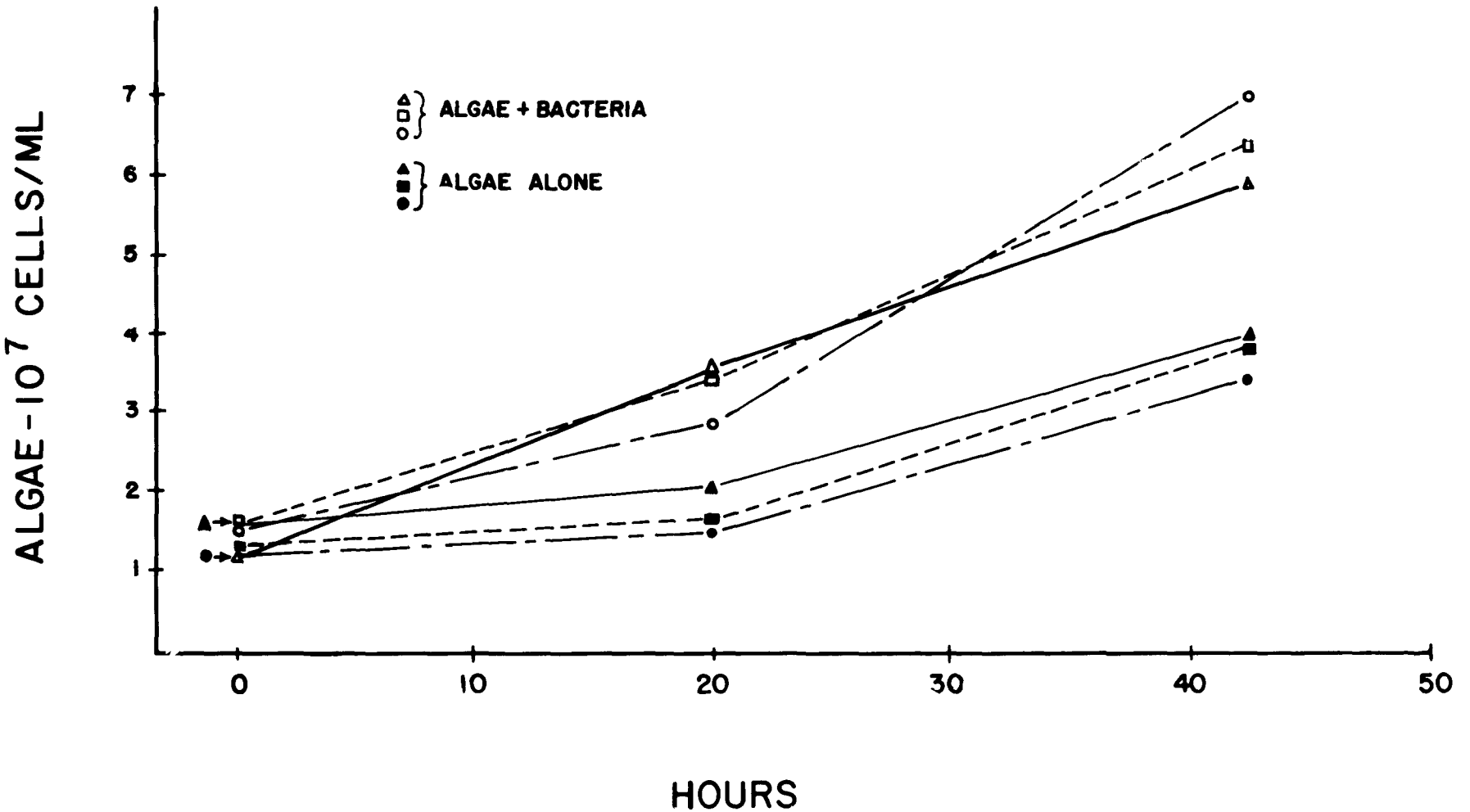
Phase II: Carbon Dioxide Requirement of *A. nidulans*.

To test the hypothesis stated in Phase I, experiments were designed to sparge the CO_2 produced in a bacterial culture through an axenic culture of algae such that it would serve as the sole carbon source for sustained algal growth.



Removal of u-^{14}C-Glucose and u-^{14}C-Acetate from the media by Anacystis nidulans. Glucose medium at pH 6.8. Acetate medium at pH 4.7.

FIG. 1



Growth of Anacystis nidulans in the presence or absence of a culture of Escherichia coli.

FIG. 2

Flasks were set up with two in series and one control in parallel (Fig. 3) in the 23°C shaking water bath under the 2000 ft-c light source. The bacterial population (Fig. 3, Flask 1) was not counted; however, the 1400 ml of Nutrient Broth was inoculated with 125 ml of a culture of approximately 40% transmittance using a 420 mm filter. The bacterial culture flask was replaced at 8-12 hour intervals to insure continued bacterial production of CO_2. The test (Fig. 3, Flask 2) and control (Fig. 3, Flask 3) algal cultures contained 1.3×10^7 and 1.4×10^7 algae/ml, respectively, at time zero.

The bacteria-algae train was gassed (300 ml/min) with CO_2-free air obtained by flowing cylinder air through an Ascarite (a quantitative CO_2 adsorber) column. This CO_2-free air passed through the bacterial culture and into the algae flask where it was released at the bottom of the algal culture. The control algae were gassed with 300 ml/min of the same CO_2-free air as the bacteria (Fig. 3).

Analysis of the gas leaving the bacterial culture showed that the bacteria were producing up to 115 mg CO_2/hr (Fig. 4, top curve). The difference between the influent and effluent gas concentrations was the quantity of CO_2 sufficient to support the growth of the algae during one cell cycle (Fig. 4, bottom curve). Repeated experiments showed that this biphasic curve is reproducible and characteristic of *A. nidulans* under the specified conditions.

By determining the area under the CO_2 uptake curve in Fig. 4, it was calculated that 1.4×10^5 ug CO_2 supported the growth of 1.3×10^{10} algae. Therefore, *A. nidulans* under the specified conditions, removed from the gas stream a calculated amount of 10^{-5} ug CO_2/cell during one cell cycle.

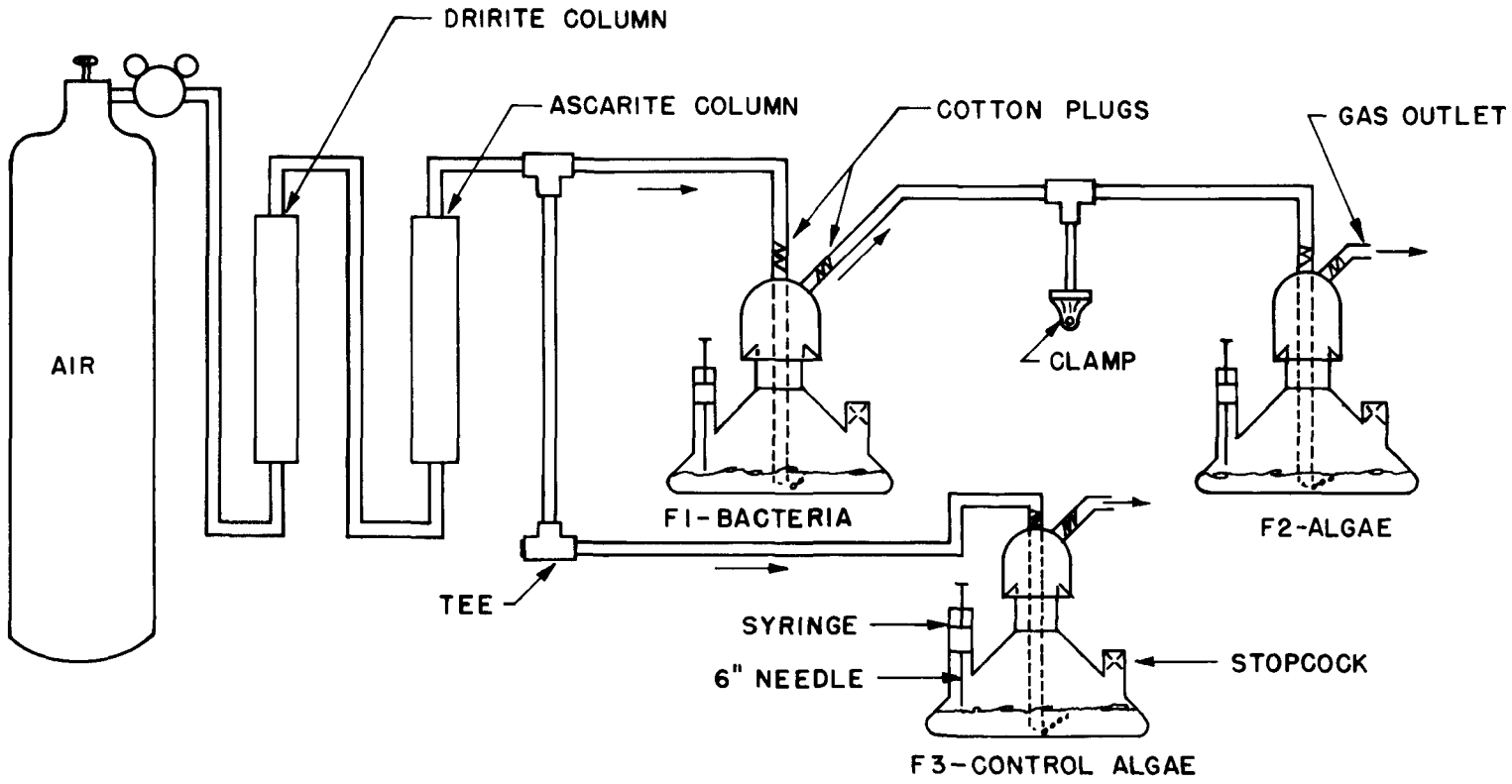
The data indicate that CO_2 was utilized continually throughout the cell cycle except for a short period prior to cell division. Since algae cannot use stored carbon for growth, they require a constant adequate supply during this uptake period for maximum growth.

In a 48-hour repetition of the same experiment, *A. nidulans* grew through four divisions (Fig. 5) when gassed with air flushed through the bacterial culture. During the same period, the control algae did not divide.

To make certain the growth stimulant was CO_2, a similar experiment was conducted with an Ascarite column inserted into the line between the two flasks to remove the CO_2 before it reached the algae. No cell division occurred in either the test or control cultures of *A. nidulans*. It was therefore presumed that the growth stimulant was bacterially produced CO_2.

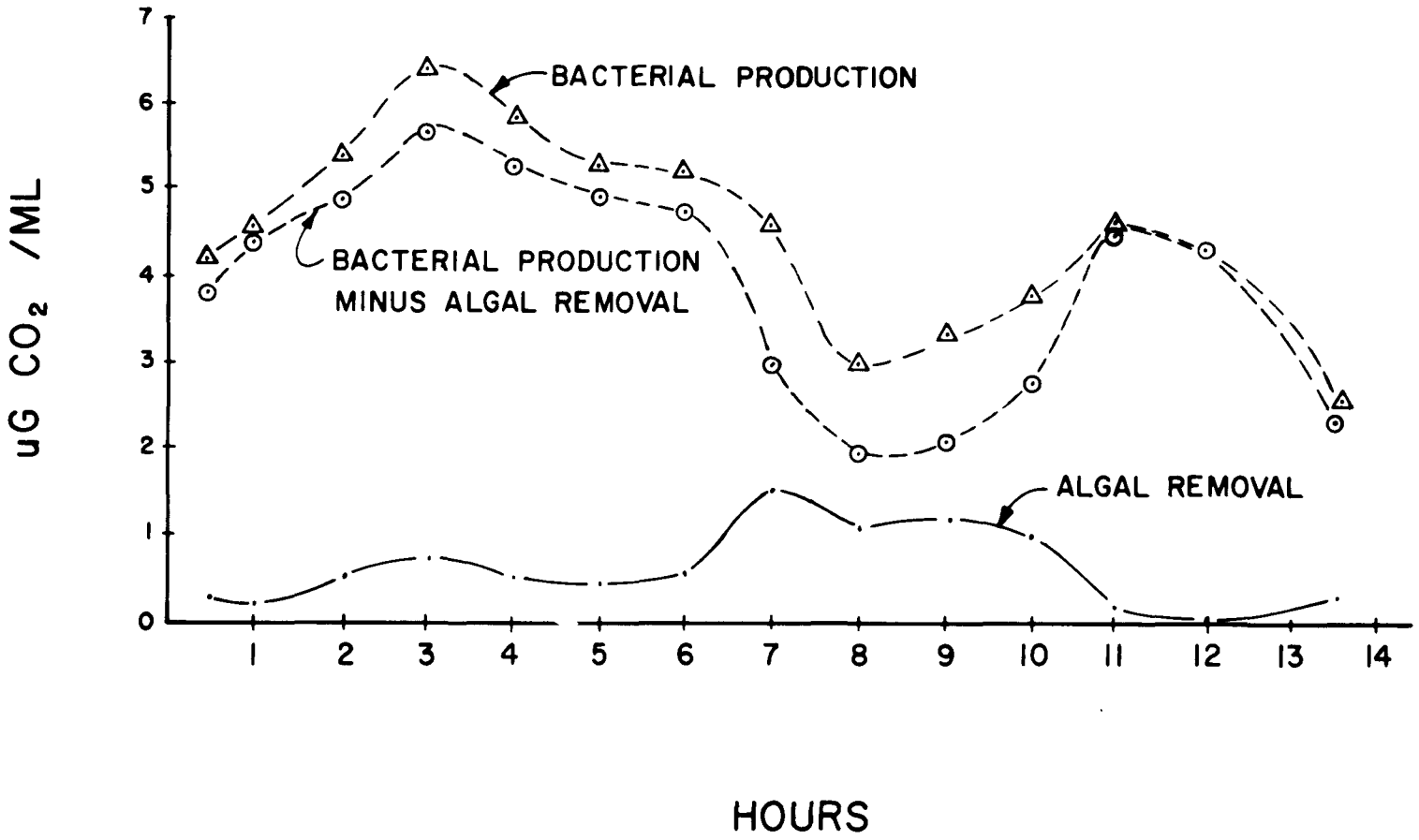
Phase III. Stimulation of Algae Growth By CO_2

Having indirectly determined that CO_2 was a growth stimulating material, experiments were performed in the laboratory to determine the effect of CO_2 concentration on the growth of *A. nidulans* where no other nutrient could be limiting.



Schematic of apparatus used to determine the influence of any volatile material produced by a bacterial culture on the growth of an algal culture. All connecting tubing was 1/4" ID Tygon.

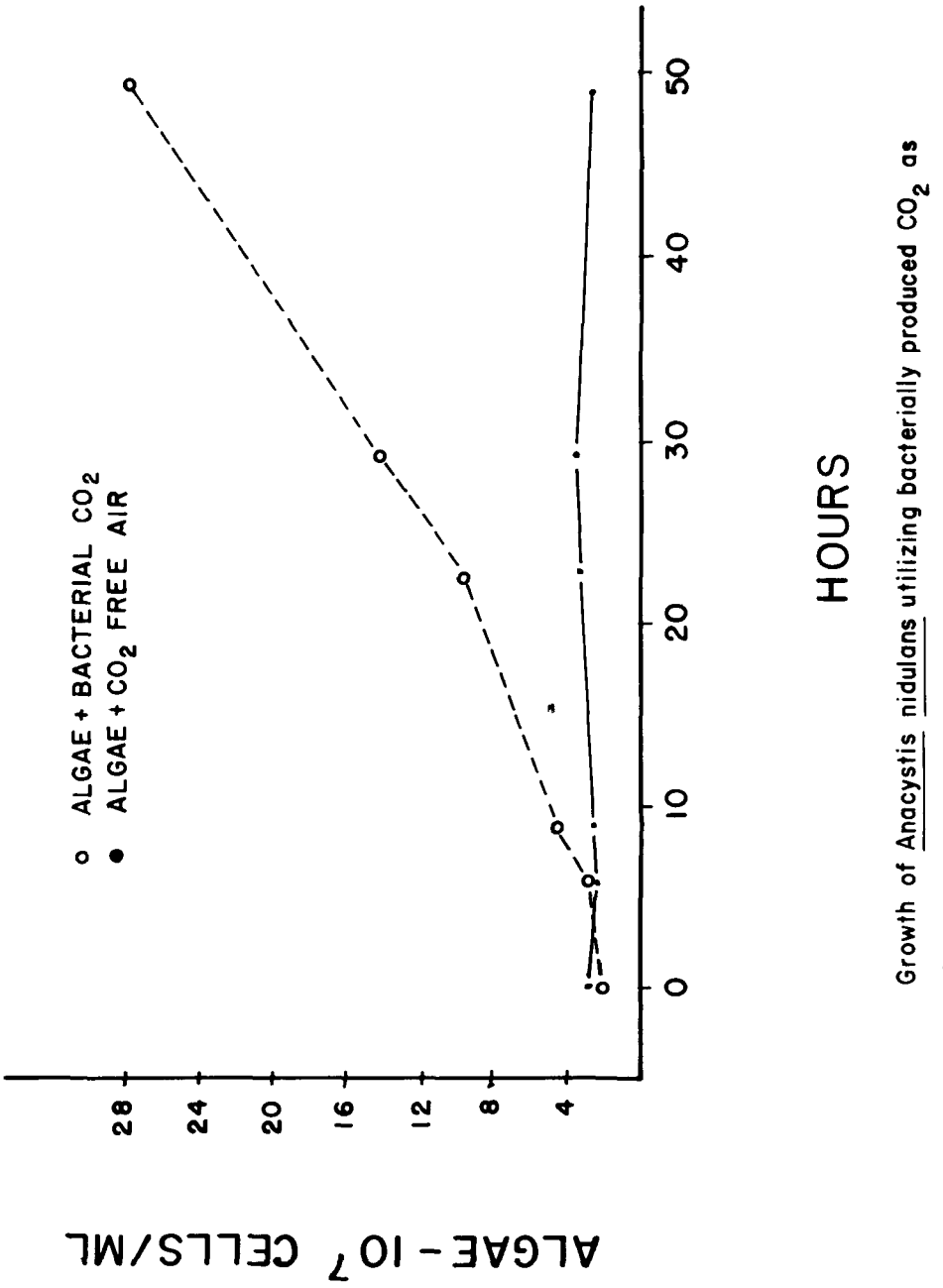
FIG. 3



CO_2 removal from the gas stream during one cell cycle of Anacystis nidulans. The gas flow rate through the 1-liter cultures was 300 ml/min. The numbers of algae in the test culture at 0 time, 8 hours, and 12 hour were 1.3×10^7, 1.3×10^7, and 2.8×10^7 cells /ml, respectively. During this same time, the numbers of algae in the control culture at 0 time, 8 hours, and 12 hours were 1.4×10^7, 1.4×10^7, and 1.1×10^7, cells/ml, respectively.

FIG. 4

FIG. 5



Growth of Anacystis nidulans utilizing bacterially produced CO_2 as the only carbon source.

The algae were grown in the standard culture medium in Fernbach flasks in the shaking water bath under the 4000 ft-c light source. Flasks 1-4 were bubbled with either CO_2-free air, air (0.03% CO_2), 1% CO_2 in air or 5% CO_2 in air. Flask 5 had air blown over the surface of the medium. The experiment was continued until the last culture stopped dividing at 168 hours. The CO_2-free air produced no growth (Table 1), while the most growth occurred in the 5% CO_2 where the algae underwent 5 doublings.

It should be emphasized that the pH was constant at 6.8 in all cultures throughout the experiment and that increased growth associated with increased concentration of CO_2 was a carbon effect that was not related to pH.

If the availability of CO_2 regulates the growth of an algae in laboratory cultures, the addition of CO_2 to natural waters should stimulate the indigenous population. Samples of water obtained from Lake Hartwell, South Carolina, and Shriner's Pond were brought into the laboratory and gassed with air or 5% CO_2 in air.

A 700-ml untreated sample of the Lake Hartwell water in a 2-liter flask was put on a rotating shaker under the 200 ft-c light source at 23°C. The water initially was clear and had a mixed diatom population of 10^5 cells/ml. Water quality data are shown in Table 2. After being bubbled with 5% CO_2 in air for four days, the water was brownish and contained 10^{10} algae/ml. The pH of this sample dropped from 7.1 to 3.5 in the first 30 minutes and remained there throughout the experiment.

Water obtained from Shriner's Pond had an initial population of 1.6×10^5 algae/ml and contained less than 5 ug each of N and P/liter. One-liter flasks containing 500-ml samples were gassed with either air or 5% CO_2 in air. From an initial population of 1.6×10^5 algae/ml the culture gassed with air alone increased to 2×10^5 algae/ml, while the culture gassed with 5% CO_2 increased to 5.6×10^5 algae/ml in 48 hours.

When these relatively unbuffered, low nutrient waters were gassed with 5% CO_2, the pH dropped precipitously so that the algae growing under these conditions had to possess the genetic potential to utilize free CO_2. If such an inoculum were not present, there would have been no increase in the algae population.

Phase IV: Phosphorus Requirements of *Escherichia coli* and *Anacystis nidulans*.

This phase was concerned with determining the P requirements of *A. nidulans* and *E. coli* and the competition between these two organisms for inorganic P.

The limiting and luxury cellular concentrations of P can be ascertained only under conditions where P is the sole nutrient regulating the growth of the organisms, and where physical parameters are not limiting. Because algal cultures must be sparged with CO_2 to insure an adequate supply of carbon, and because of the difficulty in maintaining a stable pH, it was impossible to obtain reliable uptake and growth data for *A. nidulans* in solutions containing small amounts of P. The same pH problem occurred in bacterial

Table 1

GROWTH OF *Anacystis nidulans* AT DIFFERENT CO_2 LEVELS

IN THE STANDARD MEDIUM WHICH INCLUDES 2×10^{-3} M K_2HPO_4 AND 2×10^{-2} M KNO_3

Treatment	Cells/ml		Doublings
	0 Time	168 Hours	
CO_2-free air	2.0×10^7	1.5×10^7	0
Air blown over surface of medium	9.5×10^7	21.2×10^7	1+
Air bubbled through medium	7.6×10^7	60×10^7	3
1% CO_2 in air	10.0×10^7	89×10^7*	3*
5% CO_2 in air	5.0×10^7	165×10^7	5+

*In 48 hours.

Table 2

SURFACE WATER QUALITY OF LAKE HARTWELL

NEAR CLEMSON, SOUTH CAROLINA

September 4, 1969

pH	7.1
Alkalinity as calcium carbonate	14 mg/l
Temperature	27°C
Dissolved oxygen	8.5 mg/l
Total dissolved carbon as C	5 mg/l
Total dissolved phosphorus as P	0.04 mg/l
Hardness as calcium carbonate	10 mg/l

cultures, presumably caused by secretions of CO_2 and acids by the organisms. It is difficult to measure, by the usual chemical methods, the small amounts of P (1.0 ug/liter) that are removed by growing populations of algae and bacteria.

For the above reasons, radioactive ^{32}P was used with the standard Allen and Arnon medium and the basal salts medium of Payne and Feisal to ascertain the limiting and luxury cellular concentrations of P for *A. nidulans* and *E. coli*. Since there was sufficient buffering capacity in these media to maintain pH control, more reliable data relative to the uptake of P could be obtained.

When phosphorus-starved cells are suspended in phosphate-rich medium containing ^{32}P tracer, uptake is maximal with less dilution of the ^{32}P in the medium by exchange with cellular ^{31}P than in the P-replete cells. The amount of P in P-starved cells calculated from the specific activity (specific activity = dpm/ug P) should be a more reliable measure of the P metabolized by the cells since there would be less exchange. The maximum amount of P stored by the cells would be the luxury amount of P. If the same cells containing stored ^{32}P + ^{31}P are placed in phosphate-free medium until cell division ceases (because of phosphate limitations), then the amount of P in the cells would be the limiting cellular concentration.

A. nidulans grown in Fernbach flasks in the 23°C shaker water bath was continuously bubbled with 5% CO_2 in air under the 4000 ft-c light source.

Phosphorus-deficient cells were obtained by growing them in Allen and Arnon medium with Tris buffer substituted for the potassium dibasic phosphate. When cell division ceased, they were harvested by centrifugation and washed three times with phosphate-free medium. Phosphorus-replete cells were grown in the standard Allen and Arnon medium, harvested, and washed with phosphate medium.

After harvesting, identical flasks of Allen and Arnon medium containing ^{32}P as a tracer were inoculated with deficient and non-deficient cells. Samples for cell counts and ^{32}P activity in cells and in cell-free medium were taken aseptically at 0 time, 4 hours, 8 hours, 16 hours, and 24 hours. After the first 24 hours, samples were taken daily until cell division ceased.

To determine the amount of ^{32}P in the cells and media, 1-ml aliquants of the cultures were filtered through 0.45 u Millipore filters, which were washed three times with 5 ml of 0.1 M KH_2PO_4 and three times with 5 ml of distilled water. The activity in the cells was counted by placing the Millipore filter in the planchet counter.

The initial pH was 6.8 in both cultures; when cell division ceased, the pH was 7.5 for the deficient cells and 7.8 for the non-deficient cells.

When cell division had ceased, the cells in both flasks were harvested, washed with phosphate-free medium, inoculated into separate flasks of phosphate-free medium, and grown until cell division again ceased. Samples for cell counts and for radioactivity measurements were taken on the same

schedule as in the first part of the experiment. The initial pH of the phosphate-free cultures was 7.1, and the final pH was 7.0 for the deficient cells and 6.8 for the non-deficient cells. The cultures were shown to be neither light- nor nutrient-limited by subculturing techniques.

The maximum concentration of inorganic P in phosphorus-deficient cells during the experiment was 3.0×10^{-8} ug/cell, observed at the 16-hour sampling time (Fig. 6). At 24 hours, cell number had doubled and cell concentration had dropped to 1.5×10^{-8} ug P.

By the 16-hour sampling time, the non-deficient population had doubled and the P concentration had increased from 1.5×10^{-8} to 1.8×10^{-8} ug/cell.

The concentration of P in both groups of cells was always highest just before cell division and lowest following cell division.

In the phosphate-free medium, both phosphorus-deficient and non-phosphorus-deficient cells divided twice (second and fourth days) (Fig. 7). Sampling continued through the seventh day and showed no further cell division. The cell concentration of P at the time of the last division was 0.25×10^{-8} ug P for replete and 0.3×10^{-8} ug for deficient cells. When the concentration dropped below this figure, there was no cell division. On the seventh day, the concentration per cell had dropped to 0.03 and 0.06×10^{-8} ug P for replete and deficient cells, respectively. When transferred to medium containing phosphate, these cells again started to divide.

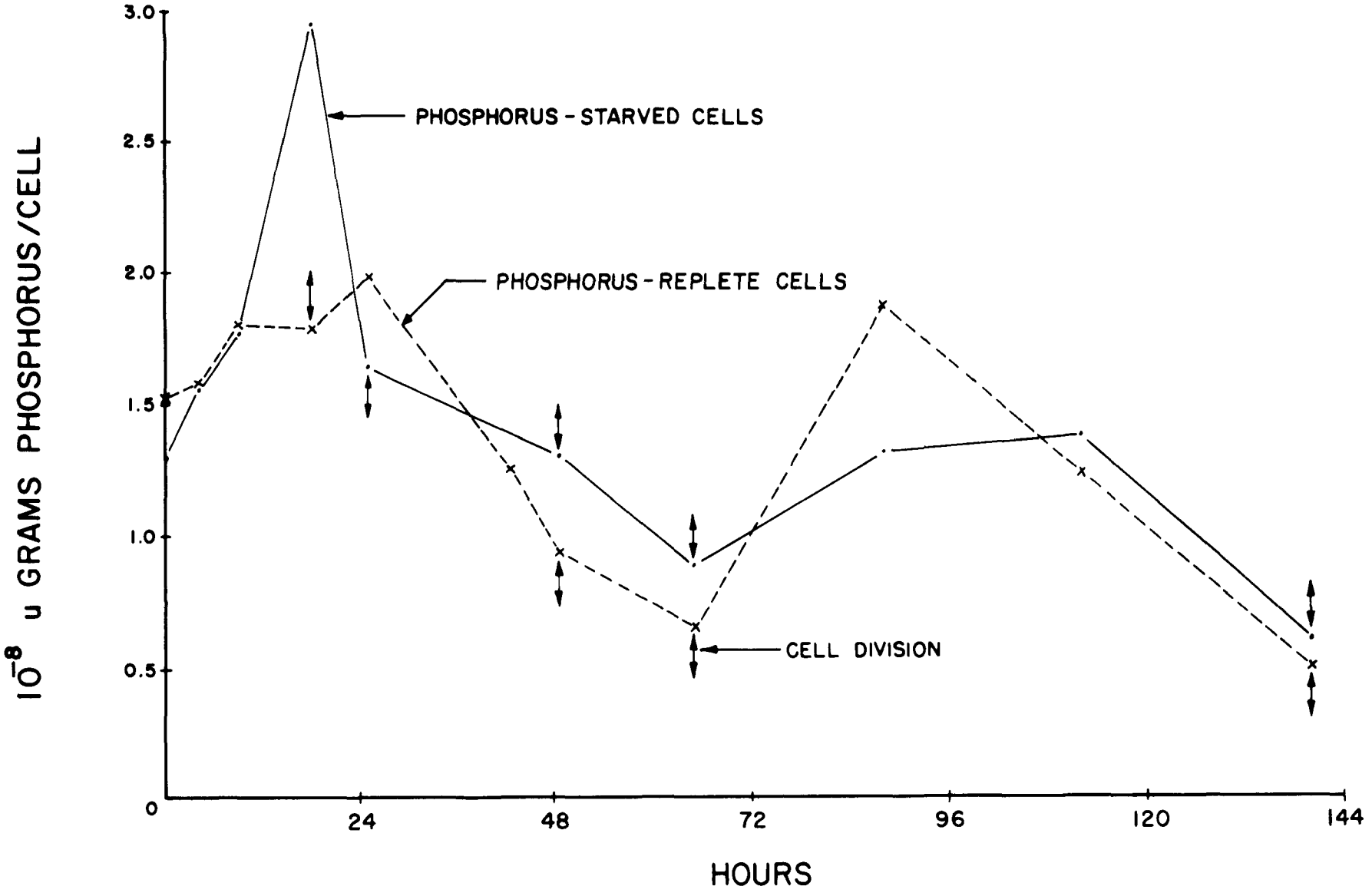
From the amounts of P in the cells under outlined experimental conditions, it would seem that *A. nidulans* required small amounts of P for cell division and that when cellular P concentration was 0.9×10^{-8} ug initially, cell division could take place in the absence of an external P source.

Similar experiments were performed using *E. coli* as the test organism. The experimental procedures were the same as described above, except the organisms were grown in different media and were gassed with air. The standard basal salts medium of Payne and Feisal plus 1% glucose was used for the P medium and the phosphate-free medium was obtained by again substituting Tris buffer for the phosphate buffer.

The initial pH of the phosphorus-containing medium was 7.0, and the final pH was 6.1 for the deficient cells and 6.0 for the non-deficient cells. The initial pH of the cells and phosphate-free medium was 7.4, and the final pH was 6.0. Samples for cell counts and for determination of radioactivity in cells and in cell-free medium were taken every 30 minutes for two hours and hourly thereafter. Cell counts for the bacteria were determined by the same direct microscopic technique used to enumerate the algae. Cells were viable at the end of the experiment.

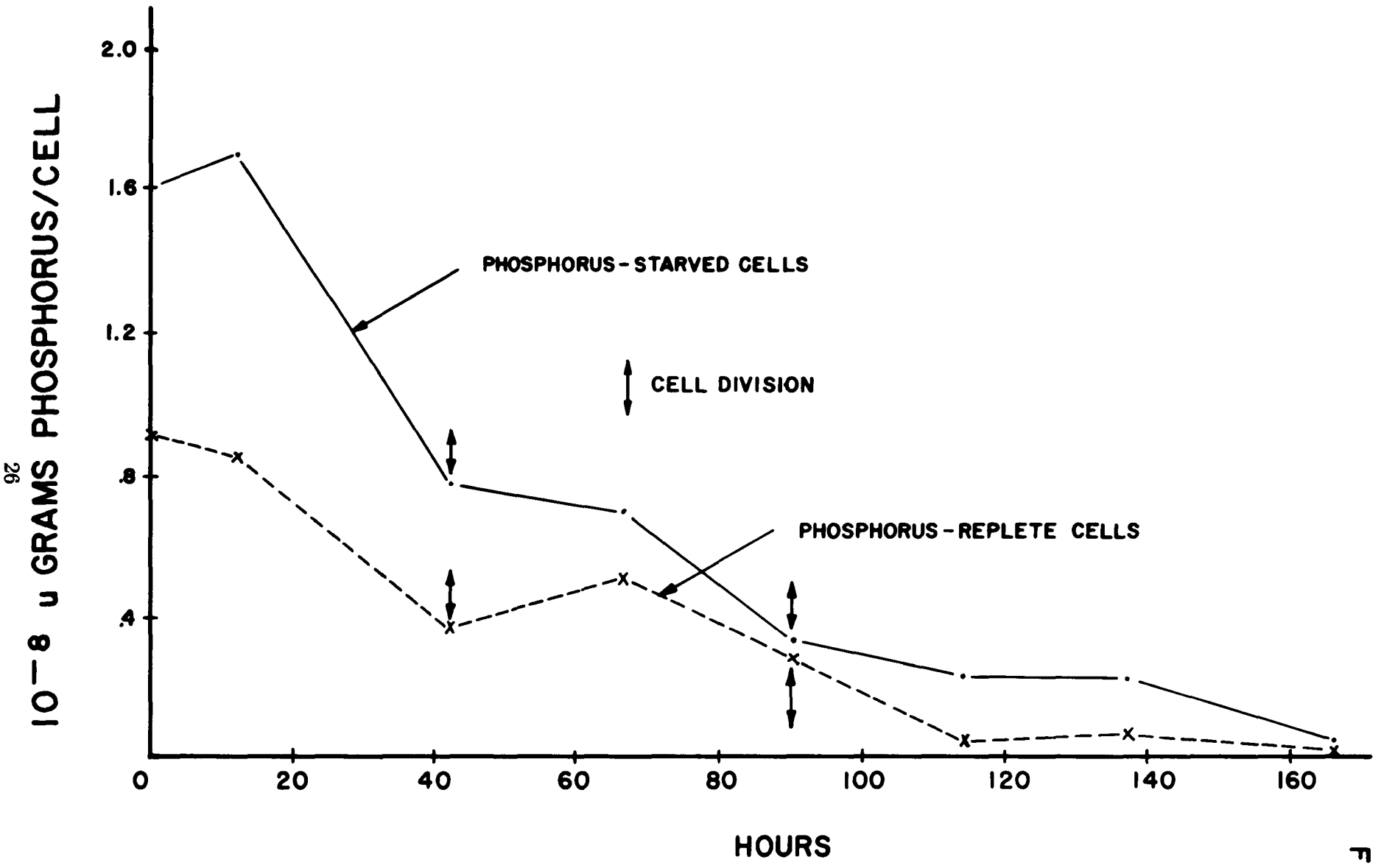
The greatest incorporation of P into starved cells was observed at the 30-minute sampling time. The cellular concentration of P-starved cells was 350×10^{-8} ug/cell at that time, and the cell count had dropped from 1.05×10^{7} to 0.7×10^{7}.

After 18 hours in medium containing P, cells containing 1.6×10^{-8} ug P/cell were inoculated into medium containing no P. In the absence of an external



Incorporation of phosphorus by Anacystis nidulans in PO_4 - medium. Cell division is non-synchronous after 64 Hours.

FIG. 6



Utilization of stored phosphorus during growth of *Anacystis nidulans* in Tris medium.

FIG. 7

source of P, about one-half of the cells of *E. coli* underwent division by the three-hour sampling time. Sampling continued for 10 hours and there was no further cell division.

Phase V: Regulation of the Growth of *Anacystis nidulans* by Additions of Carbon and/or Nitrogen plus Phosphorus to Sterilized, Infertile Pond Water.

A. nidulans was inoculated into two sterile, 1-liter flasks containing Shriner's Pond water (5 ug each of P and N/liter). To one flask, commercial 20-20-5 fertilizer was added. Both cultures were grown in shake culture at 23°C under the 200 ft-c light source. During the first 96 hours both cultures were in shake equilibrium with the air which entered the flasks through cotton plugs. After cell division ceased, air was bubbled through the flasks for another 102 hours. After the cells stopped dividing again, 5% CO_2 in air was bubbled through the cultures for an additional 66 hours.

Figure 8 shows no difference between the final sizes of the population of *A. nidulans* in the fertilized and unfertilized water. However, increasing the amount of available CO_2 stimulated growth in both cultures. In both cultures, when air was the source of CO_2, cessation of growth was accompanied by a pH in the range of 9.8 to 10.1 indicating that even though there was a constant source of CO_2 (0.03%) it was not sufficient to support larger populations of algae. The growth rate of *A. nidulans* was faster when 5% CO_2 in air was bubbled through the culture containing the fertilizer, but at the time growth ceased at 66 hours, (assumed to be due to light limitation) the maximum size of the two populations was identical irrespective of the concentration of P and N.

The difference in growth rate between the two systems is probably a reflection of the buffering capacity. Addition of fertilizer to the water does increase the buffering capacity (Fig. 9), which would tend to prevent precipitous changes in pH.

Phase VI: Regulation of the Growth of Natural Heterotrophic Organisms by Addition of Carbon and/or Nitrogen plus Phosphorus to Sterilized, Infertile Pond Water.

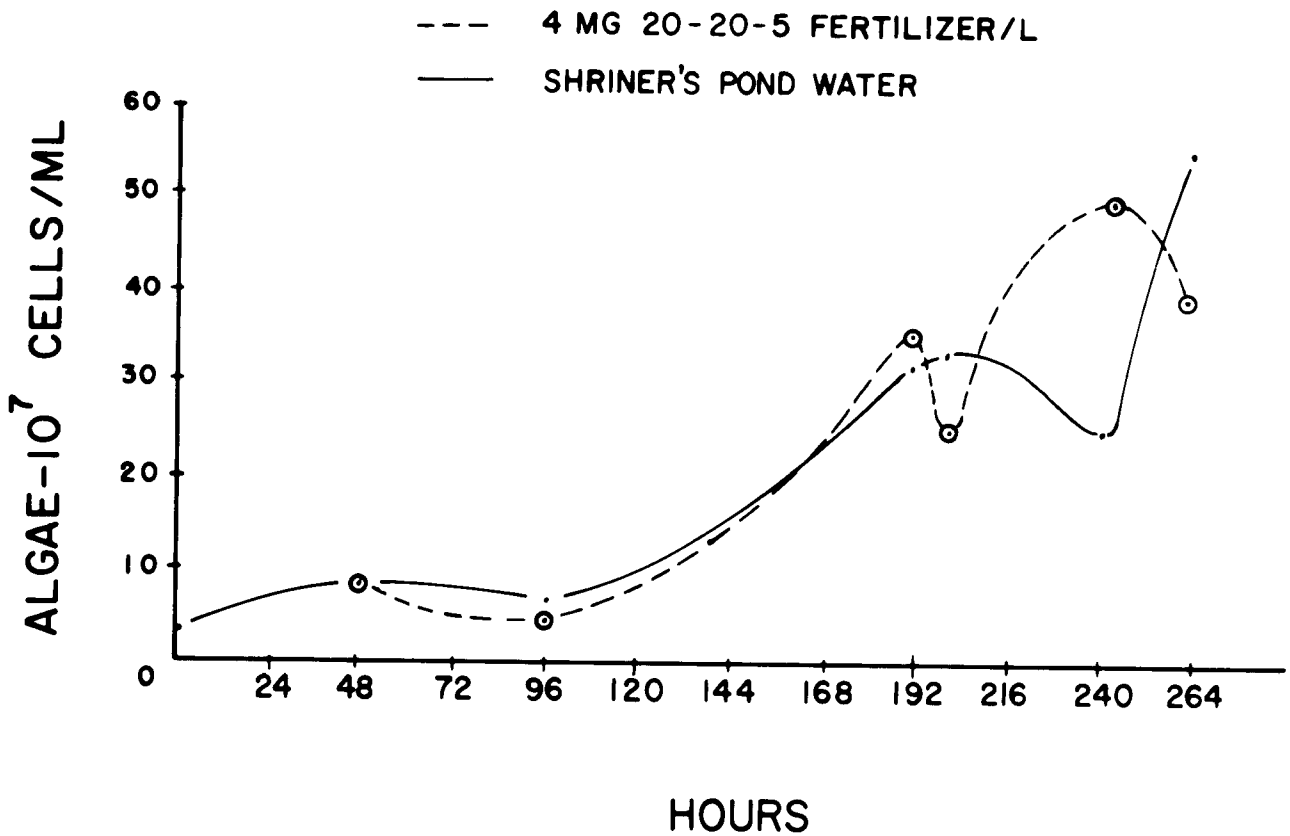
To elucidate further the relative roles of carbon and N plus P in regulating growth of heterotrophic organisms, a mixed natural population of heterotrophs was inoculated into flasks containing sterile Shriner's Pond water (SPW). Additions to the water were glucose (0.25%) and 20-20-5 commercial fertilizer. Effects of the following combinations were observed:

Shriner's Pond water (SPW) + fertilizer

SPW + glucose

SPW + fertilizer + glucose

SPW (control)

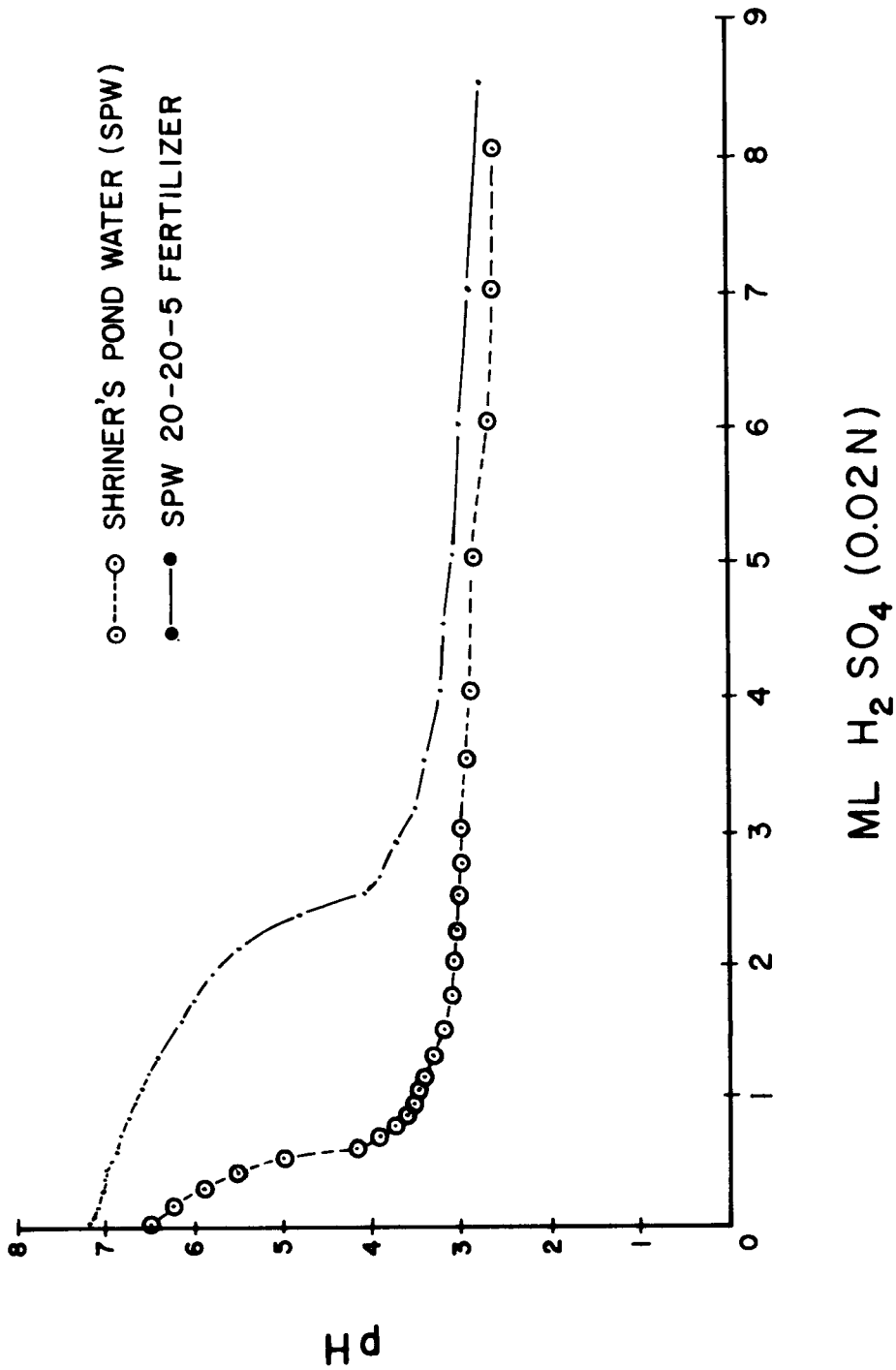


Shake equilibrium with air (0.03 % CO_2).	Bubbled air through the the culture.	Bubbled 5 % CO_2 in air through the culture.

Growth of Anacystis nidulans in Shriner's Pond water. When growth ceased at one level of CO_2, higher levels were supplied to each culture. In both cultures where air was the source of CO_2, cessation of growth was accompanied by a pH of 9.8 - 10.1.

FIG. 8

FIG. 9



Buffering capacity of Shriner's Pond water with or without added 20-20-5 fertilizer.

Carbon dioxide evolution was used as a measure of heterotrophic metabolic activity (Levin, 1963, used a similar method for determining metabolic activity in activated sludge). The heterotrophs were cultured in the dark. CO_2-free air was bubbled through the cultures and finally into saturated solutions of barium hydroxide. The cultures were grown for 72 hours, and the CO_2 produced was trapped continuously as barium carbonate. Samples of the medium were analyzed for dissolved inorganic carbon at 0 time and 72 hours. Barium carbonate was determined gravimetrically, and the amounts of CO_2 produced were calculated.

The production of CO_2 resulting from the metabolic activity of the microorganism was stimulated by the addition of inorganic N and P, glucose, and glucose plus N and P (Table 3). Duplicate experiments using reagent grade chemicals in the same proportions produced similar results.

Phase VII: Regulation of the Growth of Natural Populations of Heterotrophs and Autotrophs by the Addition of Carbon and/or Nitrogen plus Phosphorus to Non-sterile, Infertile Pond Water.

Growth of natural bacterial and algal populations in Shriner's Pond water was compared to the growth obtained when 0.4 mg of the 20-20-5 fertilizer, 0.25 g glucose, or combined fertilizer and glucose was added to 100 ml of water in 250-ml Erlenmeyer flasks. Shake cultures at 23°C were bubbled with air under the 200 ft-c light source. The initial pH of the cultures was 7.1. The number of bacteria, number of algae, and amount of dissolved CO_2 were determined at 0 time and at the end of 30 hours. Table 4 shows the increased number of bacteria and algae and the increase in dissolved CO_2 in those flasks containing fertilizer, glucose, or fertilizer plus glucose.

Additions of fertilizer and of fertilizer plus glucose resulted in the growth of such extensive bacterial populations that it was impossible to obtain reliable counts from the dilutions used for plate counts. During the same 30-hour period, the number of bacteria in the control decreased. In the culture containing the glucose, the pH was 5 at the end of 30 hours, while the pH of the other cultures varied from 6.5-7.0. The smaller number of viable cells in the glucose culture may have been due to the low pH causing either a direct inhibition of growth or lysis of the cells.

Addition of fertilizer or glucose alone resulted in a 2.5 to threefold increase of algae (largely mixed diatoms, *Chlamydomonas sp.*, *Chlorella sp.*) while the addition of glucose plus fertilizer resulted in a sixfold increase of algae. The control algal population did not grow during the 30-hour period.

Dissolved inorganic carbon decreased in the control while it increased in the fertilizer, glucose, and the fertilizer plus glucose treated flasks. It should be stressed that these amounts of inorganic carbon represent only the excess CO_2 produced by the bacteria that was not removed by the algae or lost to the atmosphere.

Table 3

CO_2 PRODUCTION BY HETEROTROPHIC POPULATION

FOLLOWING ADDITIONS OF ORGANIC AND/OR

INORGANIC FERTILIZERS TO A STERILIZED INFERTILE POND WATER

(<5 ppb each of N and P)

Treatment	$CO_2 + HCO_3^-$ (mg/l) (difference between 0 time and 72 hours)	Milligrams CO_2 trapped as $BaCO_3$
Shriner's Pond Water (SPW)	3.6	0
SPW + 20-20-5 fertilizer	10.0	0.003
SPW + glucose	9.2	0.005
SPW + fertilizer + glucose	12.0	0.03

Table 4

GROWTH OF INDIGENOUS BACTERIAL AND ALGAL POPULATIONS IN AN INFERTILE POND WATER (< 5 ppb each of N and P) FOLLOWING ADDITIONS OF ORGANIC CARBON AND/OR INORGANIC FERTILIZER

Medium	Total Bacterial Count/ml		mg CO_2 + HCO_3^-		Total Algal Count/ml	
	0 Hour	30 Hours	0 Hour	30 Hours	0 Hour	30 Hours
Pond Water	27.5×10^5	8.5×10^4	28.0	24.0	2.0×10^5	1.6×10^5
4 mg 20-20-5/1	27.5×10^5	1/	26.3	35.9	2.0×10^5	6×10^5
2.5 g glucose/1	27.5×10^5	40×10^5	25.3	45.4	2.0×10^5	5×10^5
4 mg 20-20-5/1 2.5 g glucose/1	27.5×10^5	1/	17.3	22.6	2.0×10^5	12.5×10^5

1/ Too numerous to count at the same dilutions.

Phase VIII: Growth of Heterotrophic and Autotrophic Populations and Nutrient Cycling in an Enriched Lake.

If stimulation of heterotrophic populations by the addition of carbon, N, and P is responsible for the blooms of algae occurring in enriched waters, large numbers of bacteria should be found associated with high pre-dawn concentrations of dissolved inorganic carbon, and large numbers of algae.

Since algal cultures are largely synchronous, nutrient uptake is cyclic. Algal metabolism is dependent upon light as an energy source, so diurnal and diel measurements of the amounts of carbon, N, and P in water reflect the cycling and relative importance of the nutrients in regulating the heterotrophic and autotrophic populations. Those substances necessary for the growth (increased number) of any population should be decreasing in the water during periods of active increase in size of the population.

Field studies were conducted on July 2, 15, and 30-31, 1969, in the Flat Creek area of Lake Sidney Lanier, Georgia, to follow the cycling of inorganic carbon (CO_2, HCO_3^-, $CO_3^=$), N (inorganic and total dissolved), P (inorganic and total dissolved), oxygen, algae, and bacteria.

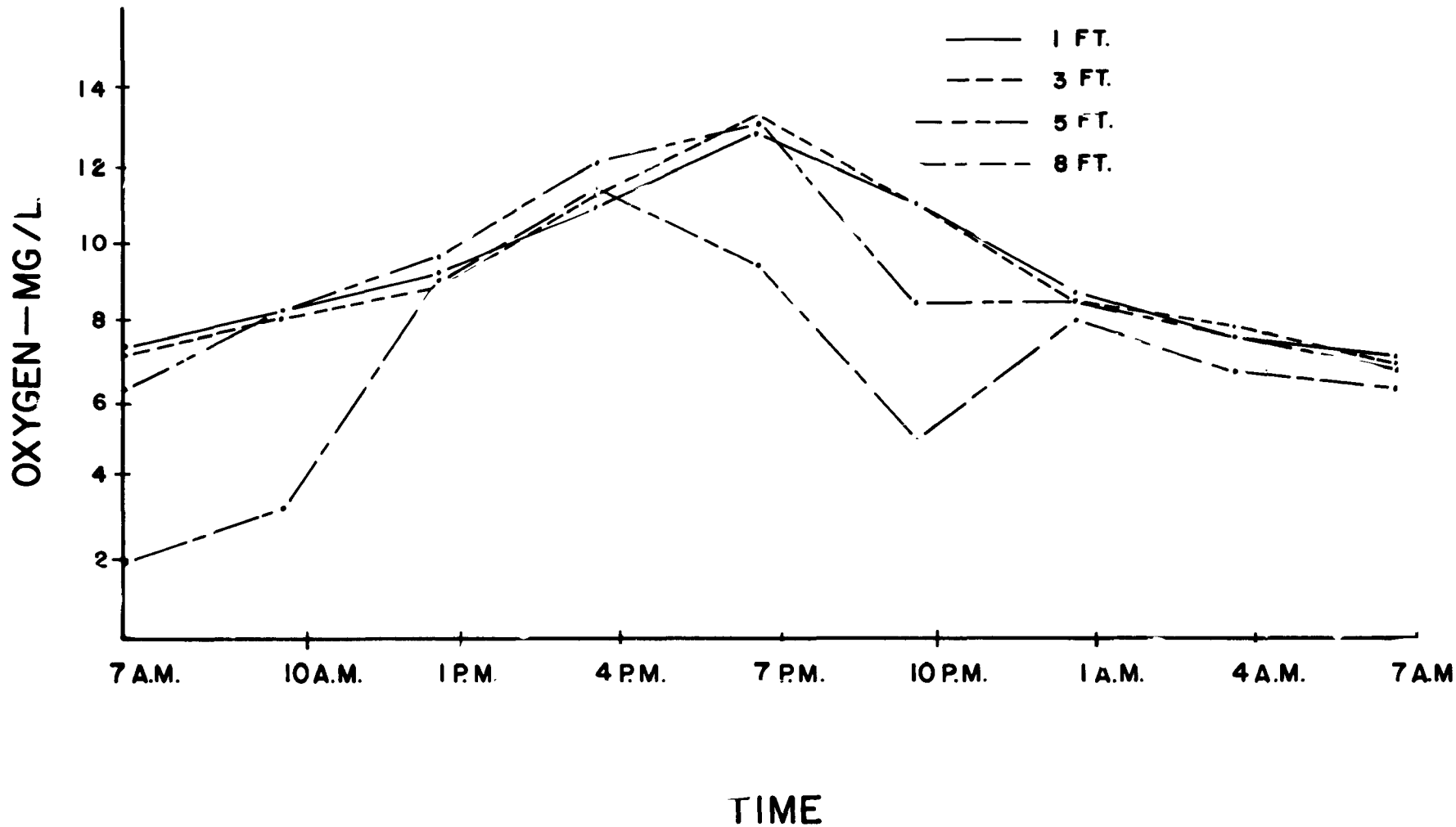
Flat Creek received the effluent from a secondary sewage treatment plant at Gainesville, Georgia, approximately seven miles upstream from the test area. Sampling stations 1,2, and 3 were located approximately 200 yards apart in the Flat Creek area of the lake. Station 4 was located one-fourth mile farther out in open water. A well-established algal bloom was present in Flat Creek from station 1 to station 3.

Water samples were taken with a Kemmerer bottle at one foot below the surface, one foot above the bottom, and at various intermediate depths, except at station 4 where no samples were taken below 20 feet (the top of the thermocline). Sediment samples were collected for bacterial analysis.

Emphasis has been placed on the cycling of carbon and P and the biological activities of the heterotrophic and autotrophic populations. Oxygen, N, and pH data are now presented. Diel cycling of oxygen (Fig. 10) occurred with the maximum in the late afternoon. Highest concentrations (12-13 mg/liter) were found at the 1-, 3-, and 5-ft levels. Minimal values occurred at all depths at 7:00 AM. The greatest fluctuation in the amount of oxygen occurred at the 8-ft depth where it ranged from 2 to 11 mg/liter.

Since both nitrate and ammonia-N were present at all times, N was not limiting the system (Fig. 11). The pH increased during the day (7:00 AM to 7:00 PM) from 6.5 to 10 at the three stations in Flat Creek, and from 8.9 to 10.6 at station 4.

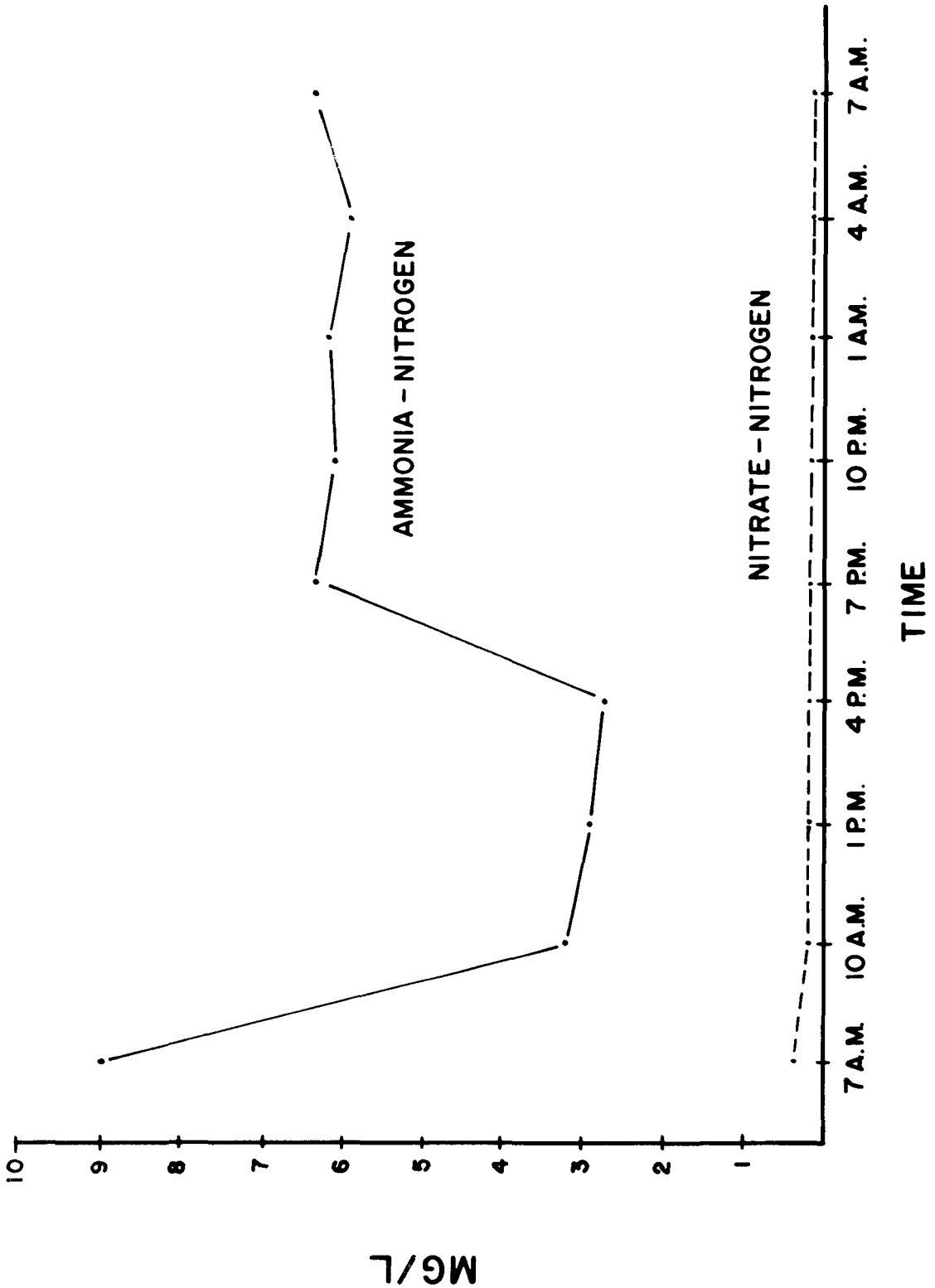
Comparison of the data from highly enriched station 1 (Fig. 12) with that from enriched station 4 (Fig. 13) revealed that approximately five times more algae (240×10^4 cells/ml) were at station 1 than at station 4 (50×10^4 cells/ml). The high, pre-dawn concentration of CO_2 (7 mg/liter) at station 1 was removed from the water by 1:00 PM. Bicarbonate decreased from 27 mg/liter at 7:00 AM to 4 mg/liter at 4:00 PM. This depletion of inorganic carbon at station 1 is especially significant because these waters



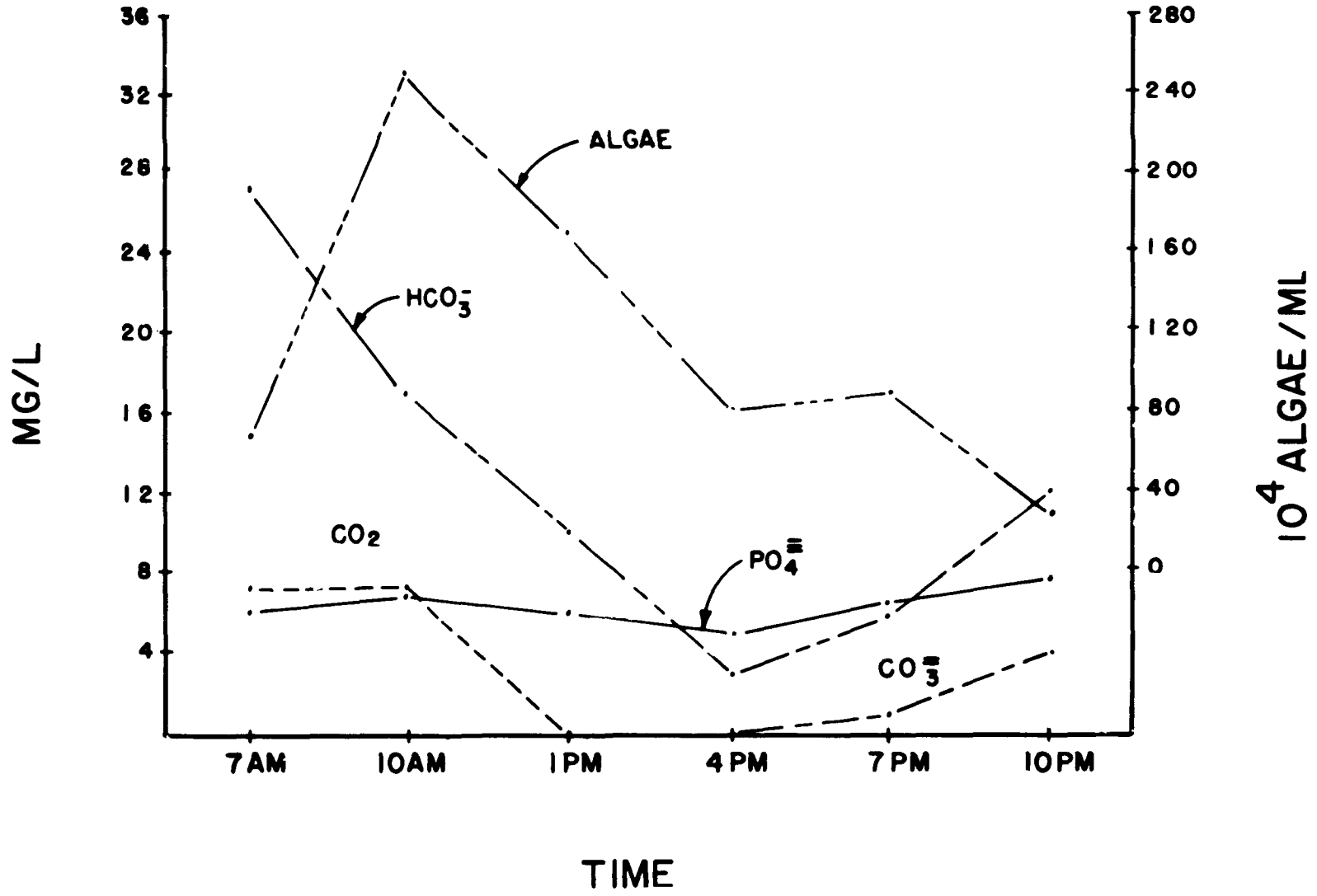
Diel fluctuations of oxygen in water of Lake Lanier at station 2 on July 30 and 31, 1969.

FIG. 10

FIG. 11

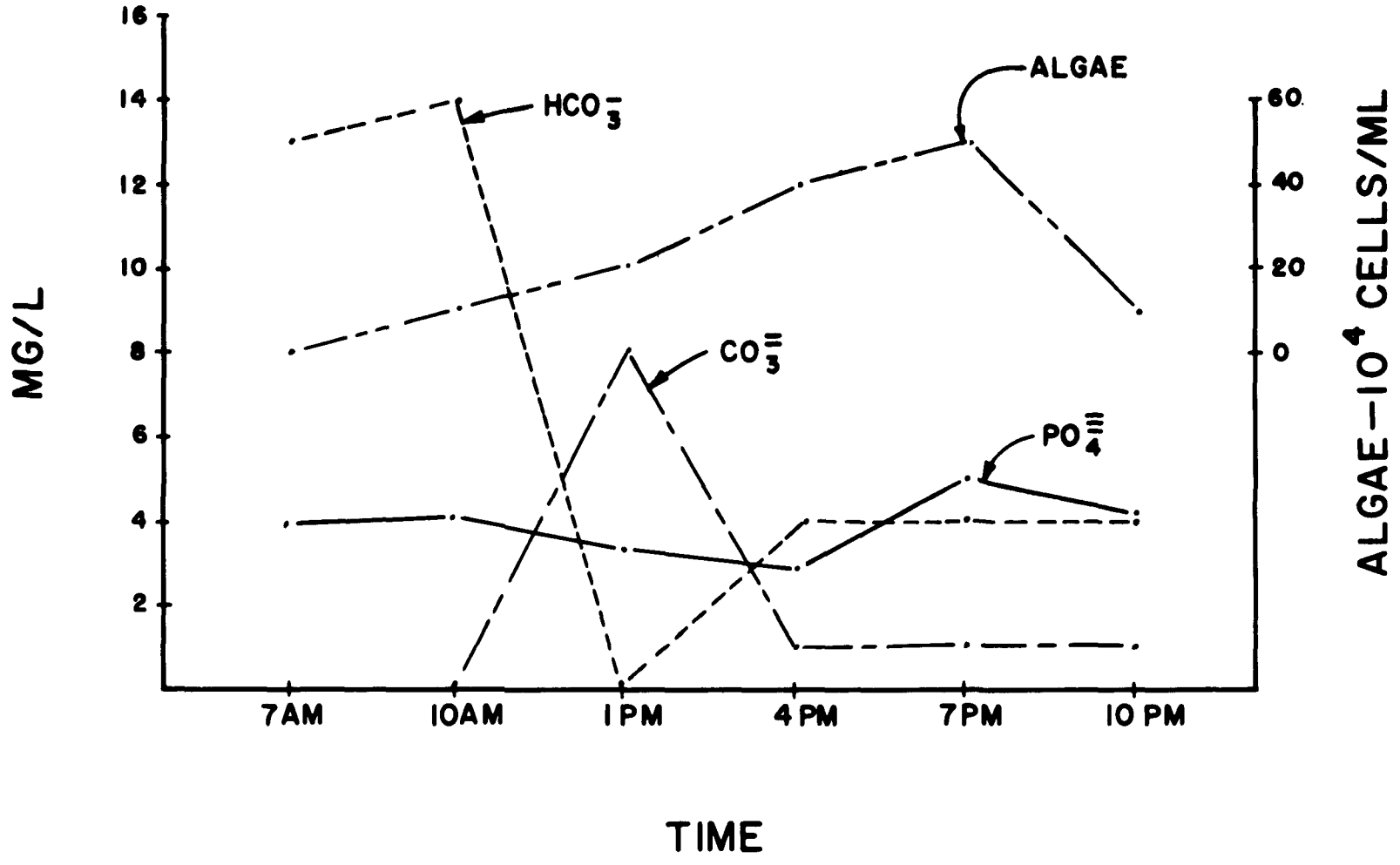


Diel fluctuations of nitrogen in water of Lake Lanier at the 1-ft depth of station 1 on July 30 and 31, 1969.



Diurnal fluctuations of CO_2, HCO_3^-, $CO_3^=$, $PO_4^{\equiv}$, and algae in the water of Lake Lanier at the 1-ft depth of station 1 on July 2, 1969. There was essentially no CO_2 in the water after 1:00 PM, and essentially no $CO_3^=$ in the water until 4:00 PM.

FIG. 12



Diurnal fluctuations of HCO_3^-, $CO_3^=$, $PO_4^{\equiv}$, algae in the water of Lake Lanier at the 1-ft depth of station 4 on July 2, 1969. There was essentially no dissolved CO_2 in this water.

FIG. 13

were constantly being gassed with bubbles containing 8-9% CO_2. Even with a constant source of CO_2, there was no accumulation of CO_2 or HCO_3^- in the water until sunset, when the uptake by the algae was at a minimum.

There was no detectable CO_2 in the 1-ft samples at station 4. Bicarbonate was removed from the water by 1:00 PM, then increased, and leveled off at 4 mg/liter by 4:00 PM. The concentration of $CO_3^=$ in this water was much higher than at station 1.

The amount of CO_2 at station 1 in the early morning is very large (7mg/liter) in comparison with the 0.4 to 1 mg/liter that can be obtained from air equilibrium. Since this is a soft-water (hardness, 30 mg/liter as $CaCO_3$) lake (Little, pers. comm. 1970), CO_2 contribution from carbonates would be minimal.

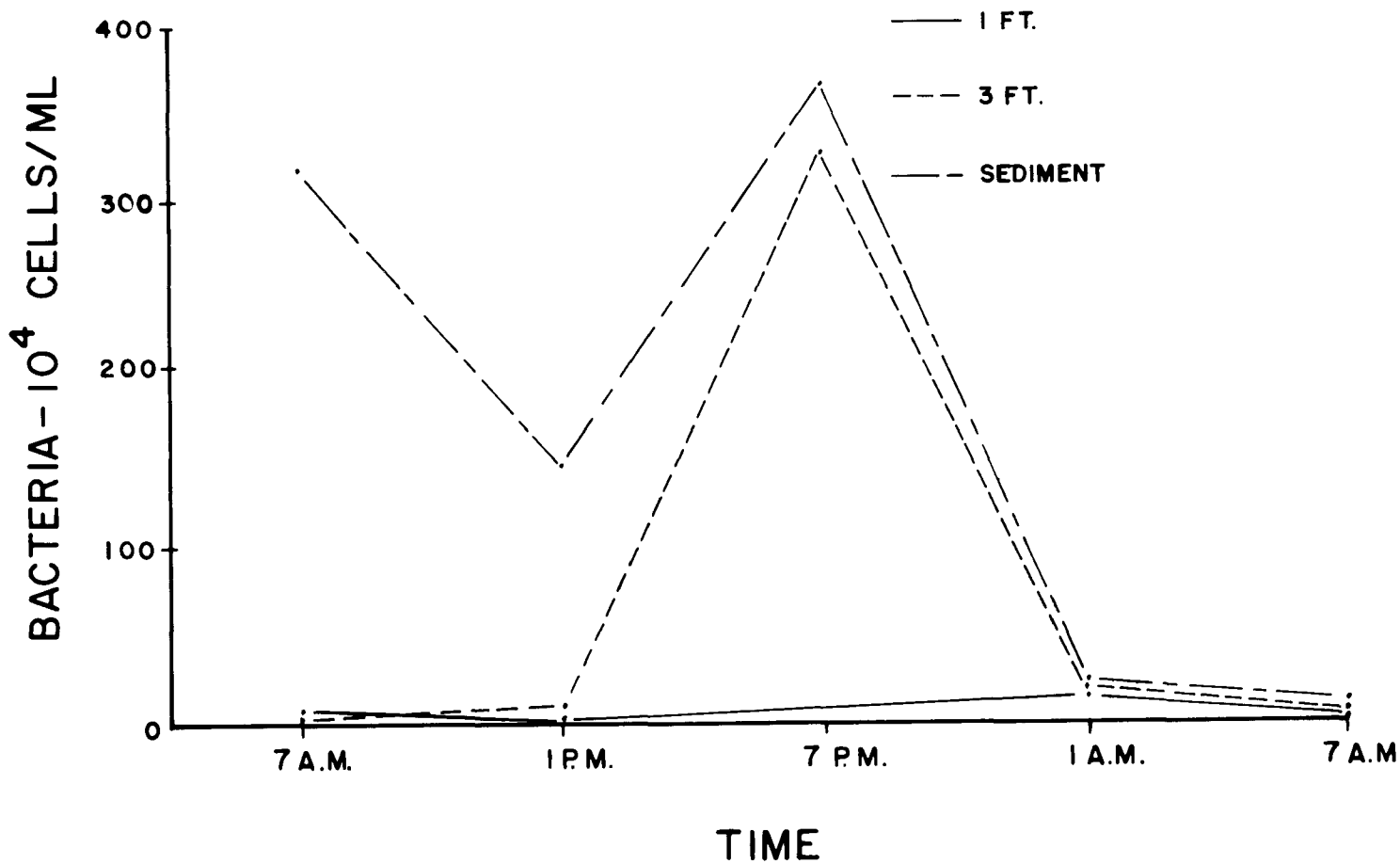
The amount of P remained constant or increased slightly at both stations during the same period that the inorganic carbon was being depleted. Phosphorus was high in these waters (1.3-2.2 mg/liter).

Figures 14 and 15 show the diel fluctuation in the size of the bacterial population at stations 1 and 4. The population density of the bacteria from the three depths at station 1 was approximately 100 times larger than the density from the five depths at station 4. At each station, the number of bacteria increased with depth. Since station 4 was 60 ft. deep, and no samples were taken below the thermocline, the reported values probably do not reflect the total biological activity. Bubbles rising through the water column indicated additional biological activity near the bottom of the lake.

The largest number of heterotrophs and presumably the majority of the heterotrophic activity were associated with the bottom, especially with the upper layers of the sediments. Metabolic activity of the benthos was indicated by the concentrations of CO_2 at the various depths of station 2 over a 24-hour period (Fig. 16). There can be no doubt that the bottom in this area was a source of large amounts of CO_2.

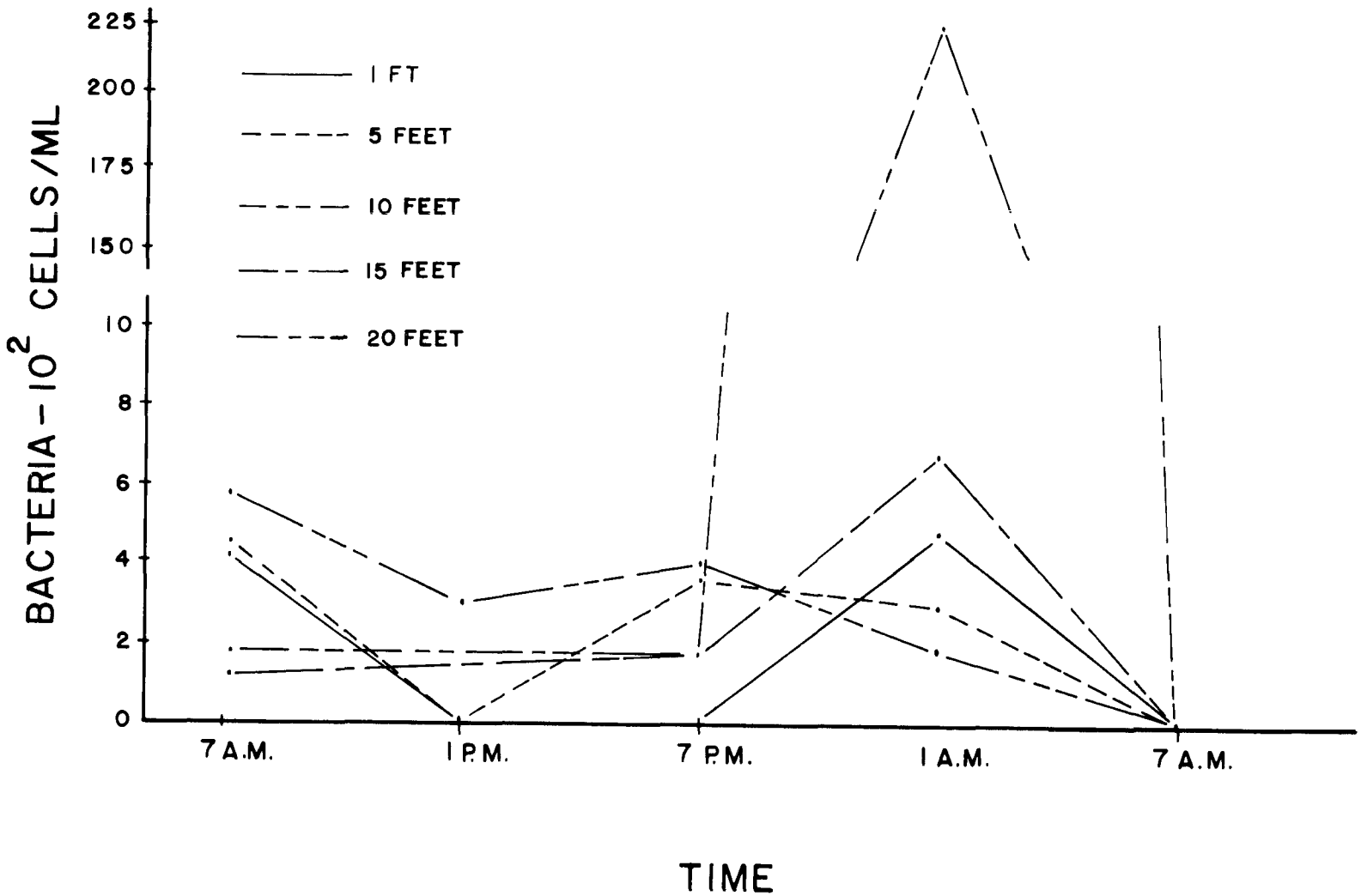
Accumulation of CO_2 in the bottom waters from 4:00 to 7:00 PM, and the build-up of CO_2 through the water column during the night was evident. Since the largest population of bacteria was found on the bottom and this is a soft-water system presumably this CO_2 originated from biological oxidation of organic substrates.

Data plotted in Fig. 17 demonstrate the importance of the physical environment (in this case, light) in regulating both biological activity and nutrient cycling. July 15, 1969, was cloudy and overcast with periods of intermittent sunshine before noon, and clear skies in the afternoon. July 2 was clear and sunny. Though the algal populations were essentially identical (240×10^4 cells/ml) on July 2 and July 15, 1969, both the rates and quantity of CO_2 and HCO_3^- removed from the water were lower on the cloudy day. On July 2, the total amount of CO_2 in solution (7mg/liter) was removed from the water by 1:00 PM; on the cloudy day, July 15, only 2.5 mg of CO_2/liter were removed by 1:00 PM. When the skies cleared on July 15, another 2.5 mg were removed in only 3 hours (1:00-4:00 PM). On July 2, 23 mg HCO_3^-/liter were removed from the waters from 7:00 AM to 4:00 PM; on July 15, only 13 mg HCO_3^-/liter were removed from solution (7:00 AM to 1:00 PM). The



Diel fluctuations of bacteria in the water and sediments of Lake Lanier at station 1 on July 30 and 31, 1969.

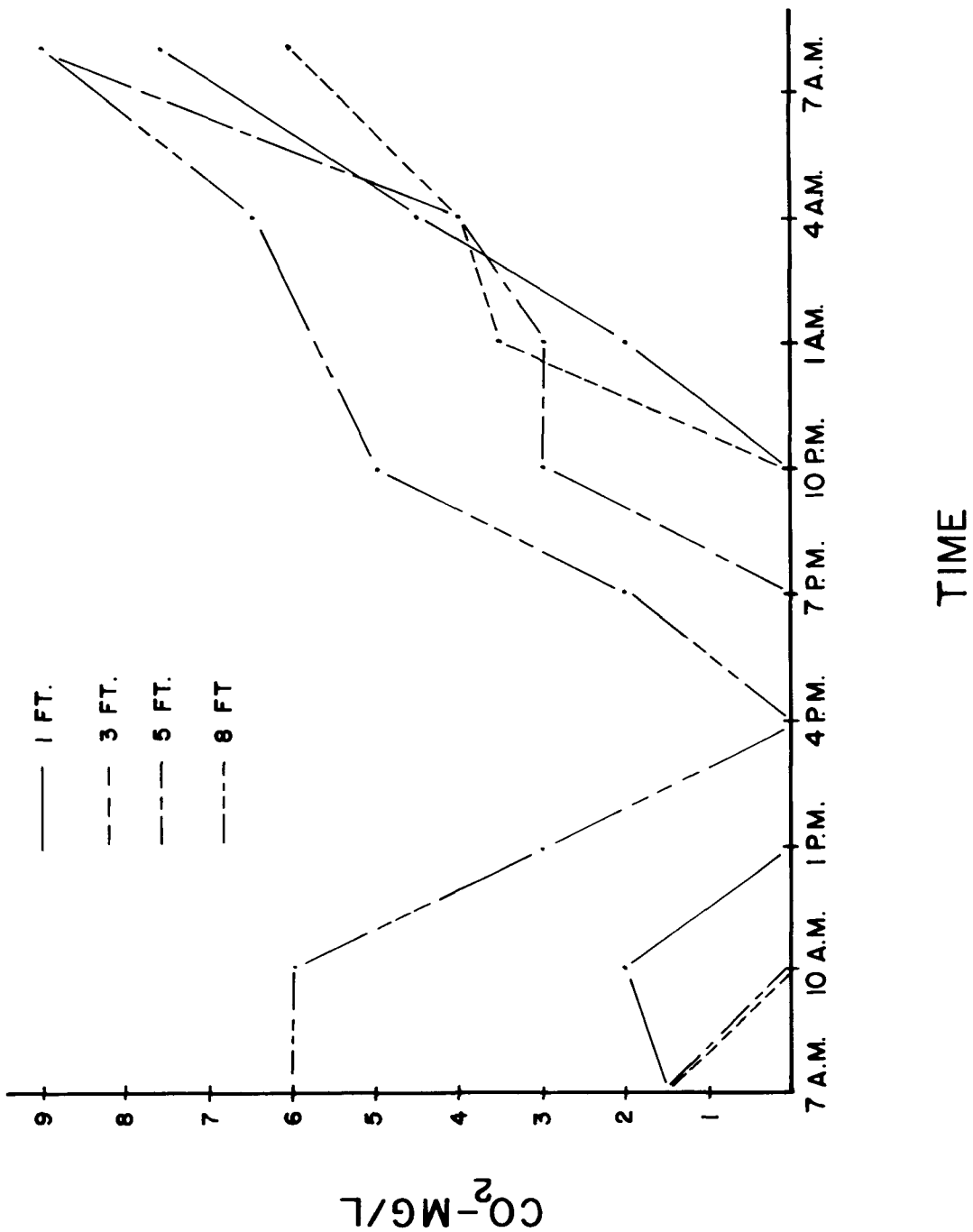
FIG. 14



Diel fluctuations of bacteria in the water of Lake Lanier at station 4 on July 30 and 31, 1969.

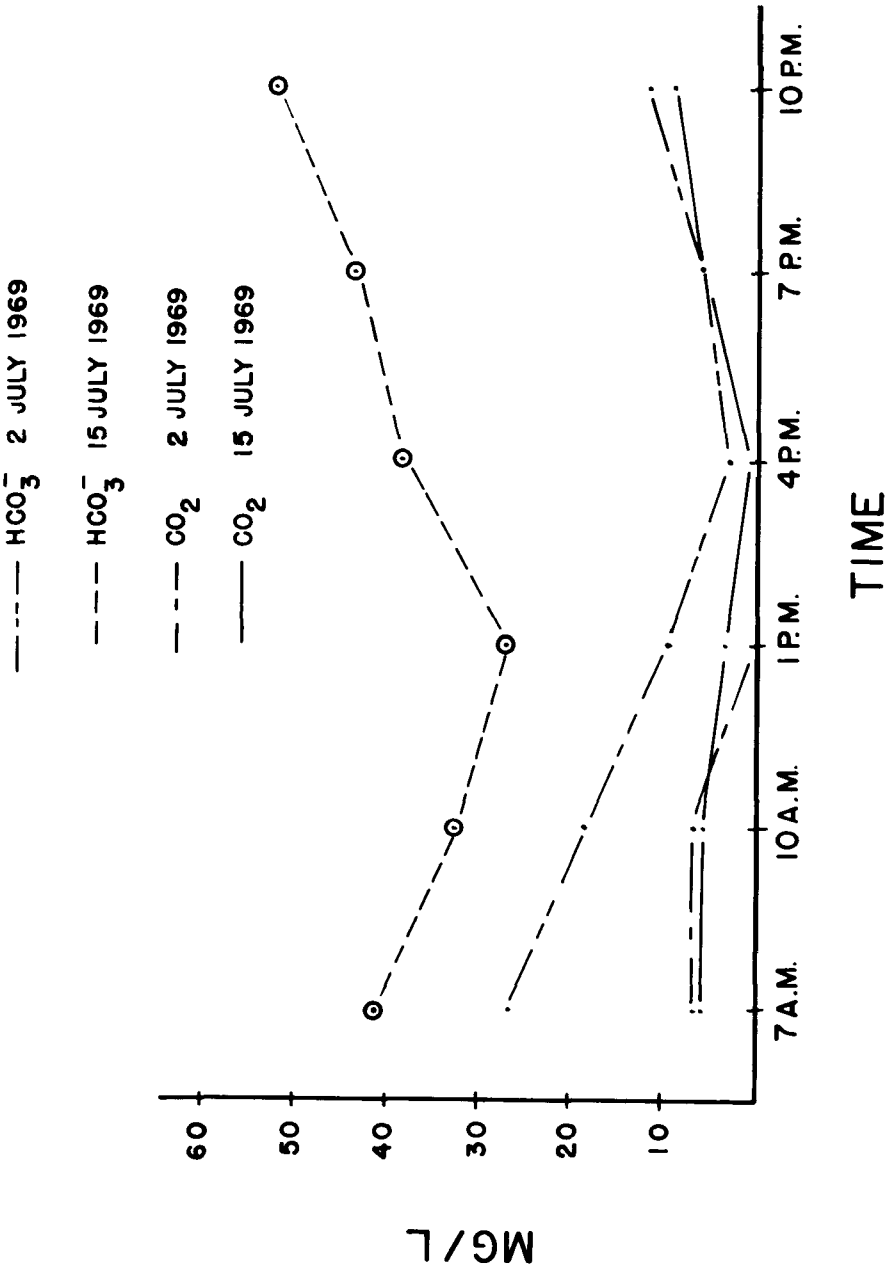
FIG. 15

FIG. 16



Diel fluctuations of dissolved CO_2 in the water of Lake Lanier at the sampling depths of station 2 on July 30 and 31, 1969.

FIG. 17



Diurnal fluctuations of CO_2 and HCO_3^- in Lake Lanier water at the 1-ft depth of station 1 on July 2 and 15, 1969.

dissolved HCO_3 was increasing during the remaining hours of the experiment (1:00 PM to 10:00 PM).

The observed differences in pH paralleled those occurring in the carbon cycling. On July 2, the pH ranged from 6.5 to 10; on July 15, the pH increased from 7.1 to 8.

DISCUSSION

Data collected in this laboratory indicate the importance of CO_2 in regulating the growth of axenic cultures of *A. nidulans* and natural populations of algae. This effect is observed when either commercial air mixtures containing CO_2, or CO_2 produced by heterotrophic oxidation of organic carbon is used as a carbon source. Lange (1967) demonstrated similar results with *Oscillatoria rubescens* and *Microcystis aeruginosa* Kutz. Allen (1955) demonstrated that *Chlorella* and *Scenedesmus* were growing in sewage oxidation ponds on the CO_2 produced by the heterotrophic oxidation of organic carbon. Cyclic uptake of carbon during a cell cycle of *A. nidulans* resulted in the calculated removal of 10^{-5} ug CO_2/cell. Birge and Juday (1911), in classic ecological studies of the lakes of Wisconsin, described the seasonal cycling of CO_2, HCO_3^-, and $CO_3^=$ in soft, medium, and hard-water lakes. The authors attributed removal of carbon available to green plants both directly by biological fixation and indirectly by chemical precipitation of carbonates as the pH rises. Birge and Juday emphasized the importance of bacterial oxidation or organic material as a principal source of CO_2 in aquatic systems. They reported that there was no diurnal, only seasonal, cycling of carbon in the lakes of Wisconsin during 1904-1906.

Carbon, N, and P compounds function both as nutrients and as buffers. Buffering capacity is important in controlling the chemical availability of carbon, N, P, and trace elements. As the data in Phase V indicate, addition of 20-20-5 fertilizer increased the buffering capacity of Shriner's Pond water. In enriched waters, the buffering capacity could be increased directly by the addition of charged compounds and indirectly by the amino acids, organic acids, and CO_2 resulting from increased biological activity. Such increases in buffering capacity could be very important in regulating algal blooms since more carbon could be held in solution in the forms (CO_2 and HCO_3^-) most available to the plants. Increased buffering capacity would also tend to "protect" the heterotrophic and autotrophic populations. Since enzymatic reactions are pH dependent, biological activity would be favored in situations where precipitous changes in pH are prevented. Thus, any increase in buffering capacity would enhance the biological generation of CO_2, its availability in solution, and the biological utilization of CO_2.

Data obtained in 1969 leave no doubt that the Flat Creek-Lanier ecosystem was in a dynamic state and exhibited diel fluctuation in nutrient cycling and biological activity. Thus, it is difficult, if not impossible, to correlate actual biological response with instantaneous measurements of dissolved nutrients. CO_2 and HCO_3^- are of extreme importance in regulating the size of the algal population in Flat Creek, but measurements of these ionic forms in this water in the afternoon revealed no CO_2 and a minimal HCO_3^- value of 4 mg/liter.

Conversely, afternoon measurements of the P concentration would yield high values of 1.3-2.2 mg/liter. Observation of the diurnal fluctuation of total P alone would reveal that there was no appreciable biological activity occurring in this ecosystem, while in fact, it is an area of intense metabolic activity.

Both Lund (1950) and Purdy (1937) cited evidence that instantaneous measurements of nutrients in water are not reliable indications of potential activity. Lund (1950) stated:

> "The amount in solution in the water at any one moment is not a reliable indication of the potential algal productivity, though it might be were other substances in excess of requirements."

Purdy (1937) reported that Mohlman *et al* in 1931, "pointed out the necessity of caution in accepting a single daily sample at a given station as representing the average conditions."

Both field and laboratory data indicated that growing populations of algae and bacteria require small amounts of P. The luxury concentration of P in *A. nidulans* is 3.0×10^{-8} ug/cell; the limiting cellular concentration is 0.25 to 0.3×10^{-8} ug/cell.

The cellular P concentration appeared to regulate the uptake of P by *A. nidulans*. Phosphorus-starved and phosphorus-replete cells were innoculated into media containing 2×10^{-3} M K_2HPO_4. Preceding the first division, the starved cells incorporated 6 times more phosphorus than the replete cells. Division of the starved cells had occurred by the 24th hour; division had occurred by the 16th hour in the non-starved cells.

Algae and bacteria possess the genetic potential to store N and P beyond their immediate metabolic needs. *A. nidulans* can undergo two doublings in the absence of an external supply of P. Both of these findings have been demonstrated earlier for the marine diatom, *Nitzschia closterium* (Ketchum, 1939).

The data from these studies support the thesis that during periods of active growth, the nutrient most critical in regulating the size of biological populations is the one that is most nearly removed relative to need from the environment. (Note the earlier discussion of removal of CO_2 and HCO_3^- from the water in the Flat Creek area while there was no measurable removal of P.)

Lund (1950) states:

> "On this view, the large phosphate concentrations in waters such as those described in Mackenzie (1940) and Gardiner (1941) may be considered to be due to there being an excess supply over that which the algal cells can take up. This may also be the explanation of the customary rise of phosphate-phosphorus in the lakes of the English Lake District in mid-winter when algal numbers are at a minimum due to lack of light."

If P and/or N are not present in the water in available forms, the growth of the population may or *may not* be regulated by these nutrients. If no P and/or N were added, the population could reach a size that would be determined by the amount of P and N stored in their cells and turned over by other organisms. The extent of growth due to P storage occurring under natural conditions is not well established, but laboratory values indicate population increases for more than a month with some algae (Levin, 1963).

Lund's (1950) data indicate that *Asterionella* containing luxury amounts of P sinks to the deeper waters where the growth of the organism is regulated by decreased light. During the spring turn-over, these cells serve as the inoculum for that season's population. Thus, the cells contain luxury amounts of P at the beginning of the growing season.

Nutrients not removed from the water are still capable of supporting the growth of other organisms, but *only if there is an added adequate supply of the nutrient that is available in the smallest amount relative to need.*

These data indicate the importance of the biological production of CO_2 in regulating the size of autotrophic populations in the three waters studied containing high (1.3-2.2 mg/liter, Flat Creek-Lanier), intermediate (0.04 mg/liter, Lake Hartwell), and low (<0.005 mg/liter, Shriner's Pond) concentrations of P. Increased production of CO_2 by mixed natural heterotrophic populations has been shown to accompany the addition of glucose, soluble N and P, and glucose plus soluble N and P to infertile water from a Georgia fishpond.

Additions of N and P to this same sterile, infertile water (<0.005 mg each N and P/liter) had no effect on the growth of an axenic culture of *A. nidulans*. Additions of increasing amounts of CO_2 increased the growth of the algae. Since the final size of the algal population was the same irrespective of whether N and P had been added, growth of the algae in this water is regulated by the amount of carbon present more than by the amount of N and P.

These findings offer a deeper understanding of the changes that can accompany the cultural (or natural) enrichment of the waters studied. Additions of N and P alone can result in transient increases in the size of the heterotrophic and autotrophic populations. Additions of organic carbon alone can stimulate the growth of both populations until either the allochthonous carbon or the N and/or P are depleted. Additions or organic carbon, N, and P can result in sustained growth of large populations of both heterotrophs and autotrophs.

Additions of organic carbon and/or N plus P to these waters directly stimulated the heterotrophic population. This stimulation increased the amount of CO_2 and HCO_3^- available for the aquatic plants. This increase in inorganic carbon is apparently directly responsible for the increased size of the algal populations in the waters studied.

SUMMARY

In our studies of the Blue-Green alga, *A. nidulans*, we observed that:

1. CO_2 produced by bacteria stimulated the growth of axenic cultures of *A. nidulans*.

2. CO_2 is important in regulating the size of the population of axenic cultures of *A. nidulans* and natural algal populations.

3. 10^{-5} ug CO_2 was sufficient for the division of a cell of *A. nidulans*.

4. The limiting and luxury cellular concentrations of P for P-starved *A. nidulans* are 0.3×10^{-8} and 3.0×10^{-8} ug/cell respectively.

5. The removal of P from solution by *A. nidulans* is dependent upon the cellular concentration of P.

6. Carbon uptake is cyclic during a cell cycle of *A. nidulans*.

7. Additions of carbon to a sterilized pond water are more important than additions of N and P in regulating the population size of axenic cultures of *A. nidulans*.

Laboratory studies of heterotrophic populations demonstrated that:

1. Additions of glucose, soluble N and P, and glucose plus soluble N and P to a sterilized pond water stimulated the production of CO_2 by isolated natural heterotrophic populations.

2. Additions of N and P, glucose, and glucose plus fertilizer to non-sterile water from an infertile farm pond resulted in increased numbers of bacteria, increased amounts of dissolved CO_2 and increased numbers of algae.

The biological and nutritional interactions studied in an enriched area, Flat Creek, receiving the effluent from a secondary treatment plant showed:

1. The diel cycling of CO_2, HCO_3^-, $CO_3^=$, O_2, and pH in these waters is indicative of an area of high biological activity. It is similar to the seasonal cycling described in the works of Birge and Juday (1911).

2. Large amounts of CO_2 were being produced by the organisms growing on the bottom of this creek. Bubbles containing 8-9% CO_2 were constantly rising through the water column.

3. In the afternoon, the water was supersaturated with oxygen which was not depleted at night.

4. Light affected the removal of nutrients from the water.

5. Growing populations of bacteria were associated with high pre-dawn concentrations of inorganic carbon and growing populations of algae.

6. Removal of certain nutrients from the waters accompanied the growth of biological populations. Inorganic carbon reached very high levels at night, but was almost depleted during the day. Phosphorus levels stayed almost constant. The nutrient, in this case, carbon, most nearly depleted, relative to need, should be the one regulating the size of the population.

7. Repetitive diel fluctuation in numbers of both bacteria and algae was observed during these studies. The most rapid increase in algal number in the first foot of water usually occurred in the early morning and was correlated with the most rapid rate of removal of CO_2 from the water. The maximum increase and decline in the bacterial population occurred from 7:00 PM to 7:00 AM and was correlated with the peak concentration and removal of oxygen from the water. Concomitant with the increased number of bacteria and removal of oxygen from the water, the dissolved CO_2 and HCO_3^- content of the water increased.

8. Instantaneous measurements of the amounts of nutrients in the water cannot be used to accurately predict biological activity of dynamic ecosystems.

Studies on these natural waters containing low, intermediate, and high concentrations of N and P stress the importance of carbon in regulating the growth of heterotrophic and autotrophic populations.

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