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CARBON AND NITROGEN AS REGULATORS OF ALGAL GROWTH IN TREATED SEWAGE

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PREFACE

This report on carbon and nitrogen as regulators of algal growth in treated sewage presents the results of the third part of a study entitled Algal Growth and Decomposition: Effects on Water Quality, Phase 2 (OWRR Project No. A-023-KY). The results of the first two parts have been published as University of Kentucky Water Resources Institute Research Reports No. 31 (October 1970) and No. 45 (January 1972). One additional study on the decomposition and nutrient regeneration of plankton samples collected from central Kentucky surface waters has been completed and the results will be published in a subsequent research report.

ABSTRACT

Continuous flow algal cultures were grown under three different growth conditions using secondary sewage treatment plant effluent as the growth medium. The only variable within each run was the hydraulic residence time. The concentrations of growth regulating nutrients were varied between the runs so comparisons of the algal mass, composition, nutrient uptake, and genera could be made. The importance of CO_2 availability for algal growth was also studied. A kinetic theory which based algal growth on cellular nutrient concentration was verified. The second phase of the study was a batch culture study in which the same growth medium was used as in Phase 1. The objective of Phase 2 was to investigate significant similarities and differences between continuous and batch culture growth under otherwise similar growth conditions.

Carbon dioxide enriched conditions produced as much as ten times the algal mass as CO_2 deficient conditions. Algal blooms dominated by blue-green algae were found to be the result of a successional change from green to blue-green algae under CO_2 enriched, nitrogen limited conditions. In the batch culture study algae exhibited a luxuriant nitrogen uptake.

KEY WORDS: algae, algal succession, carbon, chemical composition, chlorophyta, cyanophyta, hydrogen ion concentration, nitrogen, nitrogen fixation, sewage effluents

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CHAPTER I

INTRODUCTION

The natural eutrophication of a large body of water normally takes thousands of years; however, the rate of eutrophication can be rapidly increased by introducing man-induced nutrients. These nutrients find their way into lakes through domestic and industrial wastes and through agricultural run-off. If environmental conditions are favorable, these nutrients can trigger explosive algal growths. These large algal masses (blooms) are aesthetically unpleasant, decrease water recreation, impart taste and odor problems to water supplies, hamper industrial and municipal water treatment, ar.d on occasion, become toxic to certain warm-blooded animals. Another problem is encountered when these large algal masses die and begin to deteriorate. They create a tremendous biochemical oxygen demand and can deplete all of the available dissolved oxygen. This situation can result in large fish kills.

The growth of large algal blooms is eventually limited by some growth requirement, e. g., energy, nutrients, or temperature. In most temperate climates during the tolerable temperature season, algal growth has been found to be limited by nutrient availability. In most cases, either carbon, phosphorus, or nitrogen is the growth regulating nutrient as they are generally available in smallest quantities relative to their requirement for synthesis. Thus, if the growth regulating nutrient can be determined for a particular ecosystem and controlled, the massive algal blooms can possibly be regulated. Control of this nature at the present is either impossible or prohibitively costly. Therefore, each study that can provide information pertaining to growth regulation of algae contributes toward solving the eutrophication problem and saving our lakes.

The first phase of this study was a continuous flow culture study in which algae was grown under various growth conditions. When the cultures reached steady state, they were assayed to determine the extent and rate of growth, algal genera present, nutrient utilization, and algal composition. The second phase of the study was a batch culture study which provided a means for comparison of the two types of cultures (batch and continuous) and verification of some of the conclusions of the continuous culture study.

CHAPTER II

BACKGROUND

A.   Continuous Flow Culture Theory

The use of continuous flow cultures (chemostats) dates back to the early part of the Twentieth Century. Chemostats have been used to study many different aspects of algal growth, e.g., effects of temperature, nutrient limitation, effect of light intensity, determination of optimum pH, and effects of self shading. The reason the chemostat has been so widely used is its versatility for establishing desired growth conditions, adaptability to laboratory studies, and simple and inexpensive construction.

Chemostat systems can be made to simulate natural conditions very closely by controlling the hydraulic residence time (which is regulated by feed inflow rate) and the growth environment. Because of this, the information that is derived from chemostat studies is normally considered to be applicable to natural ecosystems.

If the chemostat system is to become a standard way of growing algae for assaying, steady state conditions must prevail for each study so reproducibility may be achieved for the same growth conditions. The chemostat meets these conditions as it is a completely mixed system with a constant volume, hydraulic residence time, and growth environment.

Once a chemostat has been inoculated with a small sample of algae, rapid growth and mass increase will follow. This rapid mass increase will be climaxed by a stable algal population along with a constant specific growth rate $(\frac{dX}{dt}/X)$ and nutrient concentration. These conditions are characteristic of a steady state system. When a system is in steady state, the algal growth rate

is such that each cell that is washed out of the system is replaced. Thus the growth rate is a function of the growth regulating nutrient in the feed supply if adequate energy is supplied. This makes it possible to determine the growth characteristics of algae when it is regulated by one particular nutrient.

1. Nutrient Limitation. In the past it has often been hypothesized that algal growth rate is proportional to the growth regulating nutrient concentration in solution (6). Recent studies, however, indicate that this is not always the case and that algal growth rate is sometimes based on cellular nutrient concentration.

The phenomenon of luxuriant nutrient uptake per se has been established for many years. Al Kholy (1) in 1956 and Mackereth (24) in 1953 demonstrated phosphorus uptake and storage by algae. They further maintained that this accumulated phosphorus was used to support growth and cell division in this absence of an external supply of the element. As early as 1951 Goldberg, Walker, and Whisenand (12) found that a marine alga exhibited a luxuriant consumption of phosphorus and was capable of dividing in the absence of an external supply.

Foree and Wade (9) conducted a recent continuous flow culture study and reported that the growth regulating nutrient (nitrogen) was depleted from solution shortly after inoculation and remained at low concentrations for the duration of the study. The mass increase, however, continued for a number of days after the depletion of the nitrogen from solution. Porcella (33) in a similar chemostat study found the same phenomenon to exist for phosphorus when it was the growth regulating nutrient. He pointed out that the rate of phosphorus removal from solution was much faster than would be expected based on a constant yield of algal cells for each unit of phosphorus removed. Foree and Tapp (8) maintained, from information ascertained during a batch culture study, that algae store nitrogen during nitrogen-abundant growth periods and later convert it to protein when nitrogen in solution becomes depleted.

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Although there are different opinions as to what causes mass increase when the growth regulating nutrient is depleted from solution (whether cell division subsides and cell size increases (16) or cell division continues until a minimum critical level of growth regulating nutrient in the cells is reached (10)), both schools of thought suggest that growth is based on cellular nutrient concentration.

2. Algal Growth Kinetics. From the information obtained during a literature review and the observations ascertained from laboratory studies preceding this one, it was deducted that the most reasonable way to describe algal growth would be with an equation that described growth rate as being proportional to the cellular nutrient concentration. Foree and Wade (9) developed such an equation when their results indicated that the kinetic theory outlined in Provisional Algal Assay Procedure (PAAP) (6) did not concur with their findings. The theory hypothesized by PAAP described the algal growth rate as being proportional to the growth limiting nutrient concentration in solution and stated that growth is proportional to the removal of this nutrient. Since Foree and Wade's findings did not concur with this approach, they developed the following equation:

$$(dX/dt)/X = K (N/X - [N/X]_{min}) - b$$

where:

$(dX/dt)/X$ = Specific Growth Rate

X = Algal Mass

t = Time

K = Proportionality Constant (algal mass/nutrient mass/day)

N = Cellular Nutrient Concentration (nutrient mass/volume)

b = Respiration Rate (time^{-1})

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N/X_{min} is the concentration of cellular nutrient at which growth will no longer continue. Knowing that $(dX/dt)/X = 1/\theta$ for continuous flow conditions and letting b' equal the respiration rate plus the minimum cellular concentration, the equation may be written:

$$\frac{1}{\theta} = (dX/dt)/X = K(N/X) - b'$$

where:

b' = Effective Respiration Rate (time^{-1})

θ = Hydraulic Residence Time (time)

B.   Batch Culture

A batch culture study is conducted by inoculating a growth medium with a relatively small concentration of algae and observing the growth charac-teristics by assaying the algal crop at time intervals. The only inputs to the reactors are (usually) energy (light) and air (CO_2 deficient or CO_2 enriched). The immediate results that may be ascertained from batch culture studies are algal mass variation as a function of incubation time, nutrient uptake with incubation time, growth regulating nutrients, algal growth rates, and total algal yields.

The periodic assaying of the algal mass and nutrients provides a means of determining if nutrient uptake is luxuriant and what nutrient is growth limiting. The ultimate growth determination is also a very important evaluation in the batch culture as it reveals the maximum algal crop that can be expected under a particular growth condition. The major difference between a batch culture and a continuous flow culture is that a batch culture simulates a large body of relatively stagnant water (stagnant with respect to hydraulic residence time); whereas, a continuous flow study would simulate more closely a small body of water such as a stabilization lagoon with a constant inflow source.

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C.   Carbon Dioxide Limitation

There has been a continual discordance on the relative roles of various growth regulating nutrients of algae for years. Many have recently specified phosphorus as the ultimate growth regulating nutrient. Phosphorus is probably limiting in some cases as it does not occur abundantly in nature as carbon and nitrogen, but normally is present as a result of wastewater and nutrient rich water (from fertilizer) runoff. Algae, on the other hand, only require small concentrations of phosphorus to substantiate growth. Thus, in cases where a body of water is receiving some form of phosphorus rich waste-water or agricultural runoff, phosphorus would probably not be limiting.

In the past, carbon dioxide has been somewhat overlooked as a growth regulating nutrient because it was thought that the atmosphere would provide adequate CO_2 for algae. Algae, however, demand a tremendous amount of CO_2 during rapid growth since carbon is its primary constituent (normally > 50%). Hutchinson (15) reported that CO_2 is normally present in water at concentrations between 0.4 and 1.0 mg/l. He also indicated that the movement of CO_2 in and out of a water interface is a slow process. King (19) agreed with this statement and postulated that the major carbon reserve for aquatic plants is the carbonate alkalinity system. The equilibrium reactions for the various forms of inorganic carbon in the pH range of 4 to 11 may be expressed:

$$CO_2 + H_2O \rightleftarrows H_2CO_3 \qquad (1)$$

$$H_2CO_3 \rightleftarrows HCO_3^- + H^+ \qquad (2)$$

$$HCO_3^- \rightleftarrows CO_3^= + H^+ \qquad (3)$$
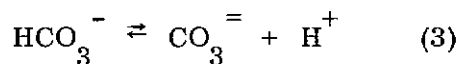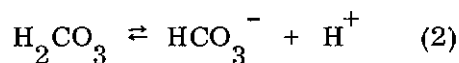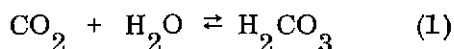
King pointed out that there are some differences of opinion as to whether algae use the bicarbonate ion directly or if they must use CO_2. In the latter case algal photosynthesis would be limited by the rate at which the bicarbonate ion is dissociated to give free CO_2.

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Clement (4), while conducting a study to determine optimum growth conditions for algae, found that improvements in the cultures resulted from reductions in $CO_3^=$ and HCO_3^- concentrations or by completely replacing them with CO_2. Kuentzel (20) pointed out that the replacement of free CO_2 by carbonate salts in slightly alkaline media is a relatively slow process and that available free CO_2 from natural inorganic sources probably never exceeds 1 mg/l. Myers (30) found that under normal conditions where free CO_2 is available it is often the preferred form of carbon used by the photoautotrophic algae.

Kuentzel (20) further noted that the conversion of carbonate salts to free CO_2 is too slow a process to supply the massive blue-green algal blooms with adequate CO_2. These rapid shifts to blue-green algae dominance and exponential growth can occur within a few hours which makes it physically impossible for enough CO_2 to be made available through the alkalinity system. Kunetzel suggests that the only way that this magnitude of CO_2 could be produced is through bacterial action on organic matter.

King (19) hypothesized that the blue-green algal blooms are the result of a succession from a green algae dominance to a blue-green algae dominance due to the diminishing of CO_2 availability in an ecosystem. This seems somewhat questionable as algae are composed of approximately 50% carbon and blue-green algal blooms are characterized by a tremendous mass increase. A more reasonable explanation for succession to blue-green algae might be nitrogen limitation as certain blue-green algae are inherently nitrogen fixers.

It would be unreasonable to take a firm stand that CO_2 is the only nutrient that limits algal growth or that phosphorus or nitrogen is always the limiting nutrient. This has been somewhat the trend, though, as a few states have gone so far as to ban the use of detergents that contain phosphates. This is obviously irrational as the effects of its substitute have not been studied adequately.

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Each micro-ecosystem contains a different set of growth conditions and any one of the three nutrients (P, N, C) could be limiting. Light and temperature are possibly limiting in many cases. The main point is that when large blue-green algal blooms are incurred, CO_2 is in great demand and probably is the regulating nutrient in most cases. This is a reasonable hypothesis as phosphorus would only be required in small amounts and the excess nitrogen needed for blooms could be provided through nitrogen fixation.

CHAPTER III

EXPERIMENTAL PROCEDURE

The objective of this chapter is to describe the growth apparatus, operational procedures, and sampling techniques used in both the batch culture and continuous flow culture phases of this study.

A. Growth Apparatus and Operational Procedures

1. Apparatus. The apparatus used in both phases of this study is illustrated in Figures 3.1 and 3.2, Figure 3.1 showing an individual reactor and Figure 3.2 the arrangement of the entire chemostat system including six reactors. The chemostat was designed to provide a completely mixed, continuous flow system and was similar to those outlined in Provisional Algal Assay Procedure (PAAP) (6). Chemostats were also used to grow the batch cultures in the second phase of the study.

The chemostats were constructed of plexiglass tubing 61 centimeters long with a volume of 2 liters. As can be seen in Figure 3.1, the influent port and air and/or carbon dioxide diffuser are located near the bottom of the chemostat, the sampling port at mid height, and the effluent port near the top. During the continuous flow study the constant inflow feed rates were arrived at by dividing the volume of the chemostat by the desired residence time. The feed was pumped into the chemostats by Chemical Rubber Company Vibrostatic pumps. The residence times used were 1, 2, 4, 8, 16, and 32 days.

For both the batch cultures and continuous flow cultures magnetic stirrers with teflon stirring bars were used to ensure complete mixing.

2. Gas Supply System. The gas supply system (Figure 3.2) was designed so that air and/or CO_2 could be supplied in approximately equal volumes for each chemostat.

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Beckman No. 39501 pH Electrode

No. 11 Rubber Stopper

Effluent Line

Plexiglas Cylinder

Sampling Port

Air Diffuser

Feed Line From Pump

1 1/2" Teflon Stirring Bar

From Air Supply

Magnetic Stirring
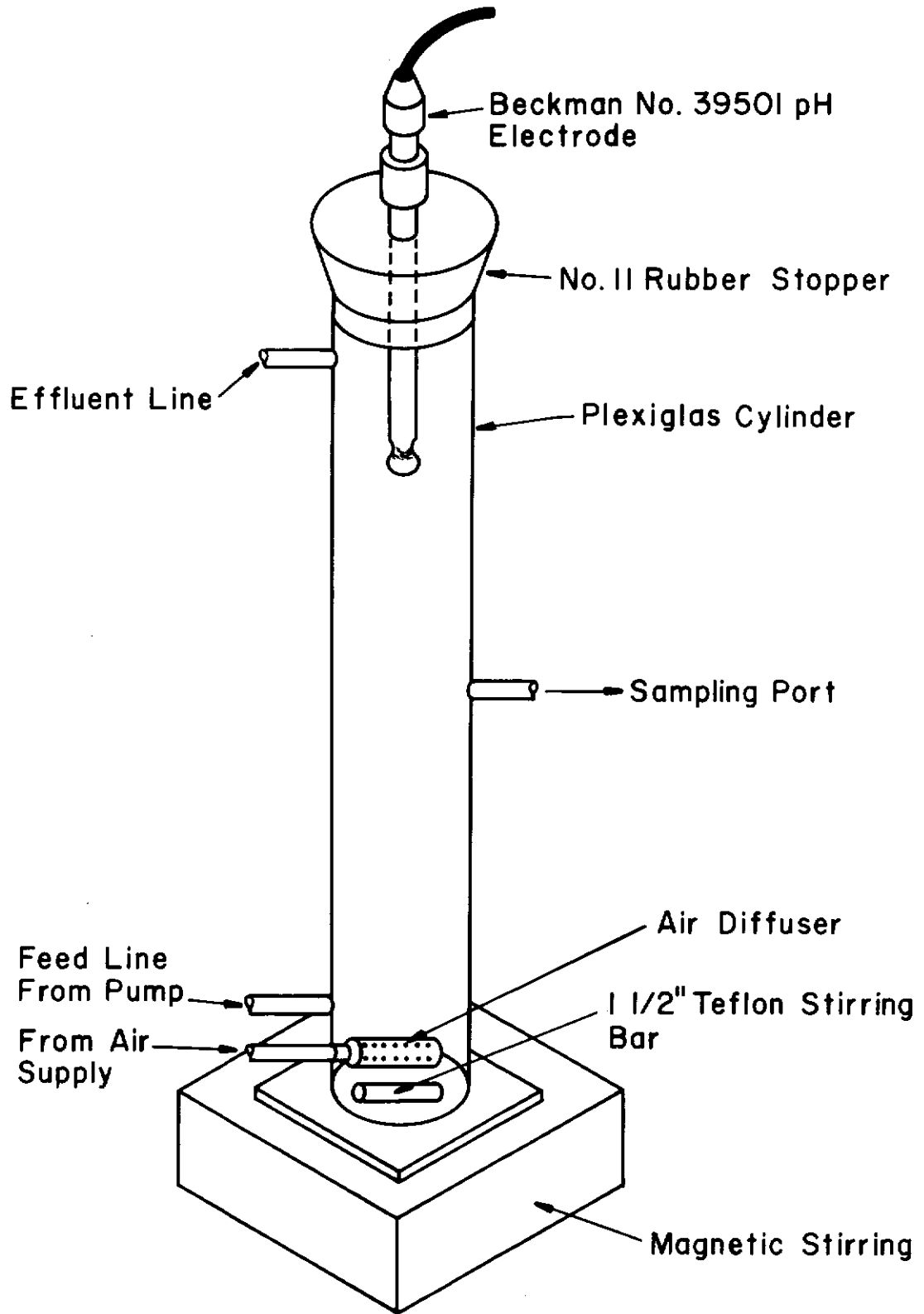
Figure 3.1   Detail of the Continuous Flow Culture Apparatus (Chemostat)

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For the continuous flow system, the air was provided from the laboratory compressed air system and the CO_2 was obtained from a bottle gas supply. The two gas supplies were joined with a glass "T" connection which led to a nine-liter pyrex bottle containing distilled water. The distilled water ensured nearly complete saturation of the gases, thereby minimizing evaporation losses in the chemostats. The flow from the bottle to each reactor was regulated by castaloy clamps. Dispersion of the air throughout the reactors was accomplished by using glass porous diffusers. The batch culture required the use of the same system for three of the chemostats, while another similar gas system was added to provide air only to the remaining three chemostats.

For Runs 1 and 3 of the continuous flow culture and for 3 of the chemostats in the batch culture, CO_2 was used to provide pH control and ample free CO_2 for algal photosynthesis. The CO_2 was adjusted to provide a pH between 7 and 8 in the chemostats. This was accomplished by adjusting the flow rate of the CO_2 so that it was approximately 1.2% of the total flow (air + CO_2). This pH range was selected as it was found to be optimum for algal growth by Foree and Wade (9) and Soltero and Lee (35) in similar studies.

Run 2 of the continuous culture phase and the remaining three chemostats in the batch culture study were controlled by acidification using 1.0 N HCl to maintain a pH in the range of 7 to 8. This was done so a CO_2 deficient growth condition could be observed at optimum pH. Foree and Wade (9) reported pH values exceeding 10.0 when algae were grown in a non-pH controlled environment.

3. <u>Light System.</u> Lighting for the chemostat system was supplied by four horizontally mounted 40 watt cool-white fluorescent bulbs. To provide diurnal lighting, which was selected to simulate natural conditions, an AMF electric 24 hour timer, that automatically controlled a 12 hour on, 12 hour off cycle, was used. The entire system was enclosed in a white plexiglass shell

with an open front. This provided an average uniform light intensity of 320 foot candles at the surface of the culture vessels.

4. <u>Temperature.</u> During growth the culture system was exposed to room temperature in the laboratory which was maintained at 22°C.

5. <u>Feed Material.</u> Secondary activated sludge effluent from the Town Branch Sewage Treatment Plant in Lexington, Kentucky, was collected periodically to use as a source of feed for the algal cultures. After collection, the effluent was allowed to settle for several hours at room temperature. The supernatant was then diluted to the appropriate dilution (10 mg/l, 5 mg/l, or 2 mg/l NH_3-N concentration). The diluting of the effluent provided a simulation of natural conditions, a constant strength feed, and a known control on a limiting nutrient (NH_3-N). Following the dilution process, the feed was stored in a refrigerator at 4° C for a maximum period of three days to minimize biological activity.

6. <u>Growth and Operational Procedure.</u> To initiate the first run, each chemostat was filled with stabilized sewage from lagoon #3 of the four lagoons in series (which received approximately 25% secondary effluent and 75% raw by-pass sewage) located near the Gainsway Sewage Treatment Plant in Lexington, Kentucky. The first two lagoons were anaerobic and contained no algae. The third and fourth lagoons contained substantial concentrations of mixed algae indigenous to Kentucky. This source of algae was selected because of its inherent adaptation to the type of feed that was used in this study. Each following run was begun by inoculating each chemostat (which was filled with feed solution) with a known amount of mixed algae from the chemostats of the previous run.

During growth the cultures were sampled periodically to determine when steady state conditions were reached. Once this condition was established, samples were withdrawn from the cultures for analysis.

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B.   Sampling Procedure

Before sampling, the sides of the chemostats were brushed down to dislodge all adhering algae. The sampling ports were then opened and closed to remove any algae in the port openings. Approximately 150 ml of sample was put in a plastic sample bottle labeled "total sample" and 150 ml more was removed to centrifuge. The latter samples were centrifuged at 17,000 RPM and 20°C for 15 minutes in an International Equipment Company Model B-20 refrigerated centrifuge. The supernatant was then passed through Whatman Glass Fibre Paper (grade GF/C) using a Millipore Filter Apparatus and stored in a plastic bottle marked "soluble fraction." The centrifuge tube containing the residue was filled with distilled water and centrifuged again. This time the supernatant was discarded and the residue was placed in a 100°C oven to dry overnight for subsequent analysis of chemical composition.

The samples marked "total sample" and "soluble fraction" were stored in a -30°C freezer and thawed at +4°C before analysis.

C.   Analysis

All of the following analyses were run at the termination of each continuous flow growth study. The analyses marked (*) were run periodically for the batch culture growth study:

* a.   Chemical Oxygen Demand
  b.   Carbon, Hydrogen, and Nitrogen Content
* c.   Total Carbon
* d.   Total Soluble Carbon
* e.   Soluble Organic Carbon
  f.   Total and Volatile Suspended Solids
* g.   Ammonia Nitrogen
* h.   Kjeldahl Nitrogen (Organic plus Ammonia Nitrogen)
  i.   Total Phosphorus
  j.   Soluble Phosphorus
  k.   pH
  l.   Alkalinity

CHAPTER IV

ANALYTICAL PROCEDURE

All tests were run in accordance with the procedures outlined in
Standard Methods for the Examination of Water and Wastewater, 13th ed. (37)
unless otherwise specified below.

A.   Alkalinity and pH.

Alkalinity and pH were run in accordance with Standard Methods,
pp. 52-56, using a Corning Model 10 pH meter with a Beckman No. 39501
combination pH electrode.

B.   Chemical Oxygen Demand (COD)

The dichromate reflux method as outlined in Standard Methods,
pp. 495-499, was used for all COD tests.   Twenty ml sample sizes and
corresponding volumes of reagents for 0.1 N standard ferrous ammonium
sulfate titrant were used.

C.   Carbon, Hydrogen, and Nitrogen Analysis

This test was utilized to determine the percent of carbon, hydrogen,
and nitrogen (CHN) in dried particulate algae samples.   After algae samples
were taken (as described in Chapter 3) from the chemostats, they were cen-
trifuged for 15 minutes at 17,000 RPM and 20°C on an International Equipment
Company Model B-20 Refrigerated Centrifuge.   The supernatant was wasted
and an equal volume of distilled water was added to the centrifuge tubes.   The
samples were then centrifuged again in the same manner.   The supernatant was
again discarded and the residue was placed in a Precision Scientific Company
Oven Model 27 to dry at 103°C overnight.   The dried samples were then
analyzed in a Hewlett Packard Model 185 CHN analyzer.   The output was

recorded as percent carbon, percent hydrogen, and percent nitrogen. The % oxygen was determined by subtracting the sum of the %C, %N, %H, and % Ash from 100%. The % Ash was determined as the difference between the total and volatile suspended solids concentrations divided by the total suspended solids concentration.

D.   Nitrogen

1. Ammonia Nitrogen. For this test a micro-kjeldahl steam distillation and nesslerization procedure was used to determine the ammonia nitrogen concentration of the samples. All reagents were made in accordance with Standard Methods, pp. 453-454.

2. Total Kjeldahl Nitrogen. The total nitrogen concentration (organic and NH_3-N) was measured by using the micro-kjeldahl digestion procedure described in Standard Methods, pp. 244-247, which converted the organic nitrogen to NH_3-N. Once the organic nitrogen was converted to NH_3-N the procedure for determination of ammonia nitrogen was followed.

E.   Phosphorus

1. Total Phosphorus. The sample along with 1.0 ml of 70 gm/l magnesium chloride reagent was pipetted into a vycor dish, dried at 100°C, and then burned for 10 minutes in a muffle furnace at 600°C. This process converted the organic phosphorus to pyrophosphate which was then hydrolyzed to orthophosphate by boiling in an acid solution. The total phosphorus concentrations were determined by the stannous chloride method for orthophosphate described in Standard Methods, pp. 530-532, utilizing the Beckman spectrophotometer.

2. Soluble Phosphorus. The soluble portion of the phosphorus in the sample was determined by conducting the total phosphorus test on the "soluble fraction" of the sample (Chapter III).

F.   Suspended Solids

1. Total Suspended Solids.   Total suspended solids concentration was determined by filtering the sample with Whatman Glass Fibre Filter Paper (grade GF/C) and a Millipore Filter Apparatus.   One or more distilled water blanks were carried through with each test.   This procedure is outlined in Standard Methods, pp. 537-538.

2. Volatile Suspended Solids.   After weighing, the dry filter pads from the total suspended solids test were placed in a muffle furnace at 580°C for 10 minutes.   The difference between the dry pad weight and the burned pad weight gave the volatile suspended solids.   Standard Methods, pp. 538-539, particularizes this procedure.

G.   Carbon

1. Total Carbon.   The total carbon was determined by injecting 20 μl of sample (or diluted sample) into a Beckman Infrared Total Carbon Analyzer Model 1R315.

2. Soluble Carbon.   Soluble carbon was found by injecting only the "soluble fraction" of the sample into the analyzer.

3. Soluble Organic Carbon.   For this determination the "soluble fraction" of the sample was acidified to pH 2.0, then purged with nitrogen for 5 minutes to drive off the inorganic carbon as CO_2.   The soluble inorganic carbon was determined by subtracting the soluble organic carbon from total soluble carbon.

CHAPTER V

RESULTS AND DISCUSSION

A.   Introductory Comments

During this study algal growth was studied in chemostats under four different sets of environmental growth conditions.   Three of the growth conditions (runs) were observed in continuous flow reactors, whereas the final growth condition was observed as a batch culture.   For convenience, the study has been divided into two phases: continuous flow cultures (Phase 1) and batch cultures (Phase 2).   Phase 2 has been subdivided into two sets: Set 1 was CO_2 enriched and Set 2 was CO_2 deficient.

The only variable within each continuous culture run was the hydraulic residence time (calculated as the volume of the reactor divided by the feed rate).   In the batch culture run, each of the three chemostats in each set was subjected to a different concentration of ammonia nitrogen (N_A).   In the first phase, the relative extent and rate of growth were observed as a function residence time along with the growth limiting nutrients, species of algae present, algal composition and algal succession.   The second phase provided a means of verifying the results from the first phase and also made it possible to ascertain the inherent differences between the batch culture and continuous culture growth conditions.

1.   Description of Runs.   The initial algal population for Run 1 was obtained from a local stabilization pond (Chapter 3) which was receiving effluent from two preceding ponds in series.   The initial lagoon influent was approximately 25% municipal secondary sewage treatment plant effluent and 75% raw sewage.   The stabilization pond provided a mixed algal culture indigenous to Kentucky and adapted to the type of feed solution that was used

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during the study. After the completion of each run, the subsequent run was initiated by inoculating each chemostat with a mixture of the algal populations from the chemostats of the previous run.

The conditions that prevailed for each run are shown in Tables 5.1 and 5.2.

TABLE 5.1

GROWTH CONDITIONS FOR CONTINUOUS FLOW CULTURES
(Runs 1, 2, and 3)

	Run 1	Run 2	Run 3
pH Control (pH $\simeq$ 7.0)	CO_2	HCl	CO_2
Feed Concentration (mg/l N_A)	10	10	2
Growth Period (days)	61	43	39
Residence Times (days)	1, 2, 4, 8, 16, 32	Same	Same

The pH was maintained at approximately 7.0 in each of the four runs, as this was found to be optimum by Foree and Wade (9) in their chemostat study. Soltero and Lee (35) also found 7.0 to be optimum when using an automatic pH controller in an algal study.

Diurnal lighting was used in each run as opposed to continuous lighting to simulate real conditions. Foree and Wade (9) found that continuous lighting at low residence times made little difference, but at high residence times, a two-fold increase in COD was noted.

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TABLE 5.2

GROWTH CONDITIONS FOR BATCH CULTURES
(Run 4)

	SET 1			SET 2		
	Chemo 1	Chemo 2	Chemo 3	Chemo 4	Chemo 5	Chemo 6
pH Control (pH $\cong$ 7)	HCl	HCl	HCl	CO_2	CO_2	CO_2
Feed Concentration (mg/l N_A)	2	5	10	2	5	10
Growth Period (days)	40	40	40	40	40	40

The temperature was held at room temperature (approximately 22°C) for both Phase 1 and Phase 2. The growth period in each case was a function of the time required to achieve steady state conditions. When the mass in each reactor (except in one or two cases when nitrogen fixation was occurring) remained constant, the cultures were sampled and prepared for analytical testing.

2. Characteristics of Feed Solution. In order to simulate a natural condition, diluted secondary sewage treatment plant effluent (Chapter III) was used as a feed solution. The magnitude of dilution was a function of the desired N_A concentration which was a controlled nutrient throughout the entire study. A characterization for a typical feed solution (10 mg/l N_A) is shown in Table 5.3. These values can only be considered approximate, as each batch collected varied in strength.

TABLE 5.3

DILUTED SEWAGE FEED CHARACTERISTICS

	Approximate Value (mg/l)	Range
Total Kjeldahl Nitrogen	13	
Soluble Kjeldahl Nitrogen	11	
Ammonia Nitrogen	10	8 - 10.9
Total COD	40	36 - 72
Total Phosphorus	3.0	2.7 - 4.0
Total Suspended Solids	< 5	
pH	7.5	
Alkalinity	130	
Total Carbon	50	
Soluble Organic Carbon	15	

3. Notation. The abbreviated symbols that were used throughout this study are listed in Table 5.4. The definition and means of determination are also provided for each symbol.

B. Continuous Culture Experiments

1. Algal Identification. Table 5.5 lists the algal genera that were present in the chemostats for the three runs of the continuous culture phase when steady state conditions prevailed. Runs 1 and 3 (CO_2 enriched) appeared to exhibit a successional change from green to blue-green algae with increasing residence time. The shift to blue-green algae first occurred in the 16 DRT chemostat for Run 1 (10 mg/l N_A) and in the 4 DRT chemostat for Run 3 (2 mg/l N_A). Presumably, Run 3 shifted at the lower residence time (4 days) because of its lower N_A concentration.

TABLE 5.4

ABBREVIATED SYMBOLS

Abbreviation		Means of Determination
θ	Hydraulic Residence Time	Volume of Reactor/Flow Rate
M_T	Total Chemical Oxygen Demand	Direct Measurement
M_S	Soluble Chemical Oxygen Demand	Direct Measurement
M	Particulate Chemical Oxygen Demand	Calculated as $(M_T - M_S)$
S	Volatile Suspended Solids	Direct Measurement
C_T	Total Carbon	Direct Measurement
C_{TS}	Total Soluble Carbon	Direct Measurement
C	Particulate Organic Carbon	Calculated as $(C_T - C_{TS})$
C_{SO}	Soluble Organic Carbon	Direct Measurement
C_{SI}	Soluble Inorganic Carbon	Calculated as $(C_{TS} - C_{SO})$
N_{TK}	Total Kjedahl Nitrogen	Direct Measurement
N_{SK}	Soluble Kjedahl Nitrogen	Direct Measurement
N_A	Ammonia Nitrogen	Direct Measurement
N	Particulate Organic Nitrogen	Calculated as $(N_{TK} - N_{SK})$
%N'	Nitrogen Content of Particulate Material Expressed as % of S	Calculated as 100 (N/S)
%N''	Nitrogen Content of Particulate Material Expressed as % of M	Calculated as 100 (N/M)
P_T	Total Phosphorus	Direct Measurement
P_S	Soluble Phosphorus	Direct Measurement
P	Particulate Phosphorus	Calculated as $(P_T - P_S)$
%P	Phosphorus Content of Particulate Material Expressed as % of S	Calculated as 100 (P/S)
%C, %N, %H, %0	Nitrogen, Carbon, and Hydrogen Content of Particulate Material Expressed as % of Total Dry Weight	Direct Measurement (%0 by Difference, See Chap. IV)

NOTE: All symbols except those preceded by % and θ represent concentrations in (mg/l).

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TABLE 5.5

ALGAL GENERA PRESENT IN CONTINUOUS CULTURES
AT STEADY STATE CONDITIONS

1 - Day Residence Time

RUN 1	RUN 2	RUN 3
Chlamydomonas - G - D	Anabaena - BG - D	Stigeoclonium - G - D
Cladophora - G	Chlorella - G	Ankistrodesmus - G
Euglena	Ulothrix - G	Oscillatoria - BG
		Chlorella - G

2 - Day Residence Time

RUN 1	RUN 2	RUN 3
Cladophora - G - D	Anabaena - BG - D	Chlorella - G - D
Chlorella - G	Chlorella - G	Scenedesmus - G - D
Chlamydomonas - G	Chlamydomonas - G	Cladophora - G
Oscillatoria - BG		Chlamydomonas - G
		Anabaena - BG
		Oscillatoria - BG

4 - Day Residence Time

RUN 1	RUN 2	RUN 3
Chlorella - G - D	Chlorella - G - D	Oscillatoria - BG - D
Cladophora - G	Oscillatoria - BG	Anacystis - BG
Oscillatoria - BG		
Chlamydomonas - G		

8 - Day Residence Time

RUN 1	RUN 2	RUN 3
Chlorella - G - D	Nostoc - BG - D	Oscillatoria - BG - D
Chlorococcum - G	Oscillatoria - BG	Gloecapsa - BG
Oscillatoria - BG	Polycystis/Anacystis - BG	Chlamydomonas - G
Anabaena - BG		Netrium - Diatom
Pandorina Morum - G		

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TABLE 5.5 (Continued)

16 - Day Residence Time

RUN 1	RUN 2	RUN 3
Oscillatoria - BG - EN	Anacystis - BG - D	Anabaena - BG - Almost pure culture
Anabaena - BG - EN	Anabaena - BG	Oscillatoria - BG
Chlorella - G - EN	Chlorella - G	Chlorella - G
		Cladophora - G

32 - Day Residence Time

RUN 1	RUN 2	RUN 3
Oscillatoria - BG - EN	Chlorella - G - D	Anabaena - BG - Almost pure culture
Anabaena - BG - EN	Oscillatoria - BG	Oscillatoria - BG
Nostoc - BG - EN	Anacystis - BG	Chlorella - G
Chlorella - G - EN		
Chlamydomonas - G		

KEY FOR ALGAE IDENTIFICATION

G - Green
BG - Blue-Green
D - Dominant
EN - Equal Numbers Present

The reactors in Run 2 (CO_2 deficient) contained predominantly blue-green algae as it was dominant in four out of the six chemostats and present in substantial populations in the other two chemostats. This same phenomenon of blue-green dominance under CO_2 deficient growth conditions was observed by King (19). The characteristics of the blue-green algal succession and dominance will be discussed in a later section.

    2.   <u>Algal Composition.</u>   The algal compositions (%N, %C, %H, %0) for the continuous culture study are shown in Table 5.6. The %N for Runs 1 and 3 generally decreased with increasing residence time for the lower residence times; however, there was an increase in %N for the 16 and 32 DRT for Run 1, and the 16 DRT for Run 2. This increase may be attributed to nitrogen fixation which is discussed in a subsequent section. The %N for Run 2 remained fairly constant except for a decrease in the 32 DRT reactor. This decrease was possibly due to the presence of a larger percentage of older cells which would be found with longer residence times and probably had less capability for luxuriant nitrogen uptake than the younger cells of lower residence times. Another noteworthy point is that the %N for Run 2 was greater for each residence time as compared to Runs 1 and 3. This resulted from the greater amount of nitrogen available from solution relative to the lesser mass produced under the CO_2 deficient conditions.

    The %C and %H remained reasonably constant with most of the values ranging between 45-50% and 7-9%, respectively. The %0 was found to range between 25% and 45%. The lower %0 values were found in Run 2 which contained a higher percentage nitrogen than the other two runs. The %0 can only be considered approximate as it was determined by an indirect method as described in Chapter IV.

    3.   <u>Mass Variation With Time of Growth.</u>   Figure 5.1 depicts the mass variation with time of growth for Runs 2 and 3. The mass in this case was assumed to be proportional to the total carbon. This was a reasonable

TABLE 5.6

ALGAL COMPOSITION FOR THREE DIFFERENT GROWTH
CONDITIONS AS A FUNCTION OF RESIDENCE TIME

% N

Run		1 day	2 days	4 days	8 days	16 days	32 days
1	% N	7.3	3.9	3.1	1.8	1.8	2.7
2	% N	7.5	7.2	6.9	6.9	6.2	2.9
3	% N	3.5	3.1	3.1	2.9	3.9	2.8

% C

Run		1 day	2 days	4 days	8 days	16 days	32 days
1	% C	47.1	45.8	48.6	51.5	52.0	50.7
2	% C	46.9	48.2	47.6	44.2	46.9	56.3
3	% C	46.1	47.5	45.7	48.3	42.9	36.8

% H

Run		1 day	2 days	4 days	8 days	16 days	32 days
1	% H	8.5	8.5	8.7	9.6	9.7	9.4
2	% H	8.8	8.7	9.1	8.8	9.2	9.5
3	% H	7.5	7.8	7.6	7.9	7.2	6.9

% 0

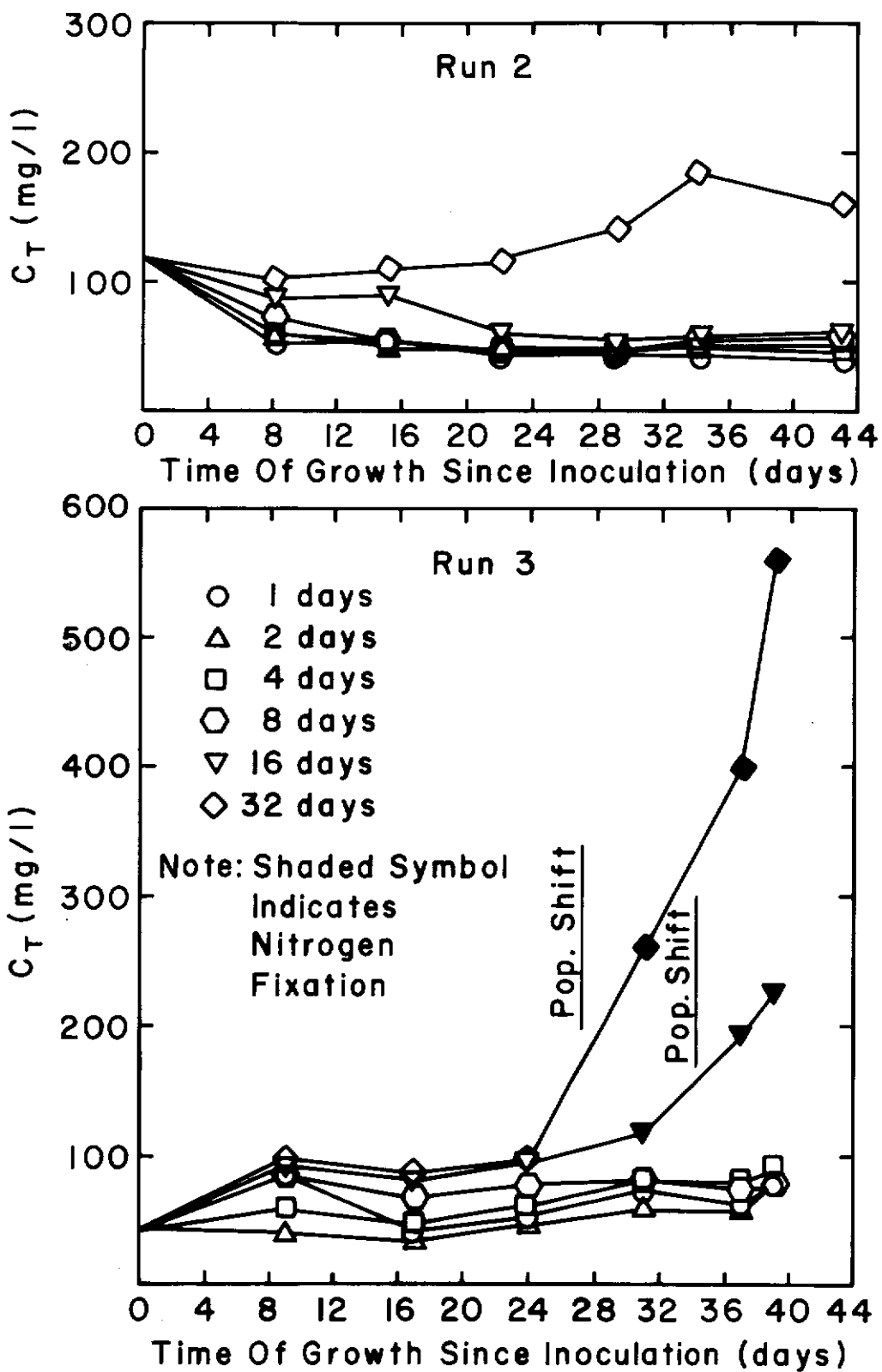| Run		1 day	2 days	4 days	8 days	16 days	32 days
1	% 0	31.1	31.5	34.3	35.9	34.0	30.8
2	% 0	25.8	25.9	32.9	29.2	25.8	25.3
3	% 0	32.4	41.6	36.4	37.8	42.1	45.8

Figure 5.1   Variation of Total Carbon Concentrations with Time of Growth
and Residence Time for Runs 2 and 3

assumption since the total soluble carbon was found only to contribute a small percentage to the total carbon. The mass variation with time was not measured for Run 1. Steady state conditions prevailed, however, as more than adequate growth time (61 days) was permitted.

For Run 2, which was CO_2 deficient and had a feed concentration of 10 mg/l N_A, the initial inoculation of algae was too high for the nutrients available. This is most easily seen from the plot which illustrates that during the first 22 days of growth the algal concentration was decreasing (or growth rate was less than washout rate). After 22 days, the algal cultures reached steady state conditions (indicated by growth curves leveling off), except for the culture with the 32 days residence time (DRT). This culture began a gradual increase after eight days of growth which continued to the 28th day, where there was a sudden increase in mass followed by a decrease. This oscillatory pattern is characteristic of a continuous flow system which is approaching steady state conditions. It may also be noted that the 32 DRT was the only residence time which was long enough to allow the growth rate to exceed the washout rate for these particular growth conditions.

Run 3 was CO_2 enriched and the feed solution was diluted to 2 mg/l N_A. The growth curves (Figure 5.1) for Run 3 increase initially (except for the 2 DRT culture, which experienced washout). Generally, the algal cultures appeared to be reaching steady state conditions after 24 days of growth. An interesting observation in Run 3 was that the reactors for the low residence times were supporting concentrations of algae of the same magnitude as Run 2, with one-fifth the N_A concentration. This was a good indication that nitrogen was not limiting in Run 2. Another point of interest was the population shift that was visually and analytically observed for the 16 and 32 DRT algal cultures. The analytical results reveal a drastic increase in total carbon between 24 and 31 days of growth. After 31 days the growth appears to be increasing exponentially. The visual observation was easily seen on the 27th and 34th

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day of growth for the 16 and 32 DRT, respectively. The change was characterized by a shift from a yellow-green to a very dark green color, which was accompanied by excessive foaming and foul odors. A population shift did not occur in Run 2 which was CO_2 deficient.

Foree and Wade (9) found the same growth trends in a similar continuous flow growth study for the CO_2 enriched and deficient cases. Using total COD as a growth indicator, they observed the initial decrease in algal concentration for the CO_2 deficient culture with an eventual stable concentration prevailing. As in this study, the 32 DRT culture was the only one with an initial increase in algal concentration. For the CO_2 enriched run, they found a population shift to occur after 15 days' growth in their 16 DRT culture, which was their maximum residence time. Porcella (33) also observed this population shift phenomenon, but did not report the residence times or when the shifts occurred. Both Foree and Wade and Porcella described growth curves for similar conditions (CO_2 enriched conditions) that reached a maximum value, then decreased slightly and leveled off to steady state conditions. They found the maximum to occur between 15 and 20 days of growth. The growth curves for Run 3 display their maximum algal concentration after eight days of growth (except 2 DRT which initially experienced washout). This tendency to reach a maximum concentration faster can be attributed to the low concentration of N_A (2 mg/l) that was used in this study.

4. <u>Mass Variation with Hydraulic Residence Time.</u> Figure 5.2 presents the variation of mass with hydraulic residence time for the three continuous flow culture runs. The variation of mass with hydraulic residence time was measured by three different parameters: particulate chemical oxygen demand (M), volatile suspended solids (S), and particulate organic carbon (C). In both cases, the runs with CO_2 enrichment (Runs 1 and 3) showed greater mass production than the run which was CO_2 deficient (Run 2).

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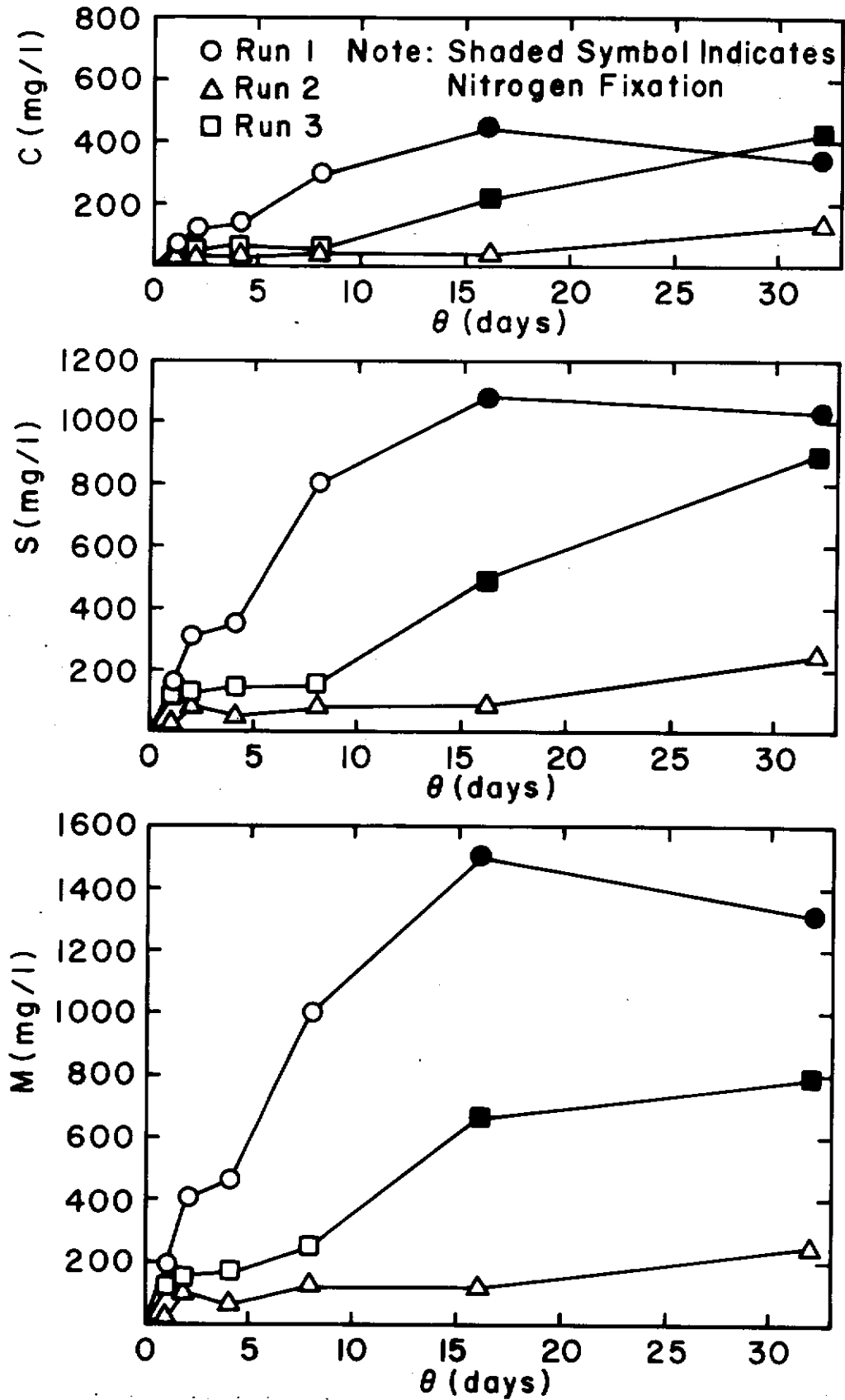
Figure 5.2   Mass Variation as a Function of Residence Time for Three
Sets of Growth Conditions

For the CO_2 enriched cultures the mass increased significantly as a function of residence time up to 16 days. For Run 1 (10 mg/1 N_A) there was actually a slight decrease from the 16 to 32 DRT while there was a significant increase for Run 3 (2 mg/1 N_A) from the 16 to 32 DRT. However, as discussed below, the population shift to blue-green algal predominance played an important role in determining the mass in these longer residence time cultures. Therefore, no concrete conclusions can be drawn pertaining to the relative magnitude of these masses as they were dependent upon the extent of the population shift and the associated nitrogen fixation and mass production.

The relatively large mass established in the 16 and 32 DRT, CO_2 enriched cultures (Runs 1 and 3) was attributed, in part, to the previously mentioned population shifts to predominance of certain blue-green, nitrogen-fixing algae. The nitrogen fixation was observed by an increase in the total Kjeldahl nitrogen (N_{TK}) which has been plotted in Figure 5.3. This plot shows that the lower residence times remained at approximately constant concentrations of 11 mg/1 and 5 mg/1 N_{TK} for Runs 1 and 3, respectively (which was near the concentrations in the feed solutions). For the residence times of 16 and 32 days a sharp increase in N_{TK} can be seen which verifies nitrogen fixation. Sixteen days appears to be close to the minimum residence time for which a population shift will occur. This seems to be so because the shift for the 16 day residence time was not completely stable, and in the early stages would oscillate back and forth from the blue-green dominance to its initial condition. The N_{TK} in study 2 showed a tendency to decrease with residence time, which indicated possible bacterial nitrification. This suggested that the excess N_A (included in N_{TK}) in solution was being converted to NO_2 - N and NO_3 - N. The decrease with residence time is what would be expected as the NH_3 conversion to NO_2 and NO_3 is a biological action and would increase with time.
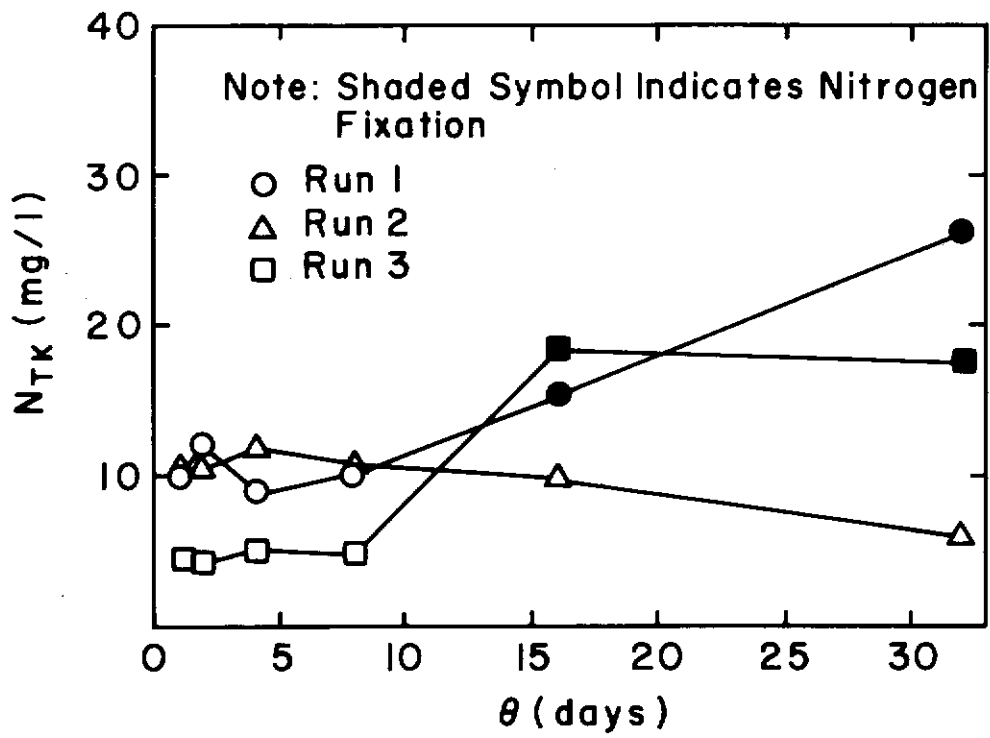
Figure 5.3   Total Kjeldahl Nitrogen as a Function of Residence Time
for Three Sets of Growth Conditions

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5. <u>Nutrient Availability.</u>   Figure 5.4 illustrates the growth limiting nutrients for Runs 1, 2, and 3 of the continuous flow study.   The possible limiting nutrients (Soluble Inorganic Carbon (C_{SI}), Soluble Phosphorus (P_S), and Ammonia Nitrogen (N_A)) are shown in their relative concentrations as a function of residence time.   The C_{SI} was further broken down into its constituents by using Figure 5.5 which depicts how the relative amounts of H_2CO_3 and free CO_2, HCO_3^-, and $CO_3^=$ vary with pH.   This figure is simply a graphical portrayal of the equilibrium equations that were presented in Chapter II.   The equal mole fraction points, which represent the first and second dissociation of carbonic acid, are at pH 6.37 and pH 10.25; however, these values may vary slightly from one water to the next (21).   Another point that should be clarified is that the proportional allocation of C_{SI} was an instantaneous measurement in Runs 1 and 3, because the pH for the CO_2 enriched runs possessed a diurnal variation between pH 6.8 and 7.5.   The CO_2 was continuously supplied and therefore was not likely to be limiting.

For Run 1, Figure 5.4 demonstrates that the C_{SI} and also the H_2CO_3 and free CO_2 tend to increase with residence time.   This phenomenon was also observed by Foree and Wade (9) under similar conditions, and was attributed to the growth rate which decreased with increasing residence time while the CO_2 supply rate was constant.   The P_S concentration has the opposite tendency, generally decreasing with increasing residence time. This is as would be expected since the P_S initially available for growth was constant per unit volume of feed, and the algal mass produced per unit volume increased significantly with residence time resulting in a greater depletion of P_S from solution.   Variations of cellular nitrogen and phosphorus concentrations are discussed later.   The minimum concentration of P_S found was 0.23 mg/l, which is well above the previously reported growth limiting minimum of approximately 0.01 mg/l.
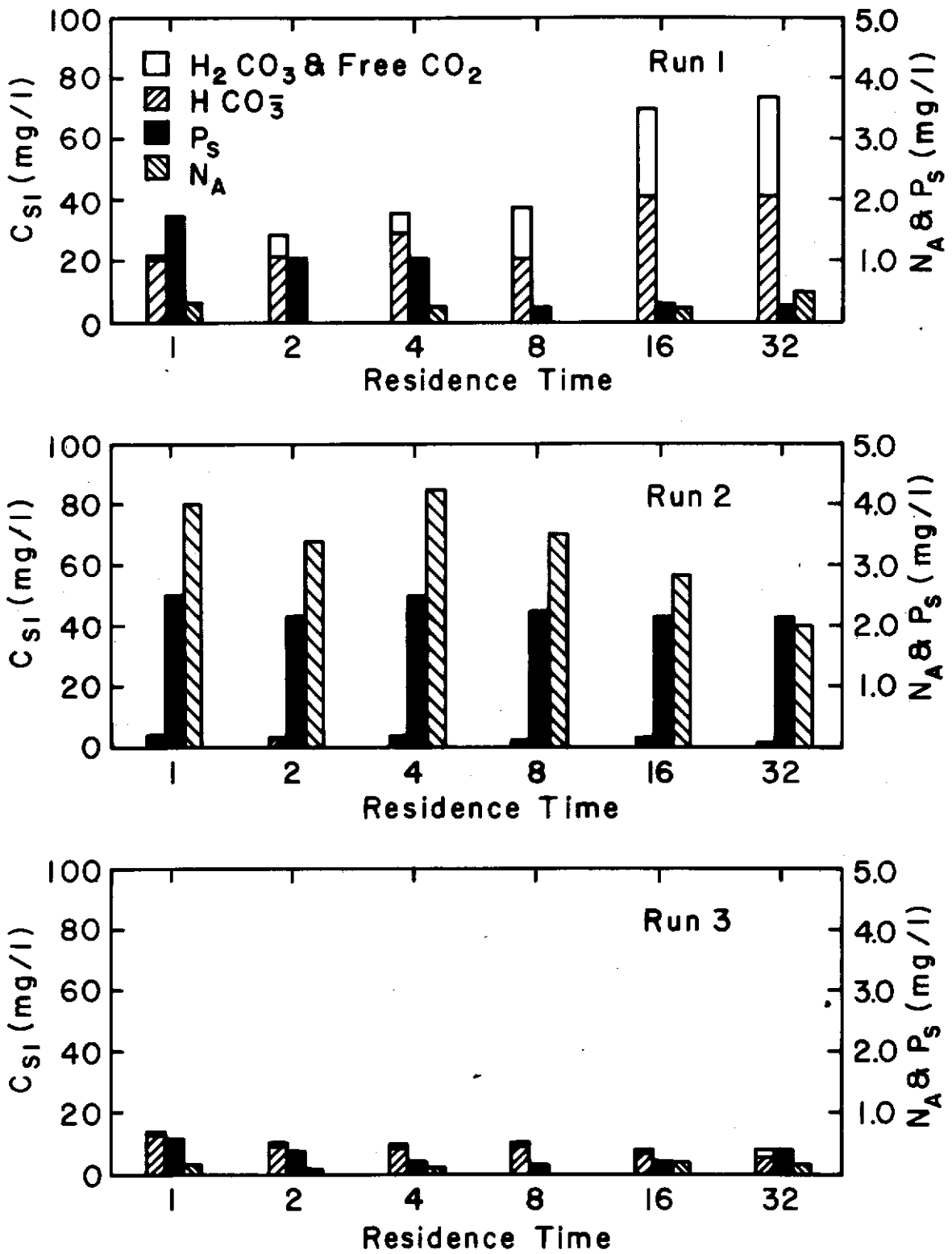
Figure 5.4   Soluble Nutrient Concentrations as a Function of Residence
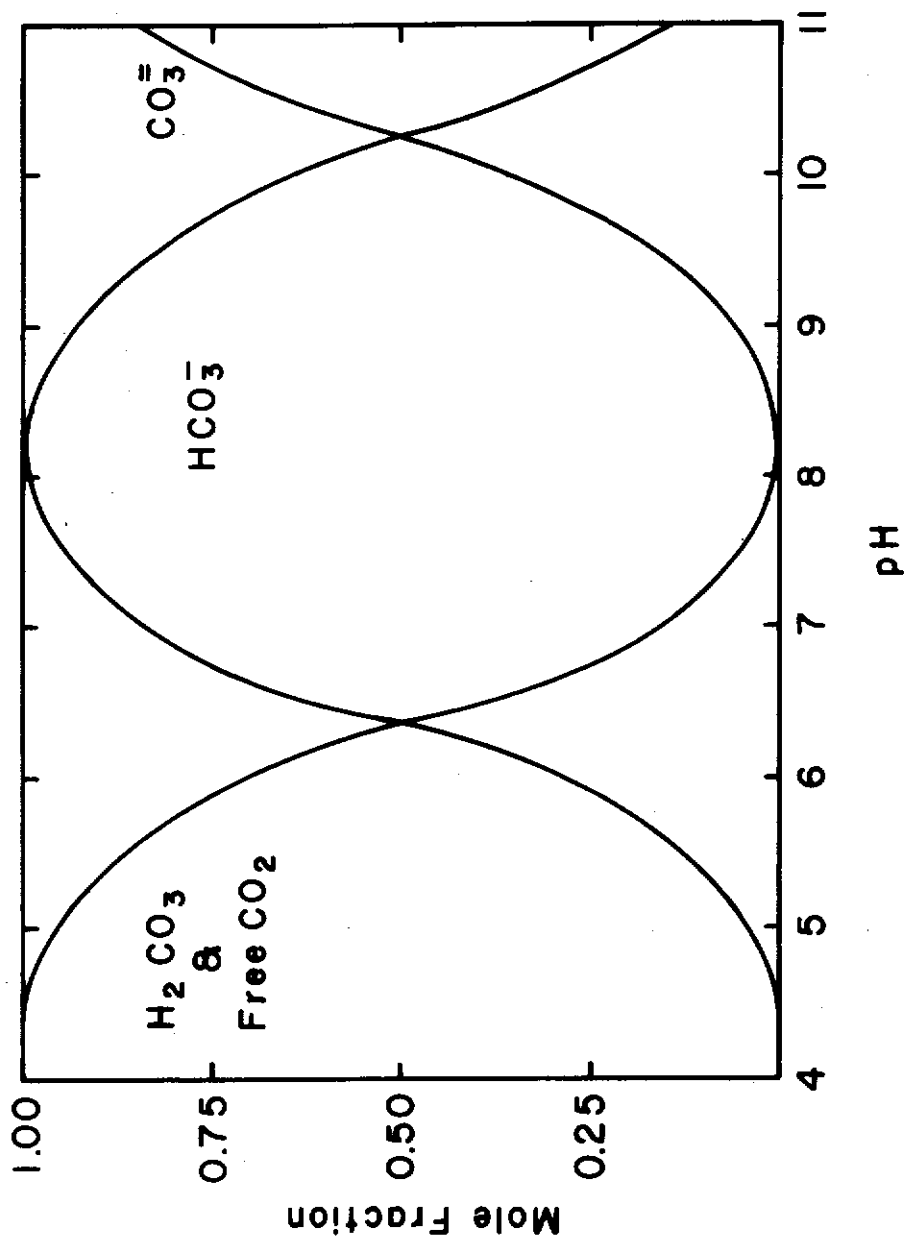Time at Steady State for Three Sets of Growth Conditions

Figure 5. 5   Relationship Between Carbon Dioxide and Bicarbonate and
Monocarbonate Alkalinity at Various pH Levels

With both C_{SI} and P_S in ample concentrations, N_A appears to be the growth limiting nutrient. This conclusion is an obvious one when observing Figure 5.4. The maximum N_A concentration was 0.3 mg/l excluding the 32 DRT. The 16 and 32 DRT cultures incurred the population shift to certain nitrogen fixing blue-green algae. The N_A limitation and population shift were also found by Foree and Wade (9) using the same environmental conditions.

For Run 2 (Figure 5.4), it can easily be determined that carbon was limiting. This was the anticipated outcome, since the cultures were not CO_2 enriched. The pH was maintained at 7 with HCl which allowed some carbonate alkalinity to be in the form of CO_2. This condition along with continuous aeration (but not CO_2 enriched) permitted the cultures to receive more CO_2 than would be the case in most natural conditions. A small amount of CO_2 could also have been generated by bacterial activity. These results indicate that CO_2 could many times be limiting in natural conditions.

The P_S concentration was relatively constant as the growth rates and mass concentrations were all very similar in Run 2 (Figure 5.1). The N_A concentration had somewhat of a tendency to decrease with residence time, which was also observed with the N_{TK} in Figure 5.3. This further verified that nitrification was taking place.

An interesting circumspection by Foree and Wade (9) was made in their chemostat study when they conducted two runs (that were similar to Run 2): one with HCl pH control and the other without. The first run was made without pH control, which resulted in an average pH of 10.8. King (19) has reported that pH's above 10.0 will cause phosphorus in solution to precipitate as $CaPO_4$ and growth may become phosphorus limited. The lowest P_S concentration that Foree and Wade noted was 0.09 mg/l, but it was still difficult to determine whether carbon or phosphorus was limiting. To determine which nutrient was limiting they made another run with the same conditions prevailing, only this time using HCl for pH control. In this run the

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minimum P_S concentration was 0.49 mg/l and the algal concentrations (measured as COD) were not any greater than in previous runs. This indicated that carbon was limiting in both cases.

Run number 3 was conducted under the same environmental conditions as Run 1, with only the feed solution being different. The feed solution was diluted to 2 mg/l N_A instead of 10 mg/l. This was done in order to see if the nutrient limitation theory hypothesized by Foree and Wade (9) would hold for low concentrations of N_A. From Figure 5.4 it is easily seen that the N_A was limiting. Since the feed solution was diluted to a lower concentration, it follows that the P_S would be present in lower concentrations. The lowest concentration observed for P_S was 0.19 mg/l. The carbon was lower in Run 3 as compared to Run 1, due to the fact that the samples were taken in late morning, which allowed the CO_2 concentration to be lower than it would have been if they had been taken early in the morning as in Run 1. The CO_2 was still in adequate concentrations, and could not be considered limiting. The C_{SI} concentration did not have a tendency to increase with residence time as in Run 1. This can be justified by looking at Figure 5.1 again, which shows that the population shift was occurring for the 16 and 32 DRT cultures. Growth was apparently in the exponential phase at the time sampled and large quantities of CO_2 were being demanded. This adds more support to the theory that blue-green algae blooms require large quantities of CO_2, which agrees with Kuentzel's (20) and Kerr's (18) thinking.

The results from the analysis of the growth limiting nutrients in this study, along with those from Foree and Wade's study, seem to indicate that carbon is in fact limiting in many natural conditions.

King (19) stated, "Blue-green algae seem to dominate after the equilibrium carbon dioxide content falls below the level required by the more desirable green algae." In other words, he projected that in a carbon diminishing ecosystem, there is an algal succession with an initial population

of green algal types and finally climaxed with the blue-green algal types that are more tolerable to carbon deficient environments. The environmental conditions of Run 2 would classify it as a carbon diminishing ecosystem. Table 5.5 shows that four out of the six cultures were dominated with blue-green algae. In culture 6, two out of the three populations were blue-green and in culture 3, there was an established population of blue-green algae. Figure 5.2 illustrated that the relative concentrations of blue-green algae in Run 2 were relatively small as the maximum was 250 mg/l (M). The maximum concentrations for Runs 1 and 3, where the population shifts occurred, were 1495 mg/l and 787 mg/l respectively. These population shifts, as mentioned previously, were accompanied by foul odors, excessive foaming, and nitrogen fixation. The blue-green algae of the CO_2 deficient run did not demonstrate these characteristics. Thus, the findings of this study concur with King in that blue-green algae will dominate in carbon limiting ecosystems; however, they disagree in that no nuisance blue-green algae blooms occurred in a CO_2 deficient environment. In fact, it was found in this study and Foree and Wade's (9) study that the blue-green blooms only occurred in CO_2 enriched cultures. Furthermore, nitrogen was limiting in this study and in Foree and Wade's study when the population shift was observed, which possibly indicates that under CO_2 enriched conditions there is a succession to nitrogen-fixing blue-green algae when nitrogen in solution becomes limiting.

Kuentzel's (20) observations seem to coincide with the findings of this study. He cites an interesting example using information derived by Mackenthum (23). Mackenthum described an algal bloom in Lake Sebasticook, Maine, which involved some 56 mg/l dry weight, most of which grew in a single August day. Using the biological mass relationship of $C_5H_7NO_2$ (5), it can be calculated that some 110 mg/l of CO_2 must have been made available to the algae for this magnitude of growth. Hutchinson (15) stated, "Both from theory and as far as can be ascertained empirically the quantity of CO_2

present will ordinarily lie between 0.4 and 1.0 mg/l." He also states that the movement of CO_2 in and out of a water interface is a slow process. If this is true, it is fairly obvious that another source of CO_2 must be available to support algal blooms of this magnitude. Kuentzel's answer to this is bacterial action on organic matter and thus provision of CO_2. He states that just 30 mg/l of organic carbon and bacterial action would supply enough CO_2 for a bloom of this magnitude with the fast growing algae providing O_2 for the bacterial degradation. Kerr (17) substantiates this theory stating, "Bacterial oxidation of organic carbon is a principal source of CO_2 in aquatic ecosystems."

It is probably safe to say that most relatively algae free lakes contain an ample concentration of carbon (whether in the form of a carbonate alkalinity or as free CO_2) to initiate algal growth. In cases such as this, phosphorus is probably the limiting nutrient as it does not normally occur naturally in concentrations capable of supporting algae. Once the lake manages to establish a concentration of phosphorus capable of supporting a substantial algal growth, it will (in most cases) no longer be limiting. The reasons for this are that algae require a very small concentration of phosphorus to support large growth and that phosphorus can be regenerated or recycled from bottom sediments. Thus from the indications of this study, it appears that once an algal population has established itself in a lake, in most cases carbon will be the limiting growth nutrient. The results also show that blue-green algal blooms require a luxurious supply of CO_2 which is possibly supplied by bacterial action on organic carbon in natural surroundings.

6. Variation of Cellular Nitrogen and Phosphorus Content. A plot of percent nitrogen versus residence time is shown in Figure 5.6 and illustrates how the percentage of cellular nitrogen varies as a function of residence time. The percent N for Runs 1 and 3 had the tendency to decrease with residence time (with the exception of the 16 DRT for Run 3 and 32 DRT for Run 1). The primary reason for this is that when a nutrient (N or P)
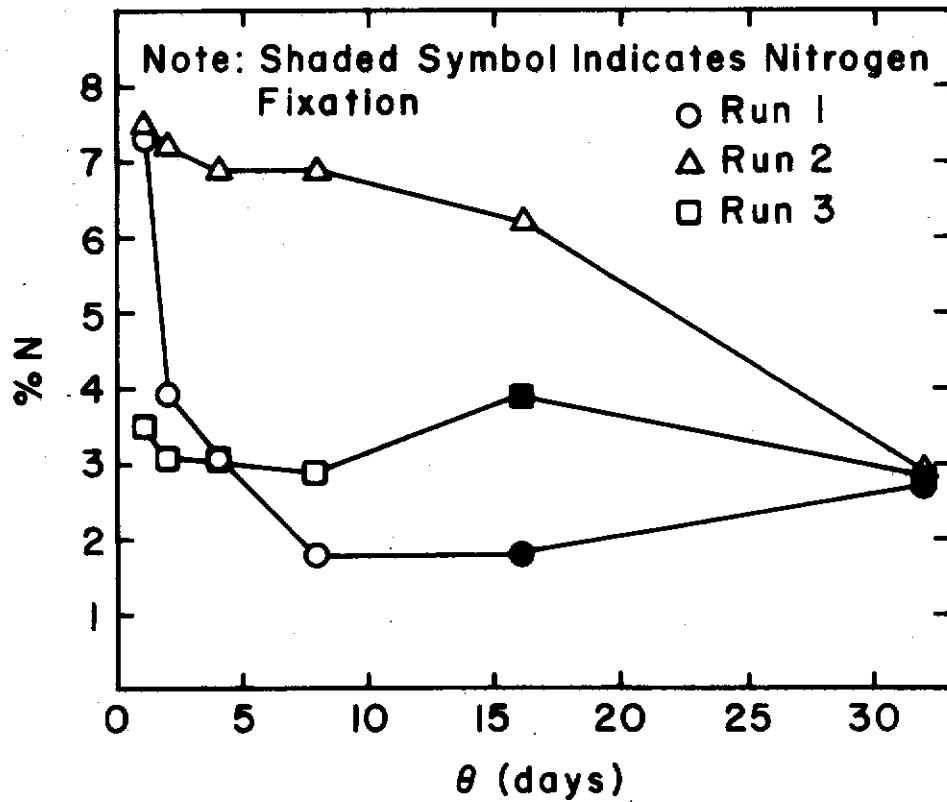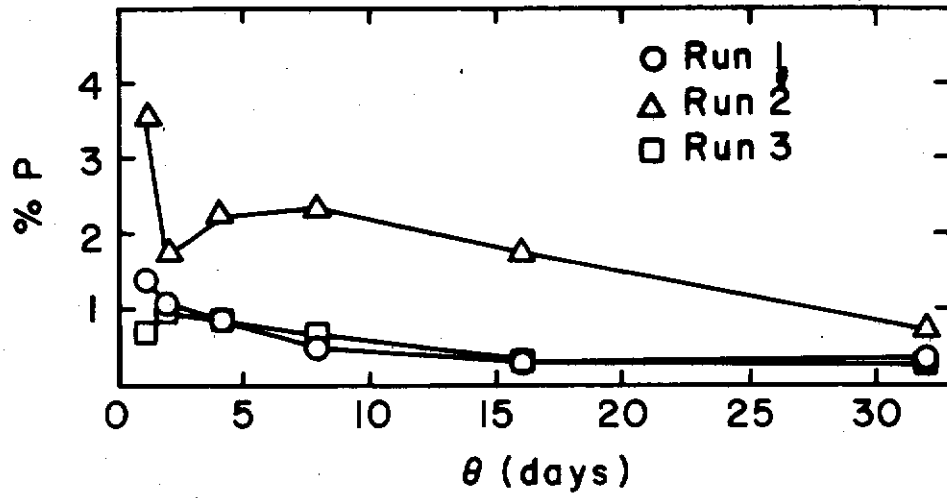
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Figure 5.6   Percentage of Cellular Nitrogen and Phosphorus in Algal
Cells as a Function of Residence Time for Three Growth
Conditions

becomes limiting, the additional mass that is synthesized with increasing residence time is produced as lipids and carbohydrates that contain little or no nitrogen or phosphorus. Foree and Wade (9) explained that when this takes place with algal nutrients depleted, the result is smaller percentages of nutrients in the cell masses.

The reason that Run 1's decrease seems much more drastic than Run 3's decrease is the difference in the initial concentrations of N_A. The tendency of the 16 and 32 DRT to increase may be justified by the nitrogen fixation from the blue-green algae.

The percentage cellular nitrogen values were significantly greater for the CO_2 limited cultures (Run 2) than for the nitrogen limited cultures (Runs 1 and 3). This was due to the lower mass production in the CO_2 limited cultures and the relatively greater availability of nitrogen from solution. The cellular nitrogen percentage generally decreased with increased residence time for Run 2 since the total cellular nitrogen remained nearly constant while the mass increased. At 32 DRT the percent N was about the same for all three runs. This was possible because in the nitrogen limited cultures (Runs 1 and 3) the establishment of the larger masses at the 32 DRT was accompanied by atmospheric nitrogen fixation and the maintenance of a percent N level of about 3%.

The same phenomenon was observed for the phosphorus, as was found for the nitrogen (decreasing percent P with increasing hydraulic residence time, Figure 5. 6), The phosphorus was below 2% for residence times of 16 days and longer in Run 2. The phosphorus content for 16 and 32 DRT in the CO_2 enriched runs was below 0.4%. The nitrogen percentages were below 3% for the 32 DRT for all cases. These percentages fall close to the results that Foree and Wade (9) found. They are also in agreement with values found by Foree and Tapp (8) for a Kentucky mixed sample of algae under batch culture conditions.

PAAP (6) proposed a theory that stated the mass yield of algae is directly proportional to the quantity of growth limiting nutrient depleted from solution and the growth rate is a function of the limiting substrate nutrient concentration. Wade and Foree (9) found this not to be in agreement with the results of their study of continuous flow, CO_2 enriched cultures where the total COD and N_A were measured periodically. Their results indicated that most of the N_A was depleted after 5 days, whereas the mass continued to increase until 14 days for the 16 DRT. In agreement with their findings were Gerloof and Skoog (10) and Jewell and McCarty (16), who stated that algal growth is limited by cellular nutrient content, not the nutrient content found in solution. Foree and Tapp (8) also concluded that algae can store nutrients when they are available in excess quantities in solution and then utilize them when the nutrients are depleted from solution.

7. <u>Continuous Culture Kinetics</u>. The kinetic theory explained in Chapter II hypothesized essentially what was developed in the previous sections: that algal growth rate is proportional to the concentration of the growth limiting nutrient contained in the cells. (This phenomenon is verified in the section on batch cultures). An equation hypothesized by Foree and Wade (9) for completely mixed, continuous flow systems may be written:

$$1/\theta = (dX/dt)/X = K(N/X) - b'$$

b' = Effective Respiration Rate (time^{-1})

θ = Hydraulic Residence Time (time)

N = Cellular Nutrient Concentration (nutrient mass/volume)

X = Algal Mass

t = Time

K = Proportionality Constant (algal mass/nutrient mass/day)

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This equation states that the specific growth rate $(dX/dt)/X$, which is equal to the reciprocal of the hydraulic residence time $(1/\theta)$, is proportional to the cellular nutrient concentration minus an effective respiration rate (b').

In order to check the linearity of the results and determine if the equation was applicable, a plot of $1/\theta$ versus N/X (where X is particulate COD) was made in Figure 5.7. Figure 5.7A illustrates the findings for $1/\theta$ versus N/M for two runs (both CO_2 enriched) made by Foree and Wade along with Run 1 of this study. The values for the slopes (K) and y intercepts (b') are listed in Table 5.7. The N/M values were found to be somewhat smaller for corresponding values of $1/\theta$ in this study as compared to Foree and Wade's study. Consequently, the b' and K values were approximately twice as large as Foree and Wade's values. This might be attributed to the fact that the total growth period for their runs was approximately 30 days as compared to 61 days for Run 1 in this study; however, this should be minimal since all systems were in steady state conditions.

TABLE 5.7

KINETIC GROWTH PARAMETERS AS DETERMINED FROM FIGURE 5.7

Run	K $\dfrac{(\text{mg X})}{\text{mg N day}}$	b' (day^{-1})
Run 1	23.6	0.14
Run 1 (Foree and Wade)	10.9	0.05
Run 2 (Foree and Wade)	11.4	0.07

Figure 5.7B portrays the plots for all three runs of Phase 1. The Run 1 plot is a repetition of the plot observed in Figure 5.7A. Run 3 is shown as a vertical line which indicates that the percent N was constant with respect
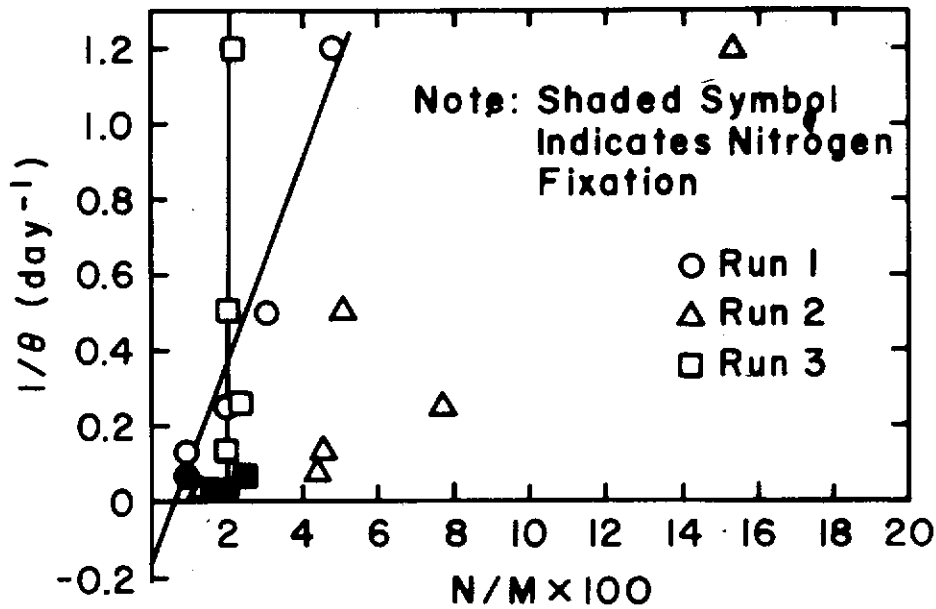
Figure 5.7B   Cellular Nitrogen Concentration on Particulate COD Mass
Basis as a Function of the Reciprocal Residence Time
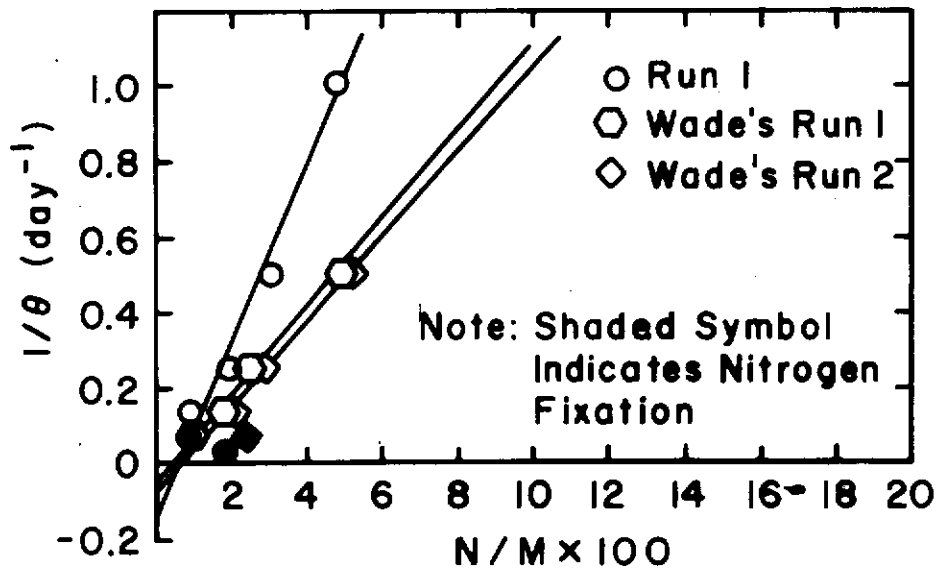for Runs 1, 2, and 3

Figure 5.7A   Cellular Nitrogen Concentration on Particulate COD Mass
Basis as a Function of the Reciprocal Residence Time
for Run 1 Compared with Results of Previous Study

to hydraulic residence time. The N_A concentration for this nitrogen limiting run was 2 mg/l. This appears to be in the vicinity of a minimum boundary for the theory developed. That is, the concentration of N_A in the feed solution cannot go below a certain value and substantiate growth under luxuriant nutrient uptake growth conditions. Another justification that the minimum lies in this range is that for Run 1, N/M decreased linearly with decreasing $1/\theta$ until a minimum value of 0.94 was reached after which N/M began to increase with decreasing $1/\theta$.

Run 2 exemplified somewhat of a random scatter, which made it impossible to apply the theory. This scatter was expected, though, as the theory may only be applied to nutrients such as phosphorus and nitrogen which incur a luxuriant uptake. Carbon growth limitation is regulated by the inorganic carbon in the feed solution because lipid and carbohydrate synthesis require a source of extracellular carbon.

The theory presented in Chapter II appears to be applicable for nutrients that experience a luxuriant uptake, i.e., phosphorus and nitrogen. The major point of interest, however, is the concentration range in which the theory is valid. Run 3 (with 2 mg/l N_A limiting in the feed solution) was found to be in the range of the minimum for which the theory could be applied. There should be no practical maximum concentration for applicability of the theory since luxuriant uptake would continue with increasing concentration. The 10 mg/l N_A in the feed solution for Run 1 in this study and for the two runs in Foree and Wade's study was found to fall within the range of applicability of the theory.

C.   Batch Culture Experiments

A batch culture study was conducted subsequent to the continuous flow culture study to provide information for comparison and verification of the results found in the previous study. As listed in Table 5.2, the batch culture study (Run 4) was divided into two sets. Set 1 was CO_2 deficient consisting of

three chemostats with initial N_A concentrations of 2, 5 and 10 mg/l. The three chemostats in Set 2 contained the same N_A concentrations as Set 1, but were also enriched with 1.06% CO_2.

1. <u>Mass Variation With Time of Growth.</u> Figures 5.8 and 5.9 illustrate the mass variation with time of growth using M and C respectively as indicators of mass. The mass for Set 1 shows an increase initially, then an oscillatory pattern. This is the same type of pattern that was observed for the continuous flow runs. Set 2 reacts basically the same as was found for the continuous flow cultures in that there was initially an increase in mass, followed by a leveling off, and then a drastic increase in mass due to a population shift. From Figure 5.8 it may be observed that the population shift for the two reactors in Set 2 containing 2 and 5 mg/l occurred after 17 days. This was sooner than the shift in the continuous culture, which occurred after 24 days growth. The reactor that contained 10 mg/l N_A in Set 2, showed apparent exponential growth for eight days at which time growth leveled off until 12 days growth time. A population shift began at 12 days and the algal mass increase continued through 40 days.

2. <u>Cellular and Ammonia Nitrogen Variation.</u> The rate at which the nitrogen was removed from the feed solution is illustrated in Figure 5.10 with a plot of N_A in solution versus incubation time. With the exception of Chemostat 3, all of the cultures exhibited a rapid removal of the N_A. A good example of the luxuriant uptake of N_A may be seen by comparison of Figures 5.9 and 5.10. Figure 5.9 shows that the masses in reactors 1 and 2 of Set 1 were practically equal through 17 days of incubation. Figure 5.10 reveals that approximately equal concentrations of N_A were left in solution for cultures 1 and 2 after five days. This indicates that a luxuriant uptake was taking place for the culture containing 5 mg/l N_A (reactor 2) as no more mass was being produced than the culture containing 2 mg/l N_A. Figure 5.11 further verifies this luxuriant uptake showing that the percent nitrogen
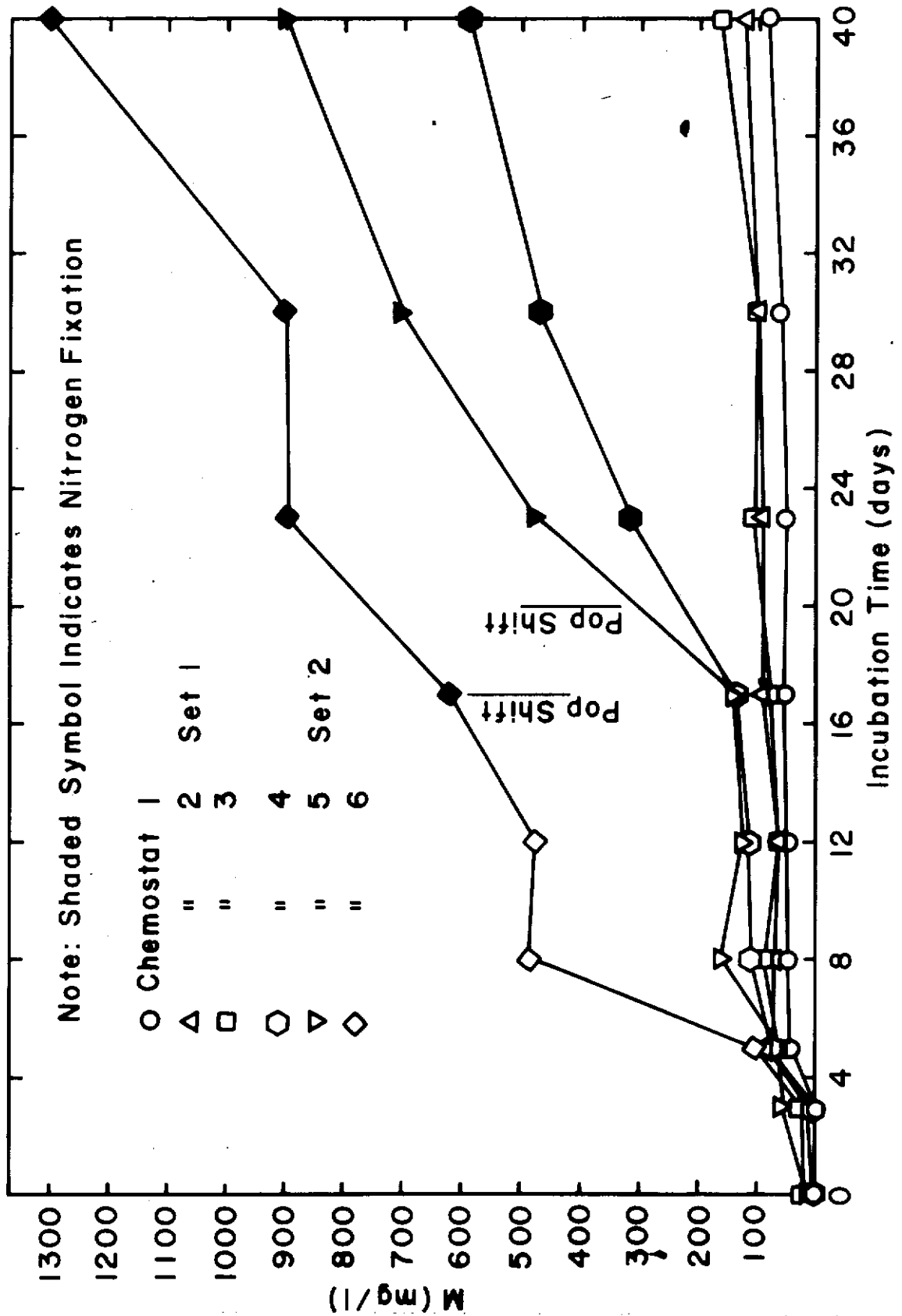
Figure 5.8   Particulate COD vs. Incubation Time for Batch Cultures
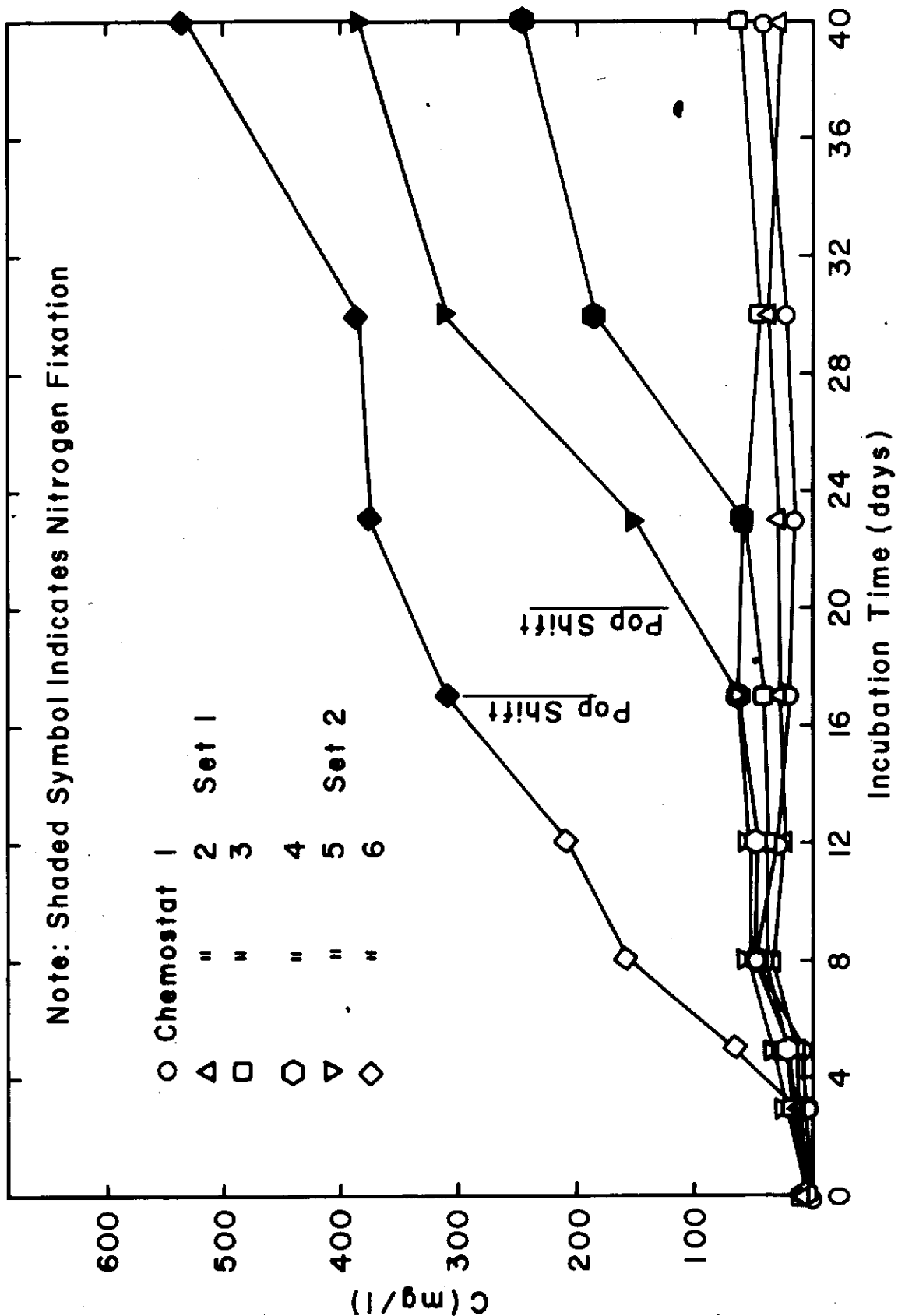
48

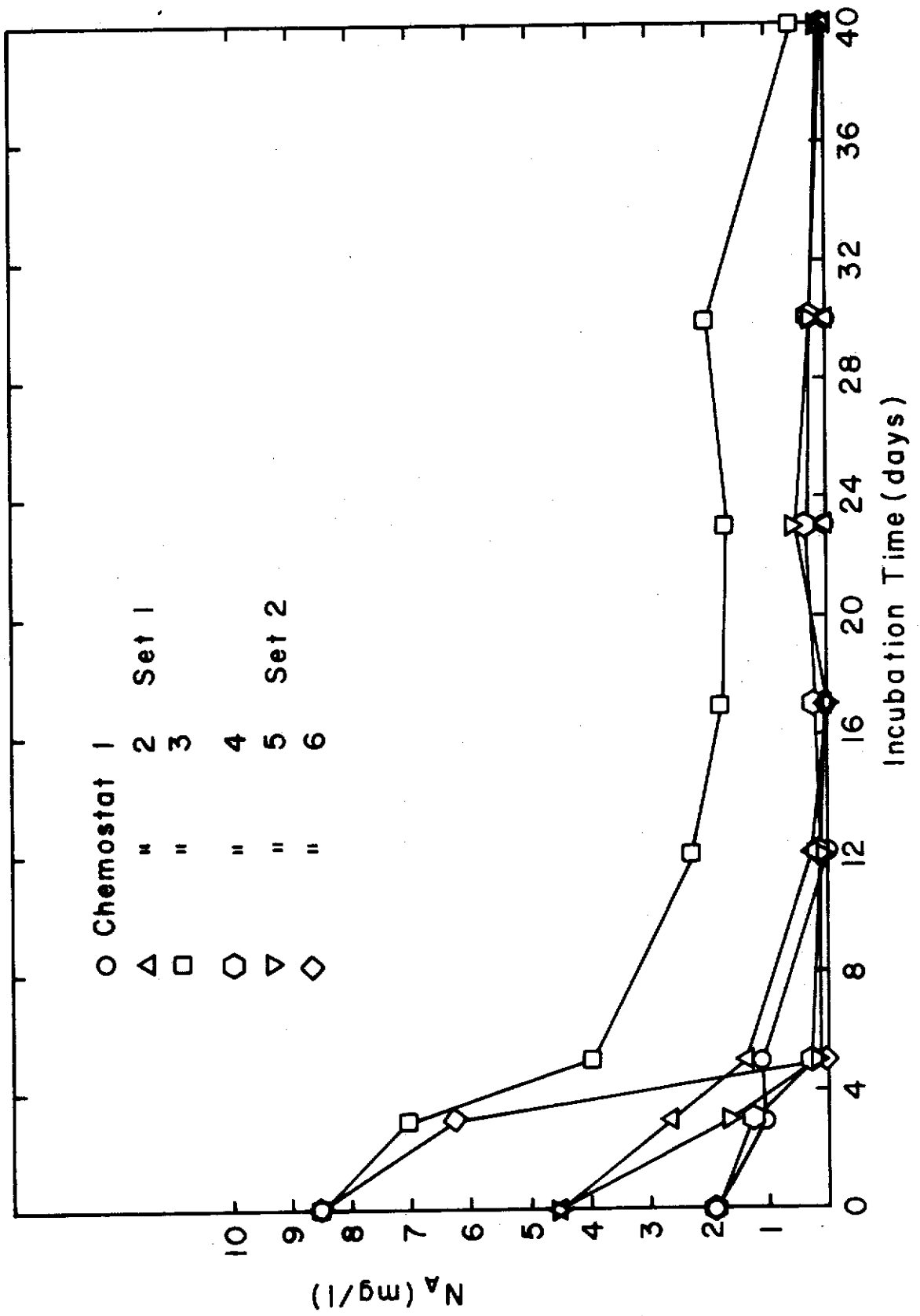Figure 5.9   Particulate Organic Carbon vs. Incubation Time for
Batch Cultures

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Figure 5.10 Ammonia Nitrogen (N_A) in Solutions as a Function of
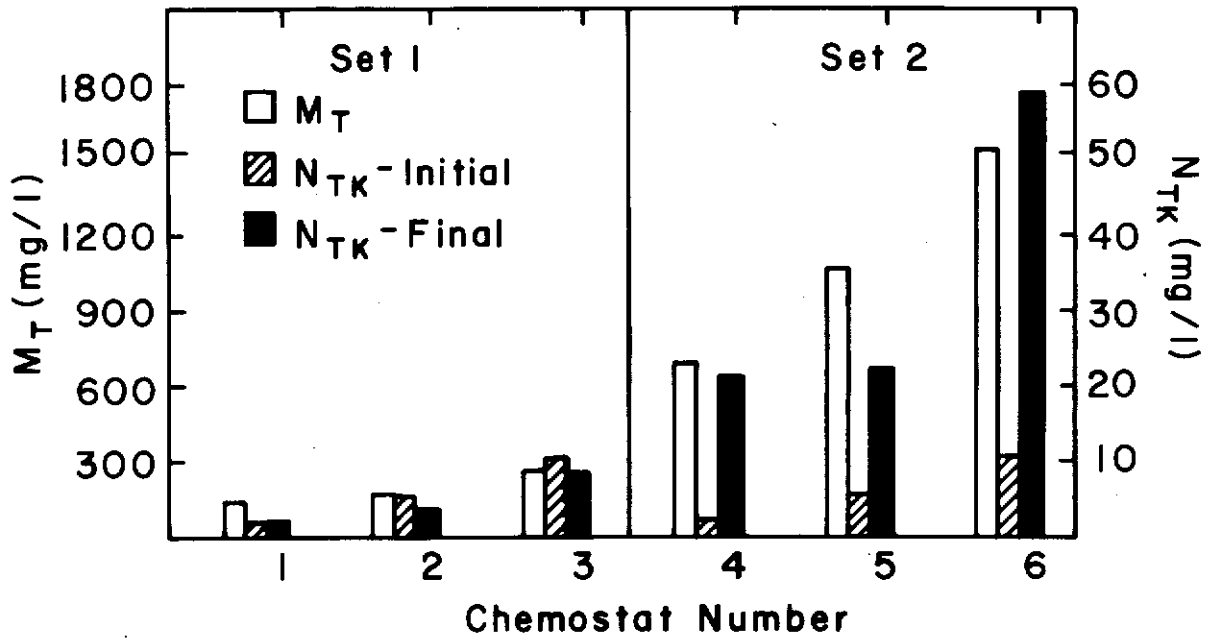Incubation Time for Batch Cultures

Figure 5.12 Nitrogen Fixation and Associated Mass Increase for CO_2 Enriched Batch Cultures
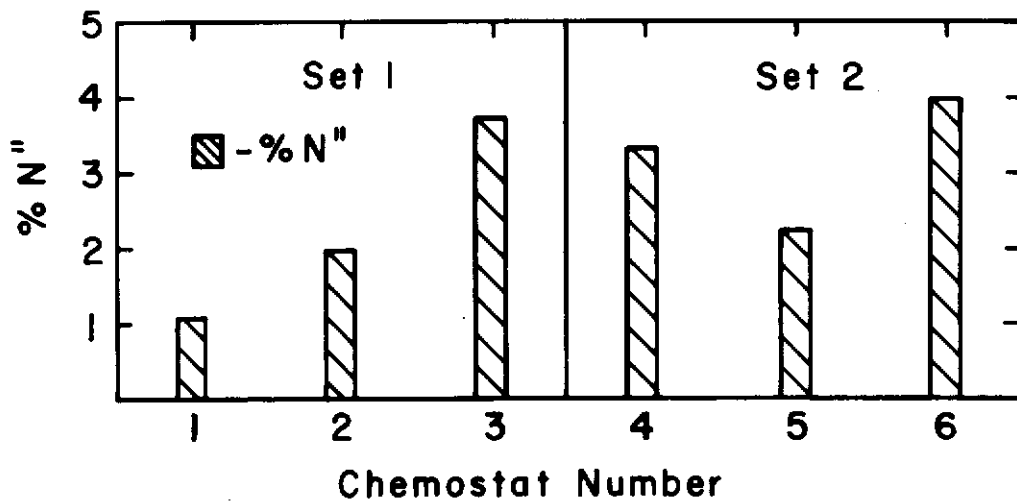

Figure 5.11 Nitrogen Content of Algae for Different Growth Conditions of Batch Culture Study

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contained in the cells for Chemostat 2 was almost twice that contained in Chemostat 1. There was a definite increase in percent N with increasing initial N_A concentration for the carbon deficient set; however, this same trend cannot be observed in Set 2, due to the CO_2 enrichment and nitrogen fixation that was taking place.

3. Comparison of CO_2 Enriched and CO_2 Deficient Cultures.

Figure 5.12 depicts a very impressive contrast between the CO_2 enriched and the CO_2 deficient cultures. Set 1 showed very little response to the increasing N_A concentrations due to the carbon limitation. Set 2, on the other hand, responded by showing healthy mass increases along with population shifts and nitrogen fixation. For the cultures which contained 10 mg/l N_A, the CO_2 enriched culture produced 5 times as much mass as the CO_2 deficient culture.

The batch culture study further verifies that CO_2 must be present in large quantities to produce the massive blooms of blue-green algae. This contradicts King (19) who contends that the blue-green blooms are normally found in CO_2 deficient conditions. As mentioned previously, for natural conditions it seems that the only possible way for algae to obtain CO_2 requirements such as this would be through the degradation of organic matter by bacteria as Kerr (17) and Kuentzel (20) have suggested.

As discussed in an earlier section, it would appear rather hopeless to limit algal growth by phosphorus control once adequate phosphorus concentrations are available to allow growth. The control of nitrogen in lakes may also be a hopeless endeavor if it is present in adequate quantities to initiate growth. This is true because of the ability of certain blue-green algae to obtain nitrogen through nitrogen fixation when it becomes limiting. Thus it may be concluded from the results of this study that nuisance blue-green algae blooms may be prevented by controlling the CO_2 availability. There is also substantial evidence (20) which indicates that the mangitude of CO_2 necessary

for algal blooms could only be supplied through the biological activities of bacteria on organic carbon. These results suggest that if massive algal blooms are to be controlled, the discharge of organic matter to waterways must be controlled since little can practically be done to control bacterial activity.

CHAPTER VI

SUMMARY

For the CO_2 enriched, nitrogen limited runs of the continuous flow cultures, a successional change was observed from green algae to blue-green algae with increasing hydraulic residence time. In the CO_2 deficient run the predominant algae were blue-green for practically all of the hydraulic residence times.

All continuous culture runs were subjected to the same growth conditions with only the dilution of feed solution concentration and CO_2 supplement varying. The runs were found to reach steady state conditions after approximately 24 days of growth. The growth curves (algal mass versus incubation time) for the CO_2 enriched cultures characteristically increased to a maximum, followed by a slight decrease and then leveling off. The CO_2 deficient run exhibited an initial decrease (inoculating algal concentration too great) followed by a leveling off.

Mass was measured by three methods: COD, volatile suspended solids, and particulate organic carbon. All three methods indicated that the growth rate and standing crop of algae were greater for the CO_2 enriched conditions. For identical growth conditions, the CO_2 enriched run (Run 1) produced almost ten times as much mass for the 16 DRT culture as the CO_2 deficient run (Run 2). The large algal masses in the CO_2 enriched runs for the 16 and 32 DRT were attributed to the population shifts to blue-green algae. These shifts were characterized by excessive foaming, foul odors, apparent exponential growth, nitrogen fixation, and a visual change from a yellow-green to a drak green color. The successional shift from green to blue-green algae was suspected to be the result of nitrogen limitation. Certain blue-green algae

are nitrogen fixers and would be more capable of propagating in a nitrogen limiting environment. The CO_2 limited run (as mentioned previously) was dominated by blue-green algae, but did not exhibit the exponential growth, foaming, or nitrogen fixation as the CO_2 enriched runs. This indicates that large quantities of CO_2 must be present for the blue-green algal nusiance blooms.

The batch culture study provided substantial evidence that there was a luxuriant uptake of the growth regulating nutrient (N_A) as it was depleted in most cases shortly after inoculation. Growth, however, continued some time after the N_A depletion from solution. The %N for the CO_2 enriched runs generally ranged between 1.8 - 3% for the longer residence times. The %P for the CO_2 enriched runs was between 0.4 - 1.4%. The %N and %P was significantly greater for the CO_2 deficient run because of the lower mass production and the relatively greater availability of nitrogen and phosphorus from solution.

The kinetic theory developed by Foree and Wade (9) and described in Chapter II was confirmed by applying the data from the CO_2 enriched runs of this study. The run with 10 mg/l N_A (Run 1) was found to fall within the range of applicability of the theory; however, the run with 2 mg/l N_A (Run 3) was found to be in the minimum range for which the theory applies. The theory was found not to apply for the CO_2 limited run (Run 2), as in this case growth was limited by the inorganic carbon in feed solution.

The batch culture study produced results very similar to those found in the continuous flow study, i.e. , greater mass with CO_2 enrichment, population shifts, and similar growth curves. The luxuriant nutrient uptake phenomenon was further verified by the batch culture study.

CHAPTER VII

CONCLUSIONS

The following conclusions are based on the information ascertained from this study of algal growth in continuous and batch cultures utilizing mixed algal populations in diluted activated sludge sewage treatment plant effluent as a growth medium.

1. Continuous algal cultures at steady state conditions had chemical compositions varying over a fairly small range as follows: 2-7% nitrogen, 45-50% carbon, 7-9% hydrogen, and 25-45% oxygen.

2. Blue-green algae were found to be predominant in CO_2 deficient conditions, but did not exhibit characteristics of nuisance algal blooms as the algae were in relatively small concentrations due to CO_2 limitation of mass production.

3. Even in sewage treatment plant effluent which contained significant concentrations of both organic and inorganic carbon, algal growth was limited by the availability of CO_2. In this study as much as a ten-fold increase in growth was observed when excess CO_2 was artificially supplied to the sewage effluent growth medium. These results support the theory that, in most instances, massive natural algal blooms must be supported by another source of carbon other than atmospheric CO_2 and dissolved carbonate and bicarbonate. The most logical source for this carbon seems to be from the action of bacteria on organic matter.

4. Luxuriant uptake of nitrogen from solution by algae was observed. This was manifested by higher percentage of nitrogen in the algal cells in the continuous cultures with shorter residence times and in the

batch cultures in the early stages of growth after inoculation.

5.   When algal growth was supported by diluted sewage effluent and excess CO_2 was supplied to the system, then the availability of nitrogen from solution became the growth limiting factor. The results of this study indicated that in natural situations where excess CO_2 might be available, but nitrogen in solution is deficient, algal succession may occur with shifts to certain blue-green algae with atmospheric nitrogen fixing capabilities.

6.   A previously developed kinetic theory which describes algal growth as being proportional to the cellular concentration of the growth regulating nutrient was confirmed when applied to the CO_2 enriched conditions with 10 mg/l ammonia nitrogen in the feed solution. The CO_2 enriched, 2 mg/l ammonia nitrogen run was found to be in the range of a minimum concentration for which the theory applies.

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