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Nutrient Uptake and Chemical Composition of Algae in Batch Culture

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PREFACE

This report on nutrient uptake and chemical composition of algae presents the results of the first phase (OWRR Project No. A-021-KY) of a comprehensive study entitled Algal Growth and Decomposition: Effects on Water Quality. The study is continuing as OWRR Project No. A-023-KY, Algal Growth and Decomposition: Effects on Water Quality, Phase 2. A study on the effects of chemical composition on the rate and extent of algal decomposition and nutrient regeneration is in the final stages and will be completed in 1970. A study to evaluate the effects of various factors on the rate and extent of oxygen uptake by decomposing algae is in the preliminary stages.

The assistance and cooperation afforded the authors during this study by Dr. Robert A. Lauderdale, Director of The University of Kentucky Water Resources Institute, are gratefully acknowledged.

ABSTRACT

The chemical composition of algae grown in batch culture depends mainly on environmental conditions, nutrient availability, presence of predators, cell age, and species. The effects of nutrient availability and cell age on the composition of three unialgal cultures (algae + bacteria) and one heterogeneous culture (algae + bacteria + microscopic animals) were evaluated. The cultures were grown in batch culture under both nutrient-abundant and nutrient-deficient conditions and the changes in compositions were observed. Luxurious uptake, where nutrients are incorporated into cellular protoplasm at levels greater than those necessary for growth, and super-luxurious uptake, where some nutrients are stored rather than converted into algal protoplasm, were observed. The commonly used model for calculating the weight percentage of protein was inaccurate when super-luxurious uptake occurred. Composition of the cultures was generally characterized by protein synthesis during the nutrient-abundant growth phase, by a fluctuating composition during transition from nutrient-abundant to nutrient-deficient growth, and by lipid and/or carbohydrate synthesis and the establishment of a relatively constant composition during the nutrient-deficient growth phase. Two unialgal cultures accumulated carbohydrates and one accumulated lipids. Soluble extracellular substances were produced in all cultures which caused high concentrations of color.

KEYWORDS: *algae, carbohydrates, *chemical composition, chlorophyta, cyanophyta, *cycling nutrients, *eutrophication, lipids, *nitrogen, *nutrient requirements, *phosphorus, proteins.

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INTRODUCTION

Wastewater discharges containing nutrients available for plant growth result in the artificial nutrient enrichment of many surface waters. This enrichment in turn results in excessive growths of aquatic plants, primarily algae, which have many detrimental effects on water quality. The evidence currently available indicates that nitrogen and phosphorus are the most serious offenders in promoting these excessive plant growths or algal "blooms". Nitrogen and phosphorus enter receiving waters through agricultural drainage, domestic and industrial waste treatment plant effluents, and various other sources. Large quantities of both nitrogen and phosphorus come from both agricultural and municipal wastewaters, but the ratio of nitrogen to phosphorus is usually considerably greater in agricultural drainage than in municipal wastewaters where large quantities of phosphorus originate from synthetic detergents.

When nitrogen and phosphorus are added in sufficient quantities to natural waters which generally contain adequate amounts of carbon and trace elements, and environmental conditions are favorable, an algal bloom will generally occur. This large growth of algae quickly consumes the available supply of nutrients in solution. Algal cell division is then believed to cease and growth during this

nutrient-deficient period is thought to occur in the form of increased cellular size and mass. Growth continues until inhibited by nutrient availability or some other unfavorable environmental factor and then decomposition commences. The extent and characteristics of decomposition are thought to depend on the compositions attained by the algae during growth. These compositions depend primarily upon the environmental conditions and the algal species involved.

One of the major efforts of current research is to find an economical way to remove nutrients from wastewaters, thus preventing algal growth and the associated water quality problems. Some authors have proposed treatment processes which utilize algae to remove the nutrients before they reach the receiving waters. These "activated" algae processes show considerable promise, but the major problem seems to be that of disposal of algae grown for the purpose of nutrient removal. A common suggestion has been digestion either by aerobic or anaerobic means. However, the digestibility seems to depend on the age of the algae and the associated environmental conditions. More commonly, algae are employed in waste stabilization lagoons throughout the world for the treatment of various types of wastes. These algae are only rarely harvested from the lagoons and thus must ultimately decompose in situ.

It was the purpose of this study to determine the changes which occur in the composition of the organic matter of algae as the cells grow and age. By becoming familiar with some of the composition changes which occur during

algal growth, especially during the nutrient-deficient growth phase, better insight can be gained for the design of waste treatment systems utilizing growing algae. A better knowledge of the organic composition of algae will also give an indication of the extent of algal decomposition that will occur under natural conditions, thereby lending insight into the water quality problems associated with algal decomposition.

CHAPTER II

BACKGROUND

A. Chemical Composition of Algae

The stoichiometry of the growth and aerobic decomposition of algae can be represented by the following equation (1):

$$aCO_2 + cNO_3^- + ePO_4^{-3} + (c+3e)\,H^+ + 1/2\,(b-c-3e)\,H_2O \underset{\text{Aerobic Decomposition}}{\overset{\text{Growth}}{\rightleftharpoons}} C_aH_bN_cO_dP_e + (a+b/4+5c/4-d/2+5e/4)O_2.$$

In this equation the coefficients a, b, c, d, and e, which determine the chemical composition of the algal matter synthesized, vary depending on the species and age of the algae, temperature, available nutrients, and other related factors (1). Many elements not shown in the above equation participate in the synthesis of algal matter. Eyster (2,3) divided the elements necessary for the growth of green and blue-green algae into two classes, macronutrients and micronutrients, depending on their relative abundance in the algal cell. The macronutrients, which are used generally as building materials, include carbon, hydrogen, oxygen, nitrogen, phosphorus, sulphur, potassium, magnesium, and sodium. The micronutrients include iron, manganese, copper, zinc, molybdenum, vanadium, boron, chlorine, cobalt, silicon, and calcium, and are commonly metal constituents of enzymes which enter into biological reactions.

1. Elemental Composition. Carbon, hydrogen, oxygen, and nitrogen are generally considered to be the major constituents of algal matter and are commonly measured as that fraction of the dry weight of algal solids which volatilizes during combustion at about 600° C (4). This fraction, called the volatile solids, is a good measure of the organic portion of algal matter and is normally 85-95% of the total dry weight of freshwater green and blue-green algae.

The fraction of algal solids not volatilized during combustion is termed the ash and includes the micronutrients as well as the macronutrients other than C, H, O, and N. This fraction is a good measure of the inorganic matter present and normally represents 5-15% of the total dry weight of freshwater green and blue-green algae. Reported concentrations of minor chemical constituents of Chlorella pyrenoidosa are illustrated in Table 2.1.

TABLE 2.1

Ranges of Concentrations of Minor Chemical Constituents in Chlorella pyrenoidosa Cells.

Constituent	(% of Total Dry Weight) Concentration Range	Reference
Total Ash	1.4-20.2	(5, 6, 7)
P	0.06-3.0	(1, 5, 7, 8, 9, 10)
Ca	0.0-1.6	(5, 8)
Mg	0.3-1.5	(5)
K	0.04-1.4	(5)
Na	0.07-0.7	(5)
S	0.4-0.8	(8)
Fe	0.02-3.4	(8)
Mn	0.02-2.6	(8)
Sr	0.0004-0.05	(8)
Cu	0.0008-0.03	(8)
Zn	0.0004-0.009	(8)

2. Organic Composition. In general the majority of the organic fraction of algal matter is comprised of proteins, carbohydrates, and lipids. However, in some instances chlorophyll may comprise a relatively significant portion of the organic matter. Spoehr and Milner (6) found that under certain conditions the chlorophyll content of Chlorella pyrenoidosa was as high as 6% of the total dry weight, but under different conditions the chlorophyll content of the same algae could decrease to 1/2000 of this value. Other investigators (11, 12) reported chlorophyll contents ranging from 3.3-6% by weight for Chlorella pyrenoidosa cells grown under various environmental conditions.

Extracellular products produced by certain algae can comprise a significant amount of the total organic matter. These extracellular products are soluble substances set free from live, healthy algal cells and are distinct from the substances liberated by autolysis or decomposition of dead algal cells (13, 14, 15). Lefèvre (16) stated that the characteristics of the extracellular products vary according to the algal species and include polysaccharides, amino acids, vitamins, steroids, saturated and unsaturated fatty acids, and toxic as well as stimulating factors.

In natural environments these substances are diffused into the water containing the algal growth and would exert some effect on the aerobic decomposition of algae which occurs in the upper layers of a natural water but, in general, would not settle into the lower layers where anaerobic decomposition takes place. Gromov (17), in a review of research on algal cultures in the

Soviet Union, explained instances in which 5-10% and sometimes even 30% of the organic matter synthesized by Chlorella was liberated into the culture medium. The algae excreted three to five times more organic matter during a lag period of growth and in the beginning of a lag phase than during exponential growth or during a stationary phase. The excretion of organic matter into the medium increased under unfavorable growth conditions and the composition of organic matter excreted by different Chlorella strains was somewhat different. Carbohydrates and acetic, formic, glicolic, pyruvic, α -ketoglutaric, and glioxilic acids were discovered in the culture medium of Chlorella, but nitrogenous organic compounds were not detected.

a. Proteins. Fowden (18) stated that the cellular nitrogen may be divided into fractions including inorganic-, free α amino-, amido-, volatile-, and protein-nitrogen. The volatile nitrogenous compounds and inorganic nitrogen are usually quantatively insignificant and protein normally constitutes the main nitrogen fraction, representing 80% or more of the total nitrogen in unicellular algae. Protein is assumed to contain a relatively constant percentage of nitrogen by weight; the value most commonly used is 16%. Hence, the percent by weight of algal protein can be calculated as 6.25 (=100/16) times the percent by weight of nitrogen (6, 19, 20).

Fowden (18) and Fisher and Burlew (21) have presented data on the amino acid composition of the bulk proteins of several algae. Fowden found eighteen different amino-acids in the bulk protein of Chlorella vulgaris and

Anabaena cylindrica, with arginine, aspartic acid, glutamic acid, leucine, and lysine being the most significant. Fisher and Burlew found ten different amino-acids in the crude protein of pilot-plant and laboratory grown Chlorella pyrenoidosa cells, with the most significant being arginine, leucine, lysine, and valine.

Gromov (17) found no significant differences in fractions of amino acids for Chlorella and Scenedesmus for autotropic and heterotrophic growth. Milner (19) demonstrated that the actual mass of protein in cultures of Chlorella pyrenoidosa was relatively constant and did not appear to change significantly as the algal cells went from the "normal" 50%-protein, 20%-lipid state to a low-protein, high-lipid state.

b. Carbohydrates. Carbohydrates are generally found in the storage materials, cell walls, and mucilages of algae. The cell wall carbohydrates can be roughly divided into water-soluble and water-insoluble materials, with the water-insoluble materials generally having a less complicated molecular structure which determines to a large extent the properties which render them suitable as skeletal materials. The water-soluble carbohydrates are generally mucilaginous or pectic substances located at greater distances from the cell lumen than the water-insoluble carbohydrates (22). Various carbohydrate constituents found in the storage materials, cell walls, and mucilages of green and blue-green algae are listed in Table 2. 2.

TABLE 2.2

Carbohydrate Constituents Found in the Storage Materials, Cell Walls, and Mucilages of Green and Blue-Green Algae.

Constituent	Type of Algae	Reference
A. Storage Materials:		
glucose	green & blue-green	(23, 24)
sucrose	green	(24)
fructose	green	(23, 24)
insulin	green	(24)
trehalose	blue-green	(24)
glycogen	blue-green	(24)
arabinose	blue-green	(24)
B. Cell Walls:		
cellulose	none given	(22)
mannan	none given	(22)
xylan	none given	(22)
alginic acid	none given	(22)
fucinic acid	none given	(22)
pectin	none given	(22)
C. Mucilages:		
glucose	green & blue-green	(25)
arabinose	green & blue-green	(15, 25)
galactose	green & blue-green	(15, 25)
xylose	green & blue-green	(25)
rhamnose	green & blue-green	(25)
mannose	green & blue-green	(25)
glucuronic acid	blue-green	(25)
galacturonic acid	blue-green	(25)

c. Lipids. Lipids are usually measured experimentally as two fractions: a saponifiable fraction (free fatty acids and their esters) and an unsaponifiable fraction, with the saponifiable fraction generally comprising the majority of algal lipids (19, 20). Various authors (19, 26, 27) found that fatty acids occurring

in algae range from 12-carbon to 24-carbon acids, with only even numbered ones being of any significance quantitatively. These fatty acids most frequently occur as mono-, di-, or tri-glycerides as opposed to free fatty acids.

B. Variations in Chemical Composition

Typical compositions of the three classes of organic compounds which comprise the major portion of algal mass are shown in Table 2.3. If the assumption is made that carbon, hydrogen, oxygen, and nitrogen comprise the entire algal mass, the composition of algal matter can vary over a limited range depending upon the relative proportions of these four fractions. The range over which carbon can vary is from 40-77%, hydrogen from 6.7-11.4%, oxygen from 11-53%, and nitrogen from 0-16%.

TABLE 2.3

Elemental Composition of Typical Organic Compounds Which Comprise Algal Mass [After Foree and McCarty (9)]

	(% By Weight)			
Typical Compound	C	H	O	N
Carbohydrate $(CH_2O)_x$	40.0	6.7	53.3	0.0
Protein (Average)	54	7	23	16
Lipid $(C_{18}H_{32}O_2)$	77.2	11.4	11.4	0.0

Foree and McCarty (9) pointed out that the chemical composition of algae is generally more dependent on the environmental conditions under which the algae are grown than on the innate characteristics of the organisms. Hence

generalizations of compositions based on chemical formulas, usually containing only the major chemical constituents, are meaningless unless the physiological history and environmental conditions under which the algae were grown are specifically defined. They illustrate by showing that the composition of Chlorella pyrenoidosa has been reported anywhere in the range from $C_{6.20}H_{10.2}O_{3.23}N$ to $C_{57.3}H_{103.2}O_{10.0}N$.

Spoehr and Milner (6) studied the effects on algal cell composition of various environmental factors such as temperature, atmospheric composition, concentration of mineral nutrients, and illumination intensity and intermittent vs. continuous illumination. The wide variation in protein, lipid, and carbohydrate fractions found by Spoehr and Milner are shown in Table 2.4. Cultures grown in media with low nitrogen concentrations and cultures illuminated with high light intensity produced algal cells of high lipid content.

Milner (19) and Fogg and Collyer (20, 28) found that the accumulation of high proportions of lipid material was more dependent on environmental conditions than on certain classes or individual species of algae. Species belonging to the same class contained similar relative amounts of crude protein, fats, and hydrolyzable polysaccharide. There were no fundamental differences in the algae belonging to Chlorophyta (green algae). Even though representatives of the Cyanophyta (blue-green algae) under certain circumstances had moderately high fat contents, they appeared to differ from the Chlorophyta in that fat accumulation was not associated with low cell

TABLE 2.4

Organic Composition of Chlorella pyrenoidosa Grown Under Various Environmental Conditions
[Calculated from data by Spoehr and Milner (6)]

(% Ash-Free Dry Weight)

Culture	Protein	Carbohydrate	Lipid
1	58.0	37.5	4.5
2	70.5	23.8	5.7
3	88.2	6.6	5.2
4	64.8	17.7	17.5
5	33.0	38.2	28.8
6	67.2	11.4	21.4
7	49.2	23.0	27.8
8	30.0	24.8	45.2
9	19.2	20.8	60.0
10	10.1	20.0	69.9
11	8.0	13.2	78.8
12	7.3	9.5	83.2
13	8.9	4.8	86.3
Mean	39.6	19.3	41.1

nitrogen content. Gromov (17) found that the correlation between carbohydrate and lipid synthesis varied depending on both the strain of algae and the environmental conditions encountered by the algae during growth.

Collyer and Fogg (28) found that the lipid content of Chlorella cells increased with culture age and the increase could be related to the exhaustion of nitrogen from the growth medium. Aach (29) found, from analysis of Chlorella cells during growth, that the lipid content of the cells increased from 22% of the dry weight on the second day of growth to 70% on the twenty-fifth day when growth had ceased. Miller (26) reported that growth (increase in cell number) can continue slowly for some time in a medium entirely depleted of nitrogen, producing cells of high fat content. However, it is generally believed that for all practical purposes cell division ceases after the nitrogen has been depleted from the growth medium.

C. Nutrient Uptake

Gerloof and Skoog (30, 31) presented results of studies on the blue-green alga, Microcystis aeruginosa, which indicated that algae might store nitrogen or phosphorus from an environment containing relatively high concentrations of these elements to permit continued growth in an environment where they are deficient. They believe that nitrogen and phosphorus would be distributed in successive cell divisions until one of the elements was reduced to its growth-limiting critical value of 4.0 and 0.12%, respectively. For cellular nitrogen and phosphorus contents of 4.0-7.7% and 0.12-0.46%,

respectively, the algal yield was constant and it was postulated that these increases above the critical levels represented luxury consumption of nitrogen and phosphorus.

In contrast, Knauss and Porter (8) found for the green alga, Chlorella pyrenoidosa, that absorption of all elements except phosphorus and sulfur was directly proportional to the concentration of that element in the nutrient solution. The quantities of phosphorus and sulfur in the algal cells were constant when the cells were grown in the higher nutrient concentrations of these elements. Borchardt and Azad (10) found that for Chlorella and Scenedesmus three regimes of phosphorus uptake may be established. For cellular phosphorus contents of 0-1% the algal yield is directly proportional to the phosphorus content. For phosphorus contents of 1-3% yield is constant and phosphorus is stored in the algal cells. When the phosphorus content rises above 3%, the cell is saturated and can no longer store phosphorus. Therefore, "luxury uptake" was defined as that phosphorus incorporated into the cells above the critical level of 1% cellular phosphorus.

D. Effect of Bacteria and Animals on Algal Yield

Under environmental conditions favorable for algal growth, the major fraction of the nutrients in a growth medium are incorporated into cellular material by algae rather than by other populations, such as bacteria and animals. These other populations can affect the natural algal yield by competing for available nutrients and predation, but the effect which the

bacteria and animals will have depends on the source of energy available to these populations (1). If organic pollutants other than algae are present, bacteria and animals may form the major fraction of the living organic mass present. However, in algal systems with light as the energy source, bacteria and animal mass seldom comprise more than 1% of the total organic matter present. In this case the nutrients used by the bacteria and animal populations are small in relation to the total quantity of available nutrients (1, 32).

E. Summary

The previously presented background material showed that the organic composition of algae is far from being a constant which can be determined and reported as such. It has been shown that the protein, carbohydrate, and lipid portions vary depending on time of growth (cell age), availability of nutrients, presence of predators (decomposers), and environmental conditions. Spoehr and Milner (6), after investigation of several environmental factors, concluded that evaluating the effect of one factor operating independently of others was almost impossible. Therefore, algal cell composition is only significant when reported along with the environmental conditions prevailing during growth. The environmental conditions to which the algal cultures of this study were subjected, as well as other growth and sampling procedures, are described in the next section entitled "Experimental Procedure."

CHAPTER III

EXPERIMENTAL PROCEDURE

The purpose of this section is to describe the procedures followed for growing and sampling the algae used in this study. The next section entitled "Analytical Procedure" describes the specific analytical analyses used.

A. Growth Procedure

1. Culture Vessels. The four vessels in which the algal cultures were grown consisted of 9-liter pyrex bottles with lower sampling nodules as shown in Figure 3.1. The purpose of the glass tubing extending to within one inch of the bottom of the vessel was to supply an air-carbon dioxide mixture to the growing algae. The shorter piece of glass tubing served as a pressure release for the system. A piece of cotton was placed in the end of the short tube to minimize contamination and evaporation from the vessel. Each vessel was housed on a magnetic stirrer which provided continuous agitation to the growing cultures by a rotating magnetic stirring bar. The glass tubing extending from the lower sampling nodule was used as a sampling port. The No. 8 stopper containing this piece of tubing was sealed to the nodule with silicone rubber. When not in use the sampling port was closed with a

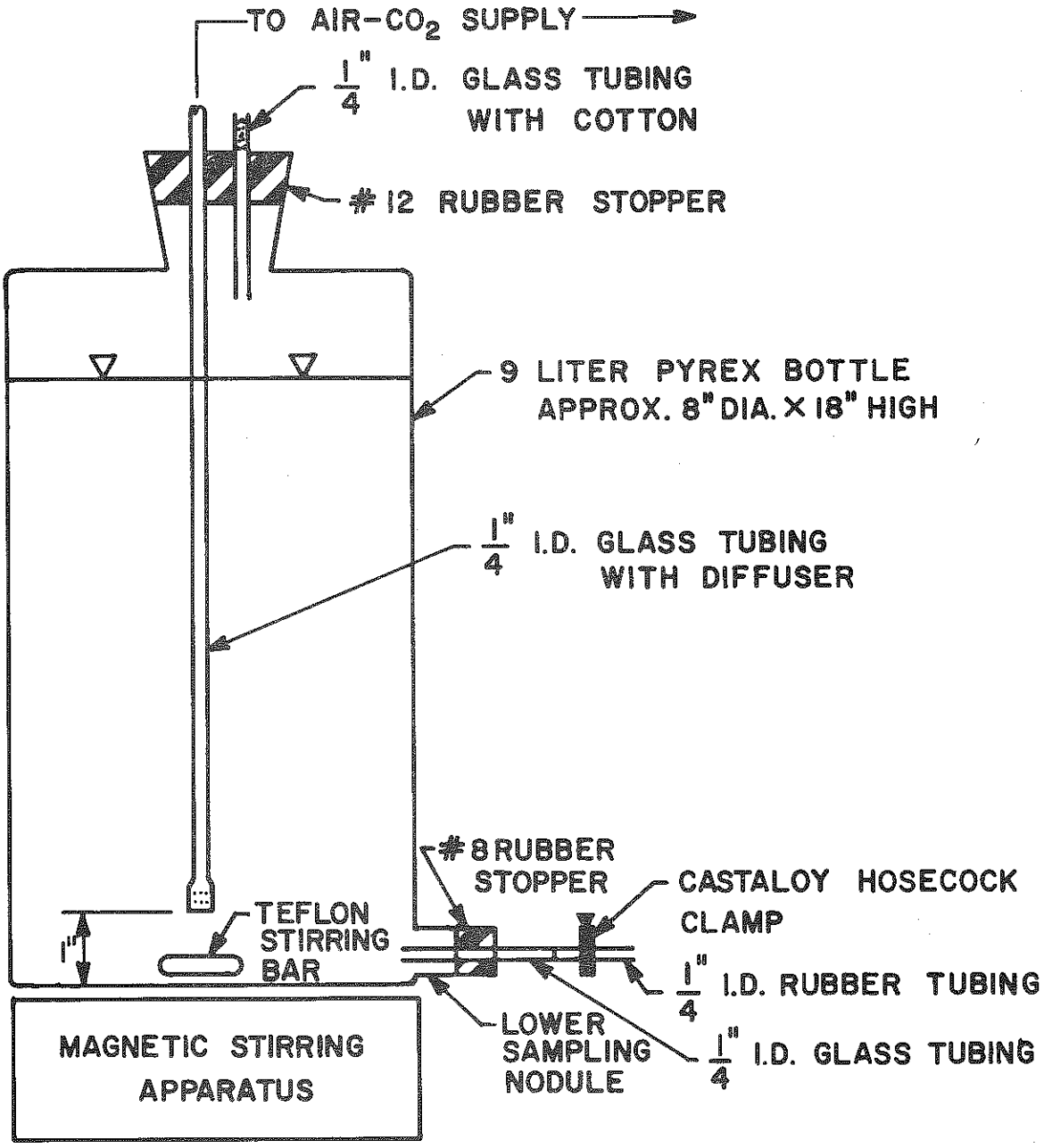

FIGURE 3.1. Diagram of Culture Vessel.

"Castaloy" hosecock clamp. Silicone rubber was also used to seal all outside contacts between glass tubing and rubber stoppers.

2. Light. Light to the algal cultures was supplied continuously by four horizontally supported 40 watt Cool White fluorescent lamps at an intensity of approximately 500 foot-candles at the outside surface of the vessels.

3. Gas Mixture and Supply System. An inorganic carbon source was continuously provided to each growing algal culture by a mixture of air and carbon dioxide at a rate of approximately 2.5 liters per minute. The pH of the bicarbonate-buffered growth medium was kept in the range 6.5-7.5 by maintaining carbon dioxide in the mixture at approximately 2% by volume.

The gas sources were the laboratory compressed air supply and pure bottled carbon dioxide. A system composed of glass T's, rubber tubing, and Nalgene twistcock connectors was used to transport the air and carbon dioxide from their respective sources to the culture vessels as shown in Figure 3.2. A Castaloy clamp was installed on the tubing leading to each culture vessel on each side on the Nalgene connector. The purpose of the clamp nearer the vessel was to regulate the flow into the vessel. The other clamp served to terminate the flow into a vessel without a pressure loss to the entire system when that vessel had to be removed from the system for manual shaking.

4. Temperature. The temperature in the room housing the growing cultures was controlled by a thermostat and maintained at approximately 25 °C throughout the duration of the study.

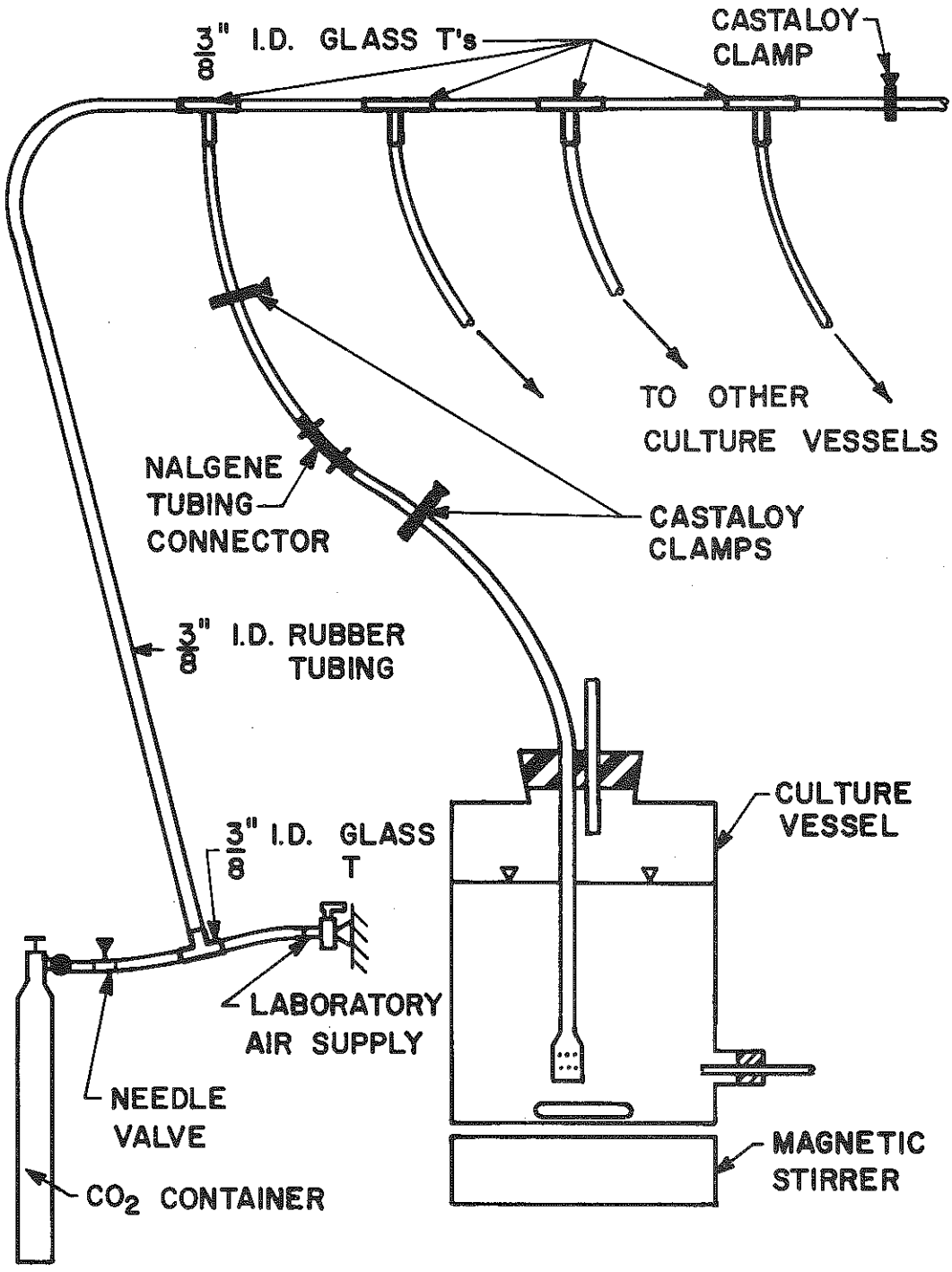

FIGURE 3.2. Diagram of Air-Carbon Dioxide Supply System.

5. Culture Growth Medium. All cultures were grown in the same synthetic medium which was designed to approximate a typical nutrient-enriched surface water. The chemical composition of the synthetic growth medium was similar to that of the FMB medium used by Foree and McCarty (9) and is shown in Table 3.1. The growth medium was prepared in each culture vessel by first adding appropriate amounts of previously prepared concentrated solutions to approximately 6 liters of distilled water and then diluting to a total volume of 8 liters. The concentrations of nutrients in the growth medium measured during the study varied somewhat from the values given in Table 3.1 due to the inaccuracy of diluting in the culture vessel when initially preparing the medium and to evaporation during the growth period.

TABLE 3.1

Chemical Composition of Synthetic Growth Medium

Chemical	Concentration (mg/l)	Comments
$MgSO_4 \cdot 7H_2O$	45.0	
$CaCl_2$	55.0	
$NaHCO_3$	250	200 mg/l Alkalinity as $CaCO_3$
$KHCO_3$	100	
KH_2PO_4	16.8	3.8 mg/l P
NH_4Cl	95.5	25.0 mg/l N
Fe, B, Mn, Mo, Si	0.1	Added as 1 ml trace element solution per liter of medium
Zn, Cu, Co, Ni, Cr, V	0.01	
EDTA	2.0	
KOH	1.0	

B. Sampling

1. Sampling Schedule. After the growth medium was inoculated and growth began, a constant check was kept on the ammonia nitrogen concentration in the culture vessels. Assuming that the initial ammonia nitrogen concentration of the growth medium was 25 mg/l, an effort was made to sample the growing algae when the ammonia nitrogen concentration was approximately 20 mg/l, 10 mg/l, 5 mg/l, and 0 mg/l. In order to check the ammonia nitrogen concentration a sample was taken and filtered through Whatman Glass Fibre Filter Paper, Grade GF/C, 4.25 cm diameter. The direct nesslerization test for ammonia nitrogen given in Standard Methods for the Examination of Water and Wastewater, Twelfth Edition (4), pp. 193-194, was run on a suitable aliquot of this check sample. The first samplings were actually taken at ammonia nitrogen concentrations of approximately 10 mg/l because initially it was not realized that precipitates formed by reaction with the Nessler's reagent resulted in turbidity which caused the direct nesslerization tests to give values approximately twice the true value. Subsequently, precaution was taken to avoid this precipitate formation. It should be emphasized that this test was used only for sampling purposes and the values obtained by direct nesslerization are not reported in the results of this study.

When the ammonia nitrogen concentration became 0 mg/l, it was assumed that essentially all the nutrients had been ingested by the algae and that growth was in the nutrient-deficient phase. Samples were taken at 5, 10,

15, 30, and 50 days after this time. A time of 0 corresponds not to the day the cultures were inoculated but to the day when the ammonia nitrogen concentration first reached 0 mg/l. Hence, a time of -1 refers to one day before the nutrient-deficient phase of growth began.

2. Sampling Procedure. The first step in sampling a culture was to dislodge any algae clinging to the sides of the culture vessel with a brush. To prevent contamination, the same brush was always used for a particular culture vessel. Next, each vessel was removed from its place on the magnetic stirrer and shaken manually. The vessel was then placed back on the magnetic stirrer and mixed vigorously throughout the sampling process. Two samples were then taken. The first sample of approximately 200 ml was placed directly into a 250 ml plastic bottle and designated the "total" sample. A second sample of approximately 250 ml was withdrawn into a 250 ml centrifuge bottle and centrifuged for about an hour on an International Equipment Company Waltham Centrifuge. The supernatant was then filtered through Whatman Glass Fibre Paper. The filtrate was collected and approximately 200 ml was placed in another 250 ml plastic bottle and designated as the "soluble fraction." Both the "total" and "soluble fraction" samples were immediately placed in a freezer and kept frozen at -30° C. For analysis, samples were removed from the freezer and thawed in a refrigerator at about +4° C.

C. Analysis

1. Total Sample. The total sample was analyzed by the procedures described in the next section entitled "Analytical Procedure" for the following:

a. Chemical Oxygen Demand

b. Total Suspended Solids

c. Volatile Suspended Solids

d. Total Kjeldahl Nitrogen (Organic plus Ammonia Nitrogen)

e. Total Phosphorus

f. Total Lipids

2. Soluble Fraction. The soluble fraction was analyzed by the procedures given in the next section for:

a. Chemical Oxygen Demand

b. Ammonia Nitrogen

c. Total Kjeldahl Nitrogen

d. Nitrate Nitrogen

e. Total Phosphorus

f. Color

CHAPTER IV

ANALYTICAL PROCEDURE

The purpose of this section is to describe the analytical procedures used for the determination of the various parameters considered in this study.

A. Chemical Oxygen Demand (COD)

The method used for determination of the COD was the dichromate reflux method. The detailed procedures used in this method are given in Standard Methods (4), pp. 510-514 with one exception: the ferrous ammonium sulfate titrant used was approximately 0.125 N instead of 0.25 N. The following volumes were used in all cases: sample plus distilled water - 20.0 ml, standard potassium dichromate solution - 10.0 ml, and concentrated acid - 30 ml.

B. Suspended Solids

The suspended solids concentration of a sample was determined using Whatman Glass Fibre Paper, Grade CF/C, 4.25 cm diameter, by the technique described by Wyckoff (33). For consistency a 10.0 ml sample was used throughout the suspended solids analysis except for the instances in which this sample size caused an excessively slow flow rate through the filter. In these cases the sample size was reduced.

1. Total Suspended Solids. To determine the total suspended solids concentration, the samples were filtered through Whatman glass pads using a Millipore Filter Apparatus. The total suspended solids concentration of the samples was determined as the weight (to the nearest 0.1 mg) of the residue left on the filter pad after drying to constant weight in a 103°C oven.

2. Volatile Suspended Solids. The filter pads from the total suspended solids analysis were burned in a muffle furnace at 580-600°C for 10 minutes. The volatile suspended solids content of the samples was determined as that portion of the total suspended solids which was lost during combustion. Care was taken to maintain the temperature in the furnace below 600°C, the melting point of the glass filter pads.

C. Nitrogen

1. Ammonia Nitrogen. The ammonia nitrogen concentration of the samples was determined by a procedure which consisted of steam distillation on a micro Kjeldahl apparatus followed by nesslerization. The reagents used in this procedure are the same as those described in Standard Methods, pp. 389-391. Each sample and 3 ml of the phosphate buffer solution were added to a 200 ml distillation flask. This mixture was steam-distilled and approximately 20 ml of the condensate was collected in a 50 ml nessler tube and diluted to the 50 ml mark with distilled water. Nessler's reagent was added, and after allowing at least 30 minutes for color development, the color was measured

photometrically on a Beckman DB Spectrophotometer with wave-length set at 410 mu. The ammonia nitrogen content (in mg) of a sample was determined from a previously developed calibration curve and, knowing the sample size, the ammonia nitrogen concentration in mg/l was calculated. When possible, sample sizes were selected so as to contain 0.05-0.20 mg of nitrogen. At least one ammonium chloride standard within this range was run with each set of samples.

2. Total Kjeldahl Nitrogen. The total Kjeldahl nitrogen concentration of the samples was determined by a micro Kjeldahl digestion procedure, which converts organic nitrogen to ammonia nitrogen, followed by the ammonia nitrogen steam distillation and nesslerization procedure described above. The reagents used are described in Standard Methods, pp. 402-404. Each sample, 5 ml acid-sulfate digesting solution, and 4 or 5 Hengar Granules were added directly to a 200 ml distillation flask, placed on a micro Kjeldahl heating apparatus, and digested until approximately 20 minutes after the samples became colorless. After digestion the samples were cooled and 25 ml of distilled water and 2 drops of phenolphthalein indicator were added. After neutralization to the phenolphthalein end-point with sodium hydroxide, the samples were distilled and the total Kjeldahl nitrogen content was measured as ammonia nitrogen by the procedure described above. A reagent blank of distilled water and at least one ammonium chloride standard were run with each set of samples.

3. Nitrate Nitrogen. The nitrate concentration of a sample was determined using the Ultraviolet Spectrophotometric Method described in Standard Methods, pp. 200-202. Even though this method was satisfactory for the purposes of this study, it is rather insensitive and may not be useful in many cases. A sample size of 15.0 ml was used in all nitrate nitrogen determinations.

D. Total Phosphorus

The total phosphorus concentration was determined by the following procedure: (a) The sample and 1.0 ml of a 70 gm/l magnesium chloride reagent were added to a vycor dish, evaporated to dryness in a 103°C oven, and burned for 10 minutes at 600°C in a muffle furnace. The purpose of this step is to convert the organic phosphorus to pyrophosphate as described by Sawyer and McCarty (34), p. 471. (b) The contents of the vycor dish were cooled and diluted to 25 ml with distilled water. One-half ml of the strong acid solution was added to the dish and the contents were autoclaved for 30 minutes at 15 psi. Boiling in strong acid hydrolyzes the polyphosphates to orthophosphates as described in Standard Methods, pp. 236-237. (c) The contents of the vycor dish were transferred with 2 or 3 rinses to a 50 ml nessler tube and the total phosphate content was determined by the stannous chloride method described in Standard Methods, pp. 234-236. The use of 50 ml nessler tubes instead of 100 ml tubes as set forth in Standard Methods necessitated a proportionate reduction of the quantities of reagents.

The color determinations were made photometrically on a Beckman DB Spectrophotometer with the wave-length set at 690 mu. The total phosphorus content (in mg) of a sample was determined using a previously developed calibration curve and, knowing the sample size, the total phosphorus concentration in mg/l was calculated. When possible, sample sizes were chosen so as to contain 0.005-0.03 mg of phosphorus. A reagent blank of distilled water and at least one potassium phosphate standard were run with each set of samples.

E. Color

The amount of color in the samples was determined by the procedure described in Standard Methods, pp. 127-129. A stock solution of Platinum Cobalt Color Standard with a color of 500 units was used to prepare the standards. The pH values recorded for this procedure were measured on a Beckman Model N pH meter.

F. Total Lipids

The total lipid concentration of the samples was determined using a modification of the wet extraction method described by Loehr and Rohlich (35). A 20 ml sample or a sample diluted to 20 ml was added to a Waring blender and acidified to pH 3. Sixty-five ml of a 50% chloroform - 50% methanol solution were added and the mixture was homogenized for 2 minutes. The contents of the blender were filtered through Grade CF/C Whatman Glass Fibre Paper and the filtrate was transferred to a 1000-ml separatory funnel. The blender

and filtering apparatus were rinsed with 15 ml of the 50% chloroform - 50% methanol solution and the rinse was added to the separatory funnel. The separatory funnel and contents were manually shaken for one minute and allowed to separate. After separation was complete, the chloroform layer containing the lipids was drawn off into a tared beaker and the total lipids content of the samples was determined as the residue remaining in the beaker after evaporation of the chloroform on a steam bath, drying to constant weight in a 103°C oven, and cooling in a dessicator. An analytical balance was used for all weight determinations with readings being made to the nearest 0.1 mg. A reagent blank was run with each set of samples.

Lipid analyses were not made on the soluble fraction because it was assumed that sufficient calcium and magnesium were present in the growth medium to precipitate any lipid material which could potentially be released into solution.

CHAPTER V

RESULTS AND DISCUSSION

A. General

Several investigations have been conducted during the last 20 years which have contributed to the understanding of the mechanisms of algal growth. However, the objectives of these studies have been quite diverse, and usually only one or two isolated pure or unialgal cultures were studied. The major objective of this study was to specifically evaluate the effect of nutrient availability on the chemical composition of three unialgal cultures, known to have different growth characteristics, and a heterogeneous algal culture containing microscopic animals. The experiments were designed to maintain optimum environmental conditions (lighting, carbon availability, etc.) other than nutrient availability during the entire growth period.

1. Algal Cultures Used. The algae studied were three unialgal cultures and one mixed culture. The mixed culture was taken from a pond in Kentucky and is subsequently referred to as Kentucky Mixed. The unialgal cultures were Chlorella pyrenoidosa (26), Scenedesmus quadricauda (76), and Anabaena sp. (B380). The numbers in parentheses are the identification

numbers in the Indiana University Collection from which the cultures for initial inoculation were obtained (Starr, 36). Chlorella and Scenedesmus are green algae which were chosen because they are typically found in waste stabilization ponds as well as in many natural waters. Anabaena, a blue-green alga capable of fixation of atmospheric nitrogen, was chosen because it is a typical nuisance causing alga found in many lakes and reservoirs of the United States (37). The mixed culture was studied so that a closer simulation of natural conditions could be obtained. The mixed culture contained a wide variety of microscopic animals indigenous to the pond where the sample was originally taken, while the unialgal cultures contained bacteria but no animals.

During the preliminary phases of the study it was noted that different algal species responded differently to the growth medium to be used, resulting in different lag growth phases for each culture. Since it was desired to keep each of the four cultures under study in approximately the same phase of growth at a given time, each culture was initially seeded with a different volume of inoculant. This was accomplished by growing a preliminary set of cultures in the same growth medium and then using various sized aliquots from these to inoculate the cultures upon which the results are based. Larger inoculants were used for the cultures which had previously exhibited longer initial lag times. The following volumes were used to inoculate the final 8-liter cultures: Kentucky Mixed - 10 ml, Scenedesmus - 10 ml, Chlorella - 20 ml, and Anabaena - 100 ml. This technique worked quite well and the growth phases

for the four cultures corresponded rather closely, thus facilitating sampling and analysis. Periodically, samples were removed from the growing cultures and analyzed as described previously.

2. Symbols Used. The symbols used in the presentation of results that follow and the method for determining the quantity which each symbol denotes are given in Table 5.1.

B. Nutrient Uptake

Of all the elements necessary for algal growth, nitrogen and phosphorus are the two which most often determine the algal growth potential of natural waters. Assuming that the other elements, commonly referred to as trace-elements, are relatively abundant in natural situations, much controversy has arisen as to which of these two elements first limits algal growth in a natural situation where they are simultaneously consumed by algae. A detailed study of which of the two is the major limiting nutrient was not an objective of this study; therefore the experiments were designed so that nitrogen and phosphorus would both be simultaneously depleted from the growth medium. This was accomplished by making the ratio of nitrogen to phosphorus in the growth medium approximately equal to the "average" ratio normally found in algae growing under nutrient-abundant conditions.

The uptake of the nitrogen and phosphorus for the four cultures is shown in Figures 5.1-5.4. These curves show that the nitrogen and phosphorus

TABLE 5.1

ABBREVIATED SYMBOLS

Abbreviation		Means of Determination
M_T	Total Chemical Oxygen Demand Concentration	Direct Measurement
M_S	Soluble Chemical Oxygen Demand Concentration	Direct Measurement
M	Particulate Chemical Oxygen Demand Concentration	Calculated as $(M_T - M_S)$
S	Volatile Suspended Solids Concentration	Direct Measurement
N_{TK}	Total Kjeldahl Nitrogen Concentration	Direct Measurement
N_{SK}	Soluble Kjeldahl Nitrogen Concentration	Direct Measurement
N_A	Ammonia Nitrogen Concentration	Direct Measurement
N	Particulate Nitrogen Concentration	Calculated as $(N_{TK} - N_{SK})$
%N	Nitrogen Content of Particulate Material expressed as a percentage of S	Calculated as 100 (N/S)
P_T	Total Phosphorus Concentration	Direct Measurement
P_S	Soluble Phosphorus Concentration	Direct Measurement
P	Particulate Phosphorus Concentration	Calculated as $(P_T - P_S)$
%P	Phosphorus Content of Particulate Material expressed as a percentage of S	Calculated as 100 (P/S)
LI	Particulate Lipid Concentration	Direct Measurement
%LI	Particulate Lipid Concentration expressed as a percentage of S	Calculated as 100 (LI/S)
%PR	Particulate Protein Concentration expressed as a percentage of S	Calculated as 6.25 (%N)
%CA	Particulate Carbohydrate Concentration expressed as a percentage of S	Calculated as (100-%LI-%PR)

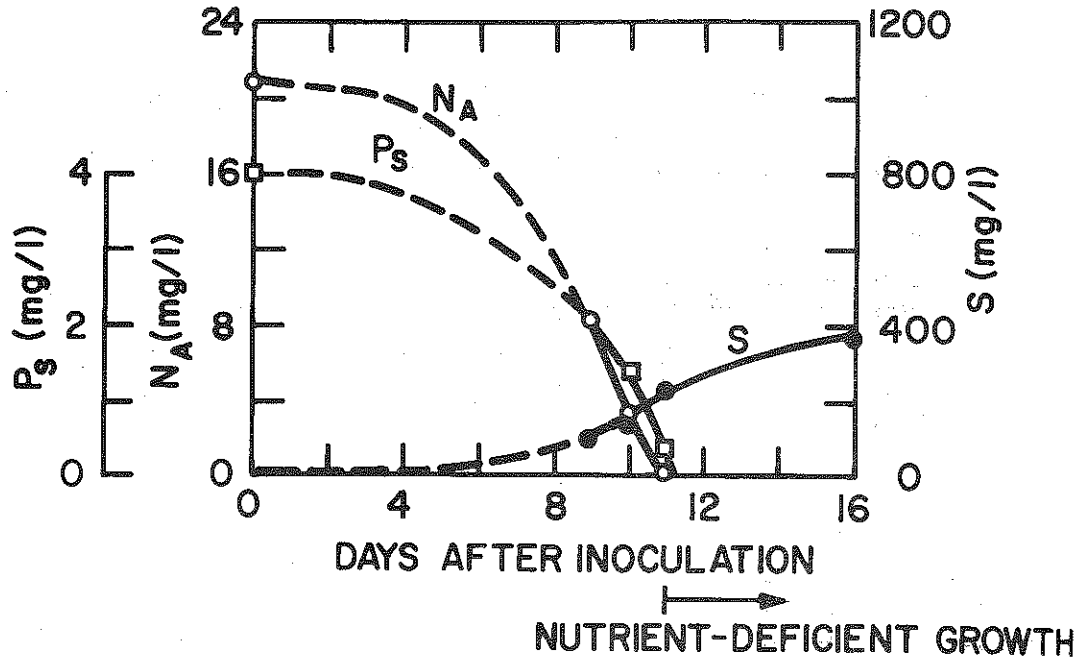

FIGURE 5.1. Uptake of Nitrogen and Phosphorus by Chlorella.

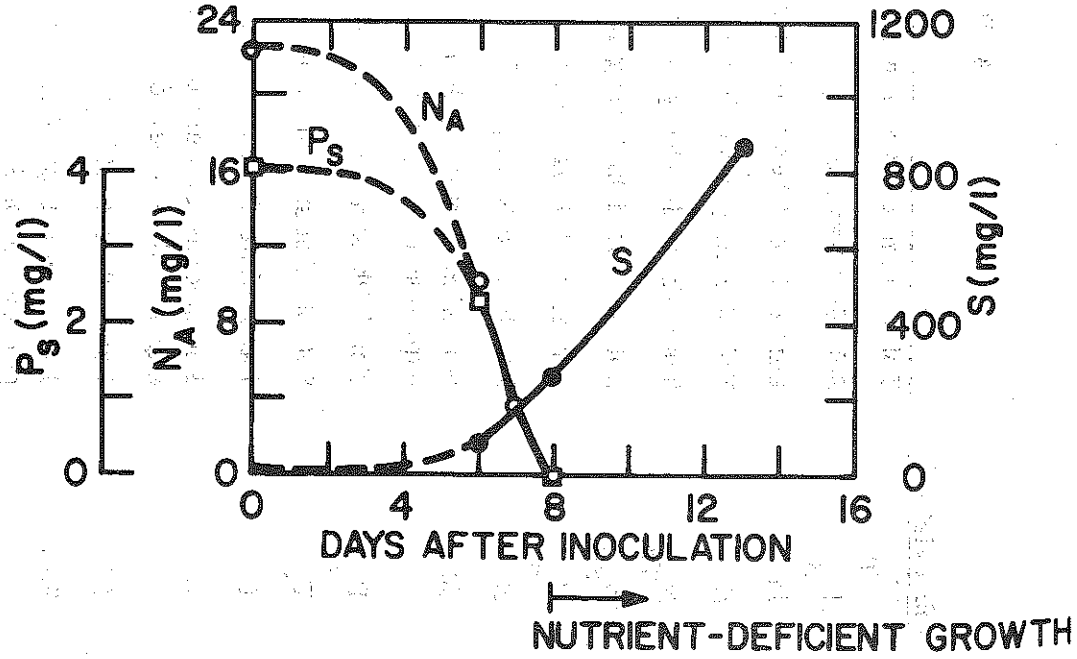

FIGURE 5.2. Uptake of Nitrogen and Phosphorus by Scenedesmus.

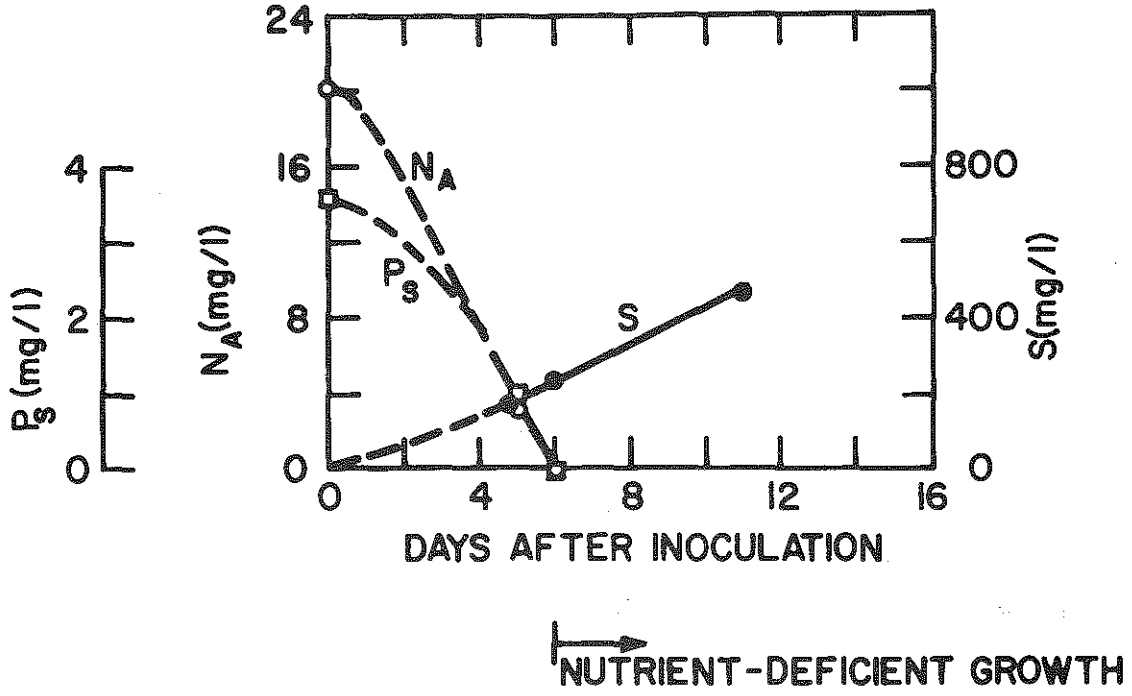

FIGURE 5.3. Uptake of Nitrogen and Phosphorus by Anabaena.

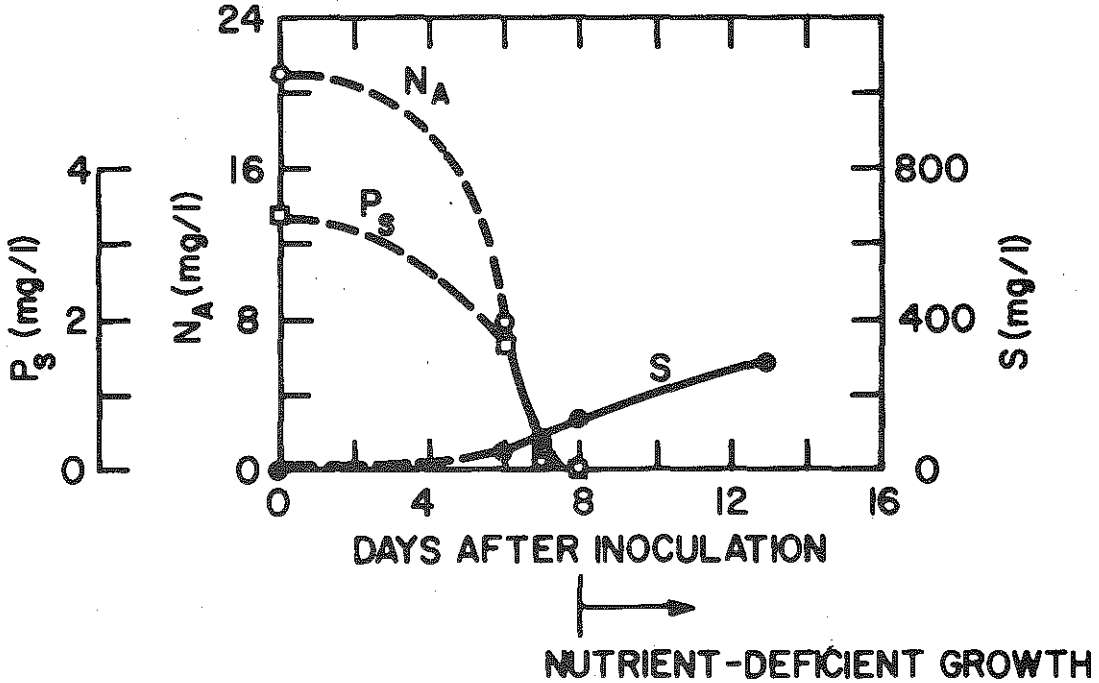

FIGURE 5.4. Uptake of Nitrogen and Phosphorus by Kentucky Mixed.

in solution were depleted simultaneously as expected. Growth subsequent to the exhaustion of ammonia nitrogen in the growth medium was defined as "nutrient-deficient" growth, as indicated in Figures 5.1-5.4. Growth prior to the exhaustion of nutrients from solution was defined as "nutrient-abundant." Indications are that the true nutrient-deficient growth phase began, not when the nitrogen and phosphorus were exhausted from solution in the growth medium, but after the nutrients stored during growth in the nutrient-abundant growth phase were converted into algal protoplasm, mainly in the form of protein. In reality, the stored nitrogen and phosphorus were converted into protein very shortly after the growth medium was void of these nutrients and for all practical purposes the nutrient-deficient growth phase began when nitrogen and phosphorus were exhausted from the growth medium.

To verify that ammonia nitrogen was the principal nitrogen constituent of the growth medium, analysis was made for nitrate nitrogen. This provided a check to determine if any of the ammonia nitrogen had been converted by "nitrifying" autotrophic bacteria to nitrate which still remained in solution in the growth medium. The nitrate analysis revealed little or no nitrate in solution at any time throughout the study period. Hence, it appeared that the algae directly incorporated the ammonia nitrogen into their cells and if any ammonia was converted to nitrate, it too was immediately incorporated into the cells.

Miller (26) reported that algal cell division can continue slowly for some time in a medium entirely depleted of nitrogen. Gerloof and Skoog (30) reported critical values of 4.0% and 0.12% for cellular nitrogen and phosphorus contents, respectively, for Microcystis aeruginosa. They indicated that when these levels are reached cell division ceases and the growth rate decreases, implying that the cellular nutrient content controls cell division and growth rate. Conversely, Jewell and McCarty (1) indicated that cell division stops when the nutrients are exhausted from solution and growth continues as an increase in cellular mass with resulting changes in the organic composition. Jewell and McCarty's concept is more generally accepted, i.e., the nutrient-deficient growth phase is characterized by algal growth without cell division.

Most researchers generally have assumed that all nitrogen taken into an algal cell is converted directly into protein, i.e. protein nitrogen is 100% of the cellular nitrogen, and it has been established that complex protein is approximately 16% nitrogen by weight. Hence, the percent by weight of algal protein can be calculated as 6.25 (= 100/16) times the percent by weight of cellular nitrogen. This method of calculation is applicable in instances when nitrogen is converted directly to protein, but not for certain stages of growth during which nitrogen is stored and converted to protein at a later time. Each algal species exhibited different nitrogen storing characteristics and only by examination of different cultures can insight be gained into this phenomenon.

1. Chlorella. The variation in cellular nitrogen content during both the nutrient-abundant and nutrient-deficient growth phases for the Chlorella culture is shown in Figure 5.5. Using the nitrogen contents in Figure 5.5, the protein contents of the algal cells during the nutrient-abundant growth phase calculated as 6.25 x %N were 68.8, 62.5, and 65.6. The corresponding weight percentage of lipids were measured to be 45.0, 62.2, and 50.0. Summing the above values of only protein and lipids gives totals greater than 100%, which are obviously impossible. A minimum carbohydrate content of about 10% would be expected (see Table 2.4) which indicates a maximum sum of lipid plus protein of about 90%. Therefore, the protein content as calculated by 6.25 times the weight percentage of nitrogen was too high, indicating that all nitrogen taken into the algal cell during nutrient-abundant growth was not converted directly to protein. This suggests three regimes of nitrogen uptake: (1) the cells only take in enough to sustain a minimum concentration necessary for growth; (2) the cells take in more nitrogen than is necessary to sustain growth but convert the nitrogen directly into protein; and (3) the cells take in nitrogen over that necessary for growth and store some of it rather than converting it directly to protein. This second type of uptake is usually defined as "luxurious" uptake and for this study the third type of uptake will be defined as "super-luxurious". The first type of uptake would be manifested by constant cellular nitrogen content during growth and was not observed for any of the cultures in this study. The data in Figure 5.5 indicate that for nitrogen contents above

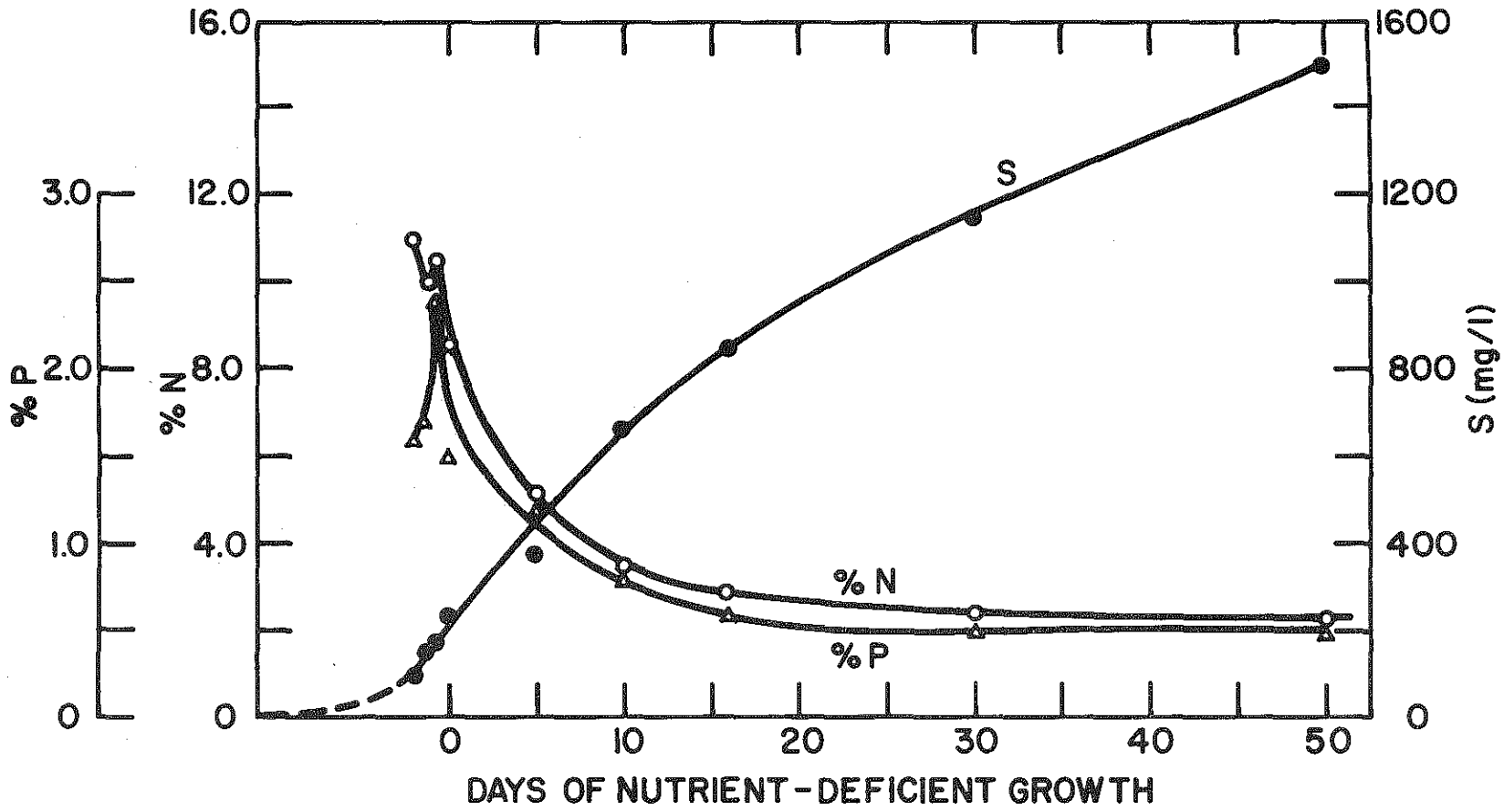

FIGURE 5.5. Variation in Mass and Cellular Nitrogen and Phosphorus for Chlorella.

approximately 9%, super-luxurious uptake occurred and some of the nitrogen taken into the cell was stored and not converted directly to protein. However, once the nutrients were exhausted from the growth medium, the algae apparently converted the stored nitrogen into protein. After this conversion was completed the model for calculating the protein content was applicable.

Fowden (18) found that young cultures of Chlorella, growing under optimum conditions, contained nearly 10% nitrogen, while in old nitrogen-deficient cultures the total nitrogen content rarely exceeded 2% and values of less than 1% were not uncommon. For a 90-day growth period Jewell and McCarty (1) found nitrogen contents of 3.5% for the green alga, Chlamydomonas, 1.9% for Chlorella, and 1.3% for the diatom, Nitzschia colosterium. Foree and McCarty (9) observed a wide range in the nitrogen content of Chlorella depending primarily on the length of the growth period under batch culture conditions, 34 and 48-day growth periods yielded nitrogen contents of 8.2% and 1.8%, respectively. The minimum nitrogen content for the Chlorella culture as shown in Figure 5.5 was 2.3%.

The minimum cellular phosphorus content shown in Figure 5.5 for Chlorella was 0.5%. Foree and McCarty (9) found phosphorus contents of 0.7% and 0.4% for Chlorella after a 48-day growth period. Jewell and McCarty (1) reported values of 0.5% for Chlamydomonas and 0.2% for Chlorella after a 90-day growth period. Borchardt and Azad (10) have defined three phosphorus regimes for Chlorella and Scenedesmus; from 0 to 1% cellular phosphorus,

growth is dependent upon the actual phosphorus content; from 1 to 3%, growth is independent of the phosphorus content and phosphorus is stored in the algal cells; and above 3%, growth is independent of the phosphorus content and the cell is saturated with respect to phosphorus. These authors defined "luxury uptake" of phosphrous as that incorporated into algal cells above the critical level of 1.0%. However, these values were determined for algal growth when phosphorus was available in solution in the growth medium and, therefore, do not apply to growth under nutrient-deficient conditions as defined for this study.

2. Scenedesmus. The cellular nitrogen and phosphorus contents of this culture are shown in Figure 5.6. The rather high nitrogen contents during the nutrient-abundant growth phase suggested the super-luxurious uptake and corresponding storage of nitrogen as was found for Chlorella. However, coupled with these high nitrogen contents and the corresponding high calculated protein contents were low measured lipid contents. The sum of the lipid and protein contents was high but always enough less than 100% to allow for the minimum expected carbohydrate content. For the Scenedesmus culture, at nitrogen contents as high as 12.5%, all nitrogen taken into the cell was apparently directly converted to protein and, therefore, this alga apparently had little or no ability to store nitrogen during growth under nutrient-abundant conditions. This was possibly responsible for the more rapid growth during the nutrient-abundant growth phase than was observed for the Chlorella and Anabaena cultures.

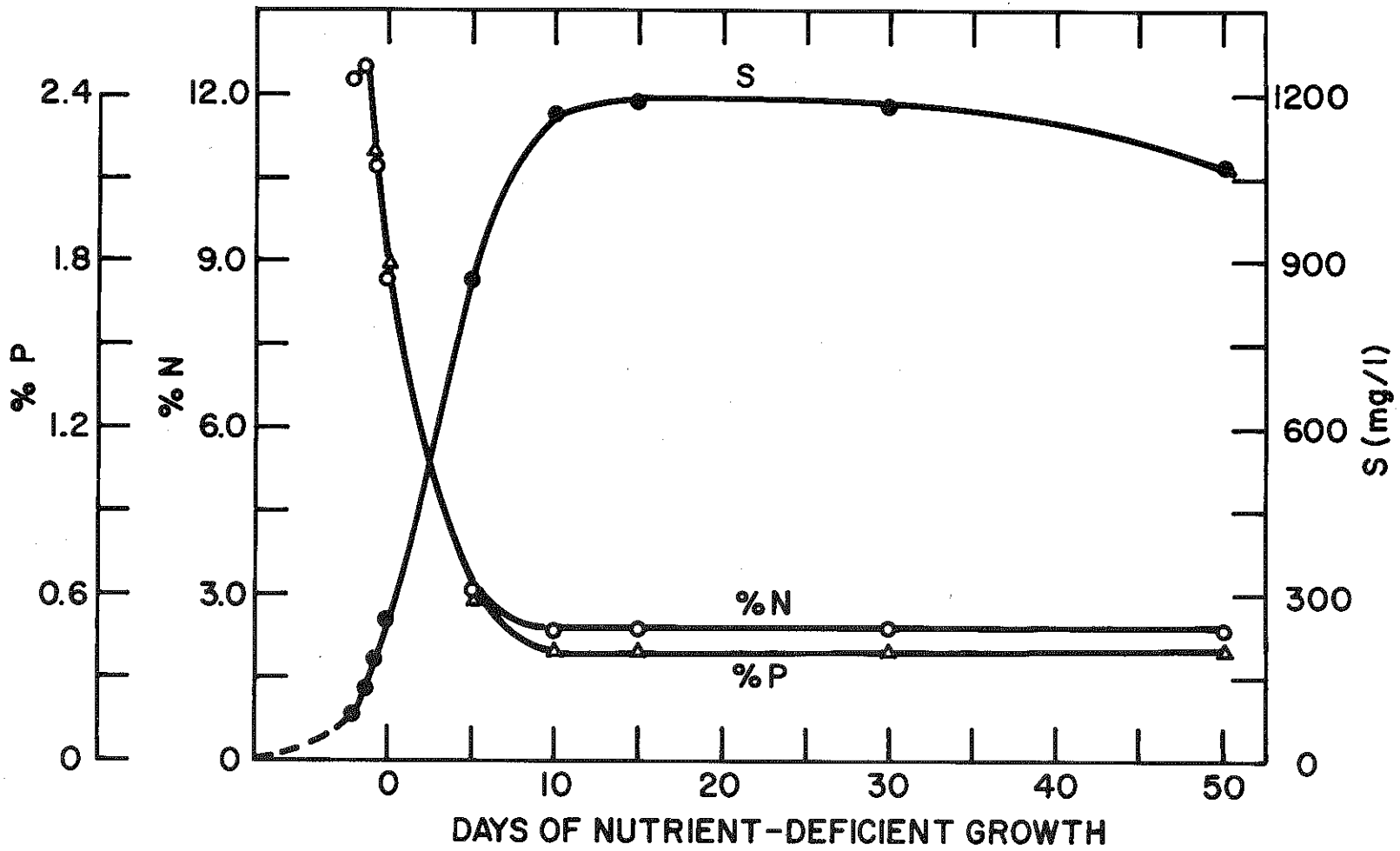

FIGURE 5.6. Variation in Mass and Cellular Nitrogen and Phosphorus for Scenedesmus.

The rapid growth rate continued into the nutrient-deficient growth phase and after day 10 began to decrease as shown in Figure 5.6. At this time the percentages of cellular nitrogen and phosphorus reached values of 2.4 and 0.4, respectively, and remained approximately at these levels throughout the remainder of the study period. For Scenedesmus, Jewell and McCarty (1) found nitrogen and phosphorus contents of 4.4% and 0.8%, respectively, after a 29-day growth period and 1.1% and 0.2%, respectively, after an 87-day growth period.

After 15 days of nutrient-deficient growth the algal mass began to decrease, as shown by the volatile suspended solids (S) curve in Figure 5.6, probably due to death of the old algal cells and subsequent autolysis and/or bacterial decomposition. This phenomenon was not observed in the other two unialgal cultures studied.

3. Anabaena. The nitrogen curve in Figure 5.7 shows a large nitrogen uptake with little corresponding growth during the nutrient-abundant growth phase for the Anabaena culture. Such a large uptake of nitrogen occurred during this period that calculation of the weight percentage of protein as 6.25 times the weight percentage of nitrogen yielded a protein content greater than 100%. This indicated the same super-luxurious uptake and corresponding storage of nitrogen in the Anabaena cells as was found for Chlorella, except on a more pronounced scale. At the beginning of the nutrient-deficient growth phase the protein content was calculated as 86.9% which added to a measured

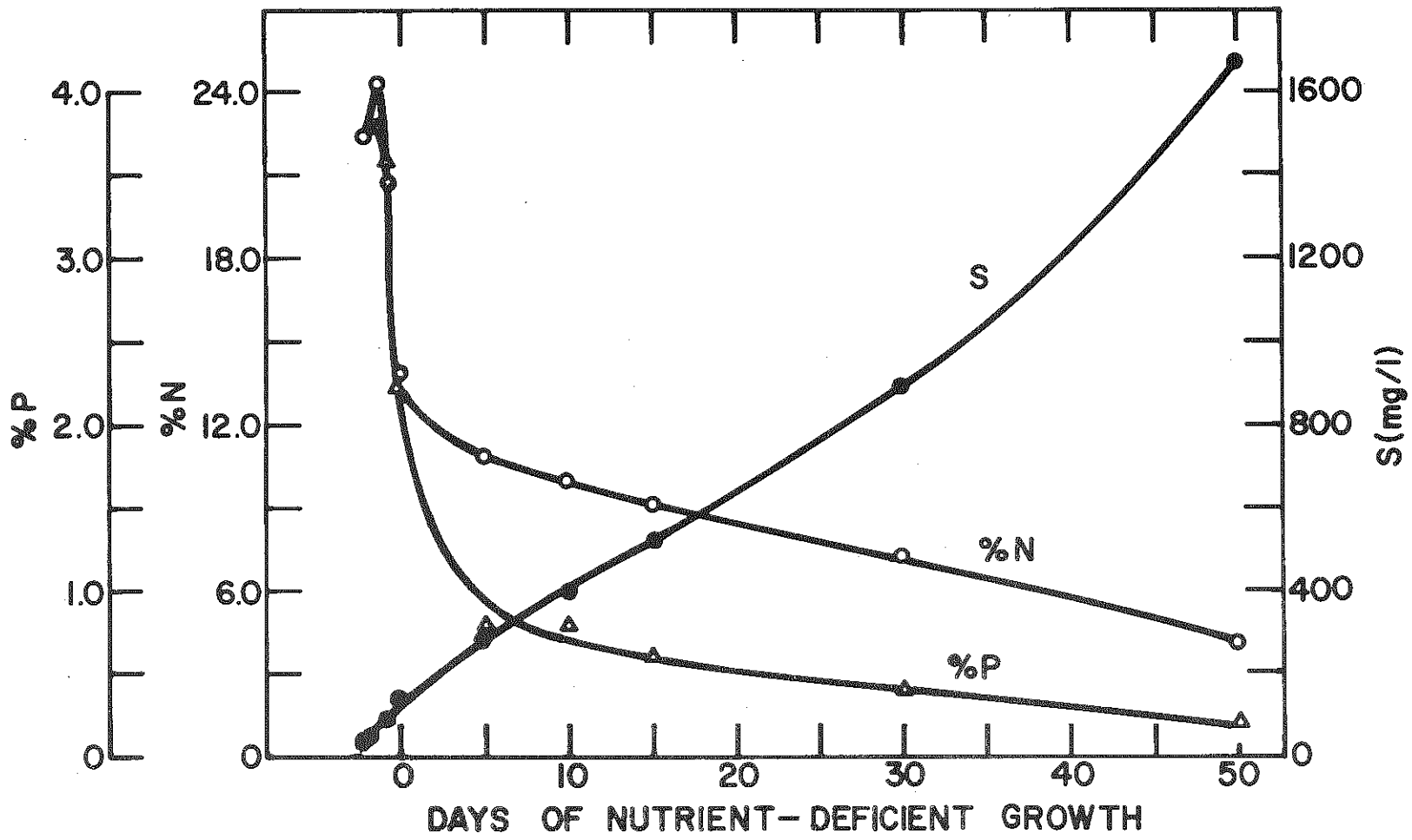

FIGURE 5.7. Variation in Mass and Cellular Nitrogen and Phosphorus for Anabaena.

lipid content of 42.3% gave a value greater than 100%. Therefore, even after the nutrient-deficient growth phase began, some of the stored nitrogen had not been converted to protein. However, after 5 days of nutrient-deficient growth, most of the nitrogen had apparently been converted to protein.

Fixation of atmospheric nitrogen occurred once the nitrogen was exhausted from the growth medium and caused the nitrogen content of the Anabaena culture to decrease at a slower rate than observed in the two green algal cultures previously described. This nitrogen fixation allowed protein synthesis to continue during the nutrient-deficient growth phase. Thus, the increase in volatile suspended solids (S) throughout the growth period as shown in Figure 5.7 was probably due to both algal cell division and lipid and/or carbohydrate synthesis.

For the Anabaena culture the cut-off between luxurious uptake, where all nitrogen is converted to protein, and super-luxurious uptake, where some nitrogen is stored and later converted to protein, appeared to be at a cellular content of approximately 12%. As can be seen in Figure 5.7, this cellular nitrogen content occurred about 3 days after the onset of the growth phase defined as nutrient-deficient. At the end of 50 days of nutrient-deficient growth, the nitrogen content of the cells had decreased to 4.1%. Foree and McCarty (9) observed nitrogen contents of 8.9% and 7.4% for Anacystis and Anabaena, respectively, after approximately 49 days of growth in batch culture.

The phosphorus curve in Figure 5.7 shows a large decrease in cellular phosphorus content at the beginning of the study, followed by a gradual decrease over the last 45 days. During the nutrient-abundant growth phase cellular phosphorus contents of 3.8% and 3.6% were observed. These values are above the maximum of 3% given by Borchardt and Azad (10). However, this 3% was determined for the green algae, Chlorella and Scenedesmus, and probably does not generally apply to other algal species. At the end of the 50-day growth period, the cellular phosphorus content had decreased to 0.2%. Foree and McCarty (9) found phosphorus contents of 0.8% and 1.2% for Anabaena and Anacystis, respectively, after 49 days of growth under batch culture conditions.

4. Kentucky Mixed. At the end of the study all four cultures were checked for the presence of animals by microscopic observation. Animals were found only in the Kentucky Mixed culture. Here a large population of rotifers along with a variety of other microscopic animals was noted. The influence of this animal population on various growth parameters is shown in Figure 5.8.

During the nutrient-abundant growth phase, the highest value of cellular nitrogen recorded was 8.6%, corresponding to a protein content of about 54%, which was in the range of protein values normally found for this type of growth. The relatively low protein content, as compared with that of the three previously discussed cultures, corresponded to a large initial growth rate and indicated little, if any, super-luxurious uptake of nitrogen. However, it must be noted that the nutrient-abundant growth phase was observed for a shorter time in

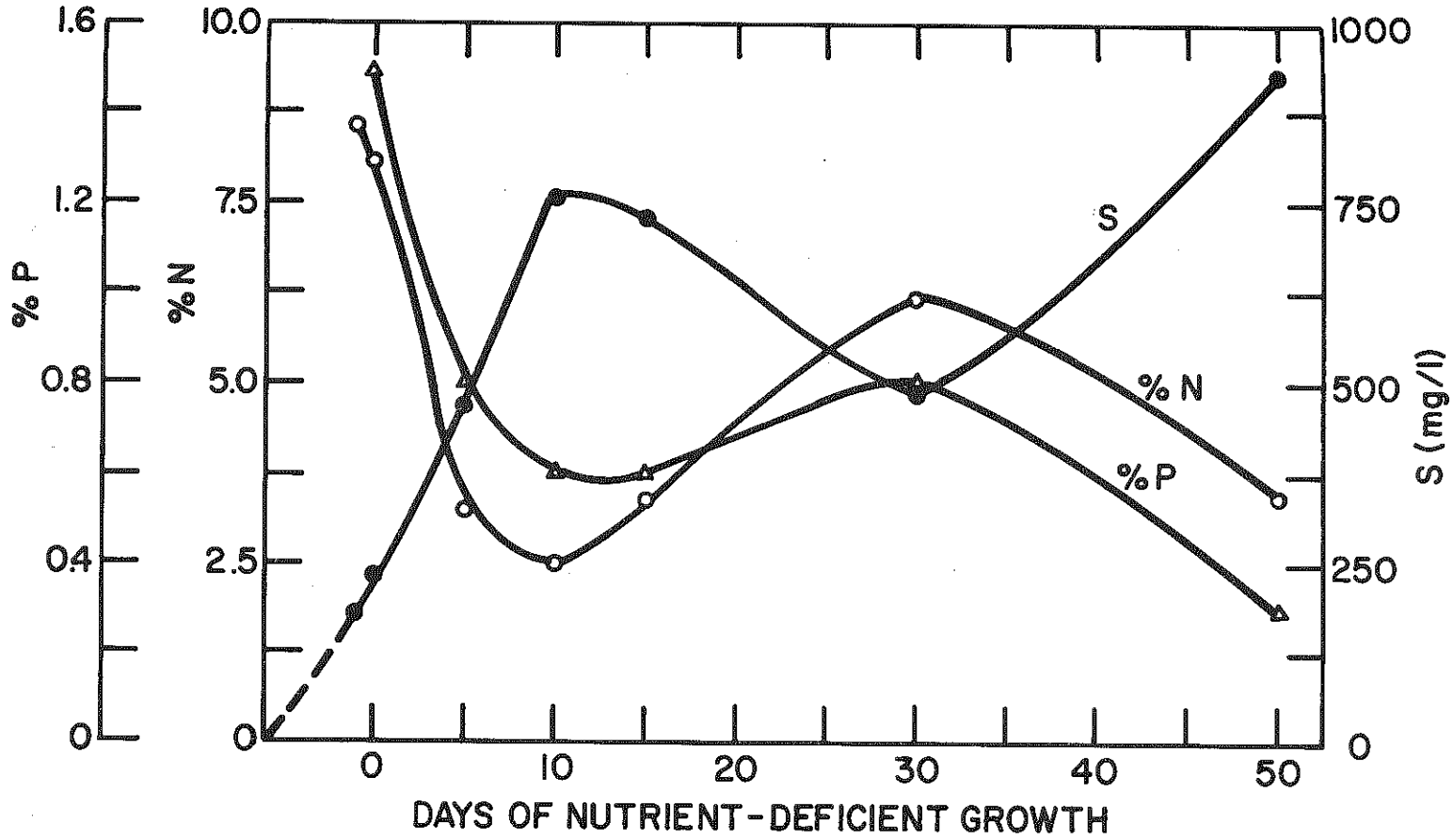

FIGURE 5.8. Variation in Mass and Cellular Nitrogen and Phosphorus for Kentucky Mixed.

this culture and super-luxurious uptake could have occurred very early before quantitative observation began.

After approximately 10 days of nutrient-deficient growth, the algal mass turned from its typical green color to yellowish-brown due to aging of the algal species which was then predominant in the mixed culture. After approximately an additional 20 days of growth in this state, the mass again turned green when conditions became favorable for growth of a different algal species. The favorable growth conditions came as a result of a release of nutrients into solution caused by autolysis or decomposition of the algal cells by animal predators. This same effect had been observed by Jewell and McCarty (1). The rotifers and other predators were mainly responsible for the wide variations in the parameters shown in Figure 5.8. By consuming the algal cells and releasing the resultant waste products into solution, they were responsible for the decrease in mass as shown by the dip in the volatile suspended solids (S) curve in Figure 5.8. The nitrogen and phosphorus contents of animal cells are generally higher than those of the algae in the culture at this time. As the algal cells were metabolized by the decomposers, the liberated nitrogen and phosphorus were converted into animal cellular matter, thus accounting for the rise in the percentage of cellular nitrogen and phosphorus of the mixed culture during the period when the mass was declining.

After 30 days of nutrient-deficient growth and the corresponding algal population change, the culture mass again began to increase with a

corresponding decrease in the cellular nitrogen and phosphorus contents. The nutrient content of the cells at the end of the study had little significance because it clearly depended on the time of sampling relative to the time of population change and would more than likely be different for each mixed culture studied.

C. Organic Composition

The three major constituents of the organic matter of an algal cell are protein, lipid, and carbohydrate. If lipid content is measured directly and protein content is calculated as a function of cellular nitrogen content as previously described, then carbohydrate content can be calculated with reasonable accuracy as (100% - % lipids - % proteins). Due to the uncertainty of calculating the protein content during the nutrient-abundant growth phase as previously indicated, the organic compositions presented in this section are only for the nutrient-deficient growth phase. This is not meant to imply that the model for calculating the protein content automatically becomes accurate once the nutrients are depleted from solution. However, once nutrient-deficient growth begins, stored nitrogen will be converted to protein relatively rapidly, thus increasing the accuracy of the model as nutrient-deficient growth progresses.

Fisher (38) found that Chlorella and some other unicellular algae contained 45-50% protein when grown with an adequate nitrogen supply, and under conditions of nitrogen starvation algae could be grown with a lipid content as high as 86% as compared with 20-25% in normal Chlorella. Von Witsch and

Harder (39) concluded from studies with Chlorella, Scenedesmus, and the diatom, Nitzschia palea, that after cessation of cell division almost all the absorbed radiation was used for formation of fat, but this fat storage (up to 70% of the dry weight) was not produced at the expense of the rest of the cellular organic matter. Milner (19) similarly concluded that the actual mass of protein in an algal culture did not appear to change much as the cells went from the normal 50%-protein, 20%-lipid state to a high-lipid, low-protein state. It may therefore be generally concluded that during the nutrient-abundant growth phase algae synthesize mainly proteins, but after protein formation is complete they synthesize fats and/or carbohydrates which add to the mass of the cell resulting in a decrease in protein content on a weight percentage basis.

Two parameters used as measures of the amount of organic material present in the particulate fraction of an algal culture were chemical oxygen demand (M), the amount of oxygen necessary to oxidize the organic matter to carbon dioxide, water, and ammonia; and volatile suspended solids (S), the weight of the organic mass. Foree and McCarty (40) derived the following theoretical M:S ratios for the three main constituents of organic matter: carbohydrate $(CH_2O)_x$-1.07, protein $(C_{3.96}H_{6.12}O_{1.26}N_{1.00})$ - 1.50, and lipid $(C_{18}H_{32}O_2)$-2.86. Therefore, the relationship between the measured M and S curves should be a function of which organic constituent was predominantly being synthesized by the algal cells. If lipids alone were being synthesized, the M curve should rise much faster than the S curve; if only proteins were being

synthesized, the M curve should rise faster than the S curve but not as fast as for lipids; and if carbohydrates were being predominantly synthesized, the M and S curves should be almost parallel. The relationships between the M and S curves presented later generally closely follow the theoretical pattern described above.

Lipid and/or carbohydrate synthesis during the nutrient-deficient growth phase depended primarily upon the algal species and is discussed on a culture-to-culture basis below.

1. Chlorella. The variations over the study period of particulate COD, volatile suspended solids, cellular nitrogen, and lipid concentration in mg/l are shown for the Chlorella culture in Figure 5.9. The lipids (LI) curve in this figure shows a high rate of lipid accumulation relative to the organic mass as measured by volatile suspended solids. The effect of the previously discussed high M:S ratio for lipids is vividly illustrated by the divergence of the M and S curves corresponding to lipid accumulation in this culture. The figure also shows a steady rise in the cellular nitrogen concentration (N), which reached a value above that originally in the growth medium. This was attributed to a noted evaporation of water from the culture vessel throughout the growth period.

The protein, lipid, and carbohydrate fractions for the Chlorella culture are shown in Figure 5.10. At the beginning of the nutrient-deficient growth phase the protein content was about 53%. It then fell off rapidly for

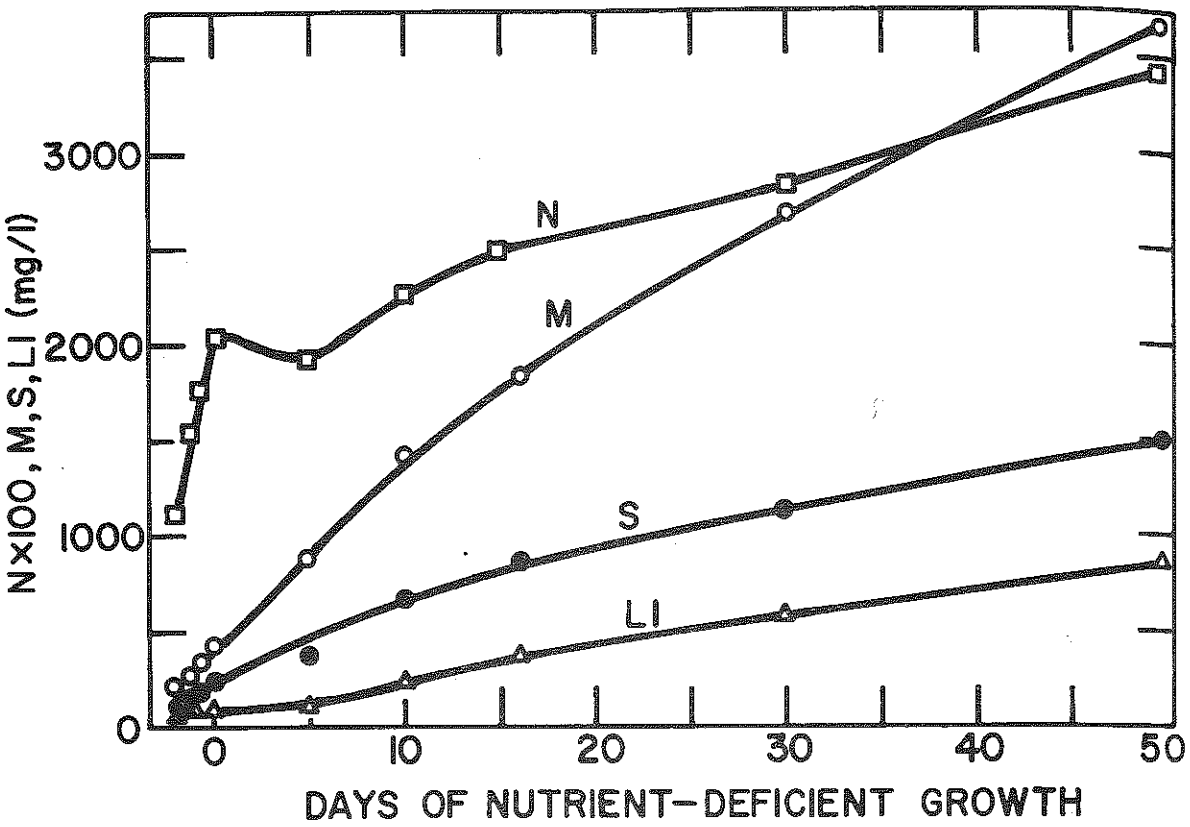

FIGURE 5.9. Variation in Growth Parameters for Chlorella.

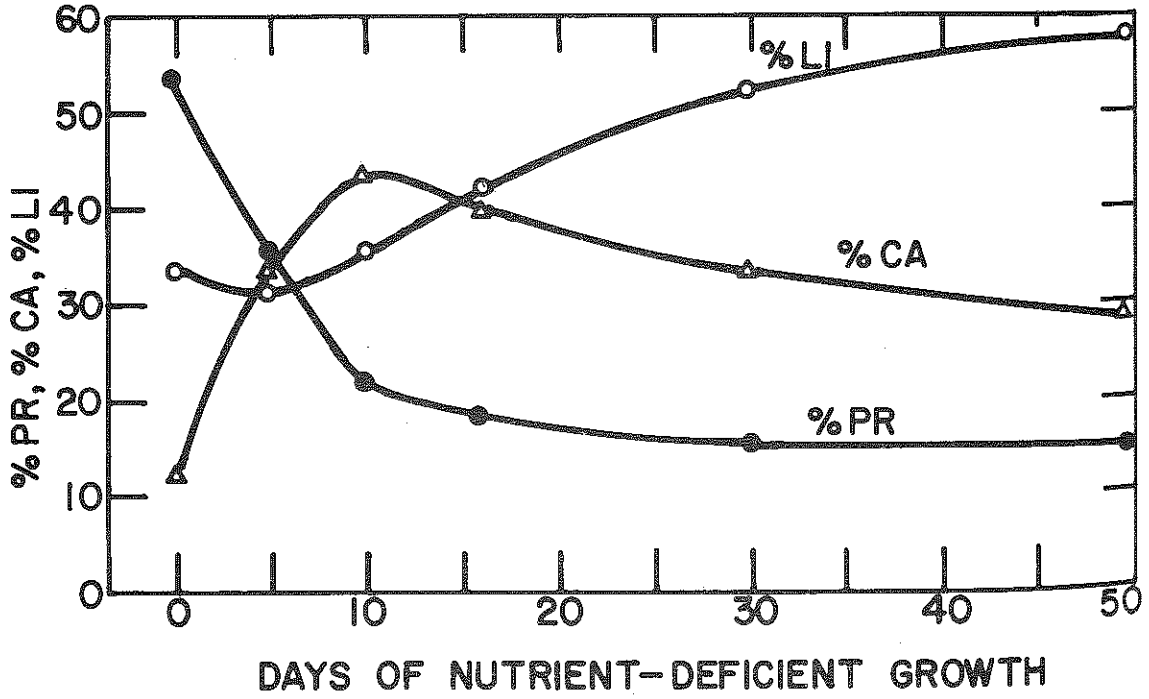

FIGURE 5.10. Variation in Organic Composition of Chlorella.

the next 10 days and gradually decreased to 14% at the end of 50 days. The rapid decrease in protein content during the first 10 days corresponded to the large carbohydrate increase (from 12% initially to 43% after 10 days). After day 10 the relatively high carbohydrate content started to decrease due to lipid synthesis, which began about day 5 and continued throughout the remaining 45 days, reaching a value of 57%. As indicated previously, this relatively large build-up of lipids under nutrient-deficient conditions is characteristic of Chlorella (19, 38, 39).

Near the end of the 50-day growth period, the Chlorella cells had changed from a bright green to a greenish-yellow color due to a decrease in chlorophyll content in the old cells. This same effect has been noted by Gromov (17) who found that the chlorophyll content of algal cells and their photosynthetic capacity decreased during prolonged exposure to a nitrogen-free medium. Syrett (41) also found that as nitrogen deficiency developed, the amount of chlorophyll in the cells decreased and photosynthesis fell, though not simply as a consequence of the drop in chlorophyll content.

2. Scenedesmus. The growth parameters for this culture are shown in Figure 5.11. The M and S curves in this figure show that maximum growth occurred during the first 15 days of nutrient-deficient growth and after this time the culture mass remained relatively constant, except for a small decrease in organic mass after 15 days apparently due to death and decomposition of the cells. The Scenedesmus culture was unlike the Chlorella culture in that

it synthesized almost no lipid material throughout the period of observation (see Figure 5.11).

The cellular nitrogen concentration curve in Figure 5.11 shows the large nitrogen uptake prior to the nutrient-deficient growth period and an increase in concentration after the nutrient-deficient growth phase began. Since this increase raised the nitrogen concentration above that originally in the growth medium and since Scenedesmus is not a known nitrogen fixer, it was attributed to evaporation of water from the culture vessel.

The organic compositions for the Scenedesmus culture are shown in Figure 5.12. At the beginning of the nutrient-deficient growth phase the culture was approximately 50% protein- 50% carbohydrate. During the first 5 days, when the growth rate was maximum, the algal cells synthesized a large amount of carbohydrate material causing an increase in the carbohydrate content and a corresponding decrease in the protein content as shown in Figure 5.12. Between days 5 and 15, the rate of carbohydrate synthesis decreased corresponding to a declining growth rate as shown in Figure 5.11. The protein and carbohydrate fractions remained relatively constant for the last 35 days of the study during which a small amount of decomposition took place.

The lipids curve in Figure 5.12 shows that also on a percentage basis there was very little lipid material synthesized at any time. Therefore, Scenedesmus did not synthesize lipids under nutrient-limiting conditions as

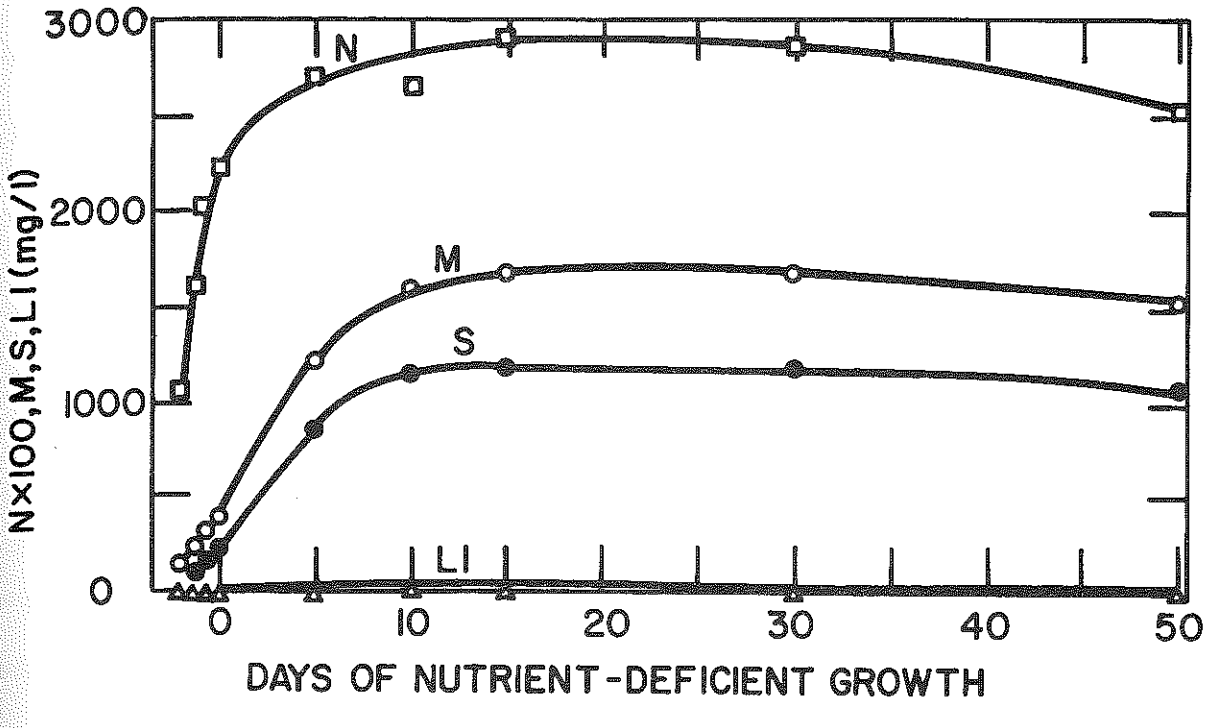

FIGURE 5.11. Variation in Growth Parameters for Scenedesmus.

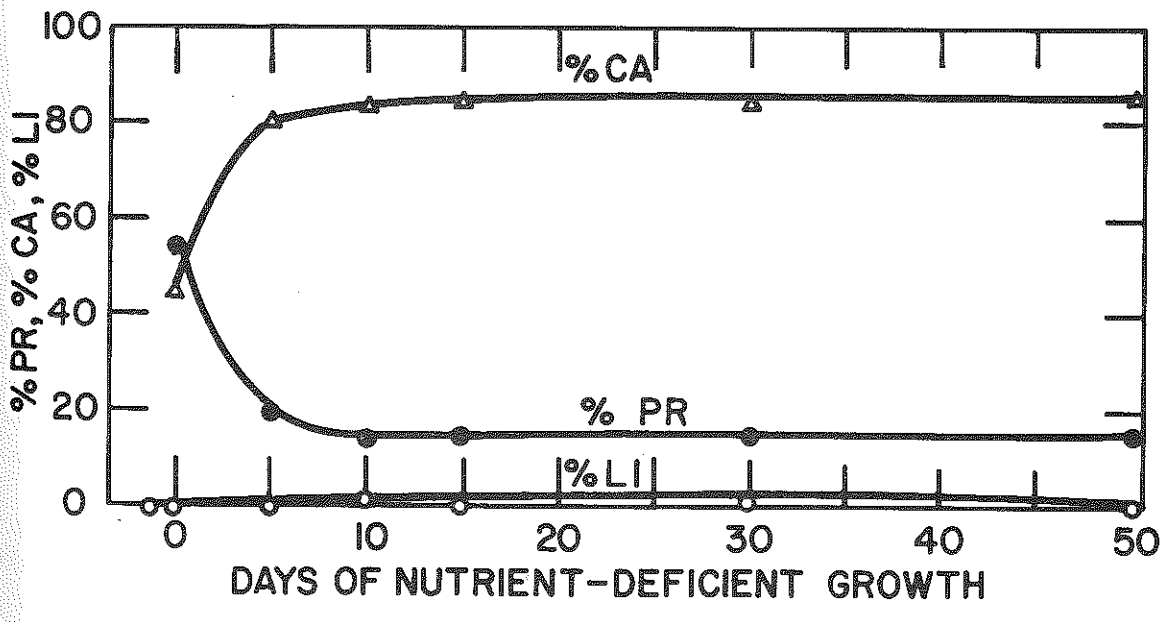

FIGURE 5.12. Variation in Organic Composition of Scenedesmus.

did Chlorella, but synthesized large amounts of carbohydrate material which amounted to 85% of the organic material present. The carbohydrate and lipid results during the last 40 days of the study agreed with those of Gromov (17) who observed no significant changes in carbohydrate and lipid content of Scenedesmus quadricauda during growth in a nitrogen-deficient medium. Toward the end of the 50 day growth period, the cells of this culture also became yellowish in color due to the decrease in chlorophyll content.

3. Anabaena. The growth parameters for this culture are shown in Figure 5.13. The nitrogen curve shows that fixation of atmospheric nitrogen by Anabaena began about the time nitrogen was exhausted from solution in the growth medium and continued at a rapid rate for 30 days. At this time the rate of fixation abruptly decreased, probably due to the exhaustion from solution of one or more trace elements, such as cobalt, molybdenum, or boron (3), known to be necessary for nitrogen fixation, or possibly due to inhibition of light penetration by a gelatinous substance which was observed in the growth medium at this time. The organic mass as measured by M and S increased at a fairly constant rate until day 30, then began to increase at a much faster rate. The lipid concentration, which had generally been increasing began to decline after day 30 and by the end of the 50-day growth period had been reduced to near zero.

The rather unusual behavior of the observed parameters which began at day 30 can be explained by examination of the organic compositions shown in

Figure 5.14. At day 30, the Anabaena cells began a rapid build-up of carbohydrates and a decrease in lipids on a percentage basis due to conversion of lipid material to carbohydrate material or due to the lipid material being burned up in respiration. The carbohydrate content at the end of the study was 74% and still increasing.

Observation of the growing culture at day 30 revealed that the culture medium had begun to change in viscosity and was becoming jelly-like. By day 50 there had been a definite change in the viscosity of the medium and the culture was very difficult to sample. The viscosity change is thought to be due to the sloughing-off of the gelatinous sheath surrounding the cells and the production of extracellular polysaccharides by the Anabaena cells. The production of extracellular substances by blue-green algae was noted by Fogg (14), who found that from 5-60% of the nitrogen fixed by Anabaena cylindrica appeared extracellularly in combined form. Lewin (15) observed this same phenomenon in some species of green algae and reported that as laboratory cultures aged, the mucilage content became so high as to change the viscosity of the growth medium.

The Anabaena culture in this study was continuously sparged with a CO_2-air mixture and, as previously shown, a large fixation of atmospheric nitrogen occurred. This is contrary to the belief of Prescott (24), who stated that experiments have shown that nitrogen will not be fixed if there is an unlimited or fully adequate supply of CO_2 or hydrogen available to the algae.

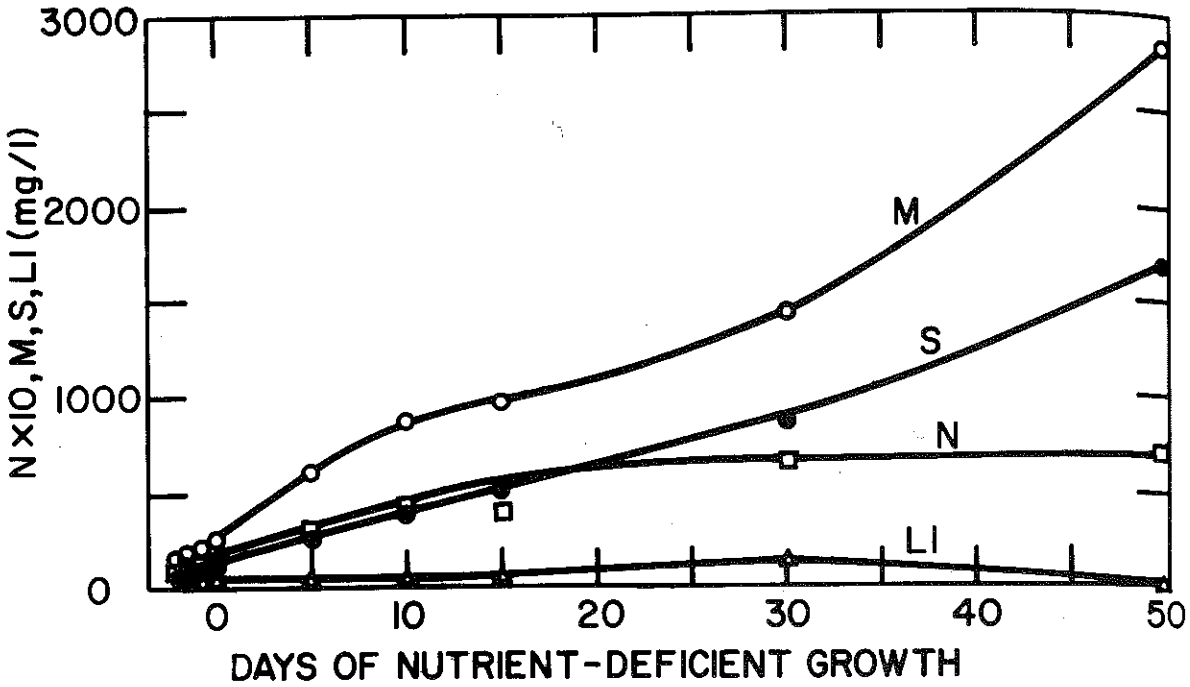

FIGURE 5.13. Variation in Growth Parameters for Anabaena.

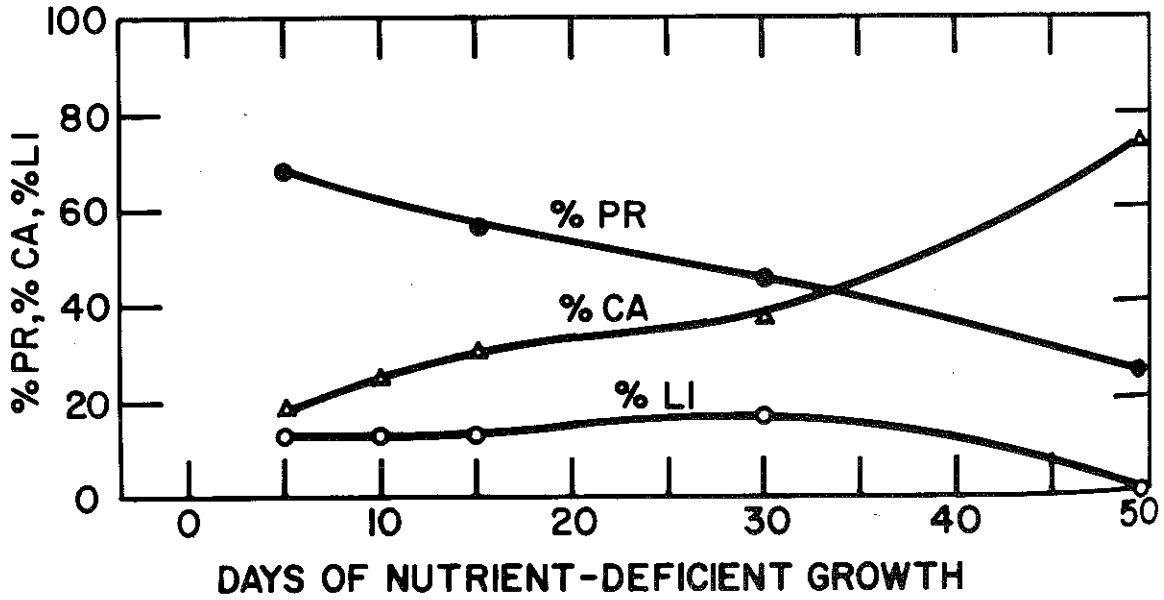

FIGURE 5.14. Variation in Organic Composition of Anabaena.

The nitrogen value of the day 15 sampling of the Anabaena culture was considerably below the curve as drawn in Figure 5.13. Examination of the data which led to this point revealed that this data point was almost certainly inaccurate and should be neglected. The nitrogen values shown previously in Figure 5.7 and the protein values in Figure 5.14 have been determined by neglecting this data point.

4. Kentucky Mixed. The curves in Figure 5.15 show the pronounced effects which the previously discussed population change had on the growth parameters of the culture. The culture mass reached a maximum after 10-15 days of nutrient-deficient growth much like the Scenedesmus culture, decreased from there until day 30, and then again began to increase due to the population change. The population change also exerted a rather large effect on the lipid concentration of this culture (see Figure 5.15). The lipid concentration increased and reached a maximum after 15 days of growth, then decreased to near zero indicating that the decomposers had metabolized nearly all of the lipid material. After day 30, the newly predominating algal species began to synthesize lipid material, hence the lipid increase shown in Figure 5.15 for this period.

The cellular nitrogen curve in Figure 5.15 shows that some nitrogen was released into solution during the first 5 days of nutrient-deficient growth but by day 10 the cellular nitrogen concentration had increased to approximately that of the growth medium. After day 10 the rather large increase in nitrogen

concentration was probably due to some nitrogen fixation by one or more algal species in the mixed culture. However, this fixation, which seemed to occur between days 15 and 30, was much less than that which occurred in the Anabaena culture previously discussed. The small increase in nitrogen concentration after day 25 was probably due to evaporation of water from the culture vessel.

The variations in organic composition of the mixed culture are shown in Figure 5.16. The wide variation in organic content throughout the study was due to the variation of biological species in this culture. During the first 5 days of nutrient-deficient growth, a large carbohydrate build-up, coupled with a large protein decrease and a smaller lipid decrease, occurred. After day 5 the algal cells began synthesizing both lipids and proteins, causing a decrease in the carbohydrate fraction. The protein synthesis can be attributed to algae capable of fixation of atmospheric nitrogen and thus ceased after day 30 when nitrogen fixation stopped. The algal species predominating in the nutrient-deficient medium was responsible for the lipid synthesis which occurred between days 5 and 15. The decrease in lipid content between days 15 and 30 was due to the action of the animals on the algae in the culture. The predomination of the new algal species caused the increase in lipid content and corresponding decrease in protein content after day 30.

After 15 days of nutrient-deficient growth, the carbohydrate fraction had reached a minimum and the initial growth of the culture was maximum. During

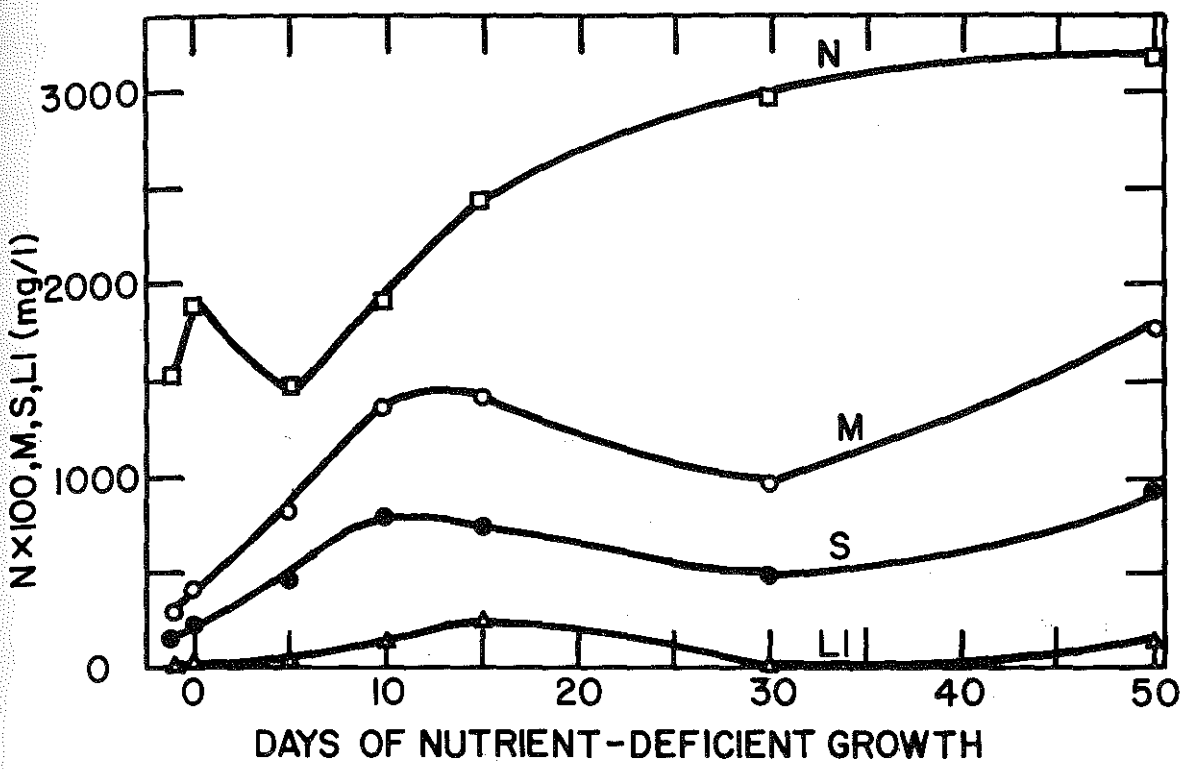

FIGURE 5.15. Variation in Growth Parameters for Kentucky Mixed.

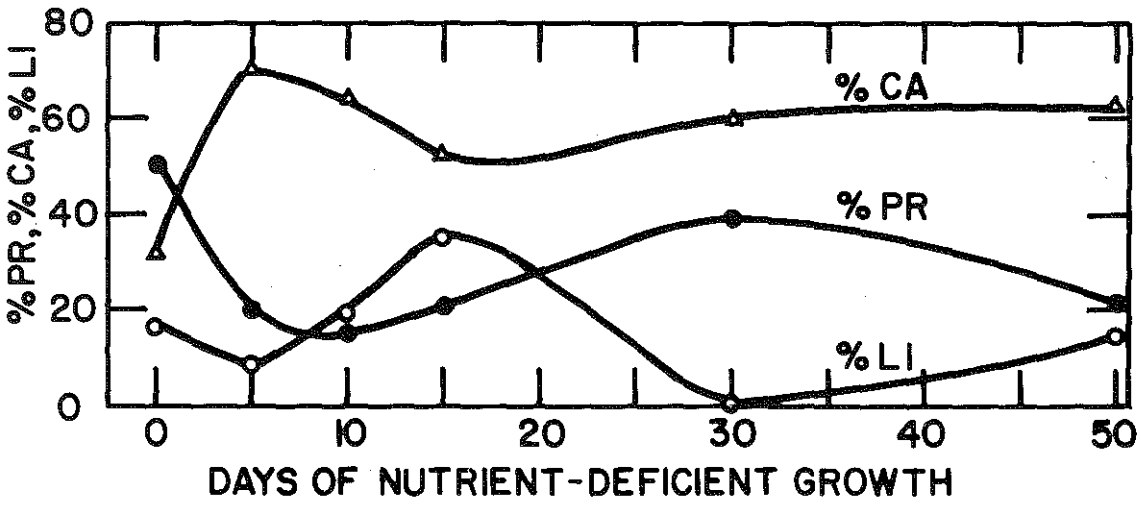

FIGURE 5.16. Variation in Organic Composition of Kentucky Mixed.

the following 15 day period (days 15-30) when the animals seemed to control the culture, the carbohydrate fraction increased at a rather rapid rate. After the population change had been completed, the carbohydrate content increased only slightly for the remainder of the growth period.

D. Significance of Organic Composition

Varma and DiGiano (42) stated that aging of the algal cell causes a shift in the metabolic pathway, causing a change in algal composition. Specifically, a young cell will be high in protein and carbohydrate while an old cell will be high in lipid content, and as a result, the young cell should be attacked more readily by the decomposers. The results of a study by Collyer and Fogg (24), showing that all algae do not accumulate lipids, were verified by the previously presented results of this study, which further showed that some algal species can accumulate carbohydrates instead of lipids as the cells age in a nutrient-deficient environment. Jewell and McCarty (1) found a generally smaller rate and extent of aerobic decomposition for old algal cultures generally high in lipid and/or carbohydrate content. Foree and McCarty (9) found that in anaerobic environments acid-forming bacteria could not degrade algal lipids, but that methane-forming bacteria and corresponding methane fermentation were necessary for degradation of algal lipids.

The above discussion suggests that the effects of organic composition on algal decomposition are significant but not fully understood. Additional studies are needed before significant conclusions can be drawn relative to the specific

effects of variations in chemical composition on the rate and extent of decomposition of algae under various environmental conditions. This study should provide a good background for future studies of this nature.

E. Water Quality Effects

Algae are known to cause taste, odor, and color in water, primarily due to excretion of extracellular organic matter. Generally, when algae grow under favorable conditions little extracellular matter is produced. However, once growth conditions become unfavorable, metabolic pathways seem to change, often resulting in excretion of extracellular organics into the growth medium. Gromov (17) found that in certain instances 30% of the organic matter accumulated by Chlorella was excreted into the growth medium and excretion increased under unfavorable growth conditions with each algal species excreting different substances.

In this study unfavorable conditions occurred when the nitrogen and phosphorus were exhausted from solution in the growth medium. Once this occurred, protein synthesis and corresponding cell division ceased causing a change in metabolic pathways, thus allowing lipid and/or carbohydrate synthesis on a large scale. It is believed that the majority of substances which cause water quality problems are excreted by the algae during the nutrient-deficient growth period.

Five tests which measure or reflect the palatability or esthetic acceptability of water are temperature, turbidity, color, taste, and odor (43).

Of these, color was chosen for this study because temperature was held constant, turbidity was removed during filtration, and taste and odor are highly subjective. The color test, as described in the section "Analytical Procedure," was run on the "soluble fraction" and therefore the color measured was "true color" as distinguished from "apparent color" which is caused by suspended matter. The color and corresponding organic mass of the algal cultures are shown in Figure 5.17.

For Chlorella the color increased from 0 units for the first observation to 100 units at the end of 50 days of nutrient-deficient growth and almost parallels the increase in mass of the culture. The Scenedesmus culture accumulated a total of 60 units of color; 30 during initial growth and the remaining 30 during the period when decomposition caused a decrease in algal mass. The Anabaena culture accumulated a total of 2400 units of color during the period of observation, 600 during the period of nitrogen fixation and the additional 1800 during the period when the production of the gelatinous extracellular matter was evident. The Kentucky Mixed culture accumulated a total of 200 units of color; 40 units during initial growth, 110 units during the decomposition period when the bacteria and animals were active, and 50 units between the population change and the last observation.

The U. S. Public Health Service recommends a maximum of 15 color units for a water supply to be used for domestic consumption. Assuming that some dilution would occur in a natural situation, a water supply containing an

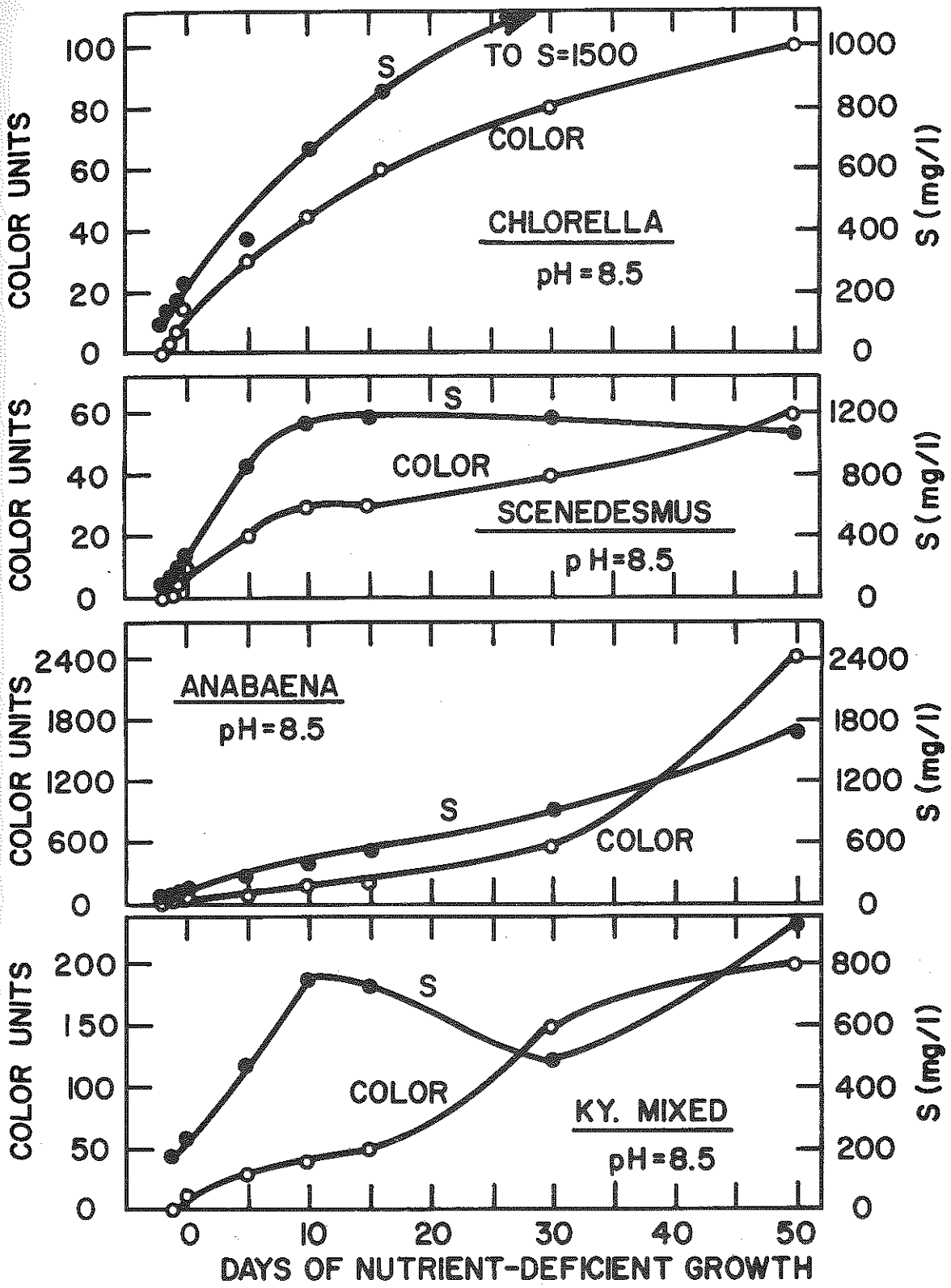

FIGURE 5.17. Color Accumulation in Algal Cultures During Growth.

algal growth similar to those of this study would require extensive treatment before use for domestic purposes.

CHAPTER VI

SUMMARY

For all four algal cultures, uptake of nitrogen and phosphorus from the growth medium occurred very rapidly. For the nutrient-abundant growth period, calculating the weight percentage of protein of the Chlorella and Anabaena cells as 6.25 times the weight percentage of nitrogen gave values too high because some of the nitrogen taken into the cells during this period was stored and not converted to protein until after the nutrients were exhausted from the growth medium. The Scenedesmus and Kentucky Mixed cultures did not exhibit this strong tendency to store nitrogen.

Some investigators have postulated that cell division and maximum algal growth continue until critical levels of cellular nitrogen and phosphorus are reached. Others have postulated that protein synthesis and cell division cease once either nitrogen or phosphorus has been exhausted from solution in the growth medium, but growth as measured by increase in mass may continue. Both theories were found to be applicable to some extent in that observed organic compositions indicated that protein synthesis and cell division ceased shortly after nitrogen and phosphorus were exhausted from solution in the growth medium, while measured growth parameters showed

that maximum growth continued after this time. Growth after cessation of cell division occurred as an increase in algal cell size and mass due to synthesis of lipids and/or carbohydrates.

The Scenedesmus culture differed from the other two unialgal cultures in that after certain levels of cellular nitrogen and phosphorus were reached, growth ceased. The Kentucky Mixed culture also exhibited this phenomenon for a period, but due to the many algal species present and the action of decomposers, a different algal species began to predominate and therefore growth continued. The unialgal cultures were characterized by synthesis of a large fraction of proteins during nutrient-abundant growth, by a fluctuating composition during transition from nutrient-abundant to nutrient-deficient growth, and by a relatively constant composition after a period of nutrient-deficient growth. The mixed culture behaved in much the same manner, but the composition changes were masked by the presence of predators in the form of microscopic animals.

The results of previous investigations, demonstrating that Chlorella accumulated large quantities of lipid material, were verified by the accumulation of a lipid content of 53% of the volatile suspended solids by the Chlorella culture. Scenedesmus, a green alga as is Chlorella, accumulated very little lipid material during either growth phase, but synthesized carbohydrate material which amounted to 85% of the volatile suspended solids at the end of observation.

Anabaena, a blue-green alga, similarly appeared to have little potential for lipid accumulation, and due to its capability of fixation of atmospheric

nitrogen, the cells never became truly nitrogen-deficient. Anabaena, like Scenedesmus, was found to favor carbohydrate synthesis. This culture accumulated a gelatinous carbohydrate substance, which changed the viscosity of the culture medium, amounting to 75% of the volatile suspended solids. The Kentucky Mixed culture, a mixture of both green and blue-green algae, accumulated a substantial amount of carbohydrate material during the nutrient-deficient growth phase. The decomposer population in this culture consumed the lipid material which had been synthesized during the early part of the nutrient-deficient growth phase. After the change in algal predomination, lipid matter was again synthesized.

The parameter chosen to demonstrate the effects of algal growth in batch culture on water quality was color which gave a qualitative measure of the amount of extracellular products excreted into the growth medium. After 50 days of nutrient-deficient growth, the color had reached 60, 100, and 2400 units in the Scenedesmus, Chlorella, and Anabaena cultures, respectively. The color causing substances excreted by Anabaena were believed to be associated with the materials which were responsible for the viscosity change in the growth medium. Color formation in the Kentucky Mixed culture medium was apparently related to the decomposition initiated by the microscopic animals, 100 of the 200 total units accumulated coming during the period of active decomposition.

CHAPTER VII

CONCLUSIONS

The following conclusions are drawn based on the results of this study of algae growing in batch culture:

1. Two regimes of uptake may be defined when excess nitrogen and phosphorus are present in the growth medium: (1) "Luxurious" uptake occurs when nutrients are incorporated into cellular protoplasm at levels greater than those necessary for growth. (2) "Super-luxurious" uptake occurs when some nutrients are taken into the cell but stored rather than converted into algal protoplasm.

2. Due to the storage of non-protein nitrogen by algae under nutrient-abundant conditions, calculating the weight percentage of protein during this period as 6.25 times the weight percentage of nitrogen may give inaccurate results.

3. The mass of an algal cell can continue to increase after nitrogen and phosphorus have been exhausted from the growth medium due to the change in metabolic pathway and synthesis of lipid and/or carbohydrate material. In most cases this increase in mass will continue at a significant rate until the

algal cells have reached nitrogen and phosphorus contents below 2% and 0.2%, respectively.

4. Composition of the unialgal cultures is characterized by synthesis of mainly protein during the nutrient-abundant growth phase, by a fluctuating composition during transition from nutrient-abundant to nutrient-deficient growth, and by lipid and/or carbohydrate synthesis and the establishment of a relatively constant composition during the nutrient-deficient growth phase.

5. Composition of a heterogeneous algal culture containing microscopic animals is characterized by the same trends as unialgal cultures, but these trends are masked by the effects of the microscopic animals.

6. All green algal species do not accumulate lipids in response to nutrient-deficient growth; some species accumulate carbohydrates.

7. Excessive growth under nutrient-deficient conditions has a detrimental effect on water quality. Soluble extracellular substances are produced which can cause very high concentrations of objectionable color.

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