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THE CRITICALITY OF THE NITROGEN AND PHOSPHORUS RATIO TO AQUATIC MICROORGANISMS, NAMELY PLANKTONIC ALGAE

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SYNOPSIS

The nutrients nitrogen and phosphorus have been incriminated as two of the more important parameters in nutritional pollution. Classically, sanitary engineers have been interested in the nitrogen to phosphorus ration, N/P, as it optimizes biological treatment processes based on the percent removal of BOD. This investigation attempts to define optimum nitrogen to phosphorus, N/P, ratio for algae-bacteria symbiotic systems analogous to sewage treatment effluent receiving streams.

Algae-bacteria systems were cultured in 150 milliliter and 2 liter Erlenmeyer flasks, and continuous systems with various concentrations of nitrogen and phosphorus. The ratio of nitrogen to phosphorus which produces maximum yield was not found to be constant at various phosphorus levels. The optimum ratio of nitrogen to phosphorus varied from 2/1 to 16/1 over a phosphorus level of 0.5 mg/l to 8.0 mg/l.

A multivariable regression analysis of the following linearized form gave a R^2 of 0.9216.

$$V(2)/ALOG(Z) = B_0 + B_1V(1) + B_2V(2)$$

Z = algae-bacteria biomass - mg/100ml

V(1) = nitrogen concentration - mg/ml

V(2) = phosphorus concentration - mg/ml

B_0, B_1, and B_2 represent constants.

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THE CRITICALITY OF THE NITROGEN AND PHOSPHORUS RATIO TO AQUATIC MICROORGANISMS, NAMELY PLANKTONIC ALGAE

INTRODUCTION AND PROBLEM DEFINITION

Excessive enrichment or eutrophication of receiving waters by nutrient rich waters is emerging as a major water pollution problem in many areas. It has been recognized for some time that ordinary domestic sewage is a rich source of the nutrients required by phytoplankton. Unusually large populations of algae, termed algae blooms, have caused deterioration of water quality. Specifically, algae blooms have made water less desirable for municipal, recreational, and agricultural purposes.

Various chemical species containing nitrogen and phosphorus have been suggested as the more important fertilizers of aquatic environments. Experience has shown that the degree of eutrophication and hence the severity of subsequent water quality problem is largely dependent upon the supply of inorganic nitrogen and phosphorus.

The algae in all natural water supplies are usually present in easily manageable numbers, but they multiply rapidly if they come in contact with nutrient sources under optimum physical conditions. This multiplication can result in an overabundance of algae in a particular

water impoundment or receiving stream. Domestic sewage, urban runoff, agricultural practices, and other sources are common nutrient contributors especially rich in nitrogen and phosphorus. Controlling the amount of nitrogen and phosphorus contributed by the various sources listed above has been suggested as a means of reducing the rate of eutrophication.

Historically, stream pollution and waste treatment research has focused on the removal of soluble biodegradable carbonaceous substances and settable solids. Commonly, these processes have been measured in the following units, BOD, VSS, MLSS, SS, and TOC. Of interest to sanitary engineers in design and operation of biological processes has been the required nutritional support of the biological degradable carbonaceous materials in sewage. In the case of domestic waste, extraneous nutritional support has not been necessary due to the nature of domestic sewage. Problems concerning nutritional support have been well founded, however, in various types of industrial wastes. Many biodegradable industrial wastes are characterized by their singularity of carbon source compounds and their limited concentration of nutrients, essentially, an imbalance in the C/N/P ratio.

Through research and application of biological and chemical treatment processes, the amount of carbonaceous material released to receiving waters has been sufficiently lowered. Because of

biological oxidation and assimilation, various bio-dynamical systems are able to reduce carbonaceous material to cellular materials and release carbon dioxide to the atmosphere by respiration. The problem of nutritional pollution has developed from the fact that domestic sewage has an excess of nitrogen and phosphorus in relation to the amount of carbonaceous material.

In any actively growing biological system, nutrient materials are continually extracted from the environment through metabolic reactions supporting respiration and cell synthesis. Rate of biological nutrient removal, other parameters being equal, is a function of the rate of cell tissue synthesis and/or cellular respiration. The amount of nutrient removed is determined by total cell composition and mineral content of the medium. The relative ratio of nitrogen and phosphorus in phytoplankton, and in other plants, has been designated the optimum nitrogen and phosphorus ratio.

The ratio of BOD to nitrogen and phosphorus is commonly called the C/N/P ratio, where the BOD is expressed in the form of carbon as correlated with a COD. Helmars (1) determined optimum and critical ratios for aerobic biological treatment systems. It was determined that 100/3/0.6 is the critical limit for nitrogen. This deficiency in nitrogen can be alleviated by adding anhydrous ammonia or some other ammonia salt to the waste.

The phototrophic organisms are able to convert solar energy, CO_2 and inorganic constituents to cellular material. The stoichiometric relationships may be expressed in the following equation (2).

$$106CO_2 + 90H_2O + 16NO_3^- + 1PO_4 + \text{light energy} = C_{106}H_{180}O_{45}N_{16}P_1 + 154.5\ O_2.$$

This equation describes an optimum C/N/P ratio for algae of 106/16/1.

The use of biodegradable detergents has increased the amount of available phosphorus for aquatic microorganisms. Even though certain esthetic and physical problems were solved by this major development, another more subtle and complex problem was intensified, that of nutritional pollution.

A paradox of modern waste water treatment complicates the problem. As measured by the classical yardsticks of suspended solids and BOD removals, the degree of treatment has increased steadily. This has resulted in more efficient mineralization of the organic constituents of sewage. Sunlight readily penetrates the clear water, and the minerals which have been released promote rapid and extensive growth of photosynthetic organisms, particularly algae. Thus, undesirable changes in natural bodies of water, which would have required thousands of years through normal eutrophication processes,

have been brought about in five to ten years by wastewater discharge.

An algae bloom is an ecological phenomena which has often been associated with the eutrophication process. It has been defined by various workers as an unusually large number of algae cells per unit of surface area (3). This increase in phytoplankton occurs in lakes, streams, reservoirs, and ponds, causing results which range from toxic effects to filter clogging problems, taste and odor problems, etc. (4). Many attempts have been made to quantify the algae bloom and associate this number with unusual populations of algae. Arbitrary values, based on rational observations and research are in the literature and serve as a comparison for regional and environmental differences. For the most part, this value can only be used as a guide or as a comparison for a particular case.

One of the most significant environmental relationships observed is the appearance of algal blooms concurrently with an increase in the nutrient concentration. The algal bloom causes an increase in turbidity due to the increase in cell concentration. The bloom usually extracts the nutrients, and in many cases quickly reduces the concentration of nitrogen and phosphorus. Some of this reduction is due directly to biological synthesis of cellular material. Chemical precipitation due to pH changes has been suggested as another means of the reduction of phosphorus, specifically during algae blooms.

Nitrogen and phosphorus are naturally added to streams, lakes, and reservoirs in the same manner as other chemical species. The edaphic effects of a particular drainage basin determine the amounts and types which will eventually find their way into the receiving body of water. The surface geology and the hydrological characteristics of the region are the two most important factors in edaphic effects. The natural productivity of the land area in the drainage basin also affects the amount of nitrogen and phosphorus which eventually reaches the water transport system. Heavily forested areas require substantial amounts of nitrogen and phosphorus and return to the soil various complex organic forms of these compounds.

Urban runoff is emerging as one of the most complex nutrient contributors from the standpoint of control. Various forms of nitrogen and phosphorus find their way to receiving streams through storm sewers and uncontrolled drainage systems. Lawn fertilizers could be considered one of the more important urban contributors of nitrogen and phosphorus.

Weible, Anderson, and Woodard (5) monitored the concentration of various chemical, biological, and physical parameters in sanitary sewage and storm water runoff. They presented their data in a ratio of the concentration of the parameters of interest in the form storm water/sanitary waste water.

Runoff from a forested watershed (6) contributes enough nutrients to support observed growths of weeds and an algal growth of bloom proportions. There is no pollution source of nutrient to augment the natural sources of nitrogen and phosphorus, natural sources being those due to edaphic effects and decomposition products of natural vegetation. The natural fertility of the land can be stated as an important factor in productivity studies and eutrophication. This information implies that any additional nutrient contribution due to man's activity would only augment the problems of eutrophication in a case as this where nutrients are already at concentrations considered critical.

In cases where man's activities have contributed to already existing productivity, problems lie in the ability of the sediments to absorb and release various chemical species upon demand, or upon physiochemical changes in the aqueous environment.

Another possible contributor of nutrients to a reservoir or lake could be due to commercial and recreational activities on and around the reservoir or lake. Points of high concentration of nutrient due to domestic sewage have been found near the marina of Chickamauga Reservoir (7) in Tennessee. Some of the bays in this reservoir exhibit different chemical and physical characteristics than the main body of water.

Irrigation return flow has also been suggested as a link in the cycling of nitrogen and phosphorus in the environment. Leaching of

nitrogen and phosphorus from the soil concentrates these compounds in the ground water flow (8).

Nitrates have been found to exist in concentration two and a half times greater in sub surface drains compared to surface drains. Sylveter reported total phosphorus compounds were highest in surface irrigation return flow drains (9).

Movement of feedlot drainage (10) through the soil and into the ground water has been shown to be a significant contributor of nutrients to ground water supplies. The water table collecting this seepage from irrigated fields sometimes contains ammonium-N (or a compound releasing ammonium) and organic compounds with offensive odors. Ammonium-N of these waters in 28 samples observed was found to contain an average of 0.2 ppm with a maximum concentration of 1.0 ppm.

Water from beneath 28 feed lots averaged 415 ppm of nitrogen as ammonium. Total soluble phosphorus was usually higher in ground water beneath feedlots as compared to irrigated water.

From time to time, interest has developed in some method of waste treatment which would remove offending fertilizing elements before discharge. Limnological investigations indicate that removal of phosphorus offers a practical and effective way of controlling algae growths in most natural waters. Phosphorus removal may be accomplished biologically and by chemical means. Either approach

TABLE I

Approximate Quantities of Algae Growth Materials (11)

(from sources either in or entering U.S. surface waters)

Source	Quantities available (millions of pounds per year) Organic matter	Nitrogen	Phosphorus
NATURAL			
Air		15-300[a]	
Rainfall (direct into surface water)	4,200-7,400 (COD)	30-590	2-17
Aquatic plants	n.a.	0-1,070	0-107
Waterfowl, fish, bottom fauna, and the like	n.a.	n.a.	n.a.
Muds under water bodies	n.a.	n.a.	n.a.
Runoff from			
Forest land (including commercial forests)	n.a.	990-2,250	243-587
Other land	n.a.	n.a.	n.a.
MAN-GENERATED SEWAGE			
Domestic			
Human and food wastes Washing wastes	5,200 (COD)[b]	1,330[b]	137-166[b] 250-280[b]
Industrial			
Food processing wastes	n.a.	n.a.	n.a.
Other wastes	n.a.	n.a.	n.a.
RUNOFF FROM			
Urban land	5,500 (COD)	200	19
Cultivated land			
Fertilized	17,900 (COD)[c]	2,040[c]	110-380
Unfertilized	n.a.	n.a.	n.a.
Land on which animals are kept	n.a.	420	170

a = 50 percent of that in rainfall, measured.
b = based on 70 percent reduction and 30 percent reduction respectively, measured.
c = estimated.
d = not available.

TABLE II

Phosphorus Entering Surface Waters With Runoff[a] From Cultivated Land (11)

	Size of Site (acres)	Phosphorus entering surface water (pounds per acre per yr.) Range	Average
Kaskaskia River, Ill.	3,320,000	0.02-0.76	0.35
Madison, Wis.	140,000	n.a.	0.40
San Joaquin Valley, Calif.	358	0.20-0.90	0.55
Yakima Valley, Wash.	265,000	0.9-8.9	1.33
Coshocton, Ohio	1.45	n.a.	1.2

[a]Includes runoff through drain tile under the crop land

Table I shows an attempt to account for all the nitrogen and phosphorus sources in or entering the surface water in the United States. This represents only an approximation, but does point out the significant contributors: rainfall, human and food wastes, and fertilizers. In this table, "a" represents approximately half the quantity entering

TABLE III

Natural and Man-generated Sources
Of Phosphorus and Nitrogen (11)

	Nitrogen		Phosphorus	
	Quantities (million pounds per year)	Per cent	Quantities (million pounds per year)	Per cent
Natural	1,035-4,210	21-51	245-711	26-41
Man-generated	3,990	79-46	686-1,015	74-57
TOTAL[a]	5,025-8,200	100%	931-1,726	100%

[a]These totals do not include what may be substantial amounts of phosphorus and nitrogen from as yet unknown and unmeasured sources. Such additional phosphorus and nitrogen may have an effect on methods of controlling algal growth.

from rainwater. The notation "b" refers to data based on the assumption of 70% removal of COD and 30% removal of nutrients in treatment plants. The notation "c" refers to an estimated value rather than a measured amount.

TABLE IV

Man-generated Sources of Nitrogen (11)

Source	Quantities (millions of pounds per year)	Per cent
Domestic sewage	1,330	33%
Runoff from		
Urban land	200	5
Cultivated fertilized land	2,040	51
Land on which animals are kept	420	11
TOTAL[a]	3,990	100%

[a]These totals do not include what may be substantial amounts of phosphorus and nitrogen from as yet unknown and unmeasured sources. Such additional phosphorus and nitrogen may have an effect on methods of controlling algal growth.

Table II shows some selected phosphorus runoff values. Table III, Table IV, and Table V indicate the effect man has on the total amount of nutrients contributed to surface waters. The contribution by man's activity is significant: nitrogen 46-74 percent, and

TABLE V

Man-generated Sources of Phosphorus (11)

Source	Quantities (millions of pounds per year)	Per cent
Domestic sewage	387-446	56-44%
Runoff from		
Urban land	19	3-2
Cultivated land	110-380	16-37
Land on which animals are kept	170	25-17
TOTAL[a]	686-1,015	100%

[a]These totals do not include what may be substantial amounts of phosphorus and nitrogen from as yet unknown and unmeasured sources. Such additional phosphorus and nitrogen may have an effect on methods of controlling algal growth.

phosphorus 57-74 percent, in both cases.

Based on these values, the hypothetical concentration of phosphorus in the surface water has been determined (Table VI). The

TABLE VI

Phosphorus Concentration (Hypothetical)
In Surface Waters (11)

	Total phosphorus (million pounds per year)	Average concentration (p.p.m.)
All estimated sources	980	0.26
All estimated sources except detergents	680	0.18
If 90% of phosphate in sewage is removed	560	0.15

values in Table VI are well within the range of phosphorus concentration in surface waters in the United States.

The concept of removing nutrients by biological means can hardly be considered as new or unique. In any actively growing biological system, nutrient materials are continually extracted from the environment through conversion of cell tissue. Rate of nutrient removal, other things being equal, is a function of the rate of cell tissue synthesis, while the amount of nutrient reduction is determined by cell tissue composition and mineral content of the medium.

Stumm and Tenny (1963) (12) found that the biological treatment of sugar wastes was highly efficient in the removal of nutrients. This was a consequence of the high amounts of carbon satisfying the carbon-nitrogen phosphorus ratio. Stumm and Tenny also considered ponds of little value if the nutrients were not removed prior to the discharge of the effluent from the pond. They found that the soluble matter could be removed most easily by biological treatment, whereas the suspended and colloidal material could be removed most easily by chemical coagulation.

Bogan (13), in an experiment with specific microbial cultures, found that nutrient removal was a function of cell synthesis rate. Various parameters such as culture media, alkalinity, temperature, and concentration of cell tissue were monitored in his experiment. Light proved to be the principal factor controlling the rate of removal of

nutrients by algae. Reductions of soluble phosphates up to 90% were achieved in detention times as short as 6 to 12 hours. Maximum light requirements were found to be around 100 to 200 ft. candles. Extension of the laboratory study to lagoons one meter or more deep exposed the problem created by the inability of the light to penetrate to any significant depth in the lagoon for effective photosynthesis.

Appreciable amounts of nitrogen and phosphorus can be removed in primary settling units. Another physical method which has proved to be extremely efficient is that of distillation of whole sewage; this, however, has certain economic disadvantages.

Fitzgerald and Rohlich (1964) (14) determined the rate of utilization in primary and secondary sewage effluents. The rate appeared to be unrelated to the concentration of the organic matter. Increased CO_2 demand by the algae shifted the bicarbonate carbonate equilibrium, causing an increase in the pH. This increase in pH precipitated the soluble phosphates, but they redissolved with a drop in pH.

Phosphorus can be reduced 90-95% by use of chemical coagulation, adsorption, sedimentation, and precipitation processes (15). These methods can also be combined with biological treatment by adding chemicals in the primary sedimentation or in an activated sludge process. Precipitation in the primary clarifier improves the biological treatment by improving clarification and BOD removal.

Addition of coagulants directly to the activated sludge stage results in the formation of slightly soluble phosphorus compounds (16). Sodium aluminate added at the rate of 1:8;, Al:P, was suggested for removal of 85-92% of the phosphorus.

Wuhrmann (1964) suggested that ion-exchange and air stripping of ammonia at a high pH could be more than ninety percent effective in removal of nitrogen from sewage plant influent (17). Various chemicals have been tested for removal of phosphates from waste water. Ferrous sulfate, ferric sulfate, cupric sulfate, diatomaceous earth, and aluminum sulfate were tested by Rohlich (1961) (18). Alum dosages of about 200 mg/l was ninety-nine percent effective in removal of soluble phosphates. Henriksen (1963) found that iron, aluminum, and calcium were effective coagulants for removal of ortho phosphates in test solution. But calcium proved to be the less effective chemical for removal of soluble phosphates from algae (19). Malhorta et. al., removed nitrogen and phosphorus with lime from the Madison, Wisconsin, sewage treatment plant. Their findings are in contrast with those of Henriksen. They found that a dosage of 600 mg/l of lime removed 99% of the soluble phosphorus at a cost of $40.00 per million gallons (20).

Economically, the chemical-biological treatment for phosphorus removal costs 5¢/1000 gallons at a 10 mgpd plant removing 90-95% of the phosphorus (21). A similar type process using the cold

lime process and recarbonation costs 10.46¢/1000 gallons.

Pollution by algal nutrients effects the ecology of recovery waters by disturbing the balance between heterotrophic and autotrophic organisms. Nutritional pollution control directs itself at the autotrophic-heterotrophic balance. Odum has suggested several approaches to this problem: (1) to reduce the inflow of algal nutrients to receiving waters, (2) to interfere with the nutrient cycle in surface waters by physical, chemical, or biological means. Among the possibilities for such unbalances are the reduction of effective light, use of algacides, and the increase of "grazing" by herbivores (22).

The consequence of nutritional pollution is accelerated eutrophication by promotion of algal blooms. As an engineering problem, certain conditions must be defined and rational assumptions made. Also, numerical values must be associated with conditions, assumptions, and the ensuing relationship which develops. The purpose of this study is to evaluate those conditions shown by the literature to be most responsive to the promotion of algae growth.

When regulations are enacted concerning the effluent or stream standard of nitrogen and phosphorus, some reliability must be built into the standard to insure success in reduction of algae in the stream or reservoir. To develop reliability in this standard, the effect of various concentrations of nitrogen and phosphorus must be known.

This involves testing for minimum growth, maximum growth, and toxic values of the nutrients.

This project approaches this particular problem from the standpoint of minimal concentrations of nitrogen and phosphorus necessary for the growth of algae. A radioactive phosphorus method was used as one of the parameters for biological mass produced and phosphorus uptake rate in an alga culturing unit. In this manner, some standard or standards, as the case may be, can be obtained to govern sewage treatment efficiency as far as nitrogen and phosphorus are concerned. This is achieved by holding one of the nutrients constant while varying the other and vice versa, sensitivity analysis. All other necessary inorganic and organic nutrients, including vitamin B_{12} and other growth metabolites, are supplied in excess to allow maximum algae reaction to the nitrogen and phosphorus.

LITERATURE SURVEY

NUTRIENTS AND MICROORGANISMS

The scope of this literature review encompasses many of the facets and types of research which have been directed to the elucidation of the elements nitrogen and phosphorus as they pertain to algae growth. Plant physiologists were the first to recognize the significance of nitrogen and phosphorus in cultivation of terrestrial plants. Much of the work of plant physiologists was conducted in controlled laboratory experiments. The nutritional studies on terrestrial plants then led to studies on higher aquatic plants and algae.

Ecologists have shown that these nutrients do exert their influence on community metabolism in aquatic environments. Some differences though exist in the minimal concentration necessary for growth in the laboratory and in the natural environment, these values being less in the latter case. Some of this can be attributed to analytical procedures; other variations are attributed to complex formation, precipitation, nitrogen fixation, etc. The competition for these nutrients in the aquatic community can explain some variation in the minimal concentration of these nutrients in the two situations.

The form in which the nutrient exists apparently offers a preferential metabolic route such as nitrogen in the ammonia, nitrate, nitrite, or organic form; phosphorus, in the ortho phosphate, metaphosphate, or organic phosphate form.

Of late, environmental engineers, in attempting to understand the cause and effect relationship in nutritional pollution, have focused their attentions on the nitrogen-phosphorus-aquatic microorganism phenomena. Table VII shows the average component of minerals for algae growth as contributed from the waste products of man.

The routine analysis of sewage for biological oxygen demand presents an interesting segment of the nitrogen cycle as it pertains to nutritional pollution. The BOD parameter is a standardized test based on the amount of oxygen utilized per amount of biologically oxidizable material. Since a heterogeneous mixture of bacteria exists in sewage, a natural component of this population will be of the nitrifying type. In the BOD test, a heterogeneous mixture of bacteria is used for seed; the nitrifying type of bacteria take place in the reaction.

The nitrifying bacteria belong to a metabolic group termed the autotrophic bacteria. This group of bacteria is characterized by their source of carbon for protoplasm construction and their source of oxygen for energy. These types of autotrophs receive their energy from oxidation of ammonia to nitrite by some species and of nitrite to nitrate by others. One other characteristic of these organisms

TABLE VII

Elemental constituents in urine and feces (23)

	Urine (g)	Feces (g)
Total-Nitrogen[a]	11.0	1.74
NH_4 - N[b]	0.67	0.06
Ca[b]	0.21	0.57
Cl[b]	8.35	0.15
Fe[b]	-	0.01
Mg[b]	0.13	0.18
P[c]	1.09	0.54
K[c]	2.47	0.39
Na[c]	3.33	0.12

a Golueke, C. G., and Oswald, W. J. Annual Review of Plant Physiology.

b Berger, E. Y. Mineral Metabolism, Chapt. 8, 249-76. (Academic, New York, 1960).

c Spector, W. S., Handbook of Biological Data, 242 (Saunders, Philadelphia, Penn., 1956).

is their slow growth and reproductive patterns, especially in the presence of organic carbon compounds. As the heterotrophic population reduces the BOD, the medium becomes more suitable for the autotrophs. The autotrophs become dominant in from six to twelve days of incubation. The BOD test is standardized as a five day test, partly to omit possibility of erroneous readings due to metabolism of nitrifiers. As the carbonaceous material is depleted, the nitrifying bacteria begin their task. First the nitrite-forming bacteria oxidize ammonia to nitrite. Then the nitrate-forming bacteria oxidize the nitrites to nitrates.

The nitrogen metabolism of higher plants and animals does not involve any gaseous nitrogen (N_2), either as a food or as a waste product. For most of the plants and for all animals, molecular nitrogen is a strictly inert material. Yet all organisms require the element nitrogen since it is a constituent of the all-essential amino acids and proteins.

Some arbitrary starting point must be chosen when discussing the nitrogen cycle. Proteins as they are found in animals and plants will be used as the starting point in this presentation.

Proteins are an essential part of every living cell, being the chief material out of which protoplasm itself is made. Analytically, proteins consist of approximately 52% carbon, 7% hydrogen, 24% oxygen, 15% nitrogen, 1.2% sulphur, and 0.8% phosphorus. Proteins differ

from carbohydrates and lipids in that they are not stored in appreciable amounts except in eggs and seeds, where they are used to build new protoplasm. It is in the making of the protein that the plant uses many of the minerals which it absorbs from the aqueous environment, particularly nitrates, sulfates, and phosphates. Carbohydrates from the photosynthetic processes serve as a source of carbon, hydrogen, and oxygen. Some bacteria can use atmospheric nitrogen; some use ammonium salts, some nitrites, and some nitrates. Still others use organic salts of nitrogen for their nitrogen source.

In the nitrogen cycle, proteins in decay are eventually reduced to water, carbon dioxide, ammonia, and free nitrogen. From the standpoint of sewage treatment, the excrement from humans and that plant and animal tissue which is disposed in the sewers is the transport system for the next step in the nitrogen cycle.

Fecal nitrogen does not appear to be related to the nitrogen of the ingested protein. Its quantity varies with the bulk of the diet and does not normally represent unabsorbed dietary protein. In the adult human, this amounts of 1 to 2 grams N/day. Perspiration, unless sweating is excessive, accounts for a loss of only 0.3 grams N/day or less (24).

Urine is the major source of excretion of nitrogen. In the average, normal human adult, the total nitrogen of the urine is about 13 grams/day, and this normally is all NPN (organic nonprotein nitrogen

nucleic acids and their derivatives). No significant quantity of protein is found in the urine normally (25). Urea (85% of the total) and ammonia (3%) vary directly with the level of protein in the diet. Creatine (5% of the total) excretion is related to the muscle mass and is quite constant for an individual. Uric acids (1% of the total) output varies with the level of dietary purines (26).

There exists a wide variety of bacteria which can hydrolize urea to ammonia and water. It is at this point that the nitrogen cycle is entering a biological treatment process. Length of time and other environmental parameters determine how much of urea has been hydrolized to ammonia.

$$\begin{array}{l} NH_2 \\ | \\ C{=}O \\ | \\ NH_2 \end{array} \quad +2\ H_2O \text{ ----------} \rightarrow \quad NH_4^- + CO_3$$

In a heterogenous bacterial population normally found in a sewage influent, there exists certain nitrifying bacteria. This bacteria (Nitrosomonas) can act upon ammonia until it is eventually combined with other elements into nitrites.

The oxidation of ammonia to nitrate takes place in two steps brought about by bacteria belonging to two different genera, as indicated in the following equation:

$$NH_3 \xrightarrow{\text{Nitrosomonas}} NO_2 \xrightarrow{\text{Nitrobacter}} NO_3$$

These bacteria are highly specialized in carrying out the above reaction. The energy that they obtain from these oxidations is used to synthesize organic material from carbon dioxide and water, as indicated by equations I and II (27).

I Nitrosomonas

$$NH_3 + O_2 \longrightarrow NO_2^- + H_2O + E$$

$$E + CO_2 + H_2O \longrightarrow \text{biomass}$$

II Nitrobacter

$$NO_2^- + O_2 \longrightarrow NO_3^- + H_2O + E$$

$$E + CO_2 + H_2O \longrightarrow \text{biomass}$$

One aspect of the nitrogen cycle reverses this process of nitrification. The bacteria which are able to do this are called the denitrifying bacteria. They are found predominantly in an environment containing excess nitrogen compounds or deficiencies in oxygen and may reverse the nitrifying process, some reducing nitrates to nitrites and ammonia, and some reducing nitrites to free nitrogen. This free nitrogen and such gaseous ammonia as escapes directly to the atmosphere are lost in the cycle.

Some organisms are capable of reducing nitrates (denitrification); for example, Ps. flourescens, Ps. denitrificans, Ps. stutzerii, and Micrococcus denitrificans are all capable of reducing nitrates and nitrites to ammonia, nitrous oxide, and molecular oxygen (28).

The nitrogen metabolism of bacteria differs considerably from that of other plants. In contrast to algae, bacteria are able to excrete water-soluble end products into the surrounding culture medium. Some bacteria form gaseous end products such as CO_2, H_2, H_2S, and NH_3 (29).

Many heterotrophic bacteria can utilize organic nitrogen compounds. The nitrogen is first removed from the compound and the product is then fermented or oxidized. Certain heterotrophics depend entirely on organic nitrogen for their source of nitrogen. The amino acids required by an organism are either synthesized or derived from its environment.

Frequently, one portion of the amino acids is oxidized while another portion is reduced. Another frequent reaction is decarboxylation of the amino acids, resulting in the formation of primary amines (30). Most of the monoamines formed by the decarboxylation of simple aliphatic monoamine acids are further metabolized; the diamines, formed from the diamine acids, are more stable and are frequently found in putrified material (31).

Algae, with few exceptions, can utilize either ammonium salts or nitrates when presented at the appropriate concentrations. Ammonium salts may have an undesirable indirect effect, however, since the pH of a poorly buffered medium falls sharply as ammonium is assimilated (32). It has also been reported that ammonium

nitrogen is often used preferentially when supplied with nitrate in that it appears to inhibit nitrate assimilation (33).

Most of the planktonic forms of algae commonly found in a bio-oxidation pond can utilize ammonium salts or nitrates if they occur at suitable concentrations. Euglena and Chlamadomonas cannot utilize nitrates, regardless of the concentration (34).

Planktonic forms of algae such as Chlorella and Scenedesmus grow well in 620 ppm solutions of nitrates or ammonium salts. This concentration is too high for many other types of planktonic forms of algae (35). Many forms of algae are inhibited by 1.8 ppm ammonium nitrogen (36). This inhibition by ammonium salts has been hypothesized to be due to an increase in internal pH due to the penetration of undisassociated ammonium hydroxide molecules (37).

Nitrites in concentrations of 0.046 ppm have also been shown to be utilized as a nitrogen source for many species. Higher concentrations than 0.046 ppm are inhibitory for these same organisms (38).

The fact that certain blue-green algae and bacteria are capable of fixing atmospheric nitrogen has been well established. Hellriegel and Wilfarth were the first workers to definitely establish nitrogen fixation by bacteria living symbiotically with leguminous plants (39). Fogg and Wolfe in 1954 showed the distribution of the blue-green algae that were known to fix atmospheric nitrogen (40). Species of

general Anabaena, Nostoc, Cylindrospermum, Calothrix, Aulosira, Tolypothrix, and Mastigocladus have all been reported to fix atmospheric nitrogen (41).

Rhodospirillum, Phodopseudomonas, Rhodomicrobium, Chromatium, and Chlorabacterium are species of photosynthetic bacteria which fix atmospheric nitrogen. Various species of Clestridium and Azotobacteria have also been shown to be nitrogen-fixing bacteria (42).

Nitrogen-fixing blue-green algae and/or nitrogen-fixing bacteria have been suggested as possible reasons for more nitrogen in the effluent than appears in the influent. Loehr and Stephenson reported that an increase in nitrogen is often found in tertiary bio-oxidation ponds. Most of this increase is due to the ammonia and organic nitrogen (43).

A search of the literature reveals no direct reference to nitrogen fixation in a bio-oxidation pond by blue-green algae. In 1961 Sawyer, et. al. (44) reported on the nitrogen fixation in natural waters by bacterial species and blue-green algae. They found that phosphorus was a key element necessary for nitrogen fixation and that excessive amounts of phosphorus could stimulate nitrogen fixation (45).

In 1961 Douglas et. al. observed that the concentration of the various forms of combined nitrogen must be at a low level for

nitrogen fixation to occur (46). In their work at Sanctuary Lake, they found a sharp rise in the Anabaena population when the concentration of inorganic nitrogen approached an almost undetectable amount. Along with this sudden rise in Anabaena, there was a rapid decline and a final disappearance of fixed nitrogen. They attribute this to the sudden rise in the ammonia concentration (47).

Fogg in 1961 (48) found that the extent of inhibition by other nitrogen sources depends upon the readiness with which they give rise to ammonia. He reported that inhibition by nitrates is sometimes incomplete. The past history of the cells is one of the most important criteria in the adaption to another nitrogen source.

Winogradsky in 1943 (49) noted that ammonium salts inhibited nitrogen fixation in the bacteria Clostridium. Azotobacter was found to be necessary for nitrogen fixation by blue-green algae. The elements calcium, boron, and molybdenum have been determined by Eyster to be essential for nitrogen fixation (50).

The element phosphorus is more likely the limiting factor for biological activity in the sea (51). Among the major elements needed by plants, phosphorus is the one most likely to be a limiting factor in world agriculture (52). Soluble phosphorus content of water may be a factor in limiting the rate of biological activity and in determining the nature of the growths when its concentration drops below 0.01 ppm (53).

The design for this experiment employs phosphorus in the form of orthophosphate associated with a sodium salt. The use of orthophosphates for the phosphorus source is based upon the work of Galloway and Krauss (54). They found that Chlorella pyrenoidosa growing actively in various polyphosphates changes the varied polymers of the phosphate to orthophosphates before absorption. The organism stimulus to hydrolize these polyphosphates is thought to be due to pyrophosphatase, which is an adaptive enzyme in the cell wall of Chlorella pyrenoidosa.

Ecological Considerations

According to the work of numerous investigators over the past twenty years (Strangenberg, (55); Sawyer, Lackey and Lorenz, (56); Lackey, (57); Lackey and Sawyer, (58); Hasler, (59); Smith, Williams and Davis, (60); Sawyer, (61); Ohle, (62); Suhrman, (63)) the phosphorus content of the waste liquids has been found to be the most important factor causing excessive enrichment - extraordinary growth of algae - and the consequent difficulties arising from the discharge of sewage effluents into waterways. It is therefore of the utmost importance that the phosphorus present in wastewater be removed or reduced to the lowest level before the effluent is discharged into any watercourse.

In considering this problem, it is necessary to examine the evidence that has accumulated on its many aspects, particularly algal

growth in waters containing phosphates. The most pertinent literature relating to this problem is reviewed below.

Wiebe (64) studied the soluble phosphorus and inorganic nitrogen in the waters of the Mississippi River and observed that phosphorus could be a limiting factor in the growth of plankton. Observations on 49 lakes in New York and Connecticut indicate that the quantity of phytoplankton in the waters depends on the concentration of phosphorus and, to a lesser extent, on the concentration of Nitrogen (Deevy, [65]). Chu (66) studied the influence of mineral composition of the medium on the growth of a few forms of planktonic algae and observed a marked inhibition in their growth when the concentration of nitrogen or phosphorus exceeded 45 ppm, while the concentrations of nitrogen below 0.1 ppm and of phosphorus below 0.009 ppm also had an inhibitory effect on the algal growth. On the other hand, Harris and Silvey (67) did not recognize any correlation between plankton growth and concentration of phosphate and nitrate. Benoit (68) found it difficult to determine critical concentrations of phosphate below which the algal blooms did not develop. Whitford and Philips (69) did not find any correlation between variations in phosphorus and phytoplankton blooms and concluded that the interaction of a complex and physical factors produces both seasonal and sporadic blooms of phytoplankton.

These conflicting results render a definite conclusion impossible. In the waters studied, other chemical, environmental, and

TABLE VIII

Selected References Concerning Nitrogen and Phosphorus Ratios and Concentrations

	N	P	N/P	
Culture Medias	.2	.1	2/1	(70)
Forested Areas	.28	.070	4/1	(71)
Surface Irrigation	1.455	.251	7/1	(71)
Urban Street Drainage	.830	.154	5/1	(71)
Multiple Use River	.495	.135	4/1	(71)
Urban Streams	1.930	.123	14/1	(71)
Eutrophic Lake	.420	.076	6/1	(71)
Southeast Wisconsin Lakes	.3	.015	20/1	(72)
Central & Eastern Oklahoma Streams	.95	.20	5/1	(73)
Sewage Effluent	50-20	13-1	7/1	(74)

meterological factors are operative. The effects of changing concentrations of phosphate phosphorus (orthophosphate) on the development of plankton algae can only be solved experimentally with algal cultures in which all the other factors are controlled.

Historically, various growth abnormalities have been observed due to phosphorus deficiencies in the environment. It is apparent that one common denominator in all these cases of phosphorus deficiencies is a growth factor. In general, phosphorus probably affects growth by participating in a number of processes at the cellular level. Included in these processes is respiration, which is tied up to the utilization of carbohydrates and various synthetic processes. When these are interfered with, secondary changes such as inhibition of salt intake, of nitrification, and loss of chlorophyll take place.

Biochemical Consideration

The initial eluciadation of phosphorus in a living system was accomplished with animal tissue and microorganisms. The research by Embden and Meyerhoff resulted in the demonstration of a complete cycle present in muscle and yeast. Through this cycle, the carbohydrates are broken down to pyruvic acid through the intermediate formation of phosphoralyzed compounds and with the modification of specific enzymes. The function of this cycle when present,

is the conversion of the energy of glucose into "ATP energy." Generally it is thought that ATP is the immediate energy source for many of the various processes which occur in cells.

The element phosphorus maintains an extremely important position in the aquatic environment in relation to productivity. Generally, many limnologists and ecologists assign phosphorus the role as the limiting nutrient in primary productivity. Much of the evidence for this opinion is based on its relatively meager natural occurrence on the earth's surface.

Marine deposits of sedimentary phosphates are found in various regions of the work, such as in England, Idaho, Utah, and Wyoming (75). Apatite and phosphatic rocks in igneous rock contributed much of the natural occurring phosphates to aquatic environments.

Phosphorus compounds are essential components of all living matter. Much of the phosphate in living organisms is in the esterified form, combined with various carbohydrates, and lipids. Adenoisine triphosphate is one of the organic phosphorus forms which is responsible for the synthesis of amino prosthetic groups and coenzymes from their vitamin precursors. The nucleic acids, RNA and DNA, also contain phosphorus as one of their components.

Phosphorus plays a major rate in photosynthesis as a phosphorylated compound which is found upon the reduction of carbon dioxide. Calvin, Basshan and Benson identified phosphopyruvic

acid and phosphoglyceric acid in photosynthesis experiments (76). The formation of ATP from glucose is termed the tricarboxylic acid cycle. The TCA cycle exists in both plant and animal and is responsible for the energy capacity of these phosphorus compounds. In some animals 60% of the initial glucose is bound in the high energy bonds of ATP (77).

TABLE IX

Mineral Requirements for Chlorella Pyrenoidosa

Based on Critical Concentrations Required in the Culture Medium

Mineral	Heterotrophic growth	Autotrophic growth
NO_3	2.5×10^{-3}M	2.5×10^{-2}M
Mg	2×10^{-2}M	2×10^{-3}M
K	4.3×10^{-3}M	4.3×10^{-4}M
P	1.8×10^{-4}M	1.8×10^{-4}M
S	2×10^{-4}M	2×10^{-4}M
Fe	1×10^{-9}M	1.8×10^{-5}M
Zn	0.77×10^{-10}M	0.77×10^{-6}M
Mn	1×10^{-9}M	1×10^{-7}M
Cl	3×10^{-6}M	3.4×10^{-2}M

EXPERIMENTAL PROCEDURE

In an attempt to simulate the natural conditions of a receiving stream, certain environmental conditions must be standardized. These assumed standard conditions are based on the classical differences underlying the laboratory approach as opposed to the ecological approach. This experimental design incorporates several facets of each of these approaches to plant nutritional study and is an attempt to control as many of the physical and chemical parameters as possible. Control means that all parameters except those of interest are held constant, and the results are measured in terms of an orderly variation in the test parameters and the effect on the particular biological system.

An environmental study selects those parameters which in the scientific background of the investigator appear to effect the natural occurrence of the species. Those parameters of interest are monitored, and a type of correlation is sought based on the selected parameters.

A hybrid experimental design termed a laboratory ecological study is based on principles from both these types of design.

Biological interaction and variation, say in this case of algae, is allowed to proceed at a quasi-normal rate without any attempt to the CO_2 concentration. Normal atmospheric gas exchange is allowed to take place without any increase in the partial pressure of the CO_2, such as bubbling 5% CO_2 through the media.

A variation in this experiment from the approach of most plant physiologists is that no CO_2 enrichment was made in the culturing units. The carbon source for the algae was in the form of alkalinity; that is the natural occurring amounts of HCO_3, CO_3, and CO_2. The relative amounts of the three species contributing to the alkalinity is indicated by the hydrogen ion concentration which is controlled externally by the natural partial pressure of CO_2 in the atmosphere. At one atmosphere CO_2 exerts 0.03% of the total pressure exerted by the atmosphere or about 1 mm of Hg. No organic carbon was added to the culture units, except in the case of showing the effect of heterotrophic bacteria and algae in uptake rate of phosphorus. Certain metabolic by-products from the algae-bacteria community will of course accumulate and possibly recycle in the media.

The humidity in the culturing room was maintained at close to saturation to depress evaporation in the culturing flasks. This was done by use of vaporizer-like devices which maintained the relative humidity between 85-90%. Temperature in the culture room was stabilized at 25°C by use of centralized heating and refrigeration

controls. The light source for culturing apparatus was afforded by ten 40 watt cool white florescent lamps. The lamps were programmed for 12 hour off-on cycle. The illumination from the cool white flourescent lights varied from 100 to 150 foot candles, depending on the geometrical positioning of the culture flask in relation to the lights. Agitation was afforded manually on a daily basis to maintain the cells in suspension and on a homogenousity of the media.

Ortho and total phosphates were determined according to the stannous chloride method (79). Total phosphates were reduced by covering and autoclaving for 25 minutes 15 PSI in the presence of ammonium molybdate.

Several methods were used to determine the concentration of the algae during various phases of the research. A colorimetric method using a Bauch and Lomb Spectrophotometer set at 580 mu was one method used to determine algal density. A calibration curve was derived relating percent transmittance with dry weight of algae and Aeral Standard Units (ASU). The colorimetric method involved a preliminary qualitative determination of maximum absorption of color produced by the algae. Some slight variations were found in the point of maximum absorbtion in the latter growth stages. This was due to the color shift of from green to yellow in the chlorophyll in the later stages of growth. For the most part the

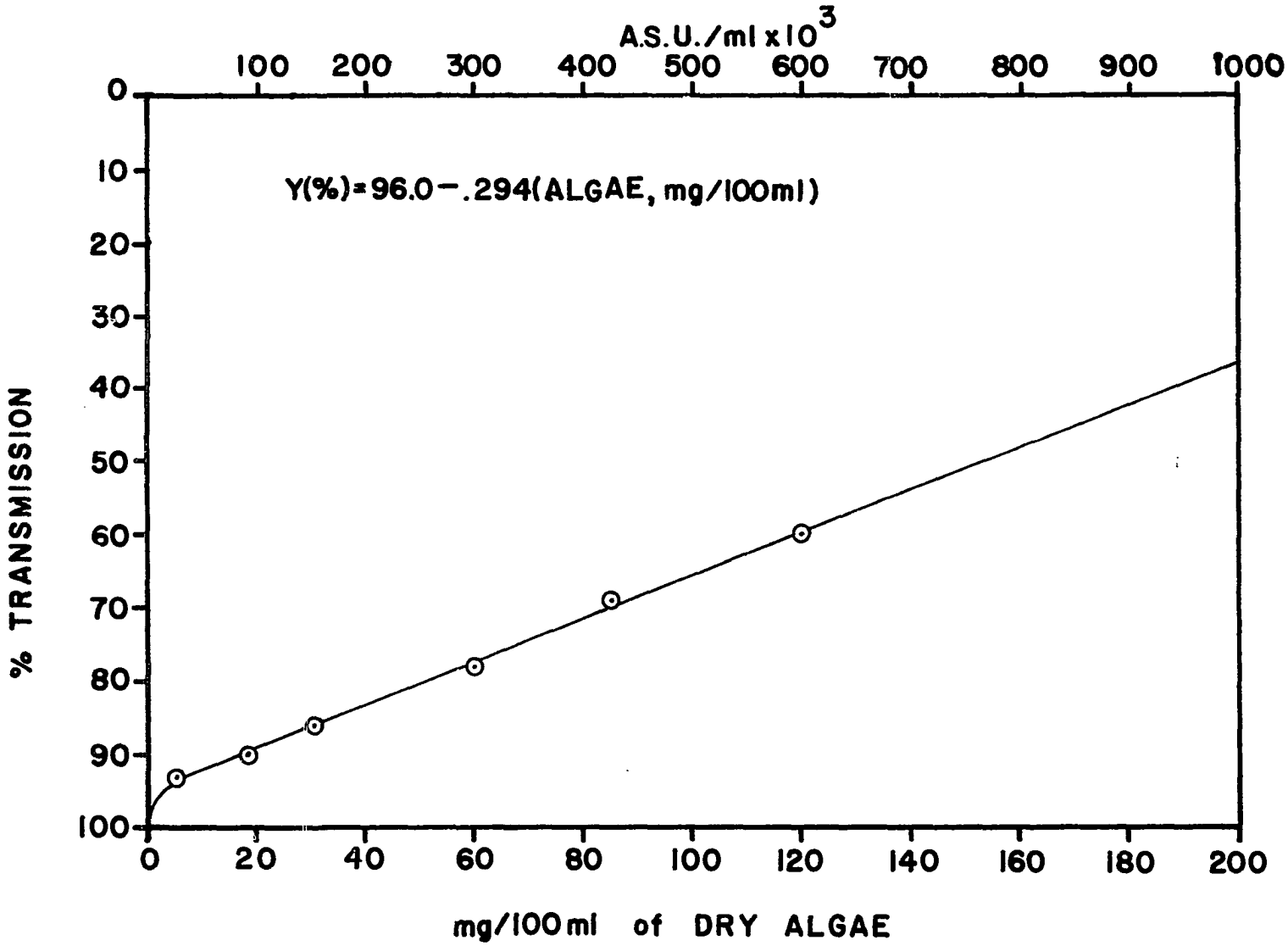

Figure 1. Standard curve for percent transmittance, dry weight, and Aeral Standard Units

necessary data for this study was found in the early active growth stages. In the initial stages of the batch cultures, especially those experiments in which organic carbon was added to the media, a turbidimetric interference with the algae color was caused by the accelerated growth of the bacteria. This did not interfere with the determination of the algae which usually were in a lag period of growth at this time.

In calibrating the per cent transmittance and dry weight, serial dilutions of a luxuriant growth of algae were dried and weighed and its per cent transmittance determined. By using dilutions of 1/2, 1/4, 1/8, 1/10, and 1/20, the wide range of possible algae densities could be considered in the calibration curve. The algae solutions were filtered through a pre-dried .45 u membrane filter, dried at 25°C for 24 hours, then dried at 35°C until weight stabilized.

A portion of the same sample was also counted using a Whipple eyepiece and a Sedgewich Rafter counting cell; the ASU were then related to the dry weight determinations and the percent transmittance.

Batch studies were carried out in 150 milliliter and 2 liter Erlenmeyer flasks. In the 150 ml flask 100 ml of media were used, and 2 liter flasks contained 1 liter of media. The culture media was composed of well water from the 1500 foot level beneath Norman, Oklahoma. The well water was used because of its consistent water

quality similar to that of surface water in the central Oklahoma area. The well water had the following significant water quality parameters: chlorides - 12.5 mg/1; sulfates - 33.0 mg/1; iron - 0.01 mg/1; Ca hardness - 19.0 mg/1 as $CaCO_3$; carbonate alkalinity - 66 mg/1; bicarbonate alkalinity - 245 mg/1; orthophosphate - 0.04 mg/1 as P; pH - 8.4; and nitrate - 0.00 mg/1. Various trace minerals and vitamins were supplied to the media by use of a commercial vitamin in dilute proportions.

Phosphorus was added in the form of orthophosphates associated with a sodium salt (Na_2HPO_4) which also served as the carrier for the radioactive phosphorus. The nitrogen was supplied to the algae in the form of sodium nitrate (Na_2NO_3) as this represented the most highly oxidized form of nitrogen in natural waters. This would more closely parallel the recovery from organic pollution rather than a stage of active decomposition. Carbon was used as a parameter in several experiments and was added in the form of glucose ($C_6H_{12}O_6$).

The ^{32}P was counted, using a deep-well integral scintillation counter (photomultiplier tube, plastic phosphorus for Beta counting) with an efficiency of 37% and a background of 80$\pm$ 4 cpm. Since geometry corrections present a problem in counting liquid samples, only 2 ml. of the batch culture was filtered and the radioactivity of the residue determined. However, after drying and counting

the residue of many samples, it was found as accurate to determine the amount of radioactivity in the biological mass by taking the difference between the ^{32}P in the batch culture and the ^{32}P in the filtrate after membrane filtration (0.45u pore diameter Millipore filters were used). Therefore, radioactivity was determined on aliquots of 0.1 ml before and after filtration and the per cent difference taken as the ^{32}P in the phytoplankton. Sampling and counting were done in duplicate, and all samples were counted to a reliable error (90% confidence level), $\pm$ .5%, Figure X.

The relationship between nitrogen, phosphorus and algal growth is expressed by a multiple regression analysis procedure, the Dolittle procedure. The Dolittle procedure determined the partial correlation coefficients and multiple correlation coefficients. The two independent variables are nitrogen, X_1, and phosphorus concentration, X_2; the dependent variable is algal mass, Y (80).

In general, the multiple regression analysis was of the following form:

$$Y = B_0 + B_1X_1 + B_2X_2$$

Where y is the estimator or the true y and B_0 represents the intercept and B_1 and B_2 represent the partial coefficients of correlation, and X 's represent the independent variables. In this case, transformations were made to account for any interdependency between nitrogen and phosphorus.

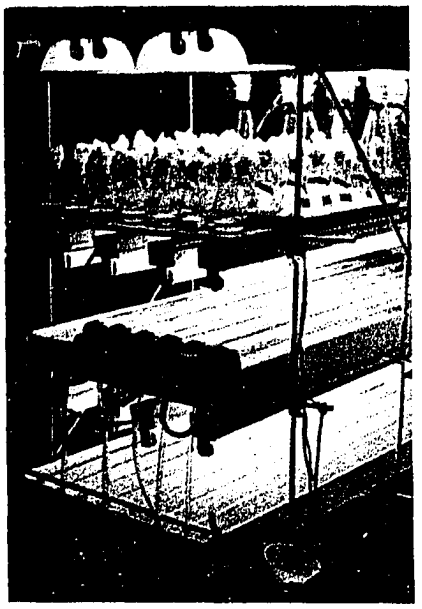

Illustration I. Algae culturing apparatus, 125 ml batch, 2 l batch, plexiglass containers, and pyrex containers

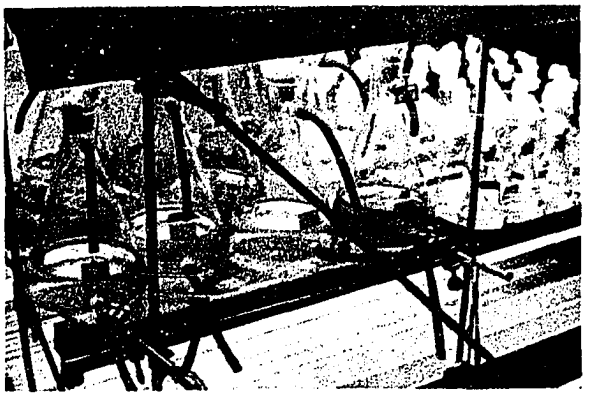

Illustration II. Close-up of batch culturing apparatus, 2 l and 125 ml flasks

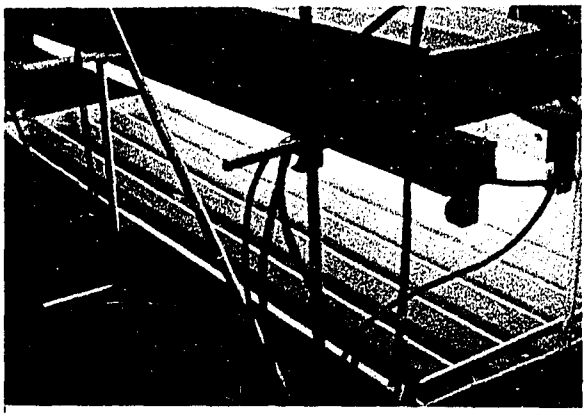

Illustration III. Close-up of plexiglass interconnected troughs with influent at the upper left corner of the bottom bank of troughs

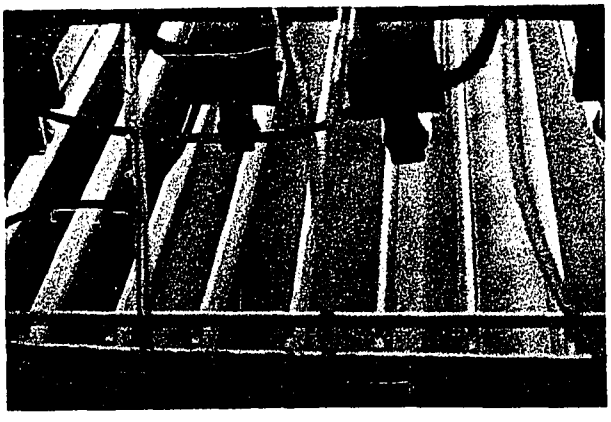

Illustration IV. Close-up of plexiglass troughs with higher algae concentrations decreasing from viewer's left to right

Continuous studies were performed in plexiglass troughs which were connected in series to simulate a reach or a river. Media was pumped through the system at various rates by use of displacement pump. By changing the pump discharge rate of the media, the velocity of the simulated stream would fluctuate in direct proportion. Total phosphate, ortho phosphate, and algal concentrations were determined at various distances on the reach on a daily basis. This culturing system was exposed to the same environmental conditions at the batch cultures such as 25°C, 90% relative humidity, cool white florescent lights, natural CO_2 atmospheric partial pressure, nutrient enriched well water, etc.

These systems were designed to follow the uptake rate of the orthophosphate, the rate of algal production, and the release rate of the polyphosphates in the continuous system. In effect by studying these parameters at various stream velocity, the wash-out rate could be determined.

The velocity in the troughs for the experiment was 0.156 ft/hr; this gave a total flow-through time for the 8 troughts of 6.5 days.

TABLE X

EFFICIENCY OF SCINTILLATION COUNTER

Sample Size 1 ml = 0.06 uc	Theoretical Counts dpm	Experimental Counts cpm	Efficiency
0.5 ml	6.65×10^4	1.44×10^4	22%
0.1 ml	1.33×10^4	5.02×10^3	37%
0.05 ml	6.65×10^3	2.80×10^3	42%
0.025 ml	3.32×10^3	1.80×10^3	54%

TABLE XI

PER CENT ERROR DUE TO ADSORPTION

Residue from	cpm	cpm/ml	Per Cent Error due to Adsorption
2 ml	896	448	
4 ml	1,650	412	8%
8 ml	2,889	361	20%
16 ml	5,666	351	22%
32 ml	10,556	330	27%

DISCUSSION

The effect of the nitrogen and phosphorus concentration on algae-bacteria symbiotic growth has been evaluated through several experimental procedures. The inherent variability of any biological system, in particular a symbiotic algae-bacteria system, demands some flexibility in experimental design if applicable data is to be obtained. Basically, three types of experiments have been performed in this research. First, ^{32}P was used to monitor growth rate and yield; a second group of experiments tried growth curves over a wide range of nitrogen and phosphorus concentrations and ratios; and the third type of experiment cultured the algae-bacteria biomass in a continuous flow apparatus analogous to a receiving stream.

The first set of experiments was designed to test the reliability of measuring algae-bacteria growth by use of change in optical density. Various other methods of enumerating algae such as dry weight and the Sedgewich-Rafter method were correlated with optical density. Figure 1 shows the standard curve which relates percent transmittance to dry weight, mg/100, mg/1 and Areal Standard Units, ASU. Initial tests indicated that an appropriate relation

exists between these parameters; thereafter, percent transmittance was used to measure bio-mass.

The use of the radioisotope ^{32}P as a tracer for the rate of phosphorus uptake and the rate of algae growth is shown in Figures 2, 3, 4, 5, 6, 7, and 8. These experiments allow an insight into the ecological interation of phosphorus, in particular with the symbiotic algae-bacteria mass. Glucose enrichment is used to expose the bacteria from the symbiotic system by forcing an intial reaction in the growth system by the bacteria. This is shown by turbidity measurement in the second and third day with an accompanying phosphorus uptake rate. Following this initial quick uptake by the bacteria, the normal algae uptake and growth rate are observed.

The effect of the glucose, which represents a BOD source in a receiving stream had no effect on the algae growth at constant nitrogen and phosphorus levels. Two possible mechanisms might be involved in obtaining essentially the same amount of algae whether carbon is included or excluded from the culture. One could be that the initial uptake by the bacteria of the PO_4-P can be released as an easily phosphoralated organic compound. In other words, the metabolic by-product or the cellular products which are released upon death are easily metabolized by the algae segment of the algae-bacteria system. The other explanation could be that the amount of phosphorus necessary for the amount of bacteria produced

is insignificant compared to the total amount of phosphorus present. This explanation would have important ecological impact in the case of a highly carbonaceous sewage plant effluent in contrast to a plant effluent which has had most of its carbonaceous material removed. From a nutritional pollution standpoint, the amount of algae that can be produced from an effluent and receiving stream condition, all other things being equal, would be the same for an inefficient sewage treatment plant as for an efficient BOD reducing sewage treatment plant.

The effect of the nitrogen to phosphorus ratio, N/P, on algae growth attempts to define the optimum ratio at a given nutrient level and to determine minimal and maximal concentrations necessary for algae growth. Growth curves for the various ratios are shown in figures 2 through 20. Algae growth is measured by percent transmittance over a period of approximately two weeks. Taking the maximum yield at each growth curve and plotting the various nitrogen concentrations at a given phosphorus level are shown in figures 21 through 28.

The ^{32}P is assimilated early in the growth period by the developing heterotrophic bacteria in the glucose enriched media while in the non-glucose enriched media, the ^{32}P uptake preceeds the growth of the algae slightly. In both cases a similar growth in terms of maximum yield is noted. This confirms the experimental

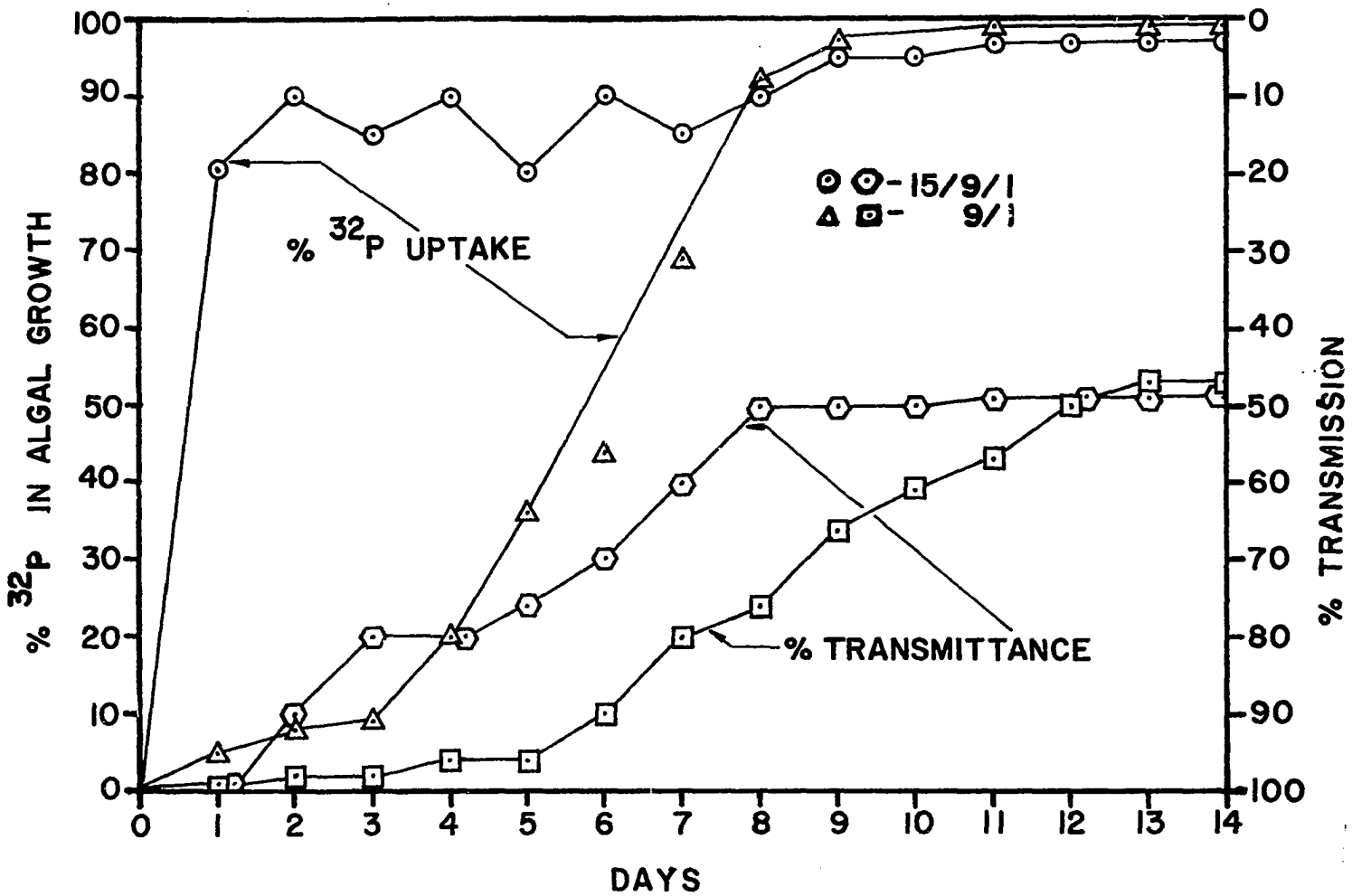

Figure 2. Percent transmittance and ^{32}P uptake for a C/N/P ratio of 15.0/9.0/1.0 and a N/P ratio of 9.0/1.0

design assumption that the algae growth is independent of the concentration of soluble carbonaceous material within the range of carbon indicated and with the particular species of algae involved. This also indicates that the phosphorus used in bacterial-mass production at the 15 mg/l at carbon level does not appreciably detract from the algae mass production at similar nitrogen and phosphorus concentrations.

Variations in the concentrations of nitrogen and phosphorus are made to obtain the ^{32}P uptake rate at various N/P ratios. The ratio of N/P is a relative figure depending on the concentration of the nutrient. Quite commonly in discussions concerning the N/P ratio, the relationship is standardized by making phosphorus unity and adjusting the nitrogen concentration appropriately. Two N/P ratio notations will be used in this discussion. If referring to the unstandardized ratio, a decimal point will be included denoting the actual concentration of nitrogen and phosphorus in mg/l. If the ratio is in whole number notation, the ratio has been adjusted to a unity expression.

Figures 3, 4, and 5 indicate a 4/1 ratio of nitrogen and phosphorus at concentration levels of nitrogen at 0.04, 0.40, and 4-0 mg/l. The phosphorus remaining in solution after maximal growth has occurred as indicated by ^{32}P uptake was not completely assimilated by the algae in any of the three cases. This indicates that

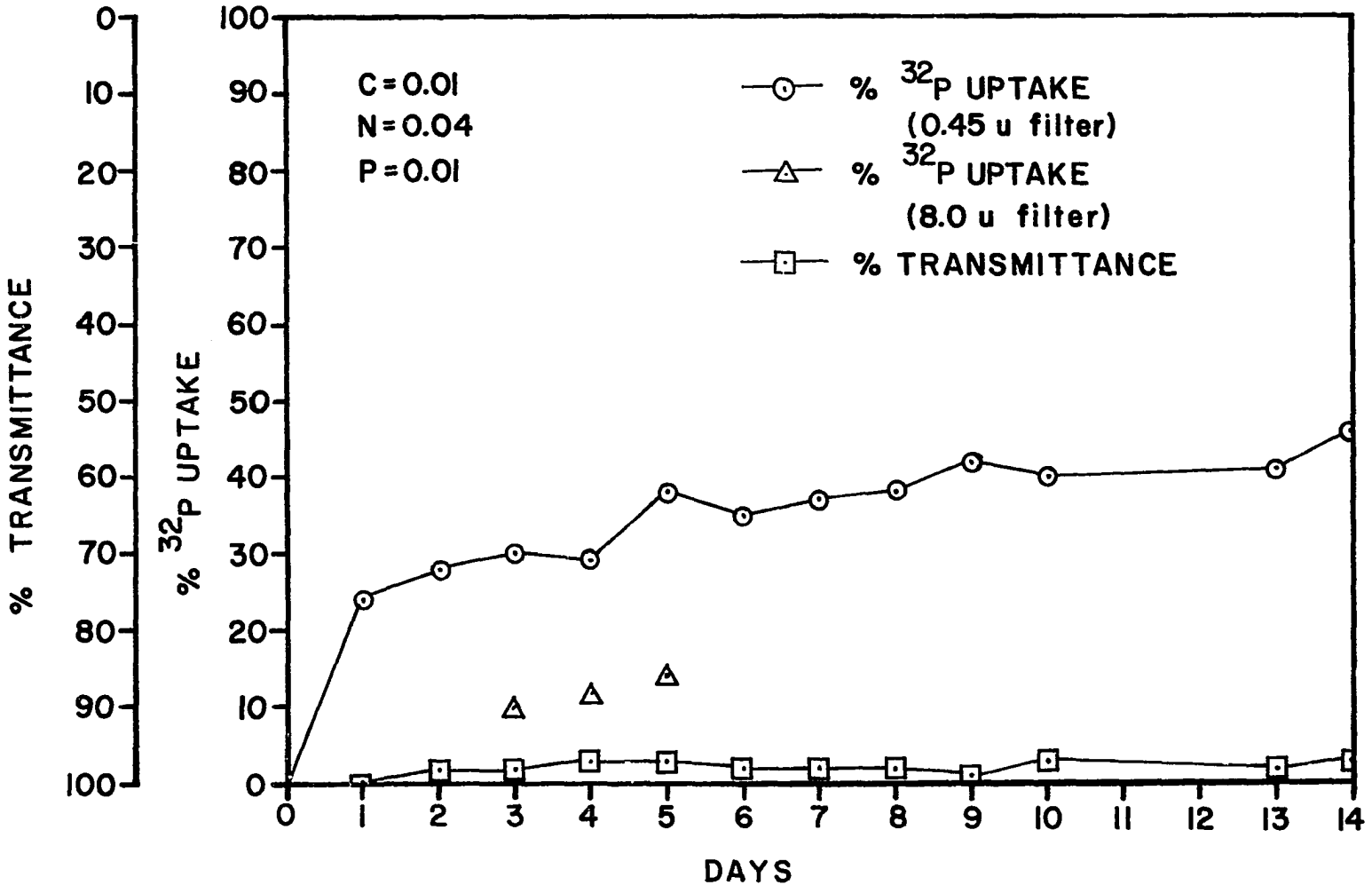

Figure 3. Percent transmittance and ^{32}P uptake for a C/N/P ratio of 0.01/0.04/0.01

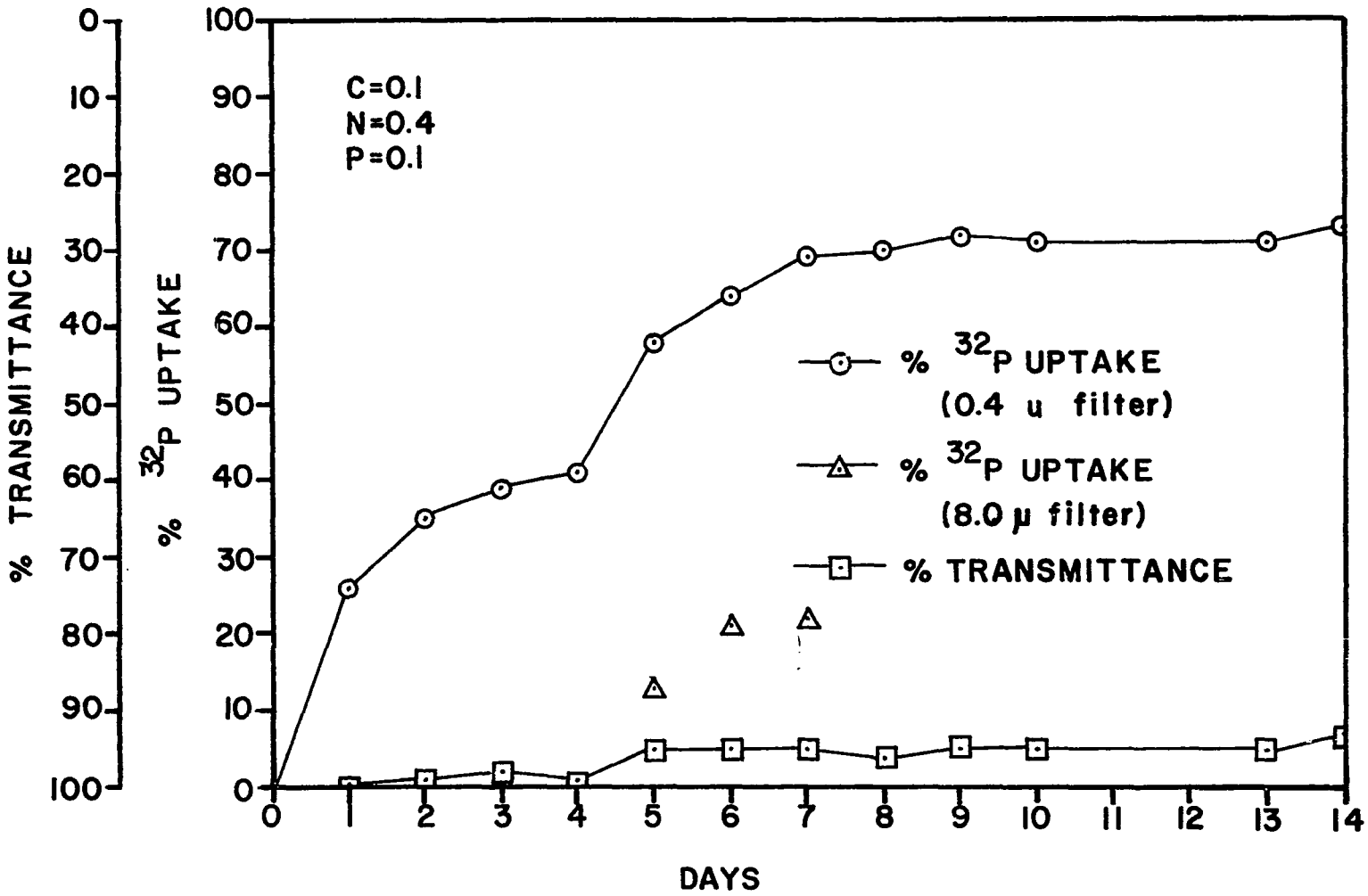

Figure 4. Percent transmittance and ^{32}P uptake for a C/N/P ratio of 0.1/0.4/0.1

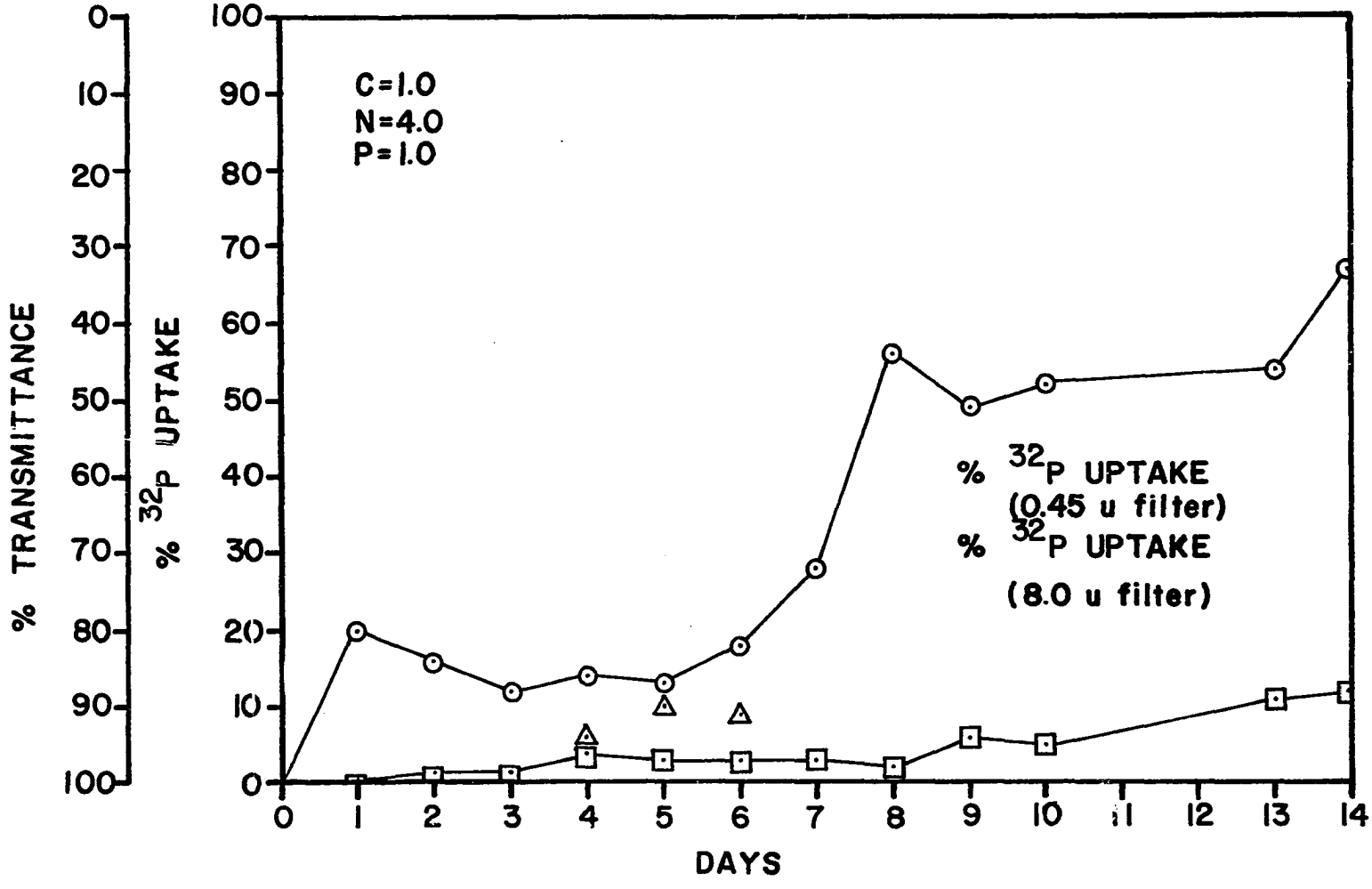

Figure 5. Percent transmittance and ^{32}P uptake for a C/N/P ratio of 1.0/4.0/1.0

the nitrogen is limiting at the 4/1 ratio level for concentrations of nitrogen indicated. Minimal amounts of algae are produced under these conditions compared to higher concentrations of phosphorus.

In Figures 6, 7, and 8, higher concentrations of nitrogen, phosphorus, and carbon are used. Again ^{32}P has been used as a tracer to measure the uptake rate of phosphorus. In these cases 90% of the phosphorus was taken up by the algae-bacteria bio-mass. The maximum yield for the three ratios is similar. In Figures 6 and 7, the nitrogen concentration is the same, 8.0 mg/1, indicating a phosphorus-limiting condition. Figure 8, in which the nitrogen concentration is 32.0 mg/1, shows the initial ^{32}P uptake due to the heterotrophic activity of the bacteria and then the phototrophic removal of phosphorus due to the algal segment of the symbiotic system.

At a concentration ratio of C/N/P of 1.0/4.0/1.0, Figure 5, a distinct lag period exists in the phosphorus uptake after the initial uptake due to the bacteria. Even though the ultimate uptake is similar, the rate of uptake increases significantly in the 4.0/8.0/4.0 culturing conditions as compared to the 2.0/8.0/2.0 conditions.

The effect of the carbon source concentration can be shown in Figure 5, as the ratio is increased to 2.0/8.0/2.0. The phosphorus is taken up completely (approximately 97%) at this particular ratio and concentration. The absolute ratio of N/P is 4/1 where phosphorus

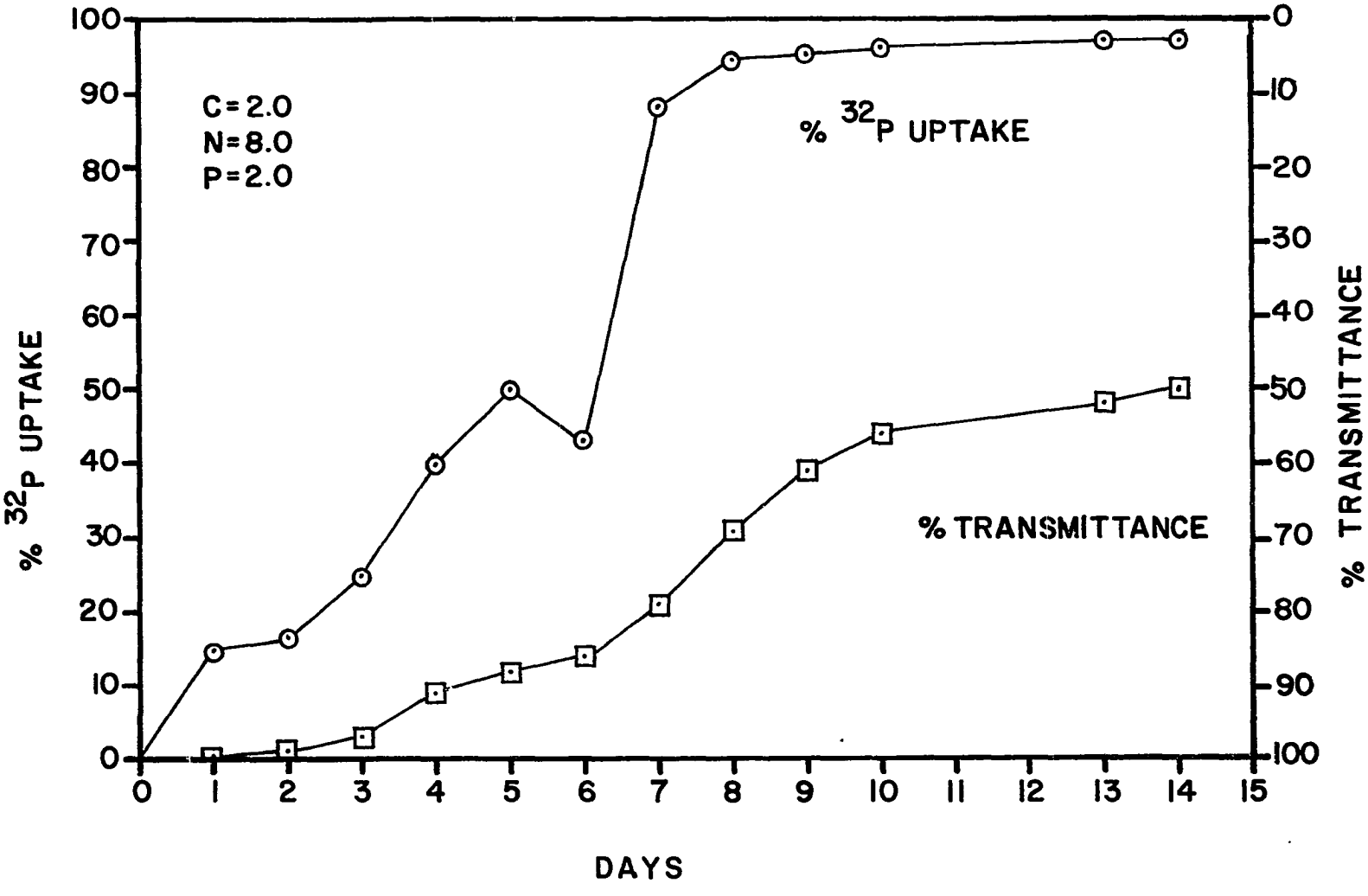

Figure 6. Percent transmittance and ^{32}P uptake for a C/N/P ratio of 2.0/8.0/2.0

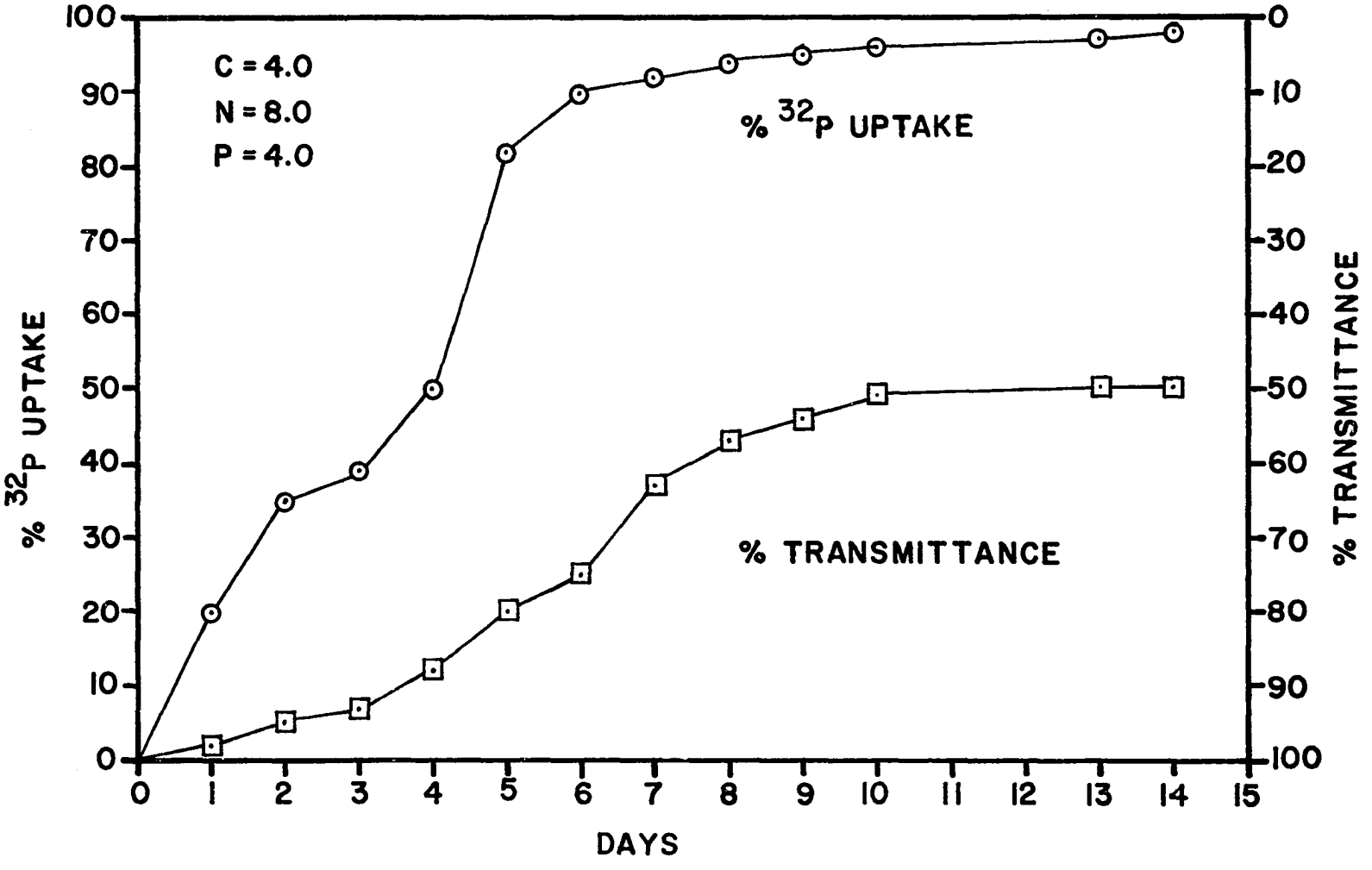

Figure 7. Percent transmittance and ^{32}P uptake for a C/N/P ratio of 4.0/8.0/4.0

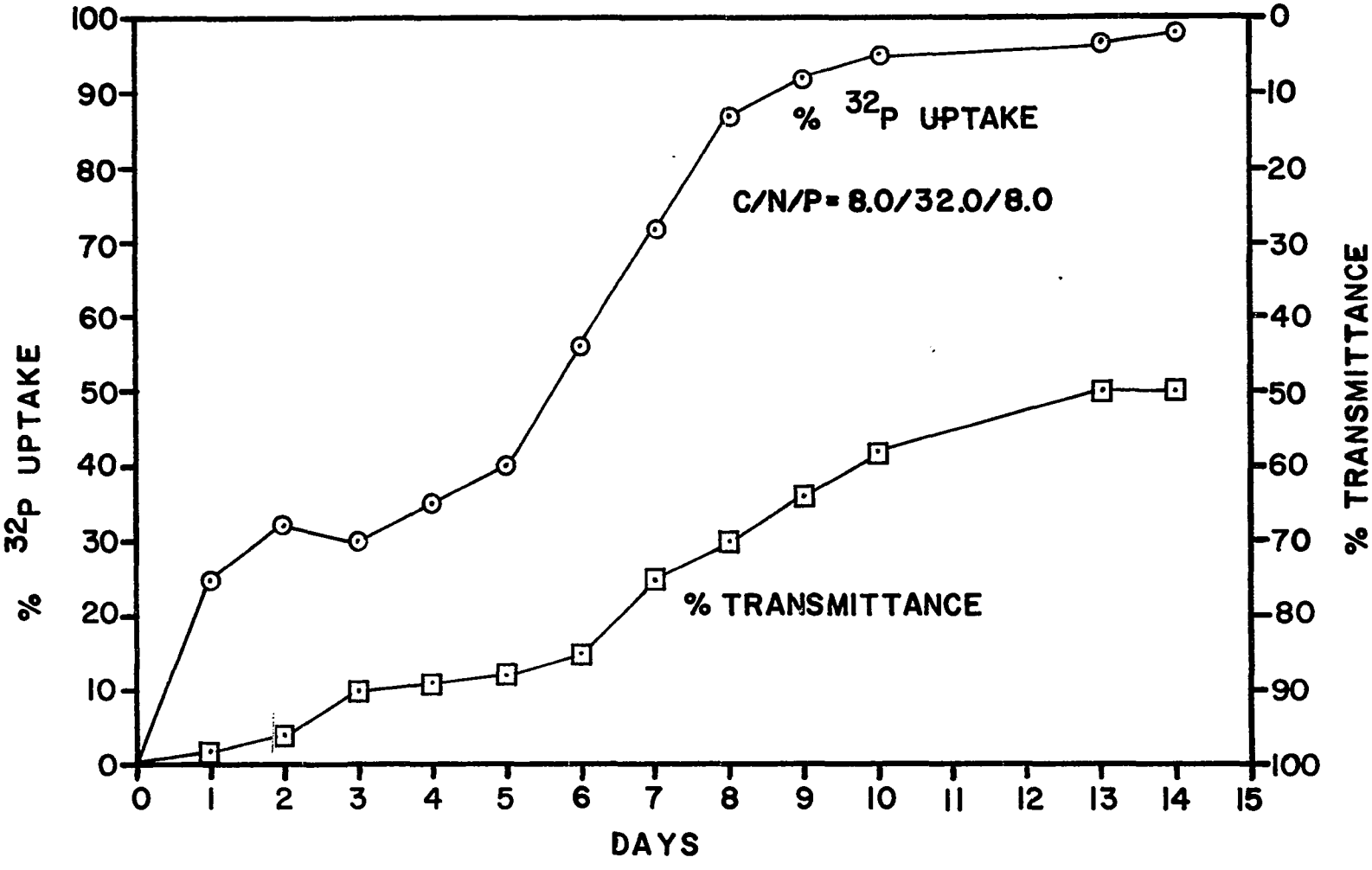

Figure 8. Percent transmittance and ^{32}P uptake for a C/N/P ratio of 8.0/32.0/8.0

is maintained at unity as in the case of Figures 3, 4, and 5. Apparently the carbon source exerts a pressure in the manner of competition for the nitrogen and phosphorus by the bacteria and algae. As the carbon concentration increases for a given phosphorus level, greater is the uptake of the phosphorus by the algae-bacteria system.

A comparison of Figures 6 and 7 shows that the phosphorus-limiting condition exists in Figure 7. The addition of 2 mg/1 of nitrogen does not substantially change the growth pattern. The phosphorus uptake level is 97 per cent with a similar transmittance trace at the N/P ratio of 8/2 growth conditions.

The rate of uptake of phosphorus in Figure 8 decreases at the C/N/P ratio of 8.0/32.0/8.0 levels in comparison with the 4.0/8.0/4.0 conditions. Maximum growth rate conditions have been altered by either the increase of carbon or by a supression mechanism at the 320 mg/1 level of NO_3-N.

The effect of glucose, in the heterotrophic bacteria population, indicated by the percent transmittance measurements in Figure 9, shows no change in the rate of growth or in the maximum yield. The apparent effect is noted on the third day where a turbidity interference due to the bacteria bloom can be detected by the spectrophotometer at 580 mu.

Figures 10 and 11 show two N/P systems at the concentration ratio of 16.0/4.0 and 8.0/2.0. The growth response is similar in

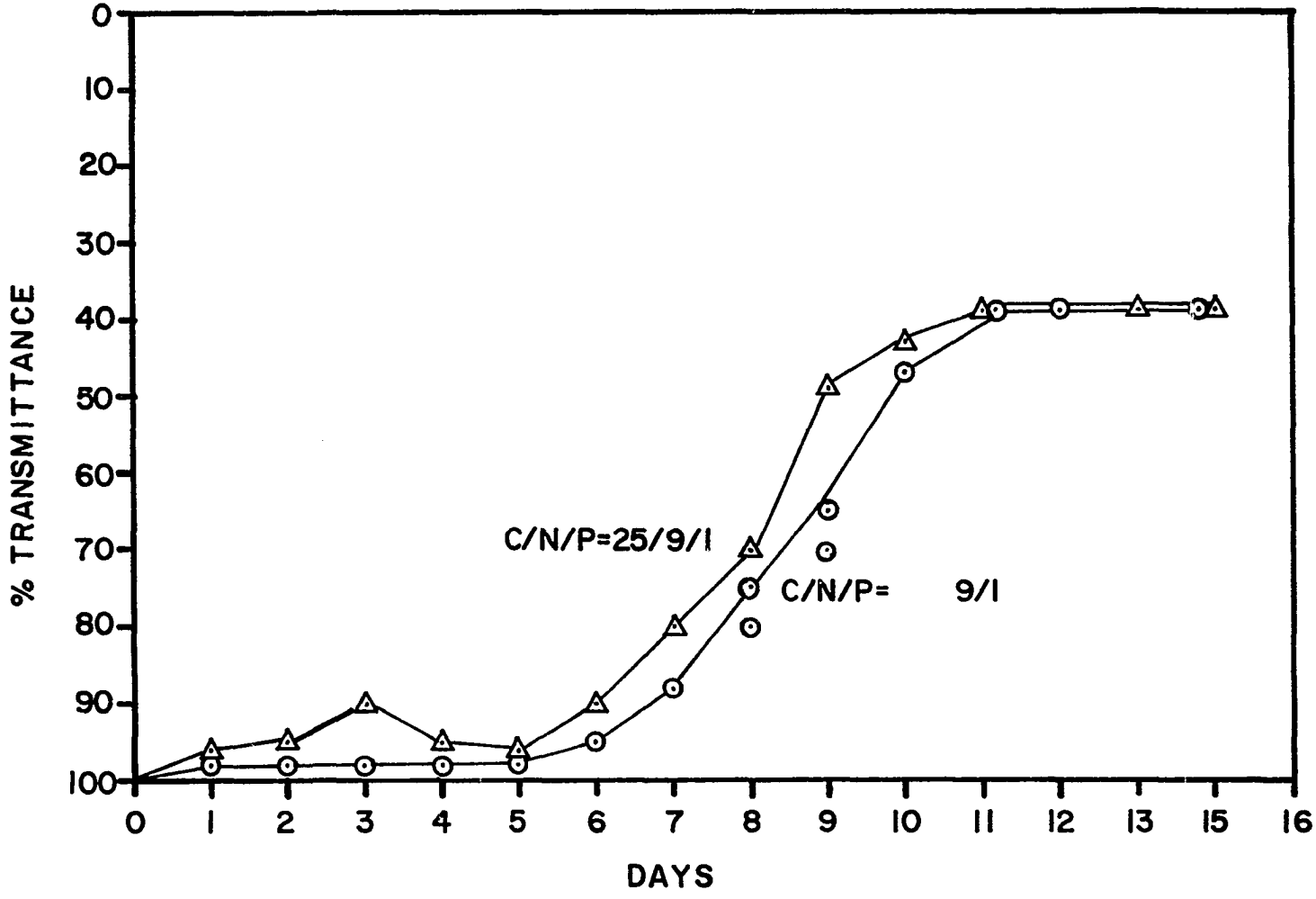

Figure 9. Percent transmittance and ^{32}P uptake for a C/N/P ratio of 25.0/9.0/1.0 and a N/P ratio of 9.0/1.0

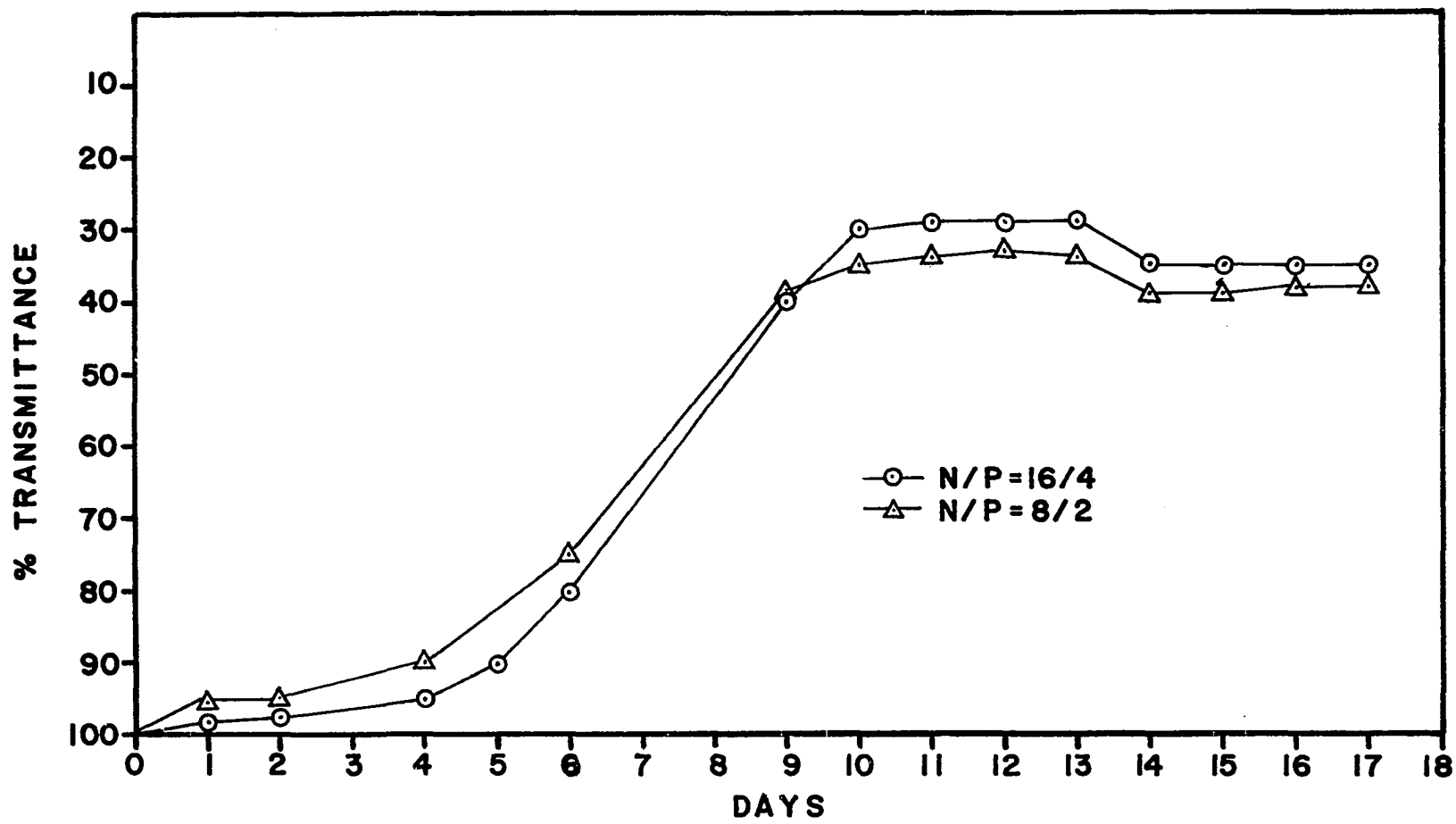

Figure 10. Percent transmittance for N/P ratios of 16.0/4.0 and 8.0/2.0

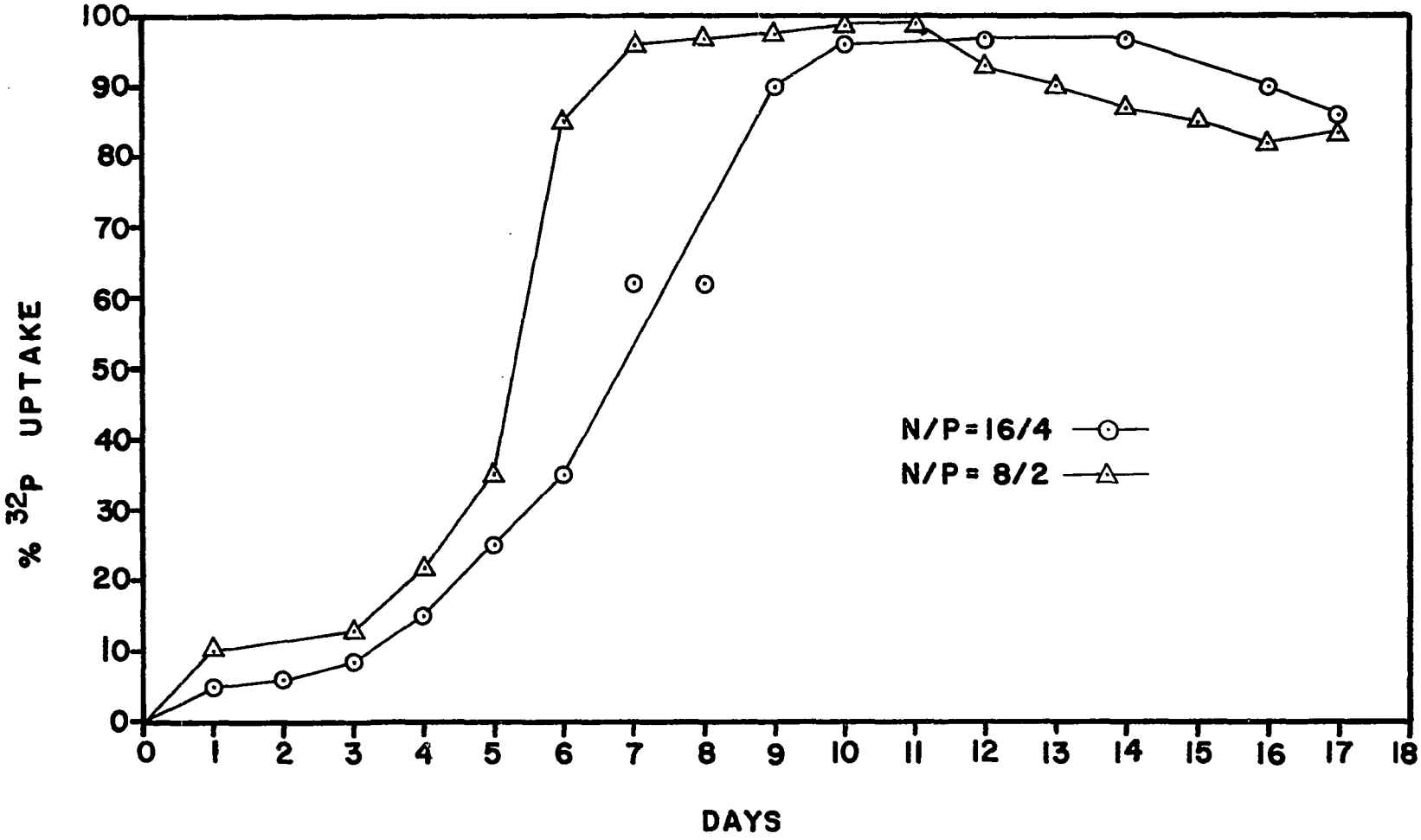

Figure 11. ^{32}P uptake for N/P ratios of 16.0/4.0

both conditions, suggesting that some other nutrient or metabolite is limiting. In this case, the concentration of phosphorus is small enough that possibly the alkalinity in the carbonate-bicarbonate equilibrium has been shifted significantly to inhibit growth at the 16/4 level. As the phosphorus uptake rate indicates, Figure 11, for this system the phosphorus is taken up more rapidly in the 16.0/4.0 condition. But the total amount of ^{32}P remaining at maximum uptake is quite similar. The N/P ratio of 8/2 systems takes up phosphorus at a high rate while the 16/4 ratio takes up radiophosphorus at a somewhat lower rate. The accelerated uptake is dampened by the release of phosphorus in the N/P, 16/4 condition after 10 days.

Incremental increases of nitrogen at N/P ratios of 3/1 to 6/1 to 12/1 with phosphorus being maintained at a level of 0.5 mg/1 is shown in Figure 12. The optimum ratio of nitrogen to phosphorus lies between the ratios of 3.0/0.5 and 6.0/0.5. In terms of a standardized ratio, this is 6/1 to 12/1. A linear relationship between the nitrogen concentration and algae growth at the ratio of 3.0/0.5 and 1.5/0.5, increasing the nitrogen with a proportional increase in the algae-bacteria mass, is noted.

Figure 13 displays growth patterns for 4 ratios at the 1.0 mg/1 level of phosphorus. The response does not necessarily follow the previously determined relationship. The optimum ratio of 9.0/1.0 in this case is within the range determined at the 0.5 mg/1 level of

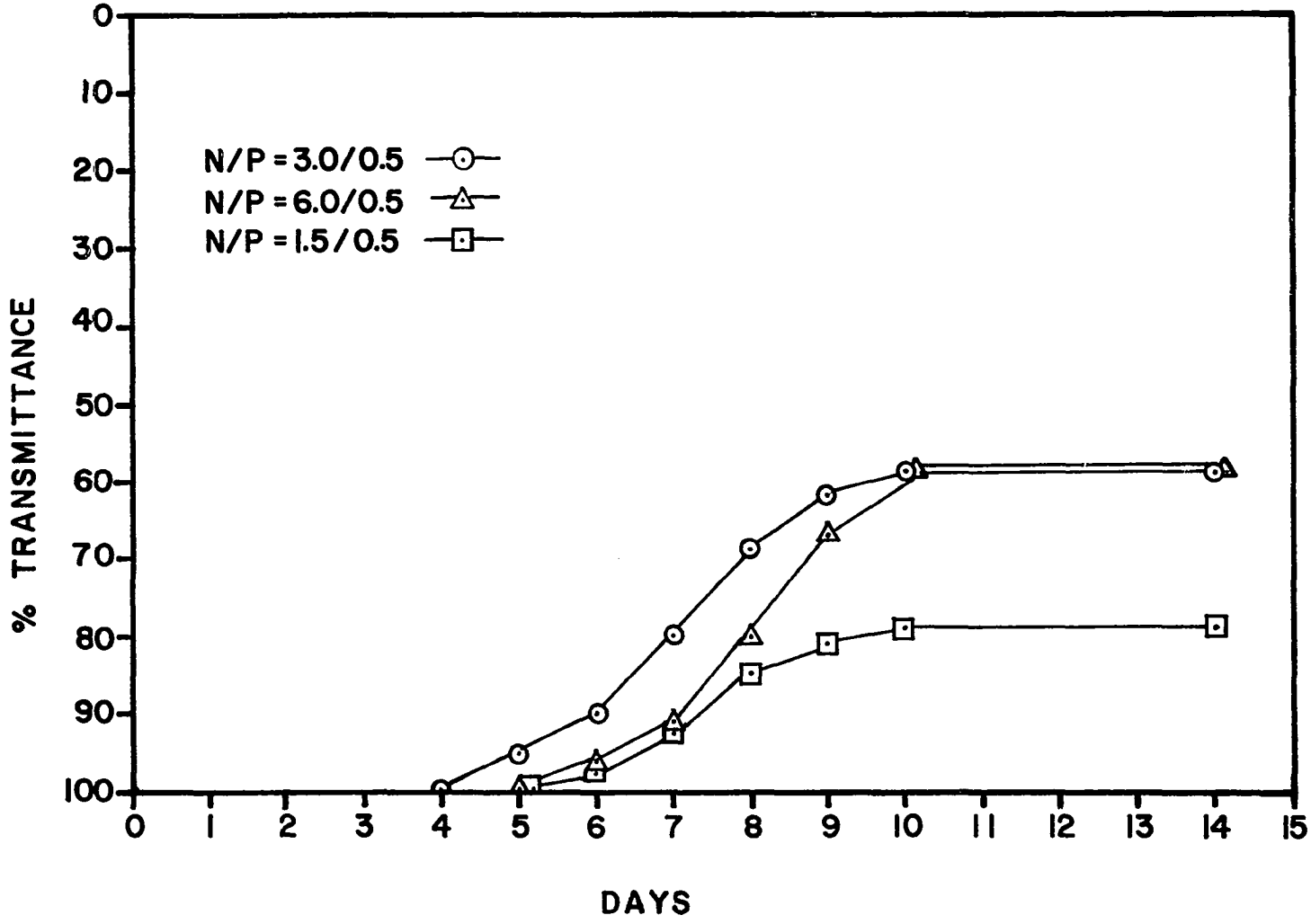

Figure 12. Percent transmittance for N/P ratios of 1.5/0.5, 3.0/0.5, and 6.0/0.5

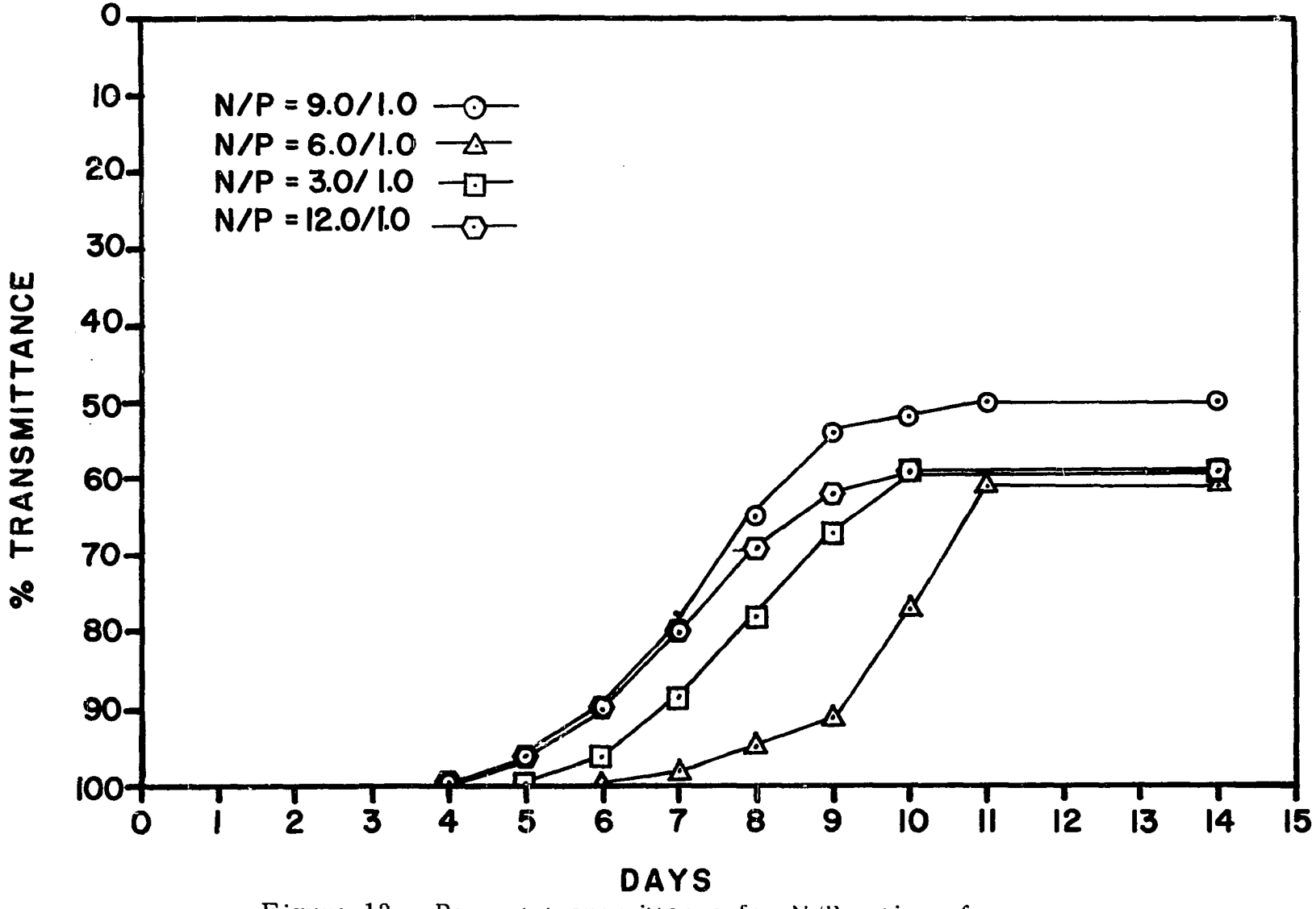

Figure 13. Percent transmittance for N/P ratios of 3.0/1.0, 6.0/1.0, 9.0/1.0, and 12.0/1.0

phosphorus. The lag in the growth at the 6.0/1.0 ratio can be attributed to the lack of acclimation of the algae-bacteria seed to this particular culture media. The rate of growth does show a progressional increase from a 3.0/1.0 ratio up to the optimum ratio and then a decreasing uptake rate to the N/P ratio of 12.0/1.0.

The optimum growth and uptake ratio of a phosphorus level of 1.5 mg/1 is 9/1. The progressional increase up to the optimum and then the decreasing growth is noted as the optimum ratio is exceeded. This phenomena suggests that the nitrogen in the nitrate form suppresses growth, or a sorbed precipitate takes the nitrate out of solution at this concentration, Figure 14.

Figures 15 through 19 show the effect on algae growth of various nitrogen concentrations at a given phosphorus concentration. The rate of growth and the maximum yield are both important criteria in interpreting these results. At the 0.1 mg/1 level of phosphorus, Figure 15, the maximum growth rate is at a N/P ratio of 8/1 and maximum yield is at a N/P ratio of 16/1. At the 2.0 mg/1 of phosphorus level, maximum yield and maximum growth rate appears to be the same with a N/P ratio of 16/1 and 8/1, Figure 16. The 3.0 mg/1 level of phosphorus shows a maximum yield and growth rate of a N/P ratio of 4/1, but the irregular pattern of growth suggests that some other element might be involved other than the two nutrients of interest.

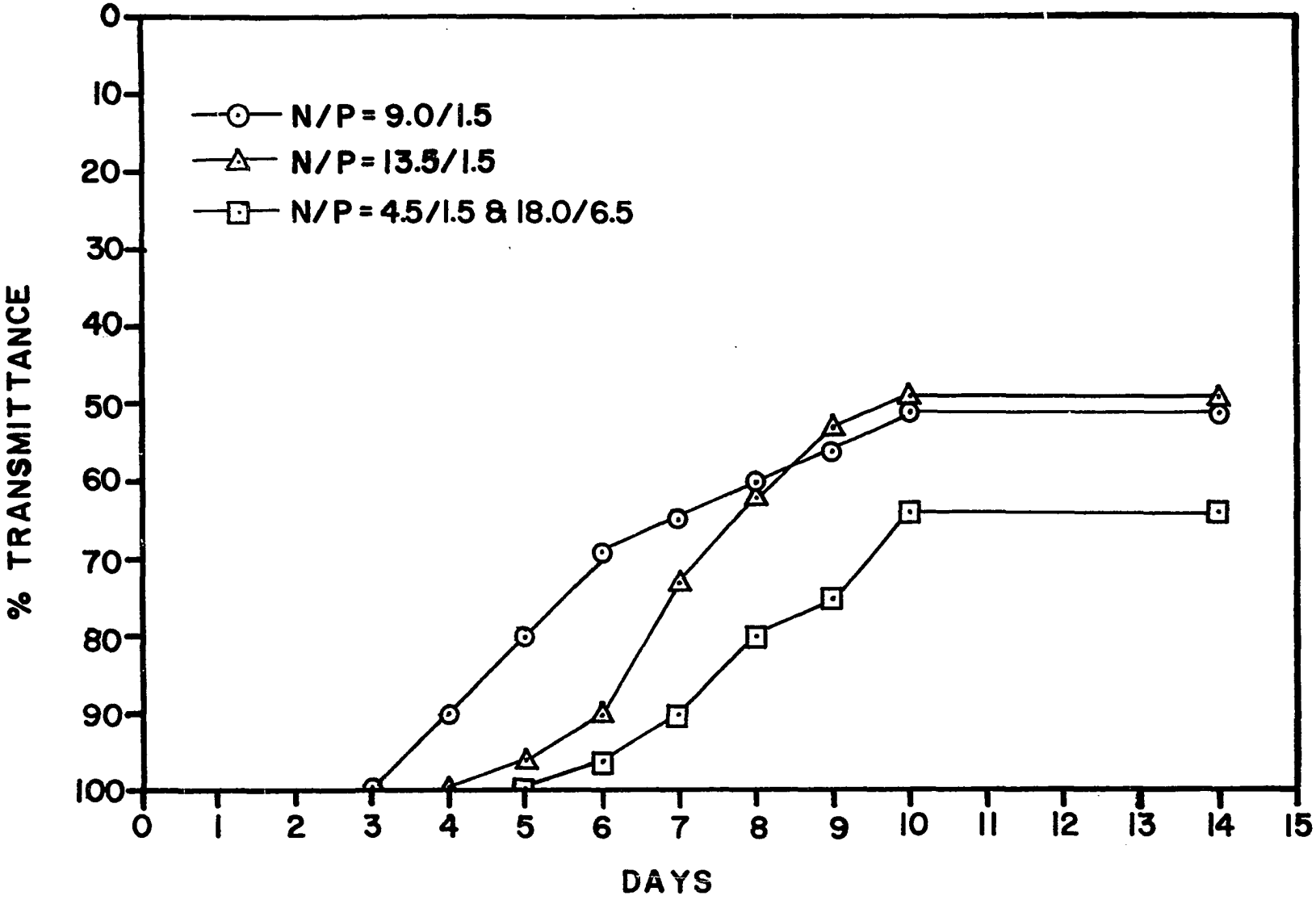

Figure 14. Percent transmittance for N/P ratios of 9.0/1.5, 13.5/1.5, 4.5/1.5, and 18.0/1.5

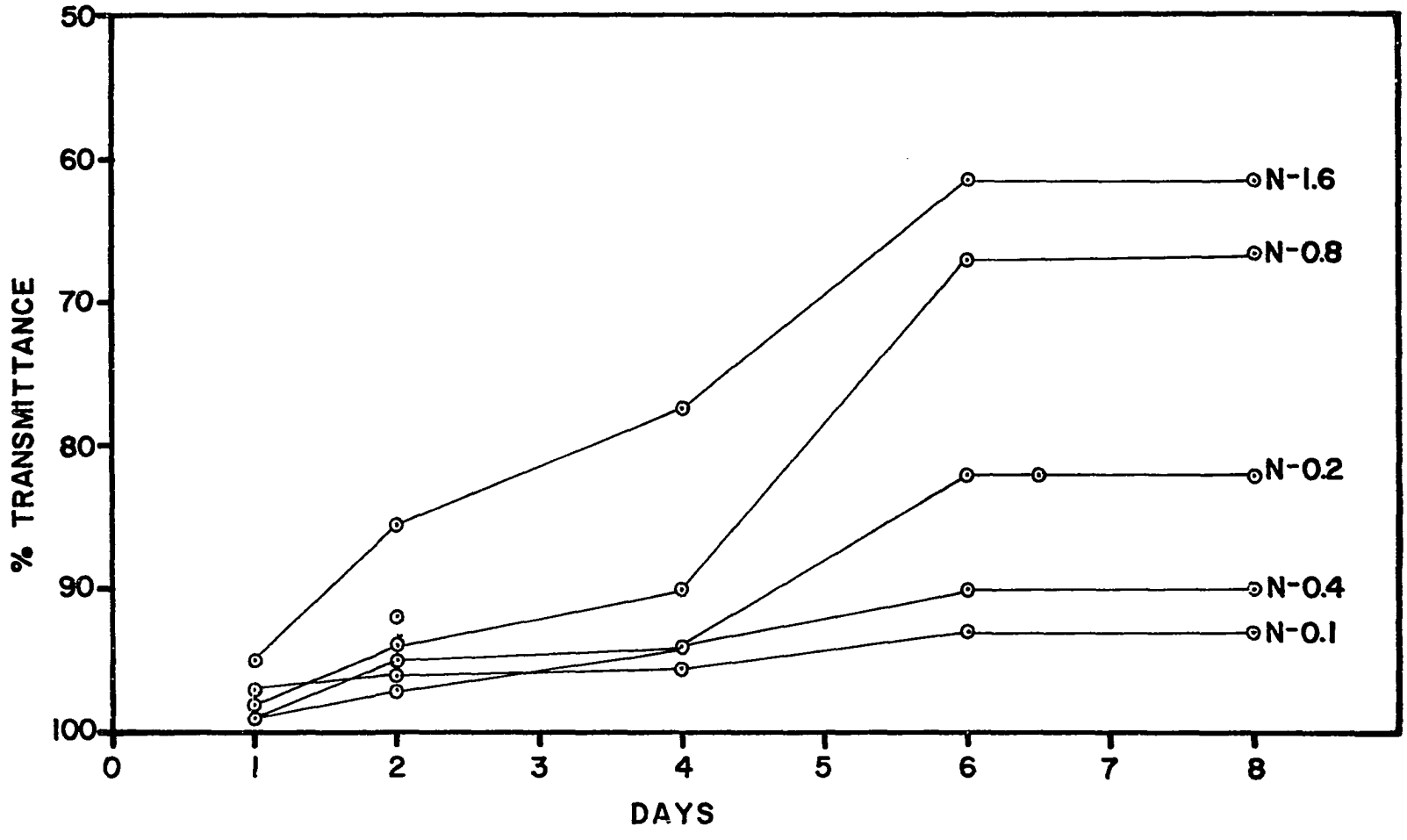

Figure 15. Percent transmittance for N/P ratios at 0.1/0.1, 0.2/0.1, 0.4/0.1, 0.8/0.1, and 16./0.1

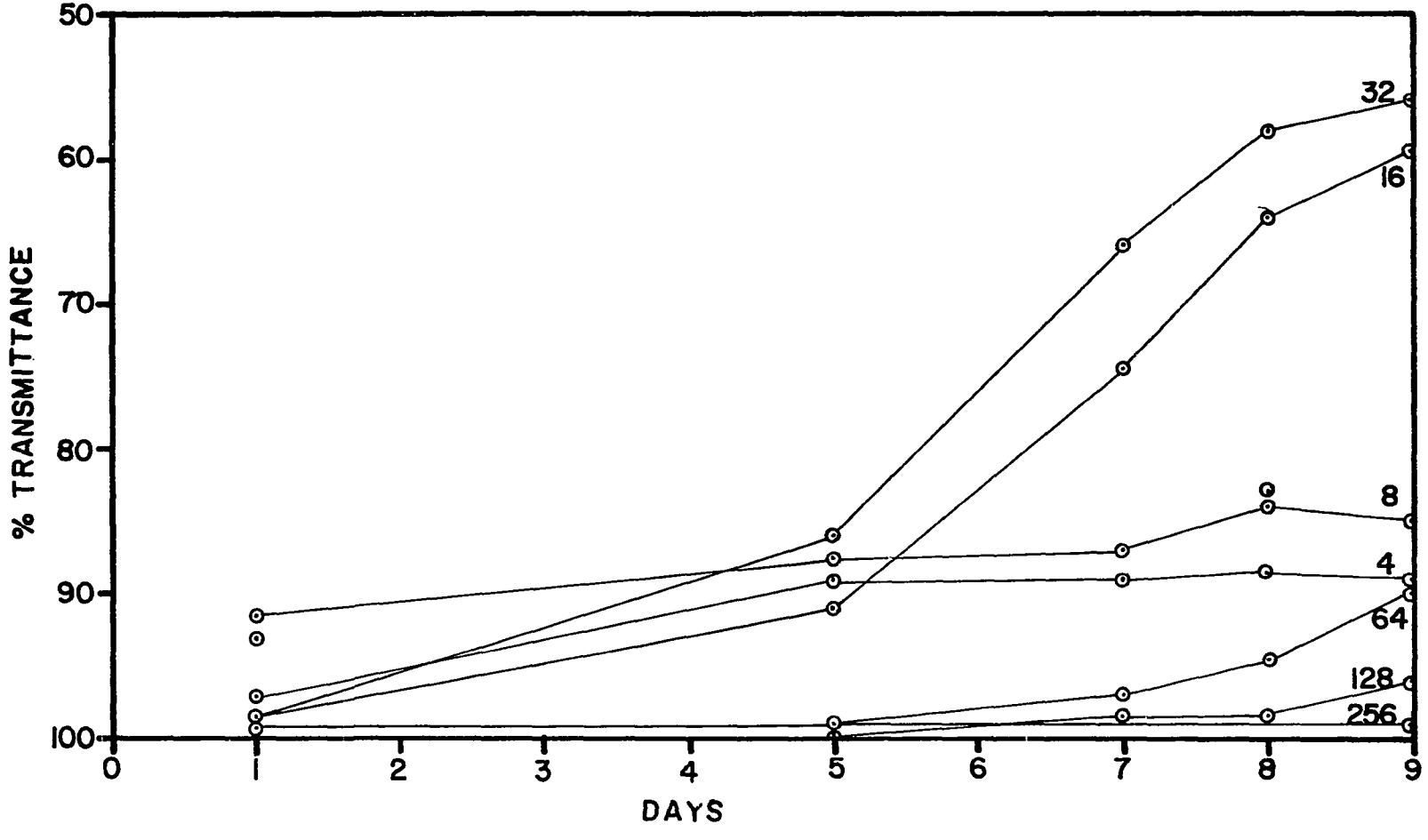

Figure 16. Percent transmittance for N/P ratios of 4.0/2.0, 8.0/2.0, 16.0/2.0, 32.0/2.0, 64.0/2.0, 128.0/2.0 and 256.0/2.0

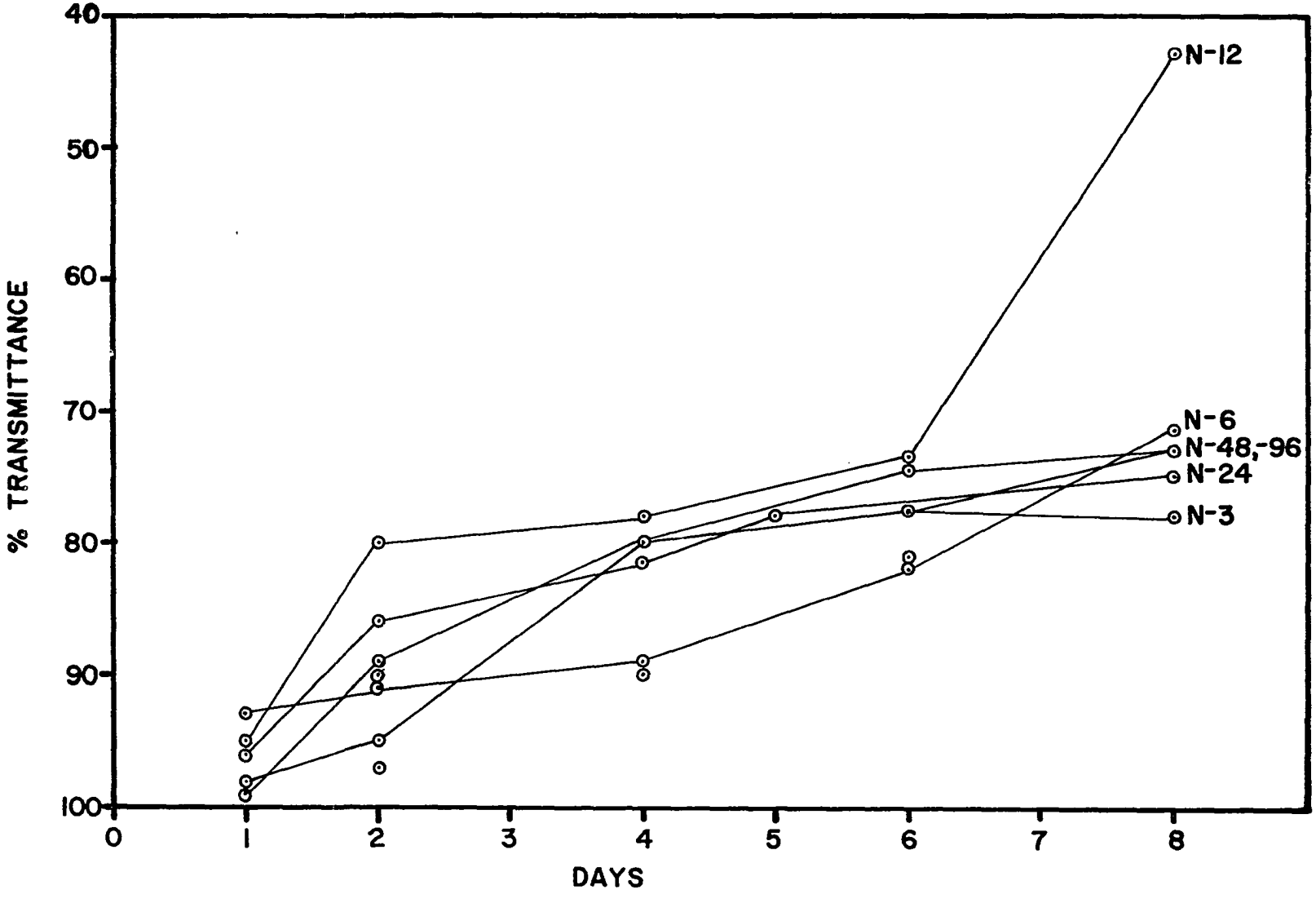

Figure 17. Percent transmittance for N/P ratios of 3.0/3.0, 6.0/3.0, 12.0/3.0, 24.0/3.0, 48.0/3.0, and 96.0/3.0.

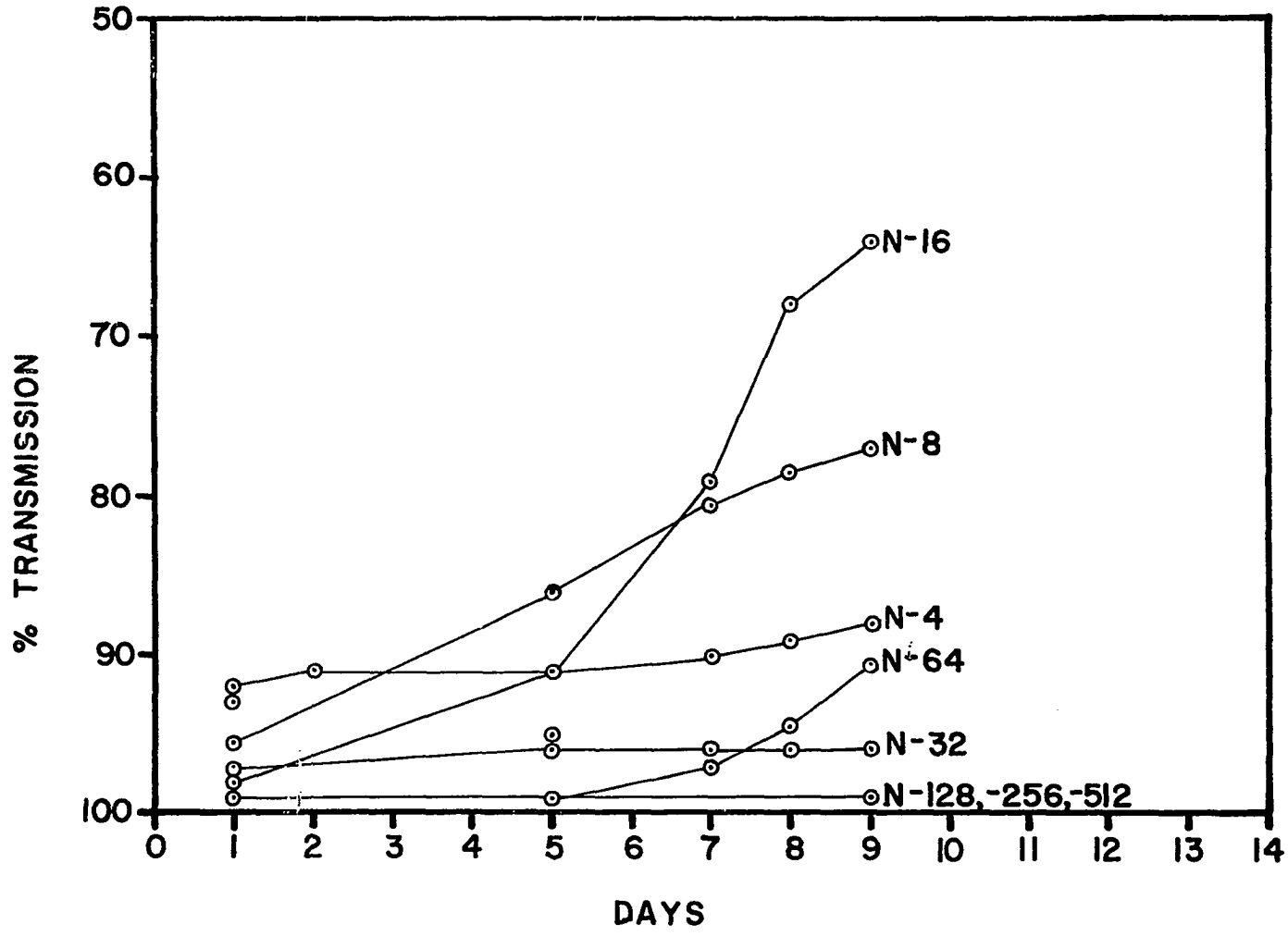

Figure 18. Percent transmittance for N/P ratios of 4.0/4.0, 8.0/4.0, 16.0/4.0, 32.0/4.0, 64.0/4.0, 128.0/4.0, 256.0/4.0, and 512.0/4.0

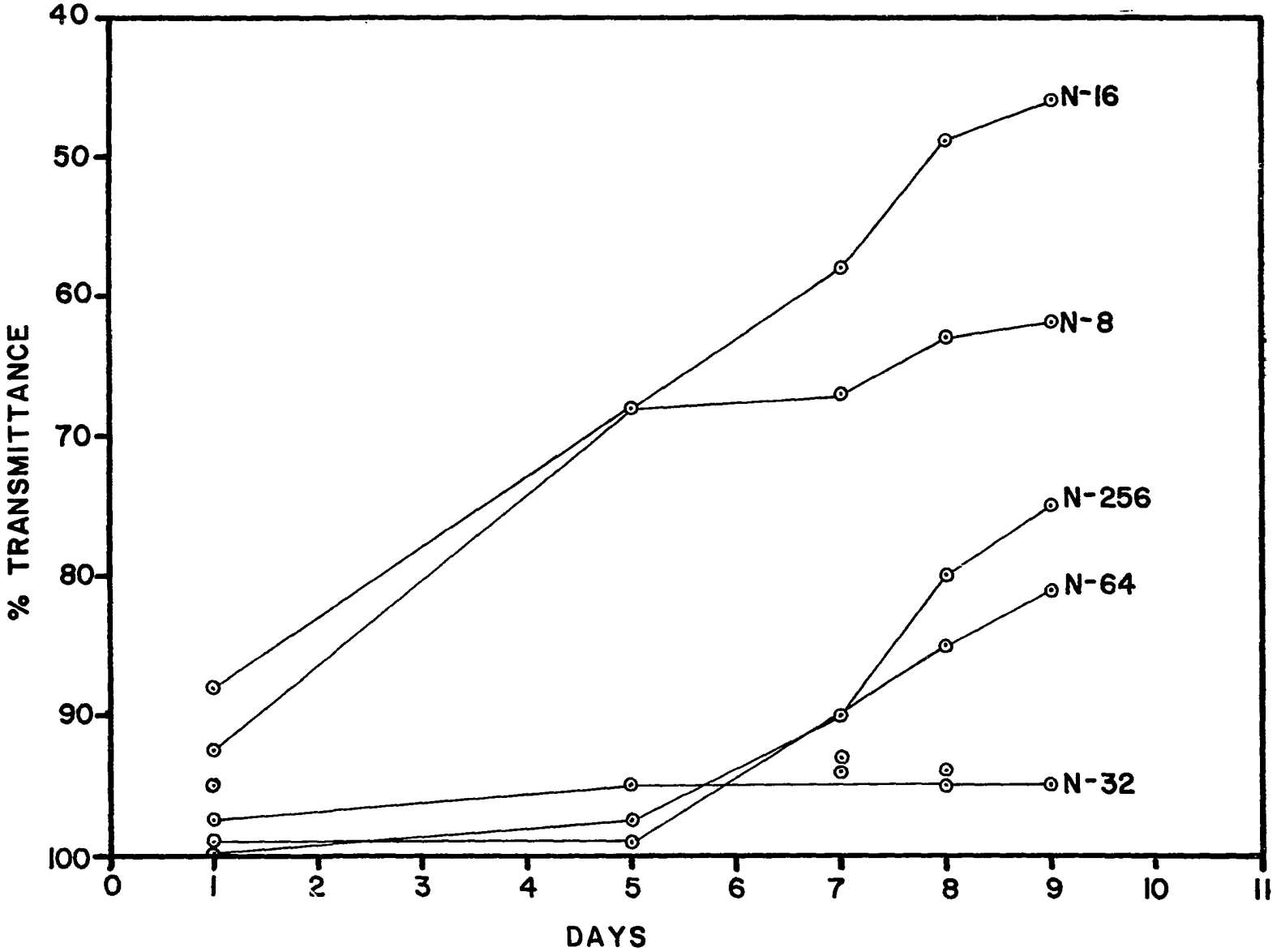

Figure 19. Percent transmittance for N/P ratios of 8.0/8.0, 16.0/8.0, 32.0/8.0, 64.0/8.0, 256.0/8.0

At the phosphorus level of 4.0 mg/1, a maximum yield is obtained at a N/P ratio of 4/1. The inhibitory effect of the NO_3 ion can be shown with diminished growth at concentrations of 128.0 mg/1, 256.0 mg/1, and 512 mg/1. The maximum yields for the various nitrogen concentration at a given phosphorus level are plotted in Figures 21 through 28. The maximum yields for the given nitrogen concentration represents the ultimate productivity regardless of time. At the PO_4-P levels chosen in this study, varied results have been obtained by analyzing data in this manner.

The PO_4-P level in Figure 21, 0.1 mg/1, reveals no optimum ratio, but rather a broad range of ratios with a gradual increase in yield with increased nitrogen.

At a phosphorus level of 8.0 mg/1, a N/P ratio of 2/1 produces the greatest yield. Again the higher concentrations of NO_3-N inhibit the growth of algae.

In Figure 22, at a PO_4-P level of 2.0 mg/1, the optimum ratio of N/P is 16/1. The optimum ratio occurs in a relatively narrow range at this level. Up to the optimum ratio, the nitrogen is limiting the growth of the algae. The algae yield is controlled by the phosphorus concentration as the optimum ratio is exceeded (at the 2.0 mg/1 level of PO_4-P, the optimum ratio of N/P is 16/1).

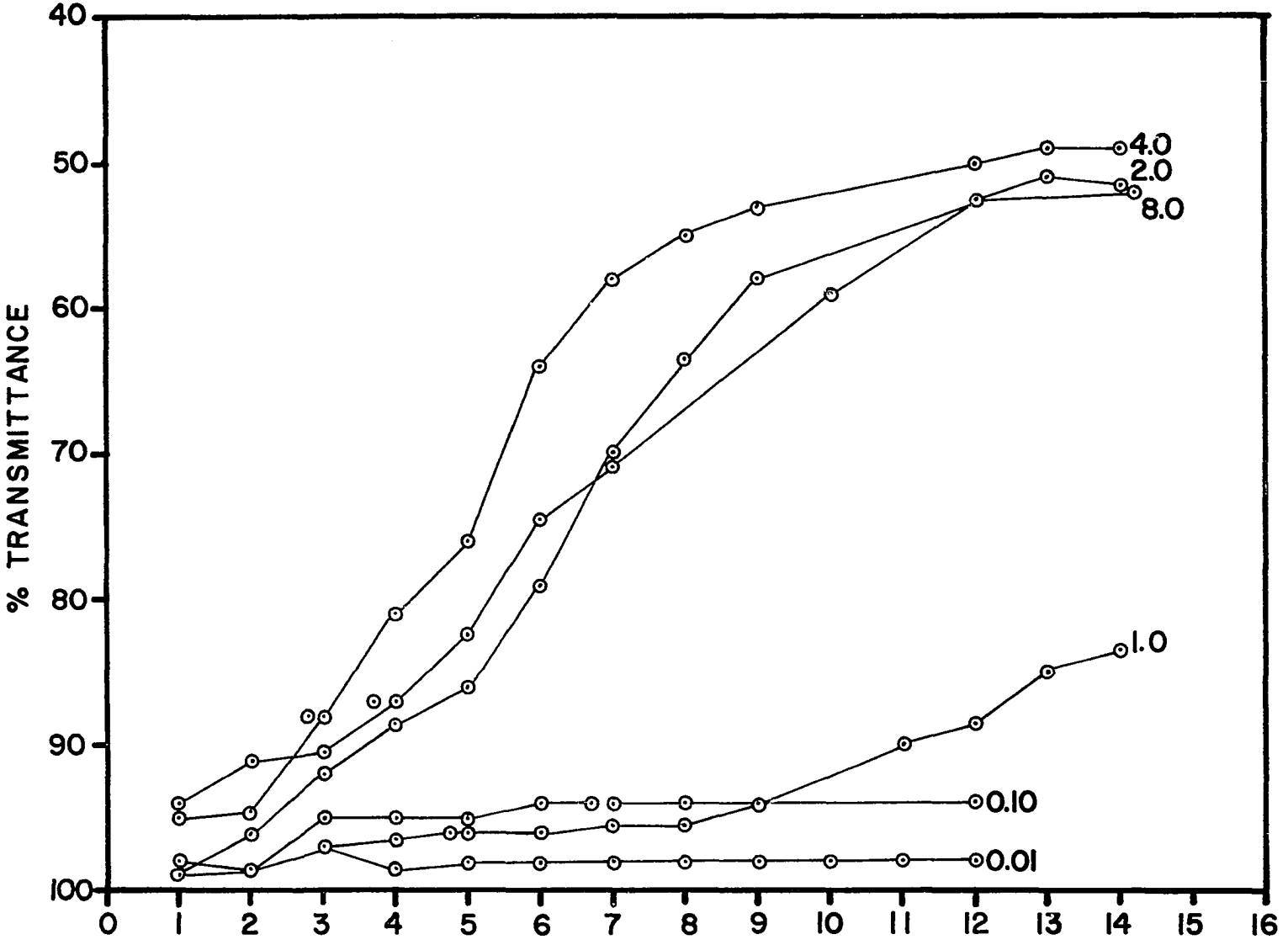

Figure 20. Percent transmittance of C/N/P ratios at 0.01/0.04/0.01, 0.10/0.40/0.10, 1.0/4.0/1.0, 2.0/8.0/2.0, 4.0/16.0/4.0, and 8.0/32.0/4.0

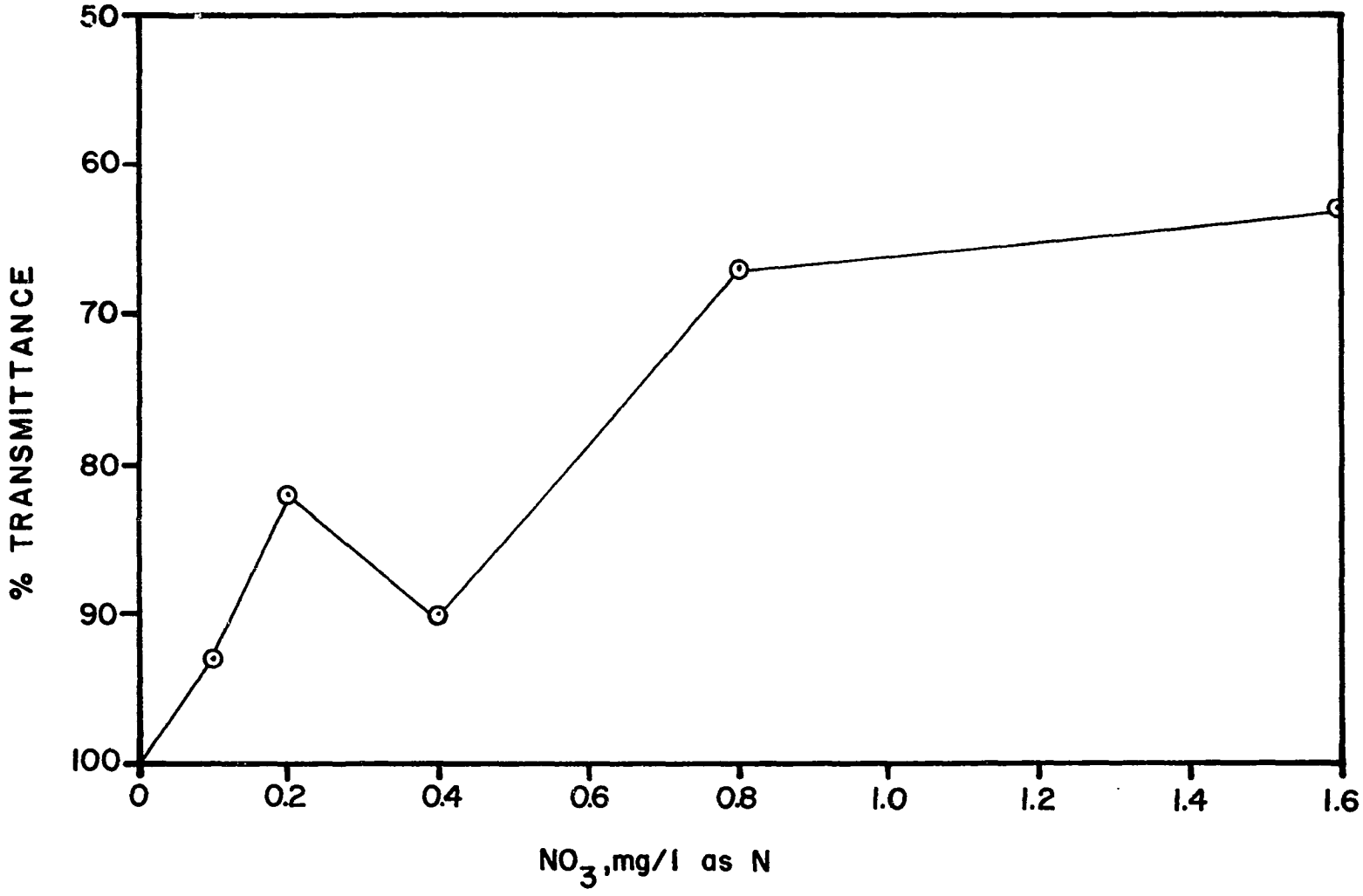

Figure 21. Maximum yield of algae at selected N/P ratios at the 0.1 mg/l level of PO_4 - P

At the 3.0 mg/1, Figure 23, of PO_4-P, a 4/1 ratio produces maximum growth. A rather stabilized yield develops as phosphorus becomes limiting above the 4/1, N/P, ratio. This is not the pattern established at the lower concentrations.

The optimum ratio of the phosphorus level at 4.0 mg/1 is 4/1, Figure 25. These ratios in this case again display the rather narrow range of ratios producing high yields. The NO_3-N concentration exerts its inhibitory influence on this system also.

The uptake of phosphorus as indicated by tracer studies with ^{32}P preceeds the production of algae cells by three to four days. The incorporation of the phosphorus into the algae cell and the subsequent anabolic processes which result in new cells, reproduction, or enlarged cells, growth, follow an extremely complicated network of reactions. The use of the radioactive tracer ^{32}P for determining the growth rate of algae and algae-bacteria systems is a useful technique and can be incorporated into nutritional pollution studies.

The effect of the carbon on the uptake of phosphorus in an algae-bacteria system does not appreciably affect the yield of algae. This suggests that small amounts of phosphorus are necessary for heterotrophic bacteria growths at the carbon concentrations used in this study. More important is that the phosphorus concentration is controlling the production of bio-mass in algae-bacteria systems.

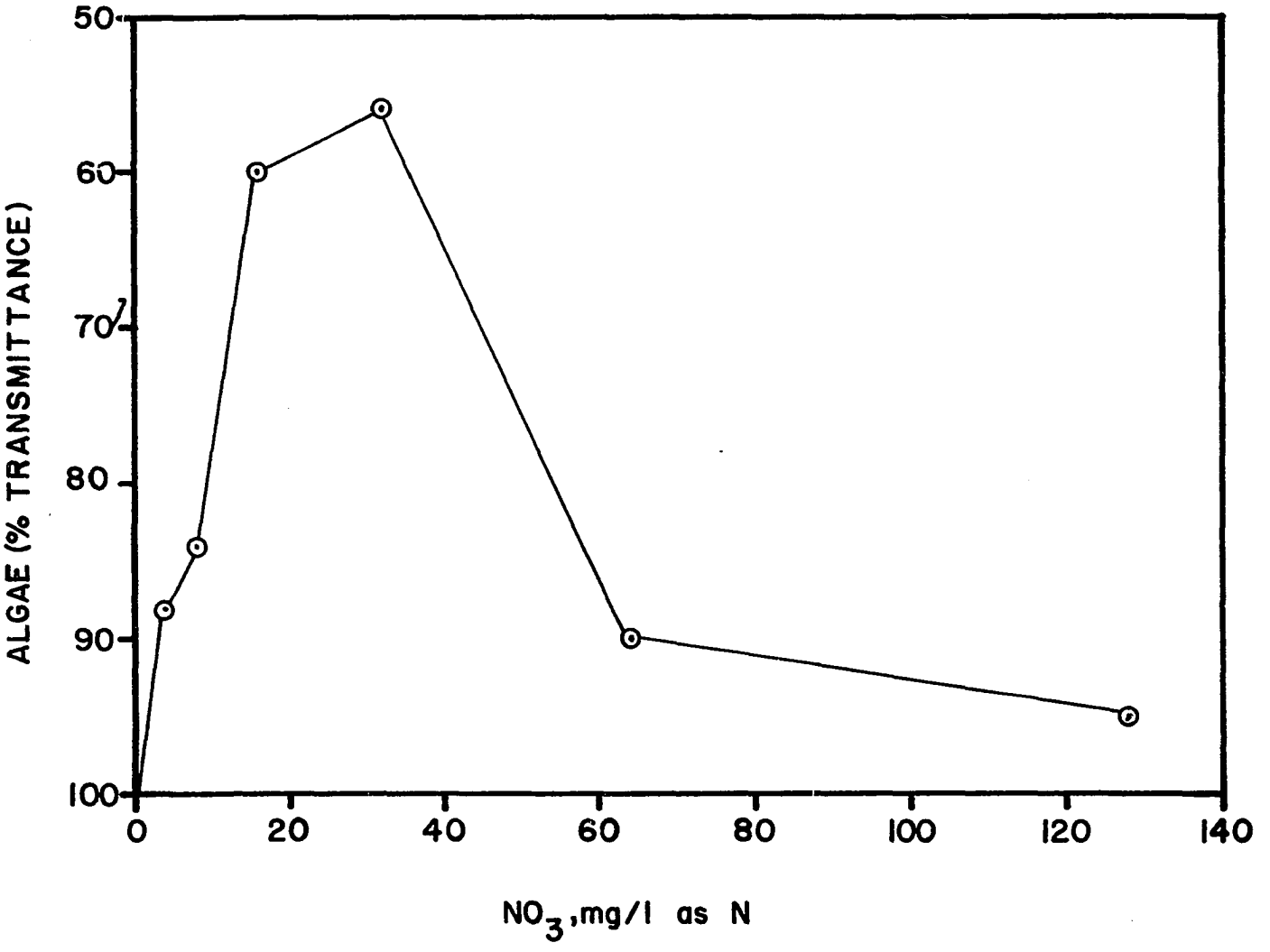

Figure 22. Maximum yield of algae at selected N/P ratios at the 2.0 mg/l level of PO_4 - P

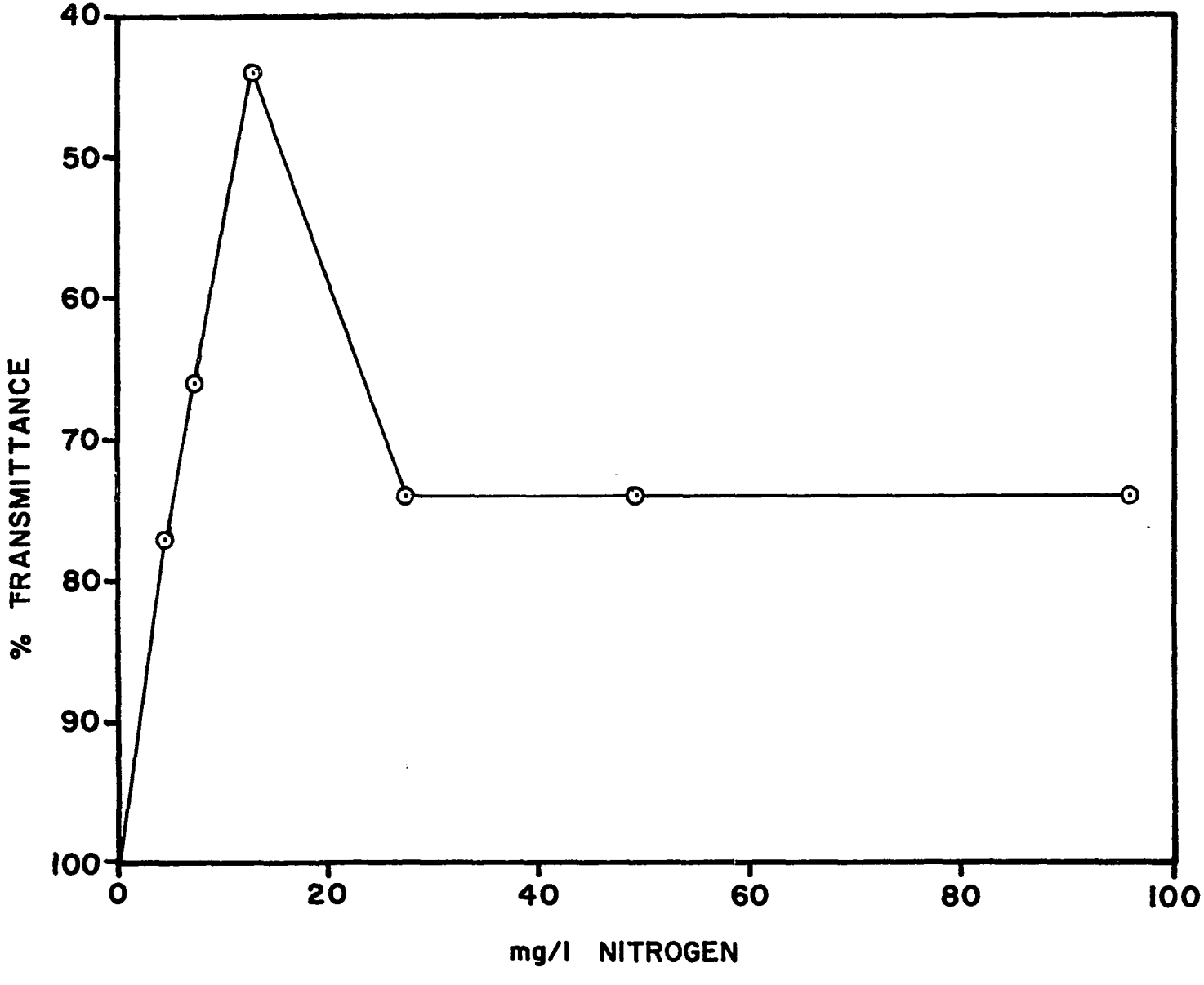

Figure 23. Maximum yield of algae of selected N/P ratios at the 3.0 mg/l level of PO_4 - P

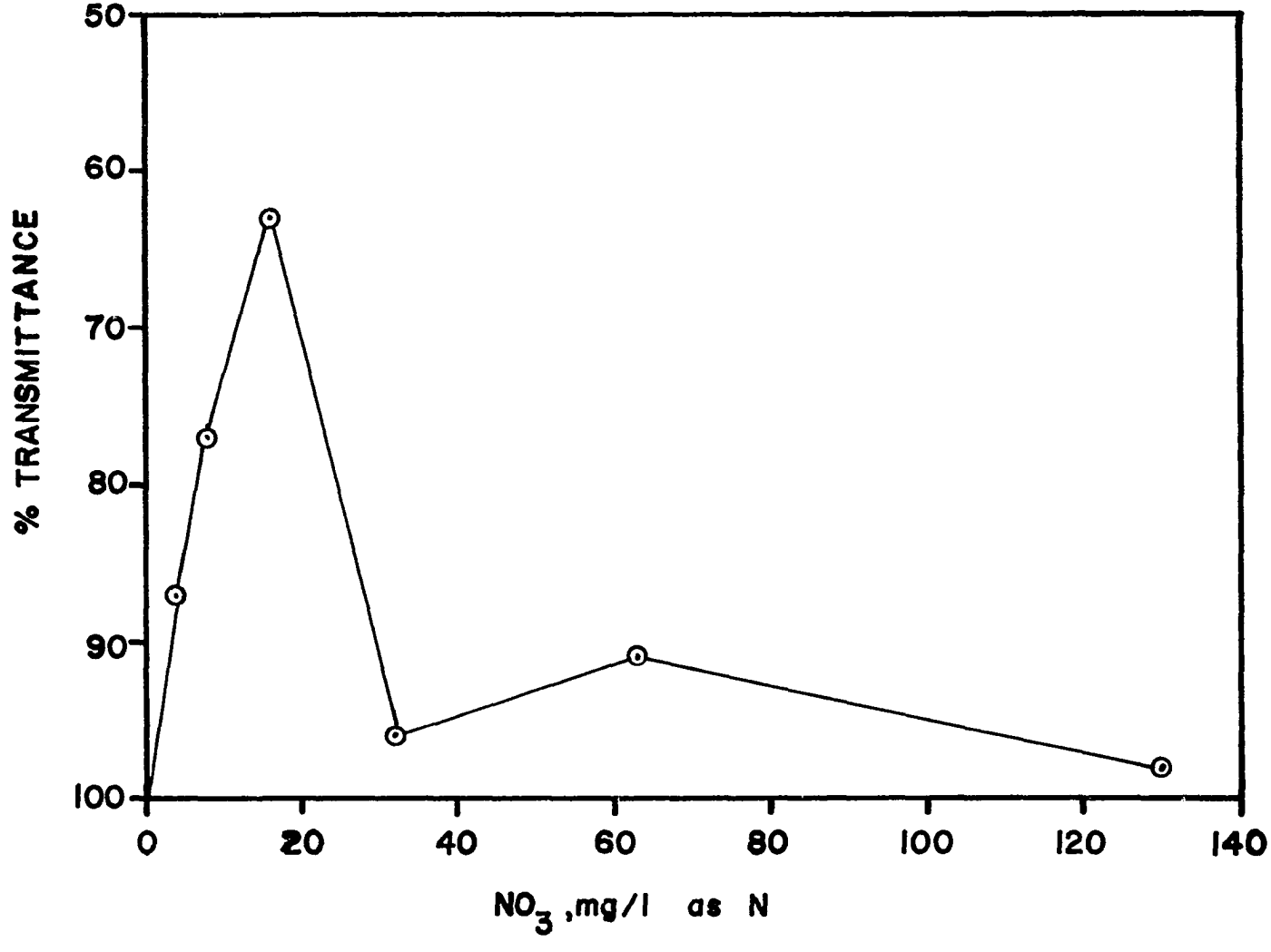

Figure 24. Maximum yield of algae at selected N/P ratios at the 4.0 mg/l level of PO_4 - P

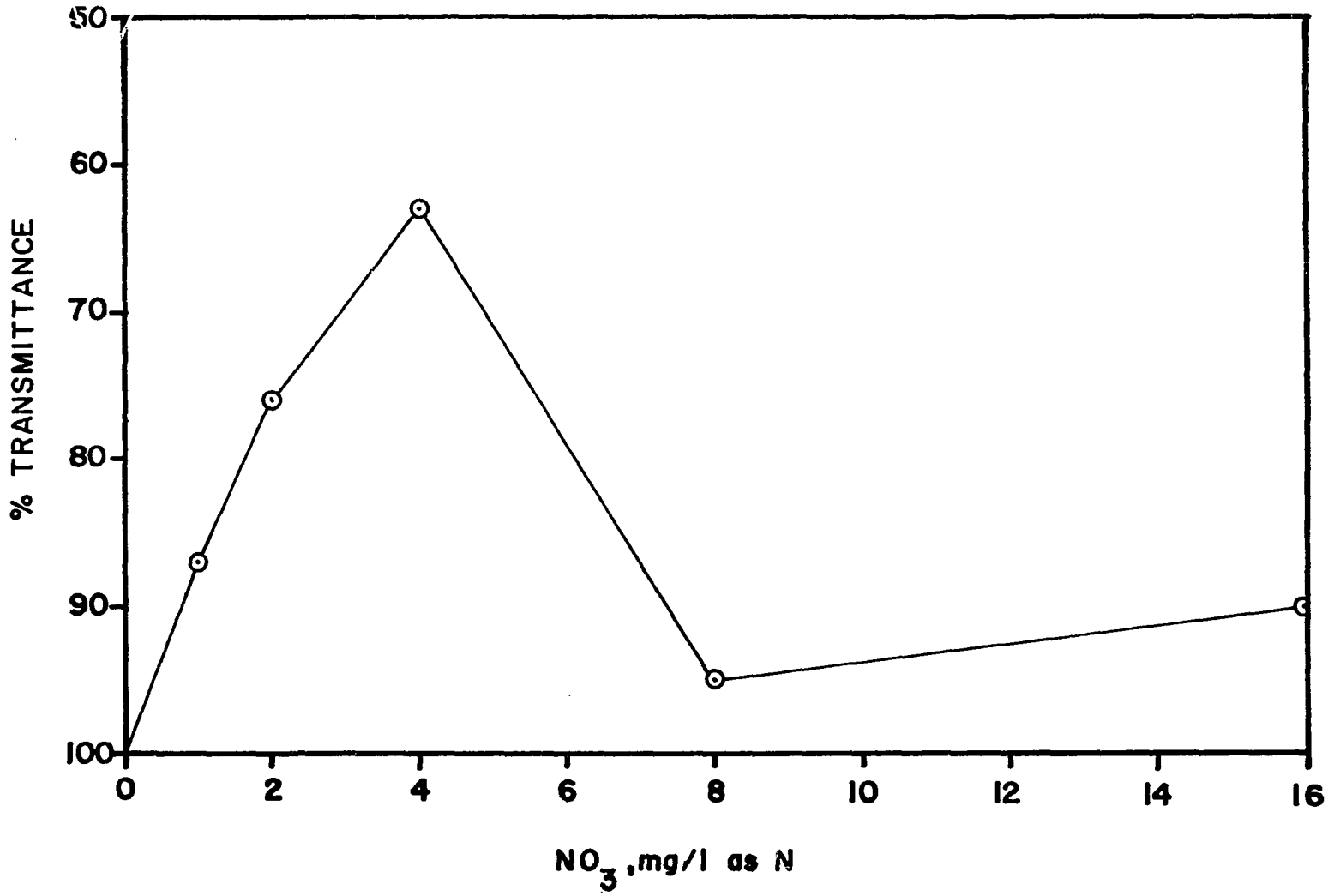

Figure 25. Maximum yield of algae at selected N/P ratios at the 4.0 mg/l level of PO_4 - P

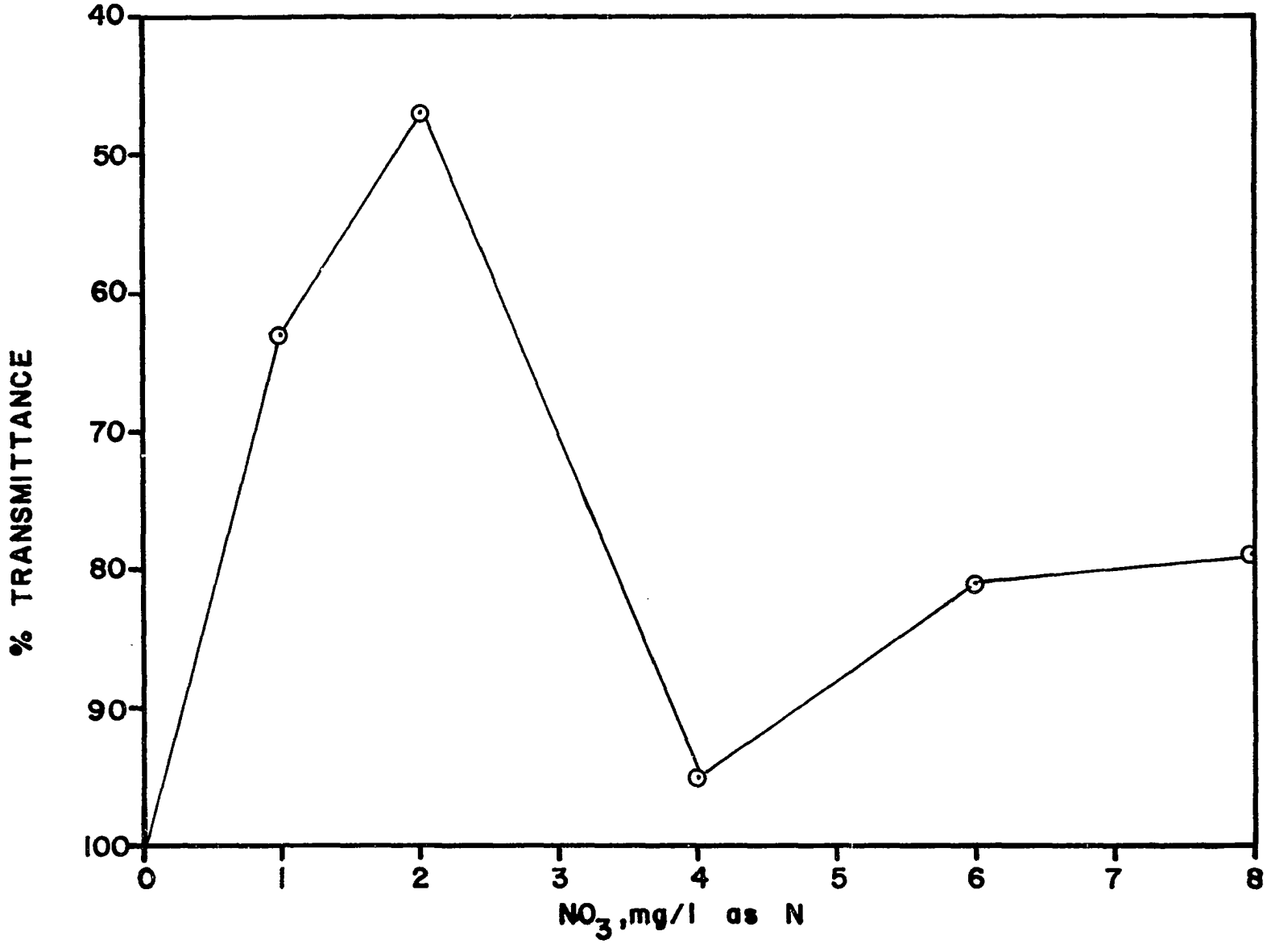

Figure 26. Maximum yield of algae at selected N/P ratios at the 8.0 mg/l level of PO_4 - P

Minimal amounts of phosphorus necessary for algae-bacteria growths of "bloom" proportion is 0.01 mg/1 of PO_4-P, Figure 3. Arbitrarily a 95 per cent transmittance level has been designated bloom conditions for this study. The 95 per cent transmittance level represents the 25,000 ASU, ml or the 5 mg/100 ml dry weight level of algae.

The relationship between nitrogen and phosphorus in algae growth is not as straightforward as some have suggested. The environmental conditions under which the algae is cultured affects the quantity of phosphorus required by the cell. Luxuriant uptake of phosphorus has been suggested by some workers, and could account for the variation in the optimum ratio of N/P for various phosphorus levels in this study. When phosphorus is in excess and other growth conditions are optimum the algae cell stores the phosphorus for adverse conditions.

The continuous growth system allowed the algae-bacteria to act as a plug flow system similar to a receiving stream. In Figure 29, the reduction of the ortho-phosphates can be observed. Ortho-phosphates were removed by biological and chemical processes exponintially at a velocity rate constant of 0.4 day^{-1}.

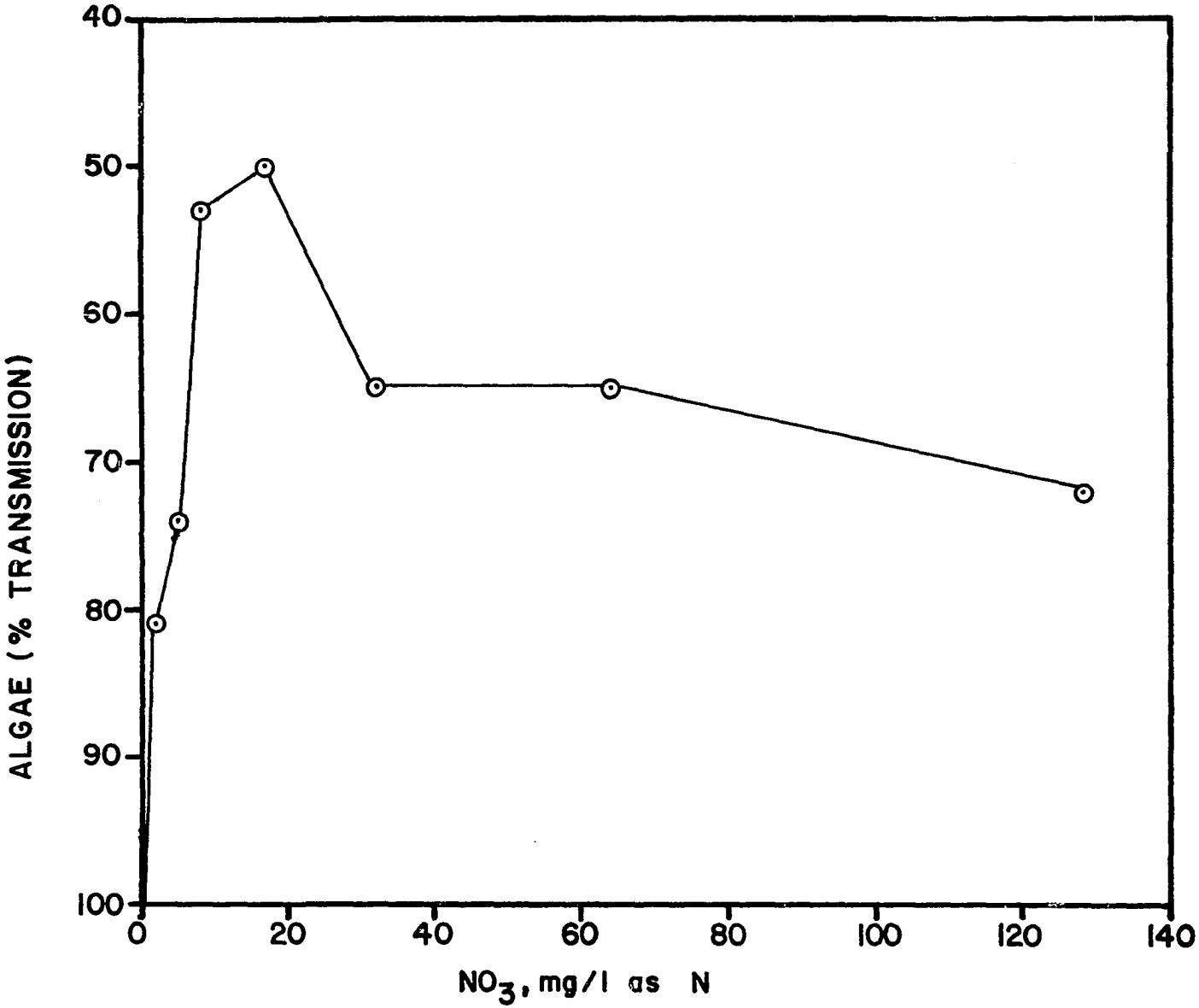

Figure 27. Maximum yield of algae at selected N/P ratios at the 2.0 mg/l level of PO_4 - P, high range

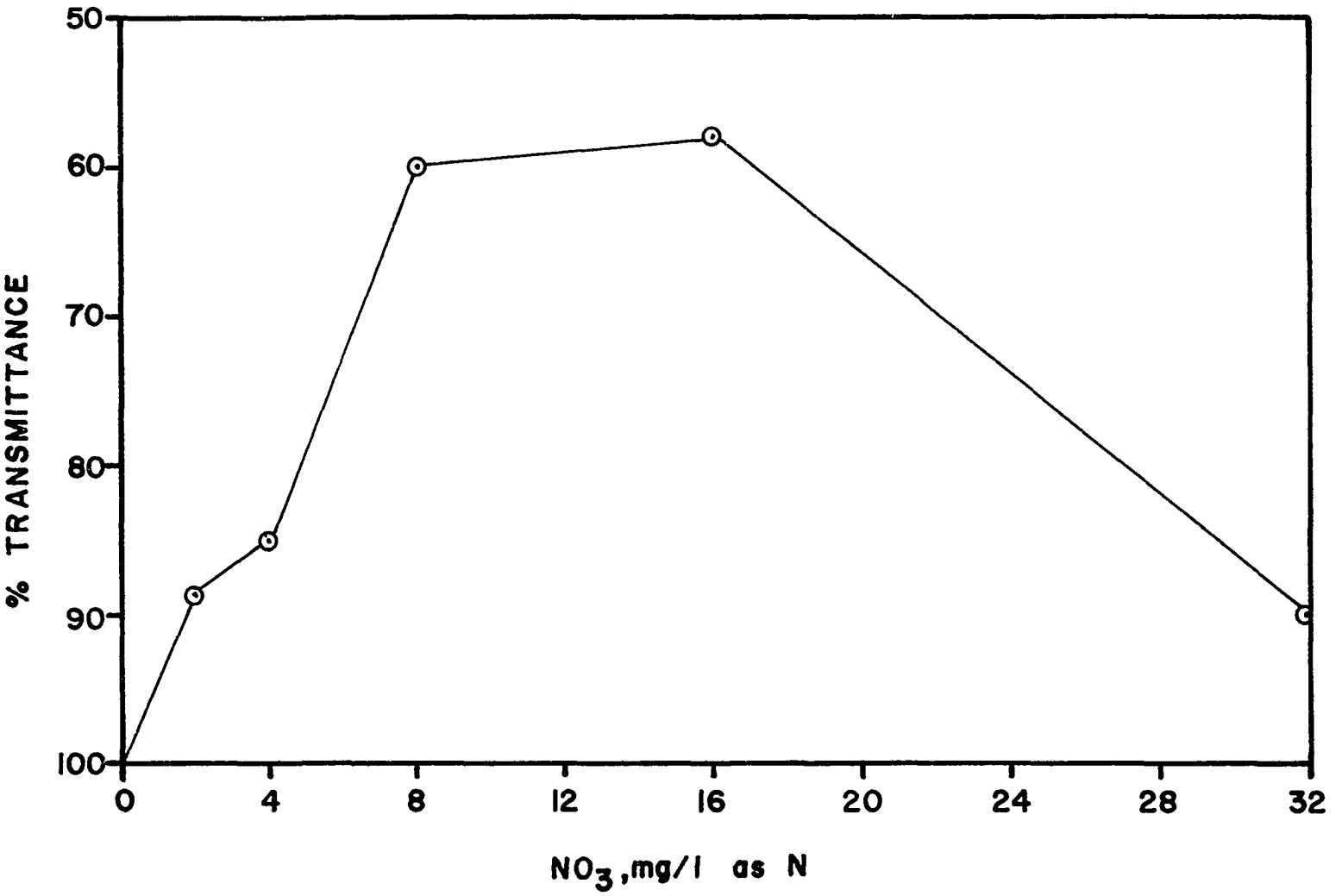

Figure 28. Maximum yield of algae at selected N/P ratios at the 2.0 level of PO_4 - P, low range

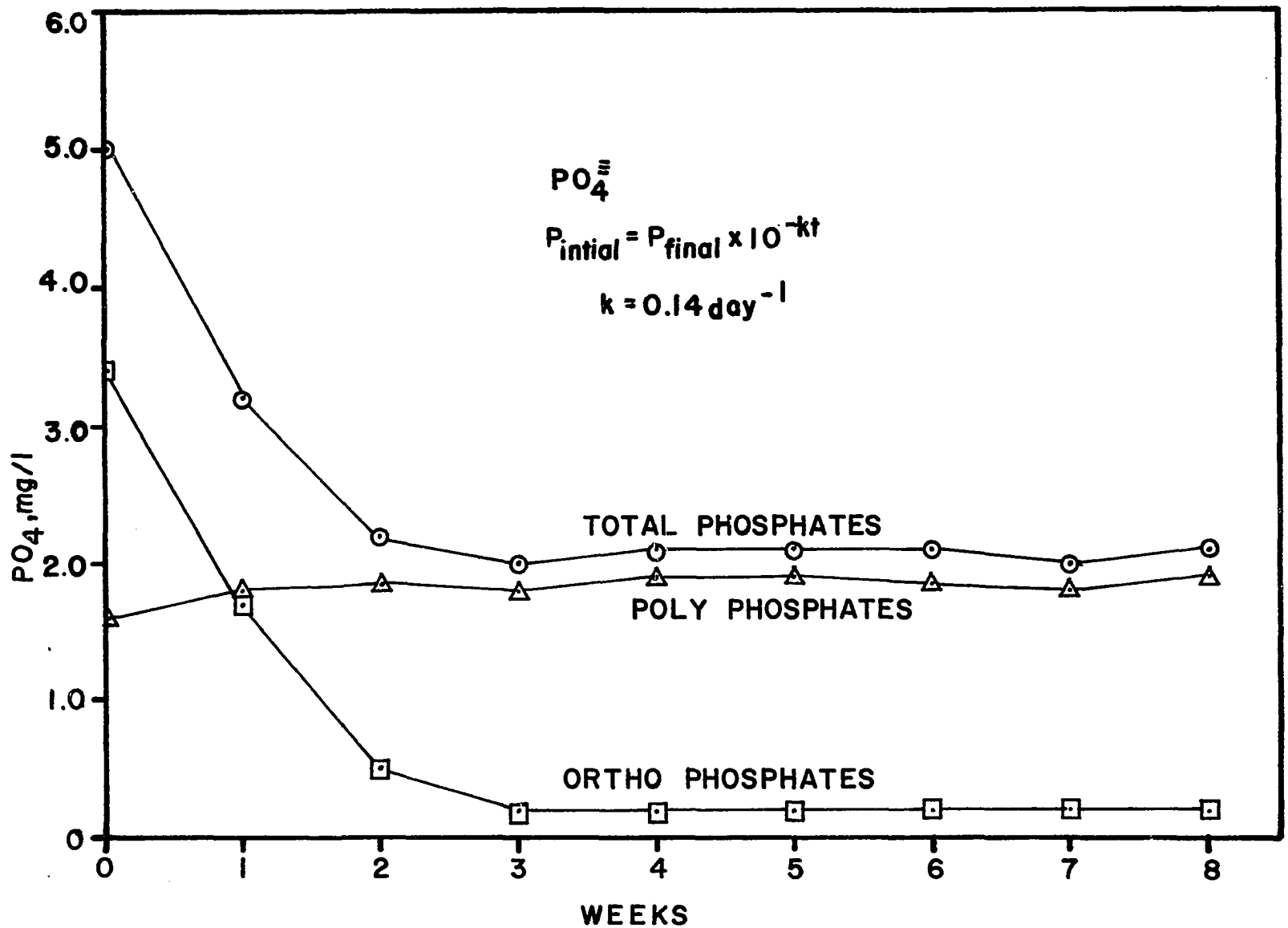

Figure 29. Assimilation of ortho phosphates by the algae in a continuous culturing device and subsequent release of meta-phosphates

TABLE XII

GROWTH RATE VELOCITY CONSTANTS AND MAXIMUM YIELDS OF ALGAE FOR VARIOUS CONCENTRATIONS OF NITROGEN AND PHOSPHORUS

N mg/l	P mg/l	N/P	Yield mg/100 ml	K day^{-1}
32	2	16/1	124	0.159
16	2	8/1	114	0.214
8	2	4/1	25	0.055
4	2	2/1	20	0.274
64	2	32/1	17	0.615
9	1	9/1	163	0.180
16	8	4/1	157	0.180
8	8	1/1	106	0.085
64	8	8/1	46	0.582
256	8	32/1	66	0.351
16	4	4/1	101	0.301
16	8	2/1	59	0.101
4	4	1/1	26	0.058
64	4	16/1	17	0.453
8	2	4/1	103	0.305
16	4	4/1	130	0.274
32	2	16/1	130	0.164
1.6	.1	16/1	107	0.190
.8	.1	8/1	59	0.204
.2	.1	2/1	39	0.419
.4	.1	4/1	17	0.233
.1	.1	1/1	7	0.118
12	3	4/1	176	0.210
6	3	2/1	79	0.168
3	3	1/1	59	0.264
24	3	8/1	69	0.173
48	3	16/1	77	0.281
96	3	32/1	77	0.252

TABLE XIII

GROWTH RATE VELOCITY CONSTANTS AND CORRELATION COEFFICIENTS, R^2, FOR 10 SAMPLES GROWN AT A NITROGEN CONCENTRATION OF 10.0 mg/l AND A PHOSPHORUS CONCENTRATION OF 1.0 mg/l

Sample #	K Day^{-1}	R^2	Maximum Yield mg/100 ml
1	0.082	0.969	46.0
2	0.046	0.987	57.0
3	0.042	0.989	38.0
4	0.058	0.941	41.0
5	0.047	0.993	52.0
6	0.044	0.989	51.0
7	0.041	0.986	44.0
8	0.095	0.926	58.0
9	0.162	0.866	52.0
10	0.043	0.969	46.0

TABLE XIV

SOME OF THE LINEARIZED TRANSFORMATIONS

USED IN REGRESSION ANALYSIS MODELS

Model	R
$V(1) / Z = B_o + B_1V(1) + B_2V(2)$	.933
$V(1) / Z = B_o + B_1V(1) + B_2V(2) + B_3V(2) / V(1)$	.950
$V(1) / Z = B_o + B_1V(1) + B_2V(2) + B_3V(1)V(2)$	.958
$ALOG(Z) / V(1) + V(2) = B_o + B_1(1.0/V(1)) + B_2(1.0/V(2))$	.965
$V(2) / ALOG(Z) = B_o + B_1V(1) + B_2V(2) + B_3(V(1)/V(2))$	.978
$V(2) / ALOG(Z) = B_o + B_1V(1) + B_2V(2)$	.978

Z = algae, mg/100 ml
V(1) = nitrogen, mg/l
V(2) = phosphorus, mg/l
ALOG(Z) = log of the algae

CONCLUSIONS

The effect of the nutrients nitrogen and phosphorus on algae has been determined for the conditions selected in this study. Of importance is the symbiotic consideration with the heterotrophic bacteria in an ecosystem, such as a sewage treatment plant's effluent receiving stream. The carbon enriched nitrogen and phosphorus studies reveal the following:

1) A bacterial bloom occurs within the second or third day in contrast to the algae growth which usually requires six to eight days.
2) ^{32}P uptake studies show immediate uptake of the soluble phosphate, 80-90% in the first day.
3) The maximum yield of algae is the same for carbon enriched and non-enriched conditions.
4) Based on the above, phosphorus metabolic by-products are easily metabolized by the algae.

The effect of the nitrogen to phosphorus ratio, N/P, appears to manifest itself in different phosphorus levels. No straightforward relationship was developed by this study concerning the N/P ratio.

Each series of N/P ratios tested produced an optimum concentration of nitrogen and phosphorus (Table XV).

Variation in the optimum ratio for an algae-bacteria symbiotic system based on this study could be due to the following:

1) The phosphorus is reused in the case of autotrophic organisms as the phosphorus is cycled through the system, promoting secondary populations of algae.
2) The physiological condition of the seed population of algae and bacteria, such as the age of the innoculate.
3) The luxuriant uptake of phosphorus by the algae-bacteria which would produce a lower optimum ratio.
4) Competition between the heterotrophic and autotrophic segments of the population.

The minimum concentration necessary for algae growth in this study was found to be above 0.01 mg/l of PO_4-P or lower. The 0.01 mg/l level is the smallest concentration used in the experimental design. At a PO_4-P concentration of 0.10 mg/l, the algal bloom condition threshold is reached. Regardless of the nitrogen concentration, this threshold bloom condition is attained with 0.10 mg/l of PO_4-P. This narrow range of sensitivity to the phosphorus concentration demonstrates the problem in attempting to control nutritional pollution. The ability of the phosphorus to control algae growth at this lower concentration could be due to several things:

TABLE XV

OPTIMUM RATIOS OF N/P AT VARIOUS PO_4 - P LEVELS

Phosphorus Level (mg/l)	Optimum Ratio	Yield (mg/100 ml)
0.5	9/1	60
1.0	9/1	50
1.5	9/1	48
0.1	16/1	60
2.0	16/1	55
3.0	4/1	45
4.0	4/1	65
8.0	2/1	50

1) Recycling of the nutrients as metabolic by-products sustaining growth.

2) N_2 fixation by bacteria in low nitrogen concentration conditions and high phosphorus conditions.

3) Variable efficiency by the algae in using phosphorus in the energy transformation reactions--in the restricted phorphorus in storage, more phosphorus in the high energy phosphate bonds.

A multivariable regression analysis, with the algae growth the independent variable and the nitrogen and phosphorus the dependent variable, was attempted. Several linearized models based on possible functional relationships were fit with the experimental data. The correlation coefficient, R^2, the number of interactions and power of polynomial were used to evaluate the models as to their applicability and to their goodness of fit.

The following linearized form gave a correlation coefficient, R^2, of 0.9216 with no interaction inputs.

$$V(2) / ALOG(Z) = B_0 + B_1V(1) + B_2V(2)$$

Z = algae-bacteria biomass - mg/100ml

V(1) = nitrogen concentration, mg/ml

V(2) = phosphorus concentration, mg/ml

$B_0 = 0.0413$

$B_1 = 0.0060$

$B_2 = 0.2204$

The F statistic for $F_{.01(2,44)} = 99.47$ while the computed value is 497.0; therefore the variance test is significant at this level. Since: $99.47 < 497.0$.

This equation demonstrates the sensitivity of algae to the phosphorus concentration in that low concentration of nitrogen and low concentrations of phosphorus produce significant algae growth.

$$ALOG(Z) = V(2)/(0.0413 - .0060(V(1)) + .2204(V(2))$$

$$Z = 10^{V(2)/(0.0413 - .0060\,V(1) + 0.2204\,V(2))}$$

This equation does not imply a functional relationship between nitrogen, phosphorus, and algae of this form. Quite possibly, many relationships could give a correlation coefficient of this magnitude. It does imply that the variation can be explained within the limits set by R^2 for the given variables.

This research demonstrates the sensitivity of algae growth to the nutrient concentration and the interaction between an algae-bacteria symbiotic system in relation to the phosphorus concentration. Further studies combining other ecological conditions, such as turbidity, light, alkalinity, and harvesting herbivores in various combinations with the nutrient concentrations need to be made. If information is to be obtained that can be incorporated into

engineering planning studies of nutritional pollution, broad assumptions must be verified and qualified.

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Program For Dolittle Procedure used on IBM 704C

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      1122 15528 01  GEARHEART                                    069025

    WATFOR

      DIMENSION AY(13),BY(13)
      DIMENSION BETA(13),X(13)
      DIMENSION SUM(13),SCPY(13)
      DIMENSION CCPY(13),SB(13)
      DIMENSION XPAR(13),V(13)
      DIMENSION A(13,13),B(13,13)
      DIMENSION C(13,13),SCP(13,13)
      DIMENSION CCP(13,13)
C     DOOLITTLE PROCEDURE FOR MULTI VARIABLES
C     M EQUALS NUMBER OF INDEPENDENT VARIABLES
C     N EQUALS NUMBER OF OBSERVATIONS
      READ(5,998) IJK
  998 FORMAT(I5)
      IJL=0
 9998 KK=J
  999 READ(5,1)M,N
    1 FORMAT (6X,2I3)
      KK=KK+1
      IJL=IJL+1
      READ(5,995)AB,BC,CD,DE,EF,FG,GH,HI,IJ,JK,KL,LM,MN,NO,CP,PG
  995 FORMAT (10A4)
      WRITE(6,994)AB,BC,CD,DE,EF,FG,GH,HI,IJ,JK,KL,LM,MN,NO,CP,PG
  994 FORMAT (16A4)
      N1=M
      SUMY=0.0
      SSY=0.0
      CSSY=0.0
      B1=N
      DO 99 I=1,M
      SUM(I)=0.0
      SCPY(I)=0.0
      CCPY(I)=0.0
      DO 98 J=1,M
  502 C(I,J)=0.0
      SCP(I,J)=0.0
   98 CCP(I,J)=0.0
   99 CONTINUE
      WRITE(6,702)
  702 FORMAT (//1X,4HDATA)
      DO 15 K=1,N
      READ(5,2) V(1),V(2),Z
    2 FORMAT (6F6.0)
      WRITE(6,701) V(1),V(2),Z
  701 FORMAT(4X,1CF13.4)
      GO TO (301,302,303,304,305),KK
```

```
 301 Y=ALOG(Z)
     X(1)=V(1)
     X(2)=V(2)
     X(3)=V(1)/V(2)
     GO TO 306
 302 Y=ALOG(Z)/(V(1)+V(2))
     X(1)=1.0/V(1)
     X(2)=1.0/V(2)
     GO TO 306
 303 Y=ALOG(Z)/V(2)
     X(1)=V(1)
     X(2)=V(2)
     GO TO 306
 304 Y=ALOG(Z)/V(2)**2
     X(1)=V(1)
     X(2)=V(2)
     GO TO 306
 305 Y=ALOG(Z)/V(2)
     X(1)=V(1)**2
     X(2)=V(2)
     GO TO 306
 306 SUMY=SUMY+Y
     SSY=SSY+(Y*Y)
     DO 15 I=1,M
     SUM(I)=SUM(I)+X(I)
     SCPY(I)=SCPY(I)+(Y*X(I))
     DO 6 J=1,M
   6 SCP(I,J)=SCP(I,J)+X(I)*X(J)
  15 CONTINUE
     DO 17 I=1,M
     DO 13 J=1,M
     CCP(I,J)=SCP(I,J)-((SUM(I)*SUM(J))/B1)
  13 CONTINUE
     CCPY(I)=SCPY(I)-((SUMY*SUM(I))/B1)
     XBAR(I)=SUM(I)/B1
  17 CONTINUE
     CSSY=SSY-(SUMY*SUMY)/B1
     YBAR=SUMY/B1
     I=1
     AY(I)=CCPY(I)
     DO 4 J=1,M
   4 A(I,J)=CCP(I,J)
     IF(A(I,I)) 145,146,145
 145 BY(I)=AY(I)/A(I,I)
     DO 14 J=1,M
  14 B(I,J)=A(I,J)/A(I,I)
 146 DO 102 I=2,M
     L=I-1
     DO 18 J=I,M
     CFY=0.0
     CFA=0.0
     DO 147 K=1,L
     CFY=CFY+A(K,I)*BY(K)
 147 CFA=CFA+A(K,I)*B(K,J)
```

```
      A(I,J)=CCP(I,J)-CFA
      IF(A(I,I)) 16,108,16
   16 B(I,J)=A(I,J)/A(I,I)
   18 CONTINUE
      AY(I)=CCPY(I)-CFY
      BY(I)=AY(I)/A(I,I)
  108 CONTINUE
  109 DO 80 I=1,M
   80 BETA(I)=0.0
      I=M
      BETA(I)=BY(I)
   20 I=M-1
      L=I+1
      CFB=0.0
      DO 22 K=L,N1
   22 CFB=CFB+B(I,K)*BETA(K)
      BETA(I)=BY(I)-CFB
 2015 M=M-1
      IF (M-1) 21,21,20
   21 RSS=0.0
      M=N1
      WRITE(6,217)
  217 FORMAT  (1X,15X,19HDOOLITTLE PROCEDURE)
      WRITE(6,220)
  220 FORMAT (1X,23HREGRESSION COEFFICIENTS)
      WRITE(6,221)
  221 FORMAT (1X,2X,1HI,6X,7HBETA(I),9X,4HMEAN,8X,11HSTD BETA(I))
  311 DO 109 I=1,M
      RSS=RSS+BETA(I)*CCPY(I)
      STDB=BETA(I)*SQRT(CCP(I,I)/CSSY)
      WRITE(6,500)  I,BETA(I),XBAR(I),STDB
  500 FORMAT (1X,I4,3E15.6)
  109 CONTINUE
      CFIC=0.0
      DO 201 I=1,M
      CFIC=CFIC+BETA(I)*(SUM(I)/B1)
  201 CONTINUE
      YINCT=YBAR-CFIC
      WRITE (6,212)YINCT
  212 FORMAT(1X,12HY INTERCEPT= ,F15.4)
      DF2=M
      DF1=N-1
      RMS=RSS/DF2
      RESS=CSSY-RSS
      REDF=DF1-DF2
      REMS=RESS/REDF
      F=RMS/REMS
      WRITE(6,206)
  206 FORMAT (1X,17HANOV OF REGRESSION)
      WRITE (6,207)
  207 FORMAT (1X,12X,2HDF,10X,2HSS,15X,2HMS,15X,1HF)
      WRITE(6,208)DF2,RSS,RMS,F
  208 FORMAT(1X,10HREGRESSION,F5.0,3E15.3)
      SY=SQRT((CSSY-RSS)/REDF)
      WRITE(6,209) REDF,RESS,REMS
```

```
209  FORMAT (1X,8HRESIDUAL,2X,F5.0,2E15.3)
     WRITE(6,210)DF1,CSSY
210  FORMAT (1X,5HTOTAL,5XF5.0,2E15.3)
     RMCC=RSS/CSSY
     RMCC=SQRT(RMCC)
     RSQ=RMCC*RMCC
     WRITE(6,213)
213  FORMAT (1X32HMULTIPLE CORRELATION COEFFICIENT)
     WRITE(6,222) RMCC,RSQ
 222 FORMAT(1X,2HR ,F15.5,5X,10HR SQUARE  ,F15.5)
     WRITE(6,216)
216  FORMAT (1X,26HREDUCTION DUE TO LAST BETA)
     DO 29 I=1,N1
     REDBN=AY(I)*BY(I)
  29 WRITE(6,214)REDBN
 214 FORMAT(1X,E15.4)
41   M=N1
42   I=M
     J=M
     CFC=0.0
43   IF(I-J) 51,52,53
52   K5=J+1
     IF(K5-N1)  62,62,61
62   DO 154 K=K5,N1
     CFC=CFC+B(I,K)*C(I,K)
154  CONTINUE
61   IF(A(I,J)) 60,45,60
  60 C(I,J)=(1.0/A(I,J))-CFC
45   I=I-1
     IF(I) 56,44,43
44   M=M-1
     IF (M) 42,56,42
51   L=I+1
     DO 55 K=L,N1
55   C(I,J)=C(I,J)-B(I,K)*C(K,J)
53   C(J,I)=C(I,J)
     GO TO 45
56   M=N1
     WRITE(6,211)
 211 FORMAT (1X,3X,1HI,10X,2HSB,15X,1HT,12X,2HDF,5X,15HSSB(I),ADJUSTED)
     DO 200 I=1,M
     SB(I)=SQRT(C(I,I)*SY*SY)
     T=BETA(I)/SB(I)
     XSS=(BETA(I)*BETA(I))/C(I,I)
     WRITE(6,205) I,SB(I),T,REDF,XSS
205  FORMAT (1X,I4,4E15.4)
200  CONTINUE
 223 FORMAT(1X,3X,1HI,3X,1HJ,7X,6HC(I,J))
202  DO 203 I=1,M
     DO 203 J=1,M
 203 WRITE(6,215)I,J,C(I,J)
215  FORMAT (1X,I4,I4,5X,E14.8)
     WRITE (6,1414)
1414 FORMAT (1X,14HEND OF PROGRAM)
     IF (IJL-IJK) 9999,997,996
9999 IF (KK-5) 999,9998,996
 996 STOP 1
 997 STOP 2
     END
```

```
                    DOOLITTLE PROCEDURE
REGRESSION COEFFIEIENTS
  I       BETA(I)              MEAN          STD BETA(I)
  1   0.1016C5E 01    0.398196E 00    C.66248CE CO
  2   0.303062E 00    0.16C090E 01    C.397298E CO
Y INTERCEPT=          -0.0415
AOV OF REGRESSICN
            DF            SS                MS                F
REGRESSION   2.       0.265E 03       C.133E C3       0.306E 03
RESIDUAL    44.       0.191E 02       C.434E CO
TOTAL       46.       0.284E C3
MULTIPLE CORRELATION COEFFICIENT
R          0.96504     R SQUARE            0.93284
REDUCTION DUE TO LAST BETA
      0.2387E C3
      0.2659E C2
   I          SB                T              DF       SSB(I),ADJUSTED
   1      0.7786E-01      C.13C5E 02      C.44C0E 02      0.7393E 02
   2      0.3872E-01      C.7826E C1      C.44C0E C2      0.2659E 02
   1   1     0.1396397E-01
   1   2    -0.4434532E-02
   2   2     0.3454251E-02
END OF PROGRAM
```

DATA

Nitrogen mg/l	Phosphorus mg/l	Algae mg/ 100ml
32.0000	8.0000	0.1000
128.0000	2.0000	0.1000
128.0000	4.0000	0.1000
256.0000	4.0000	0.1000
512.0000	4.0000	0.1000
128.0000	8.0000	66.2000
0.1000	0.1000	10.0000
0.2000	0.1000	30.1000
0.4000	0.1000	17.0000
0.8000	0.1000	95.7000
1.6000	0.1000	120.0000
3.2000	0.1000	147.0000
1.0000	1.0000	17.0000
2.0000	1.0000	10.1000
4.0000	1.0000	13.0000
8.0000	1.0000	26.8000
16.0000	1.0000	17.0000
32.0000	1.0000	3.6000
2.0000	2.0000	10.1000
4.0000	2.0000	20.2000
8.0000	2.0000	33.4000
16.0000	2.0000	114.0000
32.0000	2.0000	124.0000
64.0000	2.0000	107.0000
3.0000	3.0000	59.6000
6.0000	3.0000	79.3000
12.0000	3.0000	170.0000
24.0000	3.0000	62.9000
48.0000	3.0000	66.2000
96.0000	3.0000	69.4000
4.0000	4.0000	26.8000
8.0000	4.0000	59.6000
16.0000	4.0000	101.0000
32.0000	4.0000	3.6000
64.0000	4.0000	17.0000
8.0000	8.0000	104.0000
16.0000	8.0000	150.0000
64.0000	8.0000	46.5000
128.0000	8.0000	66.2000
1.0000	4.0000	79.3000
2.0000	4.0000	147.0000
16.0000	4.0000	89.1000
2.0000	8.0000	143.0000
2.0000	16.0000	160.0000
2.0000	32.0000	143.0000
2.0000	64.0000	124.0000
2.0000	128.0000	82.6000