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Range and Optimum Growth Concentrations of Phosphate and Nitrate for Chlamydomonas reinhardtii

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RANGE AND OPTIMUM GROWTH CONCENTRATIONS OF PHOSPHATE AND NITRATE FOR CHLAMYDOMONAS REINHARDTII

(TITLE)

BY

THOMAS E. HILL

B. S., Eastern Illinois University, 1969

THESIS

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ABSTRACT

A study was conducted on the alga Chlamydomonas reinhardtii to determine the range and optimum growth concentrations of phosphate and nitrate. There are only a few species of algae for which this type of data is known. Inorganic nutritional data is basic to the understanding of the complexities of phytoplankton ecology and eutrophication.

The "plus" strain of this heterothallic alga was cultured in a TRIS-buffered inorganic medium with various concentrations of sodium phosphate and potassium nitrate and adjusted to a pH of 7.4. The axenic cultures were evaluated at the end of 6 days with a spectrophotometer, at a wavelength of 645 millimicrons. An environmental chamber adjusted to 12 hours day/night length and illumination of 1,400 footcandles at 22°C was employed in the study. The phosphate range of growth was 0.07 to 3,200 ppm PO_4 with optimum growth from 3 to 950 ppm PO_4. The nitrate range of growth was 2 to 9,700 ppm NO_3 with optimum growth from 33 to 5,000 ppm NO_3. These data confirm earlier work which indicated that this organism is very tolerant of organic pollution. This alga has high nutrient requirements for survival and optimum growth compared to other algae.

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I would like to dedicate this thesis to my fiancee, Jackie Schildroth, who spent many hours in the laboratory and whose understanding and patience were most appreciated. Special mention is due to Dr. L. Stephen Whitley for his continual encouragement and advice, which proved invaluable in the completion of this study. I would like to thank Dr. Richard L. Smith for his advice and generous loan of equipment. The writer is also indebted to Dr. Bill T. Ridgeway and Dr. Leonard Durham for their suggestions in writing this paper. Gratitude is also extended to Theresa Walker for proofreading and typing this paper.

INTRODUCTION

One of the greatest threats to aquatic ecosystems today is the accelerated eutrophication caused by man. The problem of eutrophication is as complicated as it is widespread. Recent recognition of this major water pollution problem has stimulated a search for information concerning the nature of eutrophication. Many studies have been made on nutrient levels and their effect on the productivity of lakes, but few studies have been made on the effects of nutrient levels on specific algae. It is important to understand the complex nature of phytoplankton ecology and to isolate and test each factor separately. The determination of the effective range and optimum levels of nutrients in axenic cultures of algae is one important factor in understanding the complex interactions of the many algae present in a dynamic ecosystem in nature. Expansion of the knowledge of phytoplankton ecology would be helpful in solving the problem of eutrophication. Reliable knowledge of the algal succession during the process of eutrophication would enable workers to determine the level and rate of eutrophication and predict future phytoplankton populations. The ability to "read" the phytoplankton would improve aquatic management and pollution detection.

Man has finally realized that his existence depends on the existence of a balanced ecosystem. Man needs a balanced aquatic ecosystem to obtain drinking water,

water for industry, for livestock and crops, recreation, aesthetics, food, transportation, and many other uses. The fact that a balanced aquatic environment is so useful to man and an unbalanced aquatic environment is so detrimental, makes eutrophication an important problem to be overcome.

LITERATURE REVIEW

Eutrophication

Most authors agree that eutrophication is the evolution of events, caused by nutrient enrichment, which eventually leads to extinction of a lake. It is important to remember that lakes tend to act as nutrient-sediment traps and reaction vessels for biological productivity (Sawyer, 1966). In some areas, sediments can fill a lake to extinction in a short time without any measurable change in productivity. Turbidity is so high in such cases that even high nutrient concentrations cause no algal blooms nor raise the light-limited productivity of the lake. Lake Mead, on the Colorado River, for example, has a life expectancy of only 150 years due to heavy sedimentation (Stevens, 1946).

Streams and rivers may carry heavy loads of organic and inorganic materials which are deposited in the still water of lakes. Sand, soil, and gravel can be moved by rivers. The driving force of a river varies as the square of its velocity. Therefore, doubling the velocity of a river increases its ability to carry sediment by four times (Coker, 1968). Even slow streams can carry and deposit huge amounts of sediment over the years. A stream with a flow of one million gallons a day and only 30 ppm suspended solids can deposit 300 lbs of sediment per day in a lake (Klein, 1962). Strom (1928) went so

far as to say that lakes receiving no sediment will have an infinite life, if only natural amounts of nutrients are available.

Lakes can be very efficient traps for nutrients, even in highly mineralized forms (Hynes, 1960). In a lake near Copenhagen, 24 tons of saline nitrogen and 4 tons of saline phosphorus entered the lake each year but only 3½ tons of nitrogen and 200 lbs of phosphorus left the lake (Berg, 1958). The Madison lakes studied by Sawyer (1947) were found to retain 30 to 60 percent of the nitrogen they received. The most productive lakes tended to retain the most nitrogen. Pearsall found that nutrients entering four English lakes were significantly higher than the amount leaving the lakes. One lake had a net annual gain of 8 tons of nitrogen which promoted the growth of 100 tons (dry weight) biomass. Nutrients that enter the lake tend to be quickly fixed in the lake's biomass (Pearsall, Gardiner, and Greenshields, 1946).

The biomass in oligotrophic lakes slowly increases as more and more nutrients and sediments are trapped by the lake. Increasing amounts of dead organisms fall to the bottom and make increasing demands for hypolimnetic oxygen. The amount of oxygen required to decompose this organic matter finally reaches the point where the hypolimnion becomes devoid of oxygen just before the fall overturn. As the yearly biological oxygen demand increases, anaerobic conditions in the hypolimnion occur

sooner after the spring overturn and last for a longer period (Sawyer, 1947, 1966).

When a lake reaches the point where the hypolimnion becomes anaerobic, the lake becomes considerably more productive and may produce nuisance blooms quite suddenly (Hynes, 1960). Once a lake reaches the eutrophic stage, the character of the lake rapidly changes, often within a few years (Strom, 1928). There is a rapid change from oligotrophy to eutrophy, called mesotrophy, with long periods of stability before and after the change (Skulberg, 1964). Lake Zurich in Switzerland changed from oligotrophy to eutrophy within a few decades (Hasler, 1947).

The decrease in the amount of oxygen in the hypolimnion coincides with the rapid increase in productivity of the lake. This phenomenon occurs during the mesotrophic stage (see Fig. 1). The decline in oxygen levels in the hypolimnion is proportionate to the amount of nutrients added to a lake (Tanner, 1960).

Anaerobic conditions in the hypolimnion are characteristic of eutrophic lakes (Mortimer, 1941; Hasler, 1947; Sawyer, 1966; Fruh, 1967; Bartsch, 1968; King, 1970). When there are aerobic conditions in the hypolimnion, the benthic mud is covered by a thin layer of insoluble iron in the ferric oxidation state. This layer seals the bottom mud and prevents the release of nutrients back into solution. In fact, it often absorbs phosphorus from the

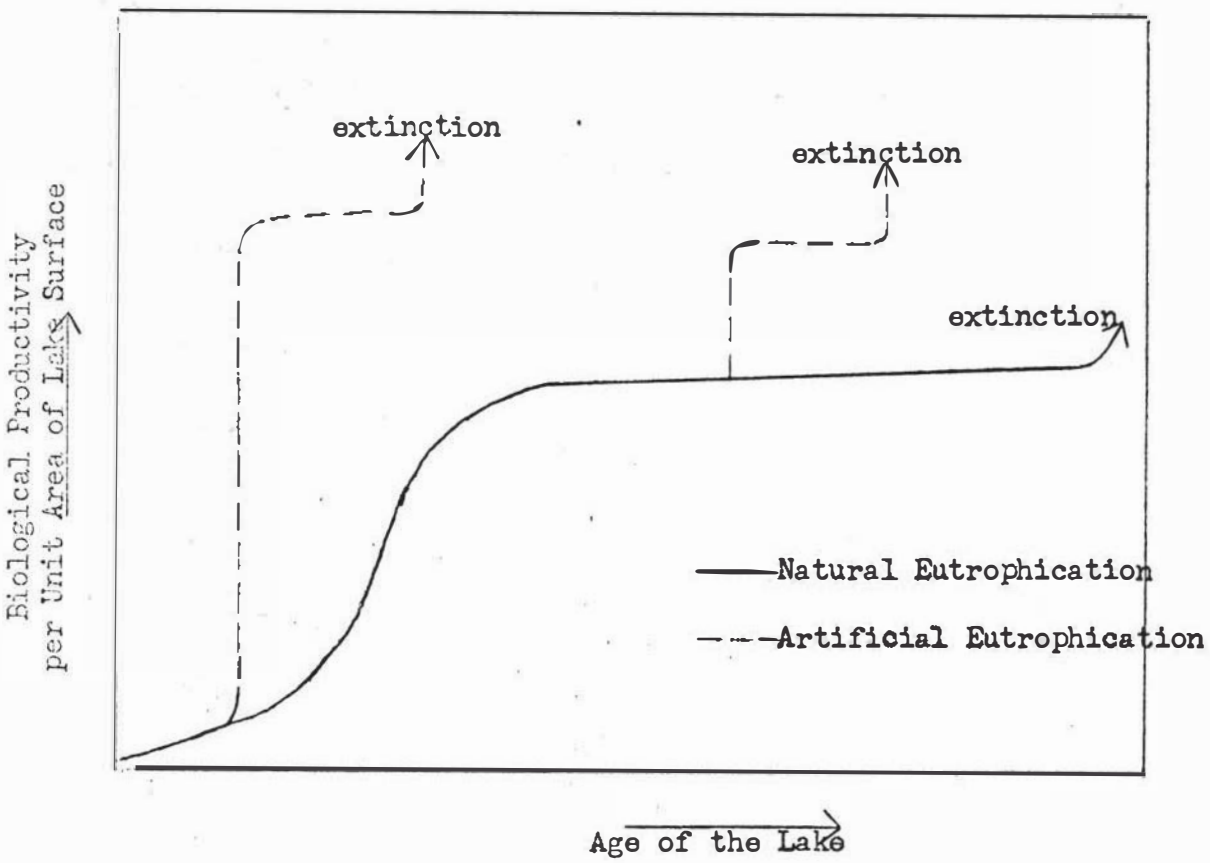

Fig. 1. Hypothetical curve of the course of eutrophication in a lake. The broken lines show the rapid increase in productivity characteristic of lakes which are artificially accelerated by man in the oligotrophic stage or in the eutrophic stage (From Hasler, 1947).

lake water. During anaerobic conditions in the hypolimnion, the iron layer becomes soluble as the iron changes to a ferrous state. The bottom mud is no longer sealed, and nutrients are released into the hypolimnion (Einsele, 1938).

When the dissolved oxygen in the hypolimnion declines from 2 ppm to 0 ppm, manganese and, later, iron are released in soluble forms. Concurrent with these releases are large quantities of phosphate, ammonia, silicate, and carbon dioxide (Mortimer, 1971). Anaerobic conditions not only release nutrients from the surface mud, but also from the deeper anaerobic decompositions which were formerly sealed at the surface (Pearsall, et al., 1946). Cultures of bacteria and other microorganisms actively release a large portion of their phosphorus within a few hours after the depletion of oxygen occurs (Mackenthun, 1968). One reason for the steep rise in the curve of eutrophy is that more nutrients are released due to anaerobic conditions (see Fig. 2) (Hasler, 1947). Along with greater abundance, Mortimer (1941) believes the process of reduction returns the nutrients in a more usable form than the process of oxidation.

After thermal stratification takes place, the hypolimnion becomes anaerobic and rich in nutrients. The epilimnion becomes poor in nutrients because of the phytoplankton uptake. Nutrients are quickly regenerated in the epilimnion, yet a large quantity of nutrients is

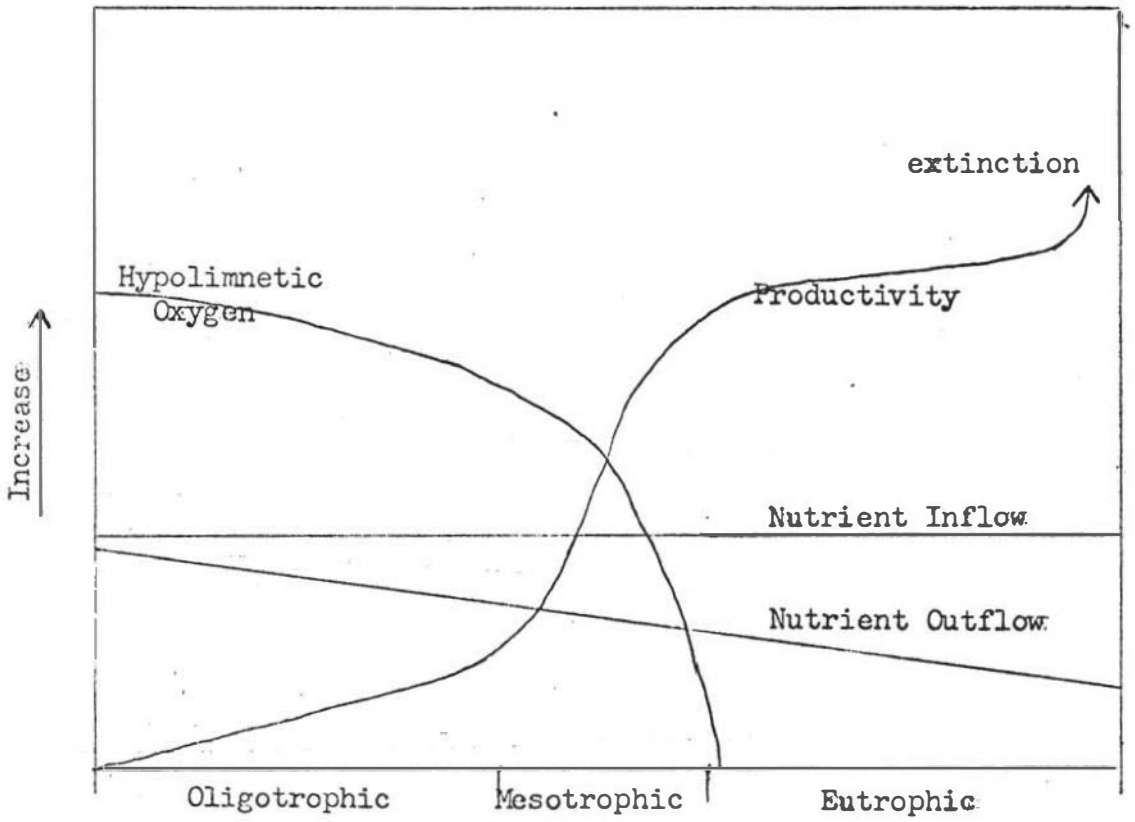

Fig. 2. Conceptualization of the process of eutrophication. In the mesotrophic stage, there is a rapid increase in productivity concurrent with a rapid decline in dissolved oxygen in the hypolimnion during stagnation. The nutrient inflow and outflow lines illustrate the progressive nutrient fixing by the lake. The productivity curve could also be labeled turbidity or nutrient availability which also change in this manner.

lost as dead organisms descend to the bottom. Several mechanisms have been discovered which return nutrients from the rich hypolimnion back into the epilimnion. Without this source of nutrients, the epilimnion could not continue its high rate of productivity. The epilimnion slowly warms and during the critical summer months the descending thermocline gradually feeds nutrient-rich waters from the hypolimnion into the epilimnion. The sooner anaerobic conditions develop in the hypolimnion, the more nutrients are released into the waters above (Sawyer, 1947). Organisms sedimented in littoral areas decompose and provide a continuing nutrient source for the epilimnion throughout the stratification period (Hasler and Einsele, 1948). Lakes as deep as 30 feet may have their thermal stratification destroyed by strong winds (Pearsall, et al., 1946).

Perhaps the first method used to assess the degree of eutrophication was the determination of dissolved oxygen after stratification. Thienemann (1928) proposed that lakes could be typed by determining the oxygen contents of the hypolimnion and epilimnion and representing them as a ratio.

Hutchinson (1938), after measuring many types of lakes, determined the rate of loss of hypolimnetic oxygen in:

oligotrophic lakes	0.004 - 0.033 mg/day/sq cm
eutrophic lakes	0.05 - 0.14 mg/day/sq cm

Mortimer (1941) found similar results:

oligotrophic lakes	under 0.025	mg/day/sq cm
mesotrophic lakes	0.025 - 0.055	mg/day/sq cm
eutrophic lakes	over 0.055	mg/day/sq cm.

Recently, Bazin and Saunders (1971) used the method of computing the rates of change of the total oxygen below the thermocline to predict future oxygen levels in the hypolimnion.

Sawyer (1966) outlined the various methods for measuring eutrophication as follows:

I. Indirect Indicators
These play no part in increasing productivity but indicate evidence of human, industrial, and agricultural wastes.
Increases of: total solids
calcium
sodium
potassium
sulfate
chloride

II. Direct Indicators (Qualitative)
A. The presence of salmonid fish indicates oligotrophy.
B. The quality of phytoplankton

III. Direct Indicators (Quantitative)
A. Hypolimnetic Oxygen
1. Dissolved
2. Rate of consumption
B. Biological Productivity
1. Standing crop
2. Volume of algae
3. Transparency
4. Chlorophyll in epilimnion
5. Oxygen production
6. Carbon dioxide utilization
C. Nutrient Levels
1. Phosphorus
2. Nitrogen
3. Nitrogen-phosphorus ratios

Welch (1952) summarized the characteristics proposed by many limnologists for the classification of oligotrophic and eutrophic lakes as follows:

Oligotrophic Lakes

1. Very deep; thermocline high; volume of hypolimnion large; water of hypolimnion cold.
2. Organic materials on bottom and in suspension very low.
3. Electrolytes low or variable; Ca, P, and N relatively poor.
4. Dissolved oxygen content high at all depths, all year.
5. Larger aquatic plants scanty.
6. Plankton quantity low; many species; water blooms rare; Chlorophyceae dominant.
7. Profundal fauna relatively rich.
8. Deep-dwelling, cold-water fishes common.

Eutrophic Lakes

1. Relatively shallow; deep cold water minimal or absent.
2. Organic materials on bottom and in suspension abundant.
3. Electrolytes variable, often high; Ca, P, and N abundant.
4. Dissolved oxygen minimal or absent in hypolimnion.
5. Larger aquatic plants abundant.
6. Plankton quantity high; quality variable; water blooms common; Myxophyceae and diatoms dominant.
7. Profundal fauna poor.
8. Warm-water fish; cold-water fish absent.

With increased levels of eutrophication, there is an increased level of productivity but a decrease in efficiency. The decrease in efficiency causes the

accumulation of organic material which fills in the lake and causes extinction (Findenegg, 1964). All of the nutrients cannot be utilized by the phytoplankton because the algal turbidity decreases the photosynthetic zone of the lake (Lund, 1969). Zooplankton will not consume many types of blue-green algae, so much of the algae dies and settles to the lake bottom (Lund, 1969). Decomposition is retarded by anaerobic conditions and a thick layer of bottom sediments builds up (Pearsall, et al., 1946). A stage is reached where the recycling of nutrients is high enough for continued nuisance algal growth (Fruh, 1967; Bartsch, 1968).

The building of bottom sediments causes more and more of the lake to become littoral in nature. Emergent vegetation contains much supporting tissue (cellulose) which cannot be used by most herbivores and decays very slowly (Russell and Hunter, 1970). The rooted aquatic plants accumulate sediment and cause the steady encroachment of the shores. The open lake becomes littoral, the littoral areas become dry land. The eutrophic lake disappears as a lake when the littoral vegetation has gained foothold throughout its bottom (Welch, 1952).

The length of time for a lake to become extinct generally varies from 100 to thousands of years. The majority of the world's large lakes will cease to exist in less than 20,000 years. An exception is Lake Geneva, which may last 40,000 years (Russell and Hunter, 1970).

The Great Lakes are estimated to be 8,000 years old. The rate at which eutrophication proceeds depends on geochemical and morphometric factors and the intelligence of man (Hynes, 1960).

Many limnologists believed that eutrophication, whether caused by man or by nature, was not reversible (Hasler, 1947). After some notable successes in reversing the trend of eutrophication, limnologists now believe that the fate of many lakes can be significantly improved. This is especially the case with artificially accelerated lakes (Edmondson, 1969; Hasler, 1969). In Lake Washington (Washington), 99 percent of the sewage entering the lake was diverted by 1967. By 1969, the phosphate concentration had been reduced 72 percent and the nitrate concentration reduced 20 percent from the 1963 record levels. The secchi disk transparency increased from 1.0 meter in 1963 to 2.8 meters in 1969 (Edmondson, 1970).

All sewage was diverted from Lake Monona (Madison, Wisconsin) in 1936, but between 1943 and 1950 sewage again entered the lake. After 1950 no sewage effluent entered Lake Monona and in 1958 all sewage was diverted from the Madison lakes. After diversion, appreciable decreases in nitrogen and phosphorus occurred. There was a shift from single species bloom of Microcystis to lesser blooms of a variety of species. The use of copper sulfate to control blooms decreased dramatically (Lawton, 1961; Hasler, 1969).

After a study made in 1963 by Rohlich and McGauhey,

the citizens voted to have the treated effluent diverted from Lake Tahoe, California. This lake has been described as the clearest lake in North America and yet the rapidly increasing sewage load threatened to destroy its aesthetic and biological value. Here is a case where nuisance conditions were stopped before they started (Hasler, 1969).

The abatement of pollution on the St..Clair and Detroit Rivers should dramatically improve conditions in Lake Erie. This is because it is theoretically possible to exchange all Lake Erie's water in three years since the lake is so shallow and its flow is so great (Hasler and Swenson, 1967).

Crecy Lake in New Brunswick is a small unstratified lake which was artificially fertilized three times in 18 years. Each time, the eutrophic conditions induced by the fertilization program reverted back to oligotrophic conditions when fertilization ceased (Smith, 1969).

Fish Indices of Eutrophication

Most suthors agree that coregonines and salmonids are the first fish to disappear as an oligotrophic lake evolves into a eutrophic type.

Kriegsmann (1955) studied the species composition of the cligotrophic Lake Obersee and the eutrophic Lake Untersee from 1910 to 1954. He theorized, by splicing the records of the two lakes together, that the cold-water fish population would suddenly decline and the coarse fish population would suddenly increase in the mesotrophic stage.

Even though the whitefish and trout populations declined and disappeared, the total fish production of the Wisconsin lakes increased with increasing eutrophication (Pearse, 1934).

Lake Zurichsee in Switzerland changed from a trout-whitefish population to a coarse fish population concurrent with heavy domestic enrichment. Restocking the lake has not been successful, indicating that the conditions for survival of game fish no longer exist (Hasler, 1947). Lake Monona and Lake Mendota cisco populations greatly declined during the 30's due to eutrophication (Hasler, 1947). Lake Erie has shown a dramatic collapse of the whitefish, lake trout, cisco, and blue pike fisheries during the period when extensive oxygen depletion in the hypolimnion occurred in the lake (Beeton, 1969). Tanner

(1960) fertilized four Michigan trout lakes at different rates. Generally, the more fertilized the lake, the greater was the reduction of oxygenated water during the summer. The epilimnion was too warm for the trout and the hypolimnion was often anaerobic. Since most trout need approximately 4 ppm dissolved oxygen, the trout were restricted to a narrow band of water in the thermocline with enough oxygen and low temperature water. It is easy to see how a trout population could be eliminated from such a lake during a spell of very hot weather and/or heavy nutrient load. Other sources of mortality may be hydrogen sulfide accumulations from anaerobic areas of the lake and egg mortality caused by anaerobic conditions during the winter (Hasler, 1969).

The succession of dominant fish species in the evolution of a hypothetical glacial lake in North America would be: cisco, trout, perch, northern pike, smallmouth bass, bluegill, largemouth bass, common sunfish, bullhead (Lagler, Bardach, and Miller, 1962). Larkin and Northcote (1969) believe the progression of fish groups would be: trout, warm-water bass and perch, plant-eating types, and finally, bottom feeders. Data such as these are helpful in determining the stage and rate of eutrophication of a lake.

Algal Indices of Eutrophication

The idea that phytoplankton could be used to determine the trophic lake type has been pursued mainly by European workers. The usual scheme for distinguishing oligotrophic plankton from eutrophic plankton is indicated in Table 1 (Unless otherwise cited, all citations belong to Rawson, 1956).

TABLE 1. Plankton of oligotrophic and eutrophic lakes

	Oligotrophic	Eutrophic
Quantity	Poor	Rich
Variety	Many species	Few species
Distribution	To great depths	Surface
Diurnal migration	Extensive	Limited
Water blooms	Very rare	Frequent
Characteristic algal groups and genera	Chlorophyceae Desmids (if low Ca) *Staurastrum* Diatomaceae *Tabellaria* *Cyclotella* Chrysophyceae *Dinobryon*	Cyanophyceae *Anabaena* *Aphanizomenon* *Microcystis* Diatomaceae *Melosira* *Fragilaria* *Stephanodiscus* *Asterionella*

The quantity of plankton is an excellent indicator of the productivity of a lake, and thus, its trophic type. Eutrophic lakes may have at least 5 times as much

plankton as an oligotrophic lake. The variety of species of plankton can be deceptive except in extreme conditions of oligotrophy or eutrophy. Eutrophic lakes may be dominated by a tremendous number of individuals of one species of blue-green algae and yet have many species with only a few individuals each. The extent of vertical migration is not easily determined nor particularly significant. The frequent occurrence of algal blooms is a universally accepted sign of eutrophic conditions.

There has been a concentrated effort by many researchers to discover algal groups or species to indicate trophic lake types. Rawson, in his study of Great Slave Lake (Canada), found phytoplankton characteristic of both oligotrophic and eutrophic lakes, although all physical and chemical data contend that Great Slave Lake is oligotrophic. Blue-green algae are scarce and no algal blooms have been recorded, yet desmind are also scarce (probably due to high calcium levels) and the diatom population is typical of eutrophic conditions. This is the problem in applying the traditional scheme of algal types to a particular lake. Large lakes may have inshore areas which are eutrophic and open water areas that are oligotrophic.

More accurate field and laboratory studies may indicate true algal indices of the trophic condition. Rodhe (1948) and Lund (1964) concluded that Dinobryon divergens and Uroglena americana thrive in low nutrient

concentrations and are inhibited by phosphate concentrations that are not characteristic of oligotrophic conditions. Indicator species of eutrophic conditions are well known. Persistent algal blooms of any blue-green algae, especially Microcystis, Anabaena, Oscillatoria, and Aphanizomenon, are reliable indices of the lake's trophic level. There are no reliable algal indicators of mesotrophic conditions.

Much needs to be done to improve field studies. Exhaustive studies should be made to determine all the species present and which species is dominant by numerical and volumetric percentages of the total community. Data should be based on collections throughout the year. Often field studies report only general classifications of algae such as diatoms, desmids, blue-green algae; sometimes genera are reported; rarely are the algae identified to species. A genus may contain species indicative of widely different conditions. There is evidence that physiological races within a single species may thrive under different nutrient conditions without showing any morphological differences. More care should be taken in taxonomic work in field studies.

Nutrient bioassays of algal species in the laboratory together with field studies should yield reliable information defining algal indicators of a lake's trophic condition. Such indicator species would be present and/or dominant only in one trophic level of eutrophication.

An exhaustive survey of the literature to determine the succession of algal species during the process of eutrophication reveals that no universal, well-defined list exists at this time. Rawson formulated the algal succession from oligotrophy to eutrophy for the lakes in western Canada. The list represents the sequential order of dominant species. The dominant species were determined by a high percentage of the plankton count over the summer season. This list is based on observations over a 25-year period.

TABLE 2. Approximate trophic distribution of dominant limnetic algae in lakes of western Canada

Oligotrophic	*Asterionella formosa*
	Melosira islandica
	Tabellaria fenestrata
	Tabellaria flocculosa
	Dinobryon divergens
	Fragilaria capucina
	Stephanodiscus niagarae
	Staurastrum spp.
	Melosira granulata
Mesotrophic	*Fragilaria crotonensis*
	Ceratium hirundinella
	Pediastrum boryanum
	Pediastrum duplex
	Coelosphaerium naegelianum
	Anabaena spp.
	Aphanizomenon flos-aquae
	Microcystis aeruginosa
Eutrophic	*Microcystis flos-aquae*

This sequence may be accurate for western Canada, but it disagrees in many ways with European data and

laboratory work of Chu (1942, 1943) and Rodhe (1948). It seems that each geographical region has its own particular sequence of succession, with the same end, blooms of blue-green algae. An accurate list of algal succession would be a quick and reliable method for determining position in, and rate of, the process of eutrophication. It is extremely important to know mesotrophic indicators, particularly species that occur just before blue-green algae become dominant. If these warning signals were known, steps might be taken to avert the problems caused by blue-green algal blooms.

Zooplankton have not received the attention that fish and algae have received; yet, when they are mentioned in connection with eutrophication, the genus Bosmina is invariably discussed. Bosmina longirostris replaced B. coregoni in Lake Zurich when it changed from oligotrophy to eutrophy (Hasler, 1969). B. longirostris is only found in eutrophic lakes in Finland (Jarnefelt, 1952). B. longirostris has replaced B. coregoni in Lake Michigan (Bartsch, 1968). The zooplankton will undoubtedly be important in characterizing the trophic levels of bodies of water when their ecology is better understood.

Algal Blooms

An algal bloom is defined as 500 or more individuals per milliliter of raw water (Lackey, 1949). It is generally accepted that increased nutrients cause increased production of phytoplankton. This increase in phytoplankton often reaches bloom levels in eutrophic lakes.

Most authors believe that of all the nutrients needed by phytoplankton, nitrogen and especially phosphorus are the most important factors in causing algal blooms (Sawyer, 1947; Lackey, 1949; Lund, 1965; Hutchinson, 1967; Mackenthun and Ingram, 1967; Bartsch 1968; Mackenthun, 1968; Hasler, 1969; Thomas, 1969; Edmondson, 1970; Schindler, 1971; Fuhs, et al., 1972; Maloney, Miller, and Shiroyama, 1972; Powers, et al., 1972; Schelske and Stoermer, 1972). Some limnologists have determined the levels of nutrients which are required to produce algal blooms (see Table 3).

Although high levels of phosphorus and nitrogen enable phytoplankton to reach bloom levels, many other factors help to determine the periodicity and succession of algal species. Mackenthun and Ingram (1967) mentioned some important factors which affect algae, including temperature, sunlight, shape and size of lake, substratum, water quality, predation, viruses, autointoxicants, extracellular metabolites, auxins, hormones, trace elements, and vitamins. Russell and Hunter (1970) determined the

TABLE 3. Minimum concentrations of nitrogen and phosphorus which can produce algal blooms

Conc.	Studied	Comments	Citation
N=300ppb P= 10ppb	Wisconsin Lakes	This conc. at the start of growing season could produce nuisance blooms.	Sawyer, 1947
N=100ppb P= 9ppb	Lab. experiments with Pediastrum, Staurastrum, Botryococcus, & 4 diatoms	Below this conc. the 7 algae were inhibited.	Chu, 1943
N=200ppb P= 10ppb	Seattle's Green Lake	Nuisance algal blooms commenced at this conc.	Sylvester, 1961
N= 70ppb P= 5ppb	Wisconsin Lakes	Minimum conc. for algal blooms	Lackey, 1960
P=100ppb/ rivers P= 50ppb/ lakes	Data from Fed. Water Poll. Contr. Admin.	Guidelines for total phosphorus in lakes and rivers	Mackenthun, 1968
300ppb NO_3/N 600ppb total N	German Rivers	Excessive algal growths in polluted rivers can be avoided if the N can be kept below these concentrations.	Muller, 1953
7ppb NO_3/N 25ppb total P	Analyses of many eutrophic lakes	Lakes which have concentrations above these will have blooms.	Prescott, 1968

essential nutrients for aquatic organisms: elements comprising 1 percent or more dry weight (in order of importance): C, O, H, N, P; comprising 0.05 to 1 percent: S, Cl, K, Na, Ca, Mg, Fe, Cu; comprising less than 0.05 percent: B, Mn, Zn, Si, Co, I, F; certain organisms may need trace amounts of Sr, Mo, Br, V, Ti, Al, Ga.

Theoretically, any one of the previously mentioned factors can be limiting (Liebig's law of the minimum) or toxic (law of the maximum) in a particular aquatic ecosystem or to any particular species within a system. In practice, phosphorus is usually the limiting factor for productivity, while the interaction of many factors seems to control the occurrence and abundance of any one species. Carbon is most likely to be limiting in those waters that are low in bicarbonate (Ruttner, 1963). Molybdenum is a limiting factor in Castle Lake, California (Goldman, 1960). Light and temperature limit Asterionella in mid-winter, but nutrient concentrations do not (Lund, Mackereth, and Mortimer, 1963). Requirements for vitamin B_{12}, biotin, and thiamine limit algal growth in some environments (Eyster, 1968).

Selective grazing by zooplankton can affect the algal species composition (Lund, 1969). Silica concentrations can determine the species of diatoms which are dominant (Kilham, 1971). Many authors believe that toxic substances produced by organisms are responsible for dominance and succession in the aquatic environment. Chlamydomonas reinhardtii produces substances which are toxic to

Haematococcus pluvialis (Proctor, 1957). Lefevre (1964) demonstrated autoinhibition in Scenedesmus cultures. Chlorella vulgaris can produce antibiotics effective against gram-negative and gram-positive bacteria.

Limiting factors may modify the effect of other limiting factors. Asterionella is severely inhibited in growth when the silica concentration falls below 0.5 ppm. In high concentrations of phosphate, the limiting threshold of silicate is reduced by an amount which depends on illumination and temperature (Lund, 1950).

Eutrophic waters have frequent and prolonged algal blooms which make the body of water less useful to man. Algal blooms in a municipal water supply often cause taste and odor problems in the drinking water (Pearsall, et al., 1946; Whipple, Fair, and Whipple, 1948; Palmer, 1962). It has been suggested that the decomposition of blue-green algae on sand filters of waste treatment plants allows toxins to contaminate the drinking water and cause gastro-intestinal disturbances (Nelson, 1941). Pipes and concrete corrode at an accelerated rate due to certain algae (Myers, 1947; Oborn and Higginson, 1954). Filters in water treatment plants will clog faster during algal blooms (Baylis, 1955). In general, frequent algal blooms cause the drinking water to be of lower quality and more costly.

Bodies of water used for recreation may be greatly reduced in value. Floating masses of blue-green algae or emergent vegetation reduce boating areas. Sport fishing

can be stopped by fish kills caused by the sudden anaerobic conditions following an algal bloom. It has been reported that certain blue-green algae can release toxins that kill fish (Ingram and Prescott, 1954). Excessive filamentous algae may reduce fish populations (Lawrence, 1958). Beaches become less popular as they become littered with decomposing algae. Swimmers avoid beaches where waters are covered with floating algal scums. Contact dermatitis and symptoms of "hay fever" have been associated with Anabaena, Anacystis, and Lyngbya blooms (Heise, 1951; Cohen and Reif, 1953). The aesthetic qualities, such as transparency and lack of color, are destroyed by opaque, green water. Turbidity increases with the increase in phytoplankton density (Welch, 1952). Decomposing algae and anaerobic conditions cause odor problems. Hydrogen sulfide gas from anaerobic decomposition can stain white lead paint on nearby houses. Eutrophic lakes can have prolific midge populations which plague visitors (Lackey, 1949; Mackenthun, 1969).

Commercial interests may be eliminated or made less profitable when a lake becomes eutrophic. Water used in the food and beverage industry must be of high quality. Eutrophic waters require much expense to purify them. Agricultural enterprises may be hurt because algal blooms can poison livestock and game birds (Fitch, et al., 1934). Freshwater fisheries are hurt because valuable commercial fish are replaced by rough fish characteristic of eutrophic waters (Larkin and Northcote, 1969).

Chlamydomonas reinhardtii in the Literature

The taxonomy and morphology of this genus began with Ehrenberg. He described the genus *Chlamydomonas* in 1833. Pascher (1927) monographed the known species. Later Gerloff furthered the systematics of the genus in 1940. The latest review of the genus was by Huber-Pestalozzi in 1961.

The first genetic studies using algae were with the genus *Chlamydomonas* (Pascher, 1918). The next major advance in algal genetics occurred when Smith and Regnery (1950) initiated the use of *C. reinhardtii* to study linkage relationships and the mechanism of crossing-over in mutant strains. From that time until today, *C. reinahrdtii* is still the major algal species in genetic research. For reviews of *Chlamydomonas* genetics, refer to Levine and Ebersold (1960), Ebersold (1962), Sager (1964), and Mattoni (1968).

Klebs (1896) reported that the suspension in distilled water, starvation, and the staling of the medium could evoke sexuality in a wide variety of microorganisms. Sager and Granick (1953, 1954) were the first to determine that both nitrogen concentration and light control gamete formation in *C. reinhardtii*. These studies set the stage for further investigation by many authors delving into the mechanism of reproduction in *C. reinhardtii* and other algae.

Artari (1913) performed the most notable early study on the physiology of *Chlamydomonas*. This was followed

by Moewus (1931, 1933) and Lutsch (1932), who also made significant contributions to the knowledge of the physiology of this alga. Recently, many studies, too numerous to mention, have been published.

Cain (1963) found that C. reinhardtii could utilize nitrate or nitrite equally well. He also found that when ammonium and nitrate were available simultaneously, ammonium was used preferentially but growth was not as good as with nitrate alone. Proctor (1957) showed that the ammonium ions were preferentially assimilated and that the nitrate ions were utilized only after the ammonium had been exhausted. Culp (1971) demonstrated that this alga grew equally well utilizing nitrate, ammonia, or a combination of the two.

Palmer (1969) rated the ability of algae to tolerate high organic pollution. One hundred-sixty-five authors were reviewed and the algae ranked by the number of authors that specified a particular genus or species. Chlamydomonas was considered the third most tolerant genus. Chlamydomonas reinhardtii ranked 45th out of 1000 species reported as tolerant of organic pollution. Palmer (1962) believes C. reinhardtii is very pollution tolerant but cautions that there are many species in this genus and many algae have chlamydomonad stages; consequently, many workers do not identify chlamydomonad specimens to species, or they do it incorrectly.

MATERIALS AND METHODS

Axenic cultures of Chlamydomonas reinhardtii Dangeard were obtained from the Culture Collection of Algae at Indiana University (CCIU). G. M. Smith isolated C. reinhardtii plus strain (CCIU no. 89) and minus strain (CCIU no. 90).

Media

Stock and inoculation cultures were grown in a TRIS-buffered inorganic medium (TBIM) devised by Smith and Wiedeman (1964) (see Table 4). The same medium with modified nitrate and phosphate concentrations was used throughout the study for test media. The media were not enriched with vitamins because C. reinhardtii has been shown not to require them (Cain, 1963).

The test media were synthesized in the following manner. A stock solution of quadruple strength TBIM (4XTBIM), but without phosphates or nitrates was prepared. NaCl (0.118 g/l) and KCl (0.150 g/l) were added to insure that the Na and K ions were not limiting at very low KNO_3 and Na_2HPO_4 levels. When phosphates and nitrates are omitted and potassium chloride and sodium chloride are added to TBIM, the abbreviation TBIM-S will signify the modification.

To achieve the desired concentration of phosphate and nitrate in a 4-ml solution, the millimolar value needed was calculated (see Tables 5 and 6). Stock

TABLE 4. Preparation of TRIS-buffered inorganic medium (TBIM)[a]

Stock Solutions	Amount
0.1 M KNO_3	20 ml
0.1 M Na_2HPO_4	10 ml
0.1 M $MgSO_4 \cdot 7H_2O$	3 ml
0.1 M $CaCl_2 \cdot 2H_2O$	1 ml
0.2 M TRIS (hydroxymethlyaminomethane)	25 ml

Each of the above is added to approximately 800 ml of glass distilled water. One ml of each of the following micronutrient stock solutions is then added and a final dilution to 1 liter made.

I.	EDTA	50.00 g	per liter glass distilled water
	KOH, 85%	31.00 g	
II.	H_3BO_3	11.42 g	
III.	$FeSO_4 \cdot 7H_2O$	4.98 g	per liter acidified water[b]
IV.	$ZnSO_4 \cdot 7H_2O$	8.82 g	
	$MnCl_2 \cdot 4H_2O$	1.44 g	
	MoO_3	0.71 g	
	$CuSO_4 \cdot 5H_2O$	1.57 g	
	$Co(NO_3)_2 \cdot 6H_2O$	0.49 g	

From Smith and Wiedeman, 1964

[a]The pH of this medium will be approximately 8.8. To adjust the pH to 7.4, HCl was added.

[b]Acidified water: 999 ml glass distilled water, 1 ml concentrated H_2SO_4.

TABLE 5. Phosphate, phosphorus, and millimolar equivalents

mM P/l	ppm P as Na_2HPO_4	ppm PO_4 as Na_2HPO_4	a 4 ml soln. contains
43.0	1,333	4,000	0.17 mM PO_4
32.2	1,000	3,000	0.13
21.5	666	2,000	0.084
10.8	333	1,000	0.042
9.7	300	900	0.038
8.6	266	800	0.034
7.5	233	700	0.029
6.5	200	600	0.025
5.4	166	500	0.021
4.3	133	400	0.017
3.2	100	300	0.013
2.2	66	200	0.0084
1.1	33	100	0.0042
0.97	30	90	0.0038
0.86	26	80	0.0034
0.75	23.3	70	0.0029
0.65	20	60	0.0025
0.54	16.6	50	0.0021
0.43	13.3	40	0.0017
0.32	10	30	0.0013
0.22	6.6	20	0.00084
0.11	3.3	10	0.00042
0.054	1.7	5	0.00021
0.011	333ppb	1	0.000042
0.0097	300	0.9	0.000038
0.0086	266	0.8	0.000034
0.0075	233	0.7	0.000029
0.0065	200	0.6	0.000025
0.0054	166	0.5	0.000021
0.0043	133	0.4	0.000017
0.0032	100	0.3	0.000013
0.0022	66	0.2	0.0000084
0.0011	33	0.1	0.0000042

TABLE 6. Nitrate, nitrogen, and millimolar equivalents

mM N/l	ppm N as KNO_3	ppm NO_3 as KNO_3	a 4 ml soln. contains
197.1	2,760	12,000	0.77 mM NO_3
180.7	2,530	11,000	0.70
164.2	2,300	10,000	0.64
147.8	2,070	9,000	0.58
131.4	1,840	8,000	0.51
115	1,610	7,000	0.45
98.5	1,380	6,000	0.38
82.1	1,250	5,000	0.32
65.6	920	4,000	0.25
49.2	690	3,000	0.19
32.8	460	2,000	0.13
16.4	230	1,000	0.064
14.7	207	900	0.058
13.1	184	800	0.051
11.5	161	700	0.045
9.9	138	600	0.038
8.2	115	500	0.032
6.6	92	400	0.025
4.9	69	300	0.019
3.2	46	200	0.013
1.6	23	100	0.0064
1.5	20.7	90	0.0058
1.3	18.4	80	0.0051
1.16	16.1	70	0.0045
0.99	13.8	60	0.0038
0.82	11.5	50	0.0032
0.66	9.2	40	0.0025
0.49	6.9	30	0.0019
0.32	4.6	20	0.0013
0.16	2.3	10	0.00064
0.082	1.15	5	0.00032
0.016	230ppb	1	0.000064
0.015	207	0.9	0.000058
0.013	184	0.8	0.000051
0.012	161	0.7	0.000045
0.0099	138	0.6	0.000038
0.0082	115	0.5	0.000032
0.0066	92	0.4	0.000025
0.0049	69	0.3	0.000019
0.0032	46	0.2	0.000013
0.0016	23	0.1	0.0000064

solutions of phosphates and nitrates were made up at 1.0, 0.1, 0.01, 0.001, 0.0001, 0.00001, and 0.000001 molar concentrations respectively. The desired concentrations were prepared using these stock concentrations.

Example: A concentration of 300 ppm NO_3 and 40 ppm PO_4

300 ppm NO_3=0.019 mM=0.19 ml of 0.1 M KNO_3

40 ppm PO_4=0.0017 mM=0.17 ml of 0.01 M Na_2HPO_4

1 ml of 4XTBIM-S was then added.

Various nitrate and phosphate concentrations were prepared in this fashion and tested with a Hach Direct Reading Photoelectric Colorimeter[1] and found to be accurate.

The medium TBIM has a concentration of 124 ppm NO_3, 28 ppm N, or 2 mM N/l, and a concentration of 95 ppm PO_4, 31 ppm P, or 1 mM P/l. Throughout this paper, concentrations of phosphorus and nitrogen are given in all three forms because of the compounds in which they are found (e.g., ortho-phosphates, nitrates, ammonia, etc.). Studies of eutrophication have shown that the total available phosphorus and nitrogen levels are more important than the forms in which they are found. Laboratory phycologists who work with many types of media find mM/l concentrations more meaningful to use.

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[1] Hach Chemical Company, Ames, Iowa 50010.

Standard Conditions

All cultures were grown in a Sherer Controlled Environmental Chamber[2], model CEL 25-7. Cain (1963) cultured a number of Chlamydomonas spp. including C. reinhardtii at 22°C with much success. In the use of C. gelatinosa as a pollution bioassay organism, Matulova (1969) recommends 20-22°C. Sager and Granick (1953) maintained their cultures of C. reinhardtii at 25°C. A constant day and night temperature of 22°C was maintained in the experiments reported herein, except for experiment number 1. Experiment number 1 had a day length of 14 hours and a night length of 10 hours. All other experiments had 12 hour day and night lengths for simplicity. Light was furnished by KEN-RAD "cool white" fluorescent lamps at an intensity of about 1,400 footcandles on the test tubes. It was found that C. reinhardtii has about 1,500 footcandles as its upper limit for optimum growth rate (Krauss, 1961). Salageanu (1967) obtained excellent growths of C. reinhardtii without agitation or the introduction of gaseous carbon dioxide, neither of which was used in the present study.

Bausch and Lomb Selected Spectronic 20 Test Tubes ($\frac{1}{2}$ in. diameter) were employed in specially made wire mesh test tube racks. These racks allowed approximately 1 cm separation between tubes and were designed to set

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[2]Sherer-Gillett Division, Kysor Industrial Corp., Marshall, Mich. 49068.

the tubes at a 45° angle from vertical to provide greater exposure to light and increased gas exchange at the surface.

The growth measured in these tests is derived strictly from asexual reproduction. C. reinhardtii is a heterothallic alga, with plus and minus mating types. Only one strain (plus) was used throughout the study, to avoid possible irregularities in growth rate due to sexual reproduction.

Six days for the length of the test cultures worked well in all tests, except experiment number 10. The growth curve study (Fig. 3) showed that C. reinhardtii had a rather constant growth rate in the range of from 92 to 27 percent transmittance. The ideal growth period should be one in which a large amount of growth (low transmittance reading) takes place and yet doesn't reach a level where the growth slows down (past 27 percent transmittance). A large amount of growth helps delineate between nutrient concentrations that promoted rapid growth and those that didn't. In experiment number 1 the average growth of both strains reached only 45 percent in 6 days, while later tests often reached a more desirable 30 percent. Examination of the test condition table (Table 7) shows that many conditions were different in experiment number 1 than in subsequent tests. One significant factor, that of pH, will be discussed later.

The plus and minus strains of C. reinhardtii grow at the same rate in TBIM. Cain (1963) found that using

Bold's Basal Medium enriched with vitamins (BBMV) and using nitrate as the nitrogen source, both strains grew equally well. Using four different concentrations of KNO_3 in TBIMV, Culp (1971) determined that both strains showed identical responses at each concentration. In experiment number 1 (see Fig. 3) the difference in growth between the plus and minus strains ranged from 1 to 5 percent. Even this slight difference in growth can be accounted for by inequities in the amount of cells inoculated into the cultures. Therefore, only one strain (plus) was used for experimentation in this study. In this way, the inoculation flasks which were derived from one stock culture flask were more nearly equal in number of organisms, and each test tube was more likely to be inoculated with equal numbers of cells. If both strains were used, it would have been very difficult to inoculate the plus tubes and minus tubes with the same number of algal cells. Thus, for reliability and efficiency's sake, only one strain was used.

Four milliliters of medium was used because that amount of medium allowed the algae to quickly reach a high density population. To use 5 ml or more of medium would tend to allow cells to become trapped in the cotton plugs during agitation and cause inaccurate Spectronic 20 readings.

Procedure

Stock cultures and inoculation cultures were grown in 50 ml cotton-stoppered flasks in the Sherer Controlled Environmental Chamber. Both were grown in TBIM under standard conditions. TBIM proved to be a good medium for this purpose because it quickly produced large amounts of high density populations for inoculation. Stock cultures were transferred axenically every week into new medium by autoclaved pipettes. Frequent transfers into new medium prevented cultures from growing erratically because of senility. Each time new cultures were prepared, they were tested for possible contamination with Bacto-AC broth medium[3] and streaked out on Bacto-Nutrient Agar[3] slants.

The desired ppm values were selected and the stock solution equivalents calculated. The day before inoculation, the media were pipetted into each tube and the pH was adjusted to 7.4 by adding HCl. The concentration of phosphate usually determined the amount of diluted 1 N HCl to be added. The pH had to be adjusted in each tube because each tube usually had a different concentration of phosphate. The pH was checked by the use of a Coleman Metrion IV pH meter[4] with a Semi-Micro Combination pH Electrode[5]

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[3]Difco Laboratories, Detroit, Michigan.

[4]Coleman Instruments, Division of Perkin-Elmer Corp., 42 Madison Street, Maywood, Ill. 60153.

[5]Scientific Instruments, Corning Glass Company.

which will fit into a ½ in. Spectronic 20 test tube.

The labeled tubes were next stoppered with cotton and autoclaved for 20 minutes at 250°F. The media were allowed to cool for 24 hours. The inoculation cultures were grown in flasks for exactly 7 days. Each flask was used for the inoculation of 20 tubes of test media. Sterilized pipettes and bacteriological procedures were used to maintain axenic conditions. One drop of inoculum was used to minimize the amount of carry-over nutrients in the inoculum.. Three to ten tubes in each test had identical medium in them (see Table 7) to increase the accuracy of the results. The data plotted in the results section are the average values of the tubes at each concentration..

Along with the tubes with various nutrient concentrations, there were control tubes with TBIM and TBIM-S, and one uninoculated TBIM tube used as a control and to zero the Spectronic 20. The amount of growth in the TBIM controls would tend to indicate differences in the amount of inoculated organisms and how other concentrations compared with a known optimum level concentration. The TBIM-S control reveals the base line growth or amount of growth due to stored nutrients from the inoculation media.

After 20 inoculations, each flask was tested with AC broth for contamination. The test cultures were then placed in the environmental chamber 6 hours into the 12-hour light phase. Exactly 6 days later and in the middle

of the light phase, the cultures were evaluated with a Bausch and Lomb Spectronic 20 spectrophotometer[6]. Each tube was agitated by a Vortex Junior Mixer[7] for 20 seconds to insure a uniform distribution of cells for a more accurate reading.

The Spectronic 20 was set at a wavelength of 645 millimicrons. At this wavelength, mainly chlorophyll-a will be measured. It must be kept in mind that an increase in chlorophyll is interpreted as an increase in biomass or growth. This is probably due to the increase in number of cells, but there is a remote chance that the cells increased in size but not in number. Data consists of values from the percent transmittance scale on the Spectronic 20. The percentage reading is inversely proportional to the amount of growth. That is, the lower the percent transmittance, the higher the growth.

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[6]Bausch and Lomb Incorporated, Rochester, New York.

[7]Scientific Industries Inc., Queens Village, New York.

TABLE 7. Experimental conditions of each of the ten experiments in this study

Exp. no.	1	2	3	4	5	6	7	8	9	10
Day/night length	14/ 10	12/ 12	12/ 12	12/ 12	12/ 12	12/ 12	12/ 12	12/ 12	12/ 12	12/ 12
pH	7.2	7.4	7.4	7.4	7.4	7.4	7.4	7.4		7.4
No. of drops inoculum	2	1	1	1	1	1	1	1	1	1
Temp. °C	20	22	22	22	22	22	22	22	22	22
ml of medium in each tube	5	4	4	4	4	4	4	4	4	4
Length of culture in days	12	6	6	6	6	6	6	6	6	8
No. of tubes per conc.	10	3	3	3	3	4	4	3	6	4
Strain used	+,-	+	+	+	+	+	+	+	+	+
Inoculation culture medium	TBIM	TBIM	TBIM	TBIM	TBIM	TBIM	TBIM	TBIM	TBIM	Depletion medium
Length of inoculation culture	7	7	7	7	7	7	7	7	7	21

RESULTS

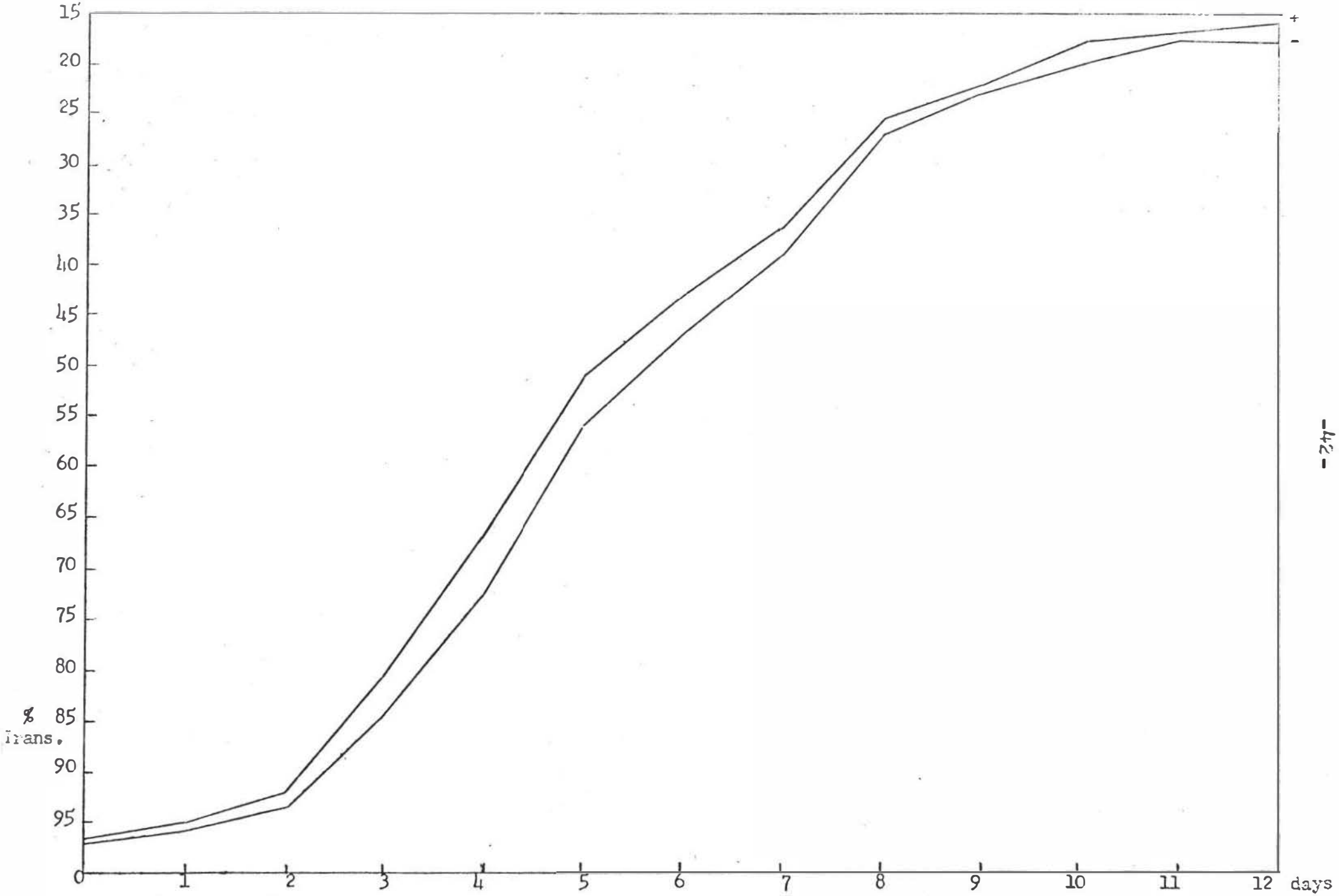

Fig. 3 (Exp. no. 1). Growth curve of Chlamydomonas reinhardtii + and - strains in LXTBIM.

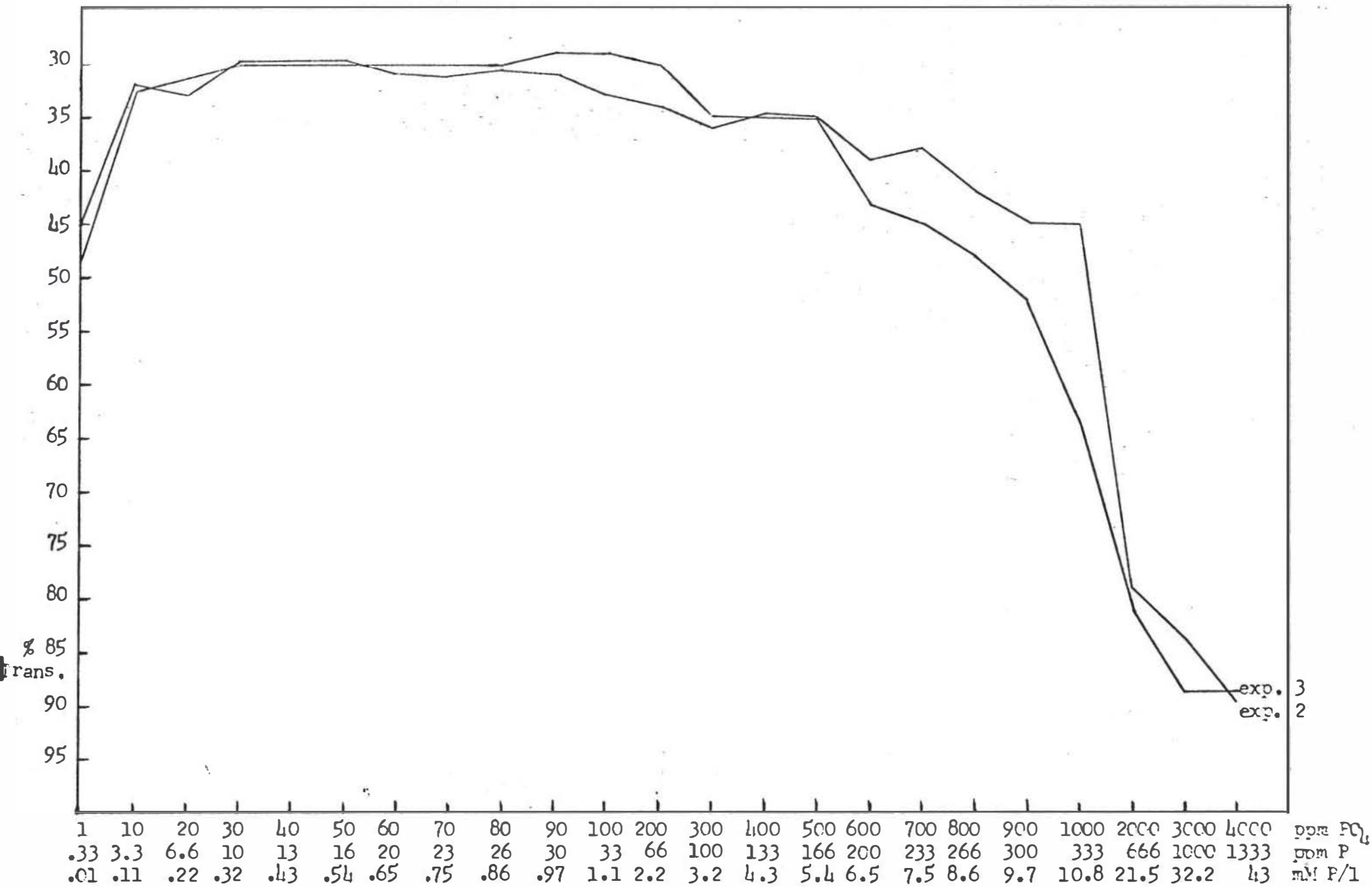

Fig. 4 (Exp. no. 2 and 3). Range of growth of C. reinhardtii (+ strain) at various phosphate concentrations, with nitrates in the optimum range.

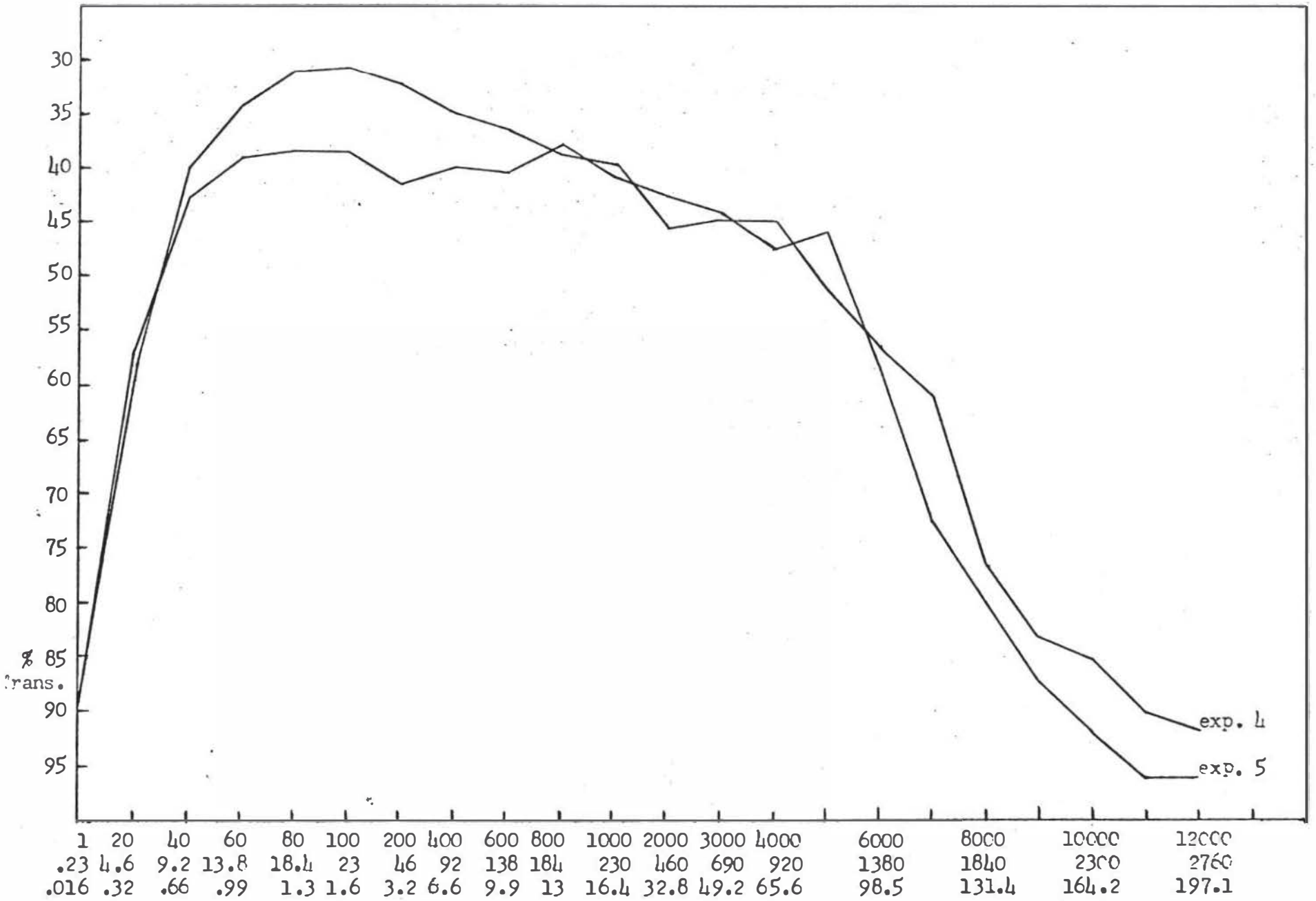

Fig. 5 (Exp. no. 4 and 5). Range of growth of C. reinhardtii (+ strain) at various nitrate concentrations, with phosphates in the optimum range.

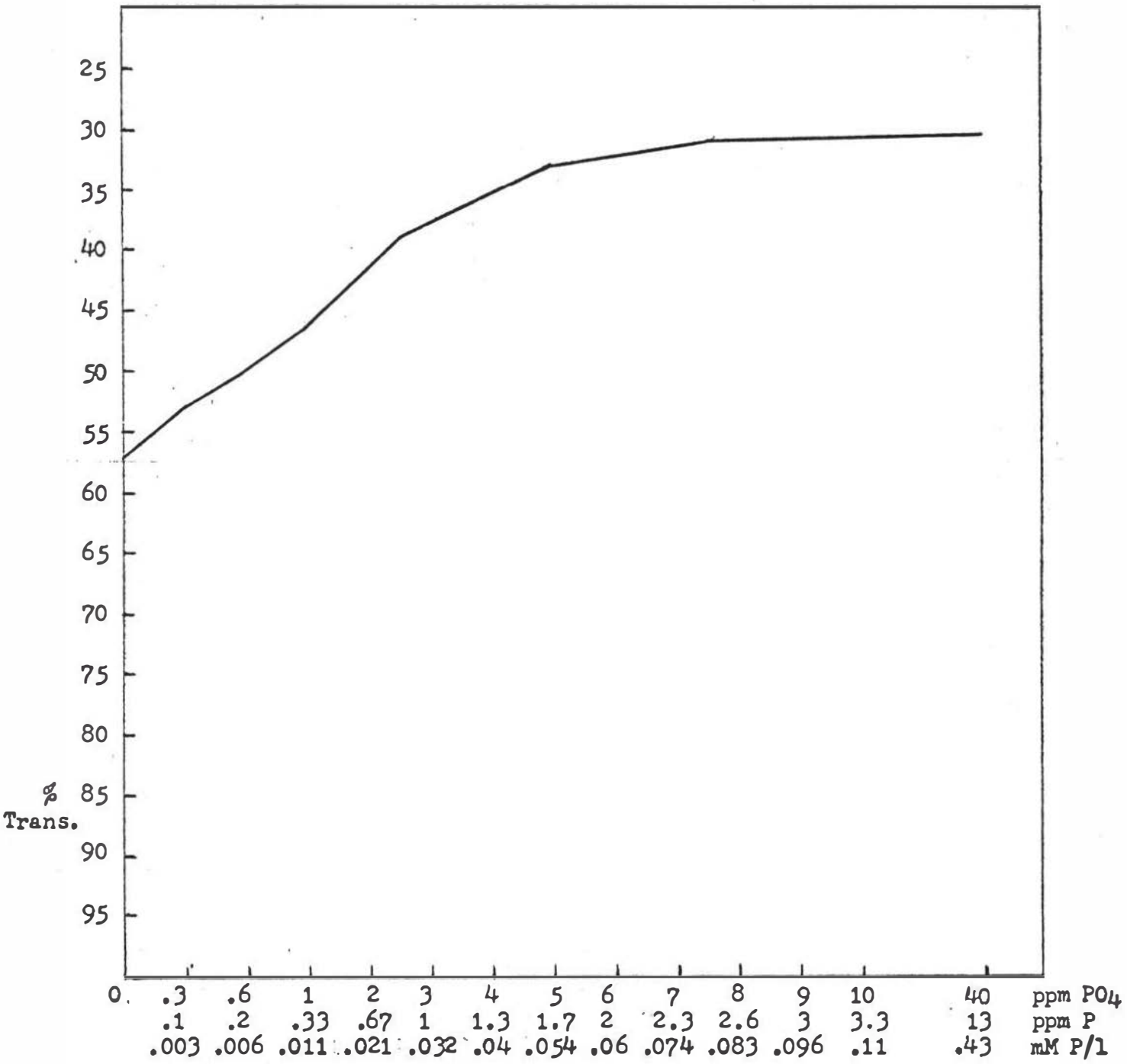

Fig. 6 (Exp. no. 6). Growth of C. reinhardtii (+ strain) in the low range of phosphate concentrations, with nitrates in the optimum range.

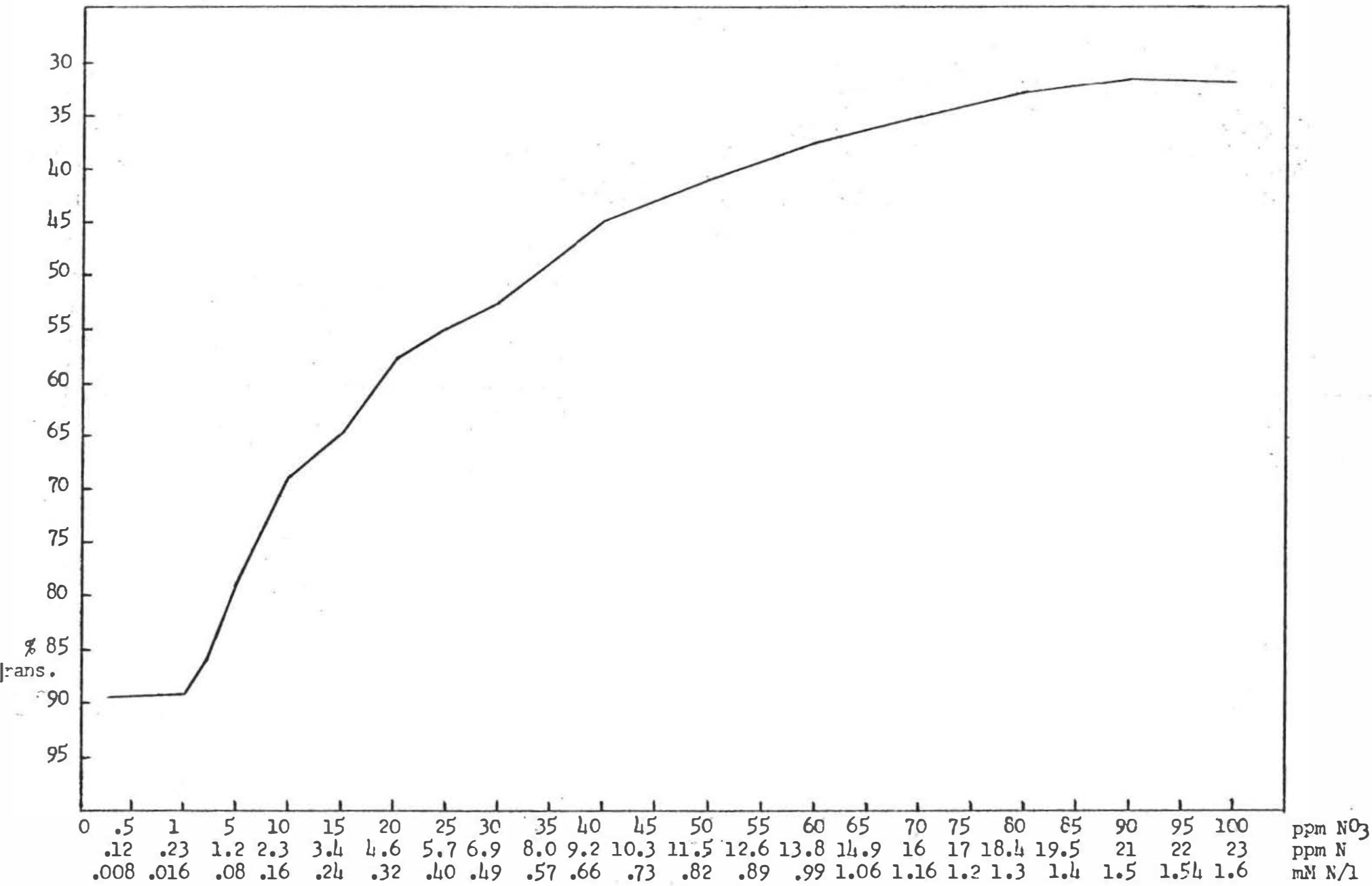

Fig. 7 (Exp. no. 7). Growth of C. reinhardtii (+ strain) in the low range of nitrate concentrations, with phosphates in the optimum range.

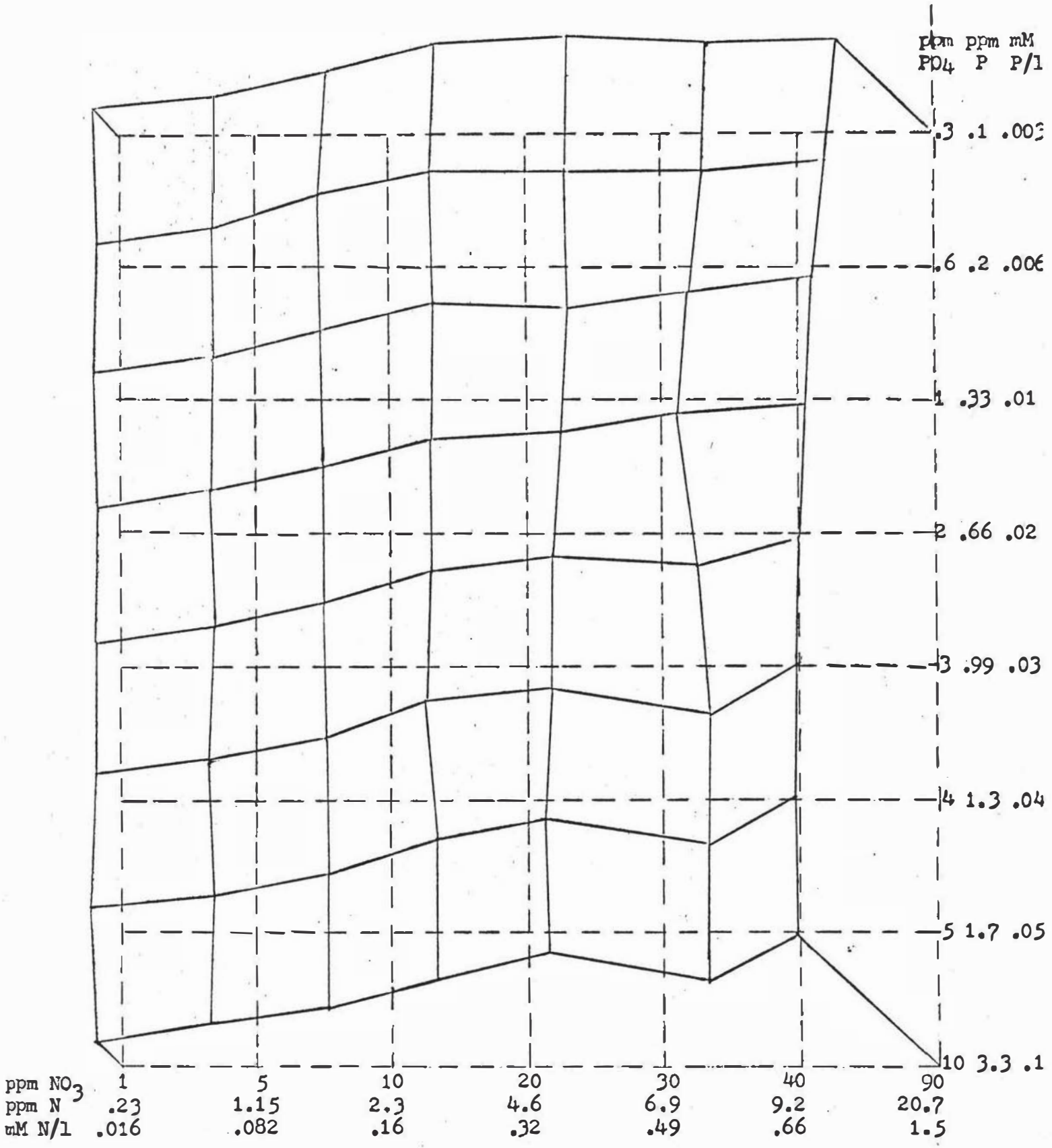

Fig. 8 (Exp. no. 8). Growth of C. reinhardtii (+ strain) at various combinations of phosphate and nitrate concentrations. The relative amount of growth of each nutrient combination (dash line grid) is represented by the amount of dislocation of the solid line grid.

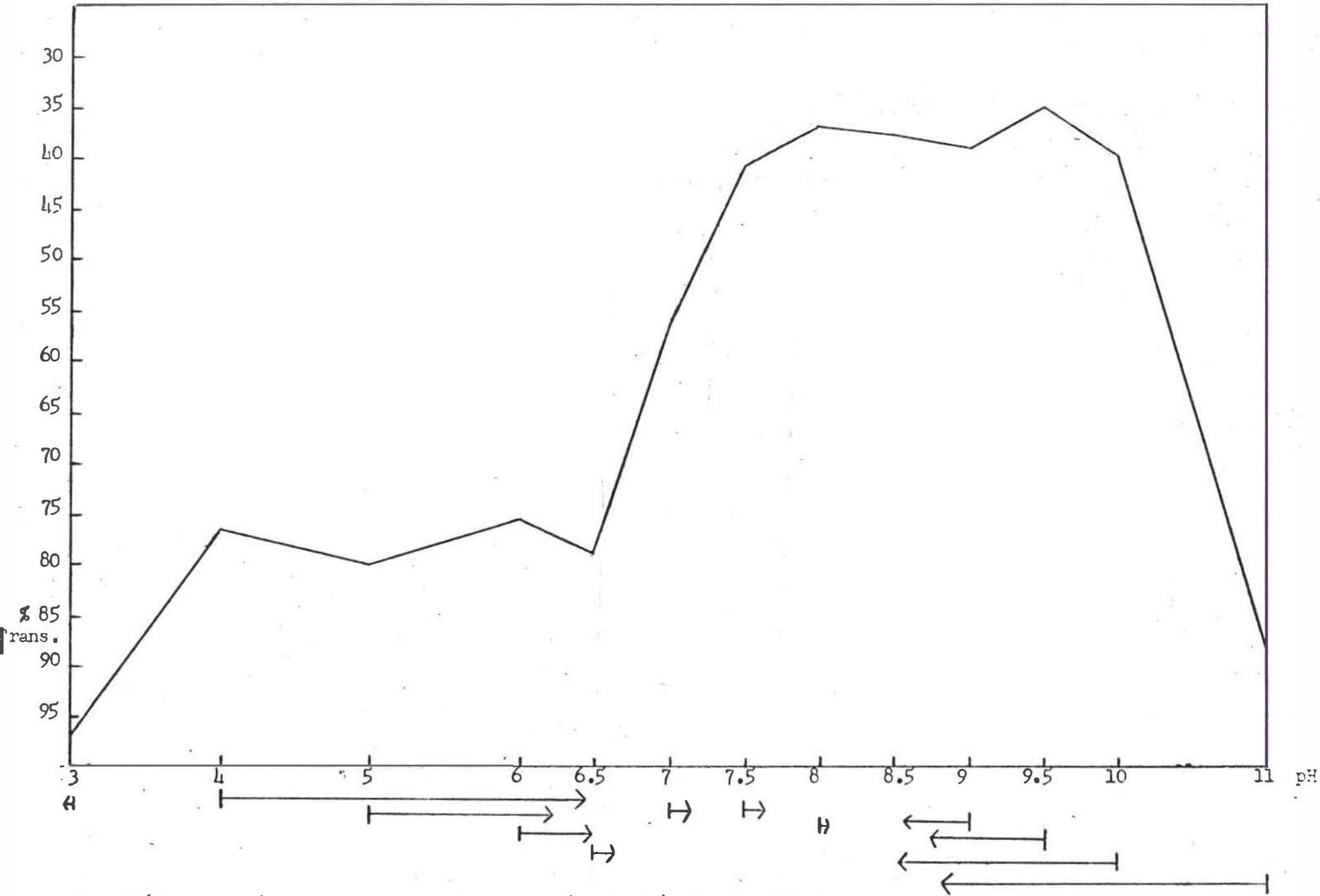

Fig. 9 (Exp. no. 9). Growth of C. reinhardtii (+ strain) at selected pH levels. Arrows denote direction and amount of pH shift, before and after culturing.

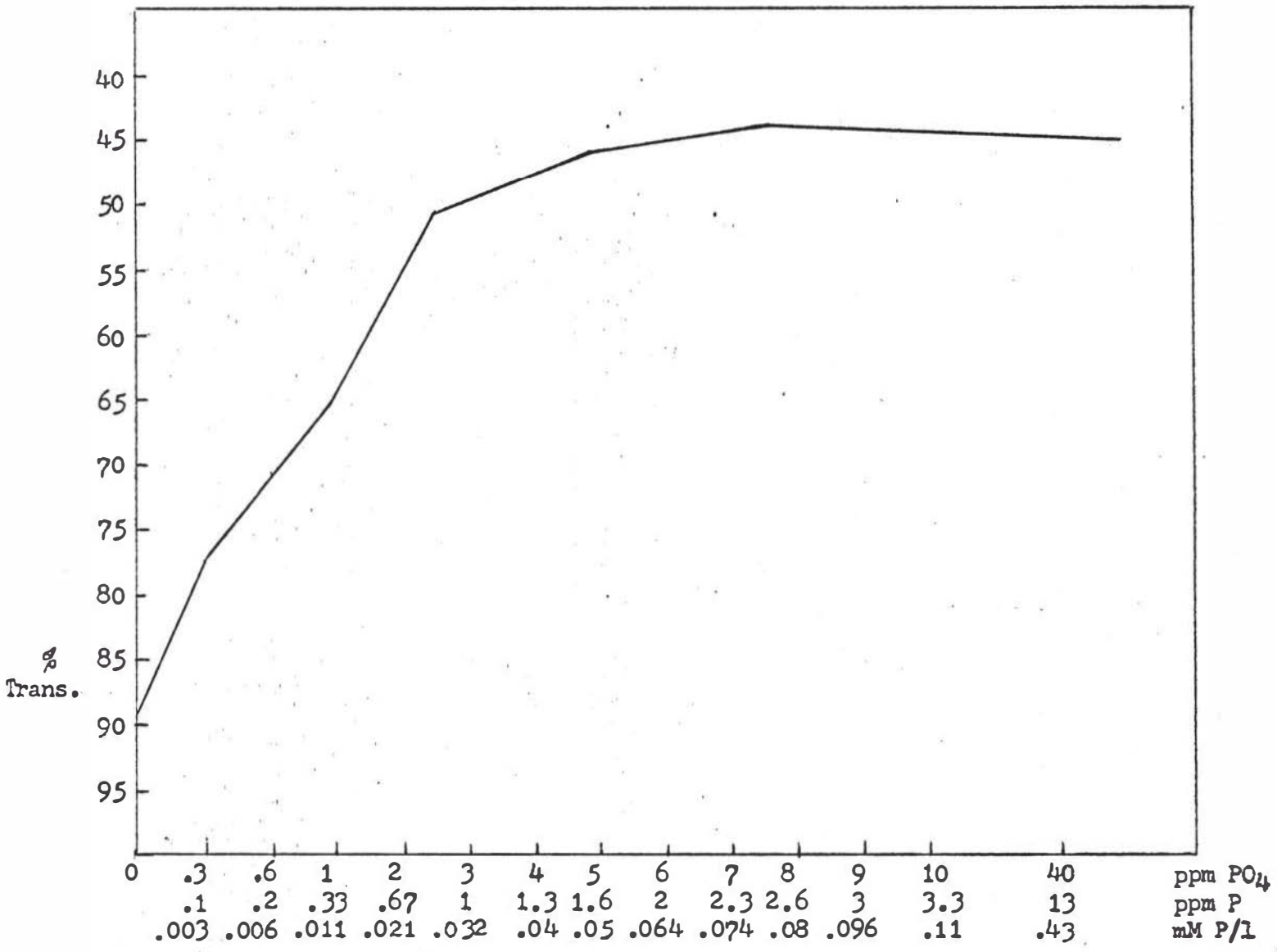

Fig. 10 (Exp. no. 10). Growth of C. reinhardtii (+ strain) in the low range of phosphate concentrations, with nitrates in the optimum range. Inoculation cultures were grown for 21 days in 10 ppm PO_4 and 90 ppm NO_3.

TABLE 8. Morphological characteristics of *Chlamydomonas reinhardtii* at different nutrient levels

High Phosphate:	Cells green; non-motile; proteinaceous sheath around each cell; 2 to 4 cells per clump; cells granular in appearance.
High Nitrate:	Cells green; large clumps of many cells; non-motile; no sheath.
Low Phosphate:	Cells nearly transparent; solitary; slightly motile; very large vacuole.
Low Nitrate:	Same as above.
Optimum Nutrients:	Cells green; highly motile; solitary; small vacuole.

TABLE 9. Results of the growth in the TBIM and TBIM-S controls

Exp. no.	2	3	4	5	6	7	8	9	10	
TBIM	32	27	27	32	31	31	51	39	44	per-cent trans.
TBIM-S	90	89	89	90	88	88	90	89	90	

DISCUSSION

Experiment number 1 established that the plus and minus strains of Chlamydomonas reinhardtii grew at the same rate. These results confirm the earlier works of Cain (1963) and Culp (1971). This experiment led to the use of only one strain (plus strain) throughout the remainder of the experiments. The results of this experiment were also used to determine the length of the culture period. It was determined that the optimum measurement of the relative amounts of growth is made in the middle of the exponential growth phase. From this experiment, it was found that after 6 days of growth, 45 percent transmittance was reached. Even in later experiments, which attained higher levels of growth, 6 days proved to be the best length for the culture period. Even the highest growth levels remained in the exponential growth rate range and were not stifled by the cell density of the senescent stage (15 to 25 percent transmittance).

Experiments 2 and 3 were run separately. These experiments employed a wide range of phosphate concentrations with only one concentration of nitrate. The nitrate concentration was the same concentration found in TBIM (125 ppm NO_3) and proved to be in the optimum growth range for nitrates. The specific purpose of experiments 2 and 3 was to determine the upper optimum growth limit and upper growth limit for phosphate.

The 50 percent transmittance reading was used to

define the upper and lower optimum growth limit. This was not merely an arbitrary decision, because cultures with this growth level were medium green and contained cell concentrations well above the bloom level (500 individuals per ml). The 87 percent transmittance reading was used to define the upper and lower growth limit. This value was chosen because controls grown with each experiment without phosphates or nitrates (TBIM-S) grew just slightly less than this amount (see Table 9). Any readings between 87 and 100 percent indicate inoculation size and growth from stored nutrients. That is why cultures were not 100 percent transparent in media without nutrients.

The average of the two experiments placed the approximate upper optimum growth limit at 950 ppm PO_4 and the upper growth limit at 3,200 ppm PO_4 with optimum nitrates.

Experiments 4 and 5 were run similarly to experiments 2 and 3, except nitrates were varied instead of phosphates. In these experiments the phosphates were in optimum range (95 ppm PO_4) and at the same concentration in which they are found in TBIM. The upper optimum growth limit was determined to be 5,000 ppm NO_3 and the upper growth limit 9,700 ppm NO_3.

The objective of experiment 6 was to test C. reinhardtii in the low range of phosphate concentrations, that is, from 0 ppm phosphates to optimum level phosphates. The nitrates were at an optimum level as with all experiments

of this type. It was hoped that this experiment would yield the lower optimum growth limit and the lower growth limit for phosphates. The results indicated (see Fig. 6) high growth even with 0 ppm PO_4. The control tubes with no phosphates or nitrates (TBIM-S) (see Table 9) yielded low growth and the optimum nutrient controls (TBIM) indicated high growth. The solution to this paradoxical problem was not found until experiment 10.

Experiment 7 was a test of the low range of nitrate concentrations with an optimum level of phosphate. The results indicate that the lower optimum growth limit is approximately 33 ppm NO_3 and the lower growth limit is 2 ppm NO_3. These data agree favorably with the results of experiments 4 and 5 which tested the same area of concentrations but less precisely.

Experiment 8 was an attempt to dramatize, with the use of a 3-dimensional graph, the growth of C. reinhardtii with various combinations of nitrates and phosphates. This is in contrast to earlier experiments which tested various concentrations of one nutrient while keeping the other nutrient at an optimum level. The concentrations used in this experiment were supposed to represent the low growth to optimum growth ranges. However, high growth was again found with the low phosphate concentrations as was the case in experiment 6. The results of experiment 8 show little difference in growth from the lowest to the highest concentrations of phosphate. At low nitrate

concentrations, the phosphate levels had no effect. At higher nitrate levels, an increase in phosphate caused a slight increase in the amount of growth. In high and low concentrations of phosphates, an increase in nitrates resulted in an increase in growth. Clearly, in all combinations, the controlling influence was the nitrate concentration.. This was nore manifest in the lower concentrations of nutrients than in the higher concentrations. The lowest growth was found in the lowest concentration of nitrate. The highest growth was found in the highest combination of nitrate and phosphate levels.

Experiment 9 tested the growth response of C. reinhardtii to a wide range of pH values. The pH in each tube was adjusted with HCl or NaOH to the desired value. One day later the pH was checked and readjusted if needed, and autoclaved. The final pH was measured at the termination of the experiment. The TRIS buffer was not added to the tubes below the adjusted pH of 6.5. The media buffered well from a pH of 6.5 to 8.5 with pH shifts toward these values by lower and higher adjusted pH values. The lower growth limit was estimated to be 3.4 and the upper growth limit, 10.9. The lower optimum growth limit and upper optimum growth limit were 7.2 and 10.2, respectively. These figures indicate a distinct preference for the alkaline pH and that its upper optimum growth limit is very close to the maximum toxic pH level. Nielson (1955) found that photosynthesis is inhibited in most algae at pH values

between 10 and 11.

Experiment 10 had the same purpose as experiment 6, that is, to test the low range of phosphate concentrations. Experiment 10 was identical to experiment 6 except that the inoculation cultures were grown for 21 days in depletion medium instead of 7 days in TBIM (see Table 7). The depletion medium had a concentration of 90 ppm NO_3 and 10 ppm PO_4, while TBIM has a concentration of 125 ppm NO_3 and 95 ppm PO_4. The lengthy inoculation culture period and the use of depletion medium would prevent the luxury uptake and storage of phosphorus from the inoculation medium. Phosphorus can be taken up by cells far in excess of present needs when the phosphorus concentration is high. This stored supply can maintain growth even when the external phosphorus concentration is very low (Mackereth, 1953). This phenomenon of luxury consumption of phosphorus would explain why Chlamydomonas grew so well, even in media devoid of phosphorus in experiment 6. The cells had absorbed enough phosphorus from the inoculation cultures to promote growth in the test cultures. This mechanism also showed up in the results of experiment 8. In the low nitrate concentrations, the phosphates were not limiting, even at the lowest phosphate concentrations. The high nitrate concentrations began to show phosphate limitation at the lower phosphate levels. This is because the stored phosphorus was not adequate for the high growth promoted by the elevated nitrate levels. Growth

levels then depended on the phosphate levels in the media. There was no such problem with nitrate carry-over because nitrates are needed in larger quantities than phosphates and are not stored by algae to any great extent (Gerloff and Skoog, 1954).

The length of the culture period was 8 days instead of the usual 6 days. This was because the inoculation cultures were grown for 21 days instead of 7 days. The long inoculation culture period increased the lag phase in the test cultures. To obtain a high yield, the length of the test culture period was increased to 8 days.

From this experiment, the lower growth limit was 0.07 ppm PO_4 and the lower optimum growth limit 3 ppm PO_4.

The summary of the nutrient limits for Chlamydomonas reinhardtii is as follows:

	Lower Growth Limit	Lower Optimum Growth Limit	Upper Optimum Growth Limit	Upper Growth Limit
ppm PO_4	0.07	3.0	950	3,200
ppm PO_4/P	0.023	1.0	317	1,066
ppm NO_3	2.0	33	5,000	9,700
ppm NO_3/N	0.46	7.6	1,150	2,231

This organism has an extraordinarily wide growth range. For this reason, it would make an excellent nutrient bioassay test alga. It is very resistant to extremely high concentrations of nitrate and phosphate. Algae tested by Chu (1943) showed marked inhibition in concentrations of phosphorus or nitrogen above 45 ppm.

The toxic levels of these nutrients with regard to phytoplankton cannot be generalized from the seven algae tested by Chu. Some scientists advocate the increase in nutrient levels of hypereutrophic waters in order to reach toxic concentrations. Acknowledgment of the resistance of C. reinhardtii to high nutrient concentrations should discourage that hypothesis.

Rodhe (1948) assigned the planktonic algae to three categories according to their optimum phosphorus limits.

	Lower Optimum Limit	Upper Optimum Limit
I. Low P requirement	less than 0.02ppm P	less than 0.02ppm
II. Medium P requirement	less than 0.02	more than 0.02
III. High P requirement	more than 0.02	more than 0.02

Rodhe (1948) and Chu (1943) are the authors cited most often for data on the nitrogen and phosphorus requirements of planktonic algae. A composite listing of the algae bioassayed by these authors, including work on Anacystis cyanea by Gerloff and Skoog (1954) and the present findings on Chlamydomonas reinhardtii, fits well into Rodhe's system.

	Lower Optimum Limit ppm P	Upper Optimum Limit ppm P	Citation
I. Dinobryon divergens		0.01	Rodhe, 1948
Uroglena americana		0.01	Rodhe, 1948
II. Nitzschia palea	0.018	8.9	Chu, 1943
Tabellaria flocculosa	0.018	8.9	Chu, 1943
III. Pediastrum boryanum	0.09	17.8	Chu, 1943
Staurastrum paradoxum	0.09	17.8	Chu, 1943
Scenedesmus quadricauda	1.0		Rodhe, 1948
Anacystis cyanea	0.2		Gerloff and Skoog, 1954
Chlamydomonas reinhardtii	1.0	317.0	

It is interesting to note that group I is composed of Chrysophyceae, group II Diatomaceae, and group III Chlorophyta and Cyanophyta.

Along with similar phosphorus requirements, Scenedesmus quadricauda, Anacystis cyanea, and Chlamydomonas reinhardtii have similar lower optimum nitrogen requirements, 5.0 ppm N, 8.0, and 7.6, respectively. Palmer (1969) ranked these three genera among the top 20 most organic pollution-tolerant genera of algae. Perhaps to Rodhe's list should be added a fourth category with a lower optimum limit of 0.2 ppm P and inclusion of nitrogen requirements as part of the criteria for categorizing algae.

Average domestic sewage has a concentration of 61.3 ppm N and 10.7 ppm P (Oswald, 1960). Both of these nutrient levels are within the optimum range of C. reinhardtii. Many species in the genus Chlamydomonas are known to inhabit sewage stabilization ponds (Eppley and Macias, 1962; Singh and Saxena, 1969). Although ecological data on Chlamydomonas reinhardtii are virtually nonexistent, the findings of this study and the general ecological preference of this genus indicate that this organism occurs in eutrophic bodies of water or other high nutrient situations.

The data show that the optimum levels of nitrogen and phosphorus for this organism are far above the minimum algal bloom concentrations (see Table 3). In fact, the lower growth limit is above all the recommended

bloom concentrations except those by Mackenthun (1968) and Prescott (1968). This reinforces the concept that this alga has high nutrient requirements.

Ecological data alone are not sufficient to determine the nutritional requirements of an alga. Blooms of blue-green algae may be found in waters with very low amounts of phosphates. The luxury consumption of phosphates or other nutrients during periods when nutrients were abundant can lead to blooms even when nutrients are scarce. Therefore, nutrient requirements of specific algae must be determined in the laboratory in order to interpret the ecological data.

Laboratory data should not be the sole basis for determining nutrient requirements either. Experimental conditions can never accurately duplicate all the environmental conditions of the field. Organisms can adapt to laboratory culturing and behave differently than they ever would in natural environments. Field observations and laboratory experiments must be examined together in order to obtain reliable results.

The nutrient parameters found in this study are accurate for this set of conditions, but how these nutrient requirements vary with different levels of these conditions or with the multitude of factors in a natural habitat is still unknown.

SUMMARY

1. Chlamydomonas reinhardtii was grown axenically in various concentrations of phosphate and nitrate to determine the range of optimum growth concentrations.
2. Nutrient data of this type is only known for a few select species of algae. The relationship between nutrient levels and algal growth is an important first step in understanding phytoplankton ecology and the problem of eutrophication.
3. A TRIS-buffered inorganic medium, adjusted to a pH of 7.4, with various concentrations of sodium phosphate and potassium nitrate, was utilized in this study.
4. The plus strain of C. reinhardtii was cultured in cotton-stoppered spectrophotometer test tubes and evaluated with a spectrophotometer at the end of 6 days of culture.
5. The cultures were grown in an environmental chamber with a day/night length of 12 hours at 22°C and illumination of 1,400 footcandles.
6. The phosphate range of growth was 0.07 to 3,200 ppm PO_4 with optimum growth from 3 to 950 ppm PO_4. The nitrate range of growth was 2 to 9,700 ppm NO_3 with optimum growth from 33 to 5,000 ppm NO_3.
7. Compared with other algae, C. reinhardtii has a wide growth range and is tolerant of extremely high nutrient concentrations. This alga has relatively

high nutrient requirements for survival and optimum growth.

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