THE NITROGEN AND PHOSPHORUS REQUIREMENTS OF MICROORGANISMS IN ACTIVATED SLUDGE

by

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ABSTRACT

A synthetic substrate of highly reproducible composition was used to study the nitrogen and phosphorus requirements of the microorganisms and settling characteristics of activated sludge

The study showed that the production of biological growth or activated sludge with maximum nitrogen and phosphorus contents, required a substrate with BOD.N and BOD:P ratios not wider than 19:1 and 81:1, respectively. To produce an activated sludge with optimum nitrogen and phosphorus contents, the BOD:N and BOD·P ratios of the substrate should not be wider than 23·1 and 168 1

A highly significant interaction existed between the quantities of nitrogen and phosphorus required by the activated sludge microorganisms

The phosphorus requirements increased logarithmically with the quantity of nitrogen consumed.

Bulking of activated sludge was caused by wide BOD:N and BOD:P ratios of the substrate and the presence of a filamentous bacterium, Sphaerotilus sp , in the sludge. Bulking was most severe when the substrate had BOD:N and BOD:P ratios wider than 37:1 and 420 1, respectively Sphaerotilus organisms were present in all sludges, but their growth was favoured by wide BOD:N and BOD:P ratios of the substrate

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I. INTRODUCTION.

When "raw" (untreated) sewage, or when sewage mixed with industrial wastes is discharged directly into natural bodies of water, the dissolved oxygen in the water is soon consumed by the microorganisms present in the sewage. Anaerobic processes develop and create a nuisance, aquatic life is reduced and considerable economic loss results. The polluted water constitutes a health hazard and is unfit for drinking, swimming or aesthetic purposes. It is, therefore, of primary and vital importance that sewage should be treated as economically and rapidly as possible.

Microorganisms are primarily responsible for the purification of sewage. Rapid purification depends on the unrestricted activities and reproduction of the organisms. Many species of water, soil and intestinal bacteria are involved in these processes. Aerobic, oxidative processes are of primary importance for the treatment of the liquid phase of sewage, while anaerobic processes are used for the digestion of the sludges.

The activated sludge process is one of the most efficient methods of sewage treatment in terms of volumetric requirements (Lackey and Smith, 1956). Activated sludge may be defined as particles of floc consisting of mixed species of microorganisms. These may include bacteria, yeasts, moulds, algae (if exposed to sunlight), protozoa, rotifers, nematodes and insect larvae, all living together in a gelatinous mass. The organisms comprising the floc are aerobes or at least facultative aerobes. It is a

certain indication that the process is not functioning properly if large numbers of anaerobes appear in the floc (Lackey and Smith, 1956).

Biological purification of organic wastes is affected by the interplay of three factors: nutrients, organisms and environment. Organic matter, present as suspended or colloidal solids, is removed by coagulation, entrainment, adsorption and oxidation. One factor which may be limiting in the overall mechanism of oxidation is the rate at which nutrients enter the cell. Therefore, the chemical composition and the physical state of a waste undergoing treatment will affect the availability of the nutrients. Sufficient quantities of available nutrients must be present in a waste water undergoing treatment in order that the microorganisms present in the activated sludge, or in a biological filter, may grow and be active.

Aerobic biological treatment methods had been applied to waste waters, largely domestic sewage, for at least forty years before any serious consideration was given to the nutrition of the microorganisms. The issue of the nutritional requirements was finally forced when industrial wastes of a wide variety and character were subjected to biological treatment in admixture with domestic sewage, or when attempts were made to treat them separately.

According to literature (Sawyer, 1956) certain industrial wastes are deficient in nitrogen and phosphorus. Domestic sewage normally contains nitrogen and phosphorus in excess of the

nutritional requirements. Addition of industrial wastes to domestic sewage causes nutritional imbalance of the biological system. This imbalance retards the rate of purification of the mixed waste water and increases the cost of treatment.

Marked changes in the composition of the sewage, aeration, pH, temperature, etc., will affect the microbial population of the activated sludge. Lackey and Smith (1956) reported that insufficient aeration or excessive concentrations of carbonaceous material, such as sugar, can cause production of a floc composed largely of the sheathed bacterium Sphaerotilus. The bulking sludge produced is frequently troublesome because it develops poor settling characteristics and passes over into the final effluent, thus making purification incomplete. In the normal temperature range and in the absence of "toxic" materials, biological floc development is limited only by the availability of nutrients and the lack of dissolved oxygen (Smith, 1953). Wider recognition of such deficiencies and their correction are necessary, especially in the aerobic process of sewage purification.

The objective of the present study was to determine the nitrogen and phosphorus requirements of microorganisms in a synthetic substrate that would produce an active sludge with an ability to settle rapidly. A synthetic substrate of highly reproducible character was used for this purpose. In the present investigation the term sewage includes domestic sewage combined with industrial wastes.

II. LITERATURE REVIEW.

Laboratory-scale activated sludge studies.

The activated sludge process may be simulated in the laboratory either by using continuous-flow pilot plants or fill-and-draw, batch-type, pilot plants. A wide variety of pilot plant procedures and construction is described in the literature. The following is modified from Symons, McKinney and Hassis (1960):

Continuous-flow pilot plant.

The sewage or substrate is supplied in a continuous flow. The mixed liquor (i.e. suspended solids plus treated liquid), is continuously removed from the aeration chamber and settled in a settling tank. The clear, supernatant liquor is removed in a continuous flow over the weirs and the settled sludge is returned to the aeration chamber at a predetermined rate.

The continuous-flow pilot plant has been used by the following workers: Smith (1952), Coe (1952), Garrett and Sawyer (1952), Hatfield and Strong (1954), Dougherty, Wolford and McNary (1955), Pasveer (1955), Schulze (1956), Garrett (1958), Busch (1959), Busch and Myrick (1960), Gaudy, Engelbrecht and De Moss (1960), Ludzack (1960), Moore *et al*. (1961), Ludzack, Schaffer and Bloomhuff (1961) and Ludzack, Schaffer and Ettinger (1961).

Fill-and-draw pilot plant.

The sewage or substrate is added batch-wise to the

settled sludge in an aeration chamber. The liquid plus solids is then aerated for a predetermined time. Aeration is stopped and the sludge allowed to settle. The supernatant liquor is removed and a fresh batch of substrate is added.

The fill-and-draw, batch-type pilot plant has been used by Sawyer (1940b), Logan and Heukelekian (1948), Weinberger (1949), Helmers et al. (1950), Rudolfs and Amberg (1953), Kountz (1953), Engelbrecht (1954), McKinney and Jeris (1955), McKinney, Tomlinson and Wilcox (1956), Symons and McKinney (1958), Morgan (1959), Busch and Myrick (1960) and Kelly, Sanderson and Neidl (1961).

Sludge acclimatization and sludge return.

The methods of preparing activated sludge and acclimating the sludge to the various substrates to be tested, vary widely. Trubnick and Rudolfs (1949), Coe (1952) and Amberg and Cormack (1957) acclimated their sludges by building up a concentration of solids from sewage and then slowly increasing the waste concentration while decreasing the quantity of sewage supplied. Rudolfs and Amberg (1953) and Hoover and Porges (1950) used cultures obtained from operating activated sludge plants. Strong and Hatfield (1954) worked with a petrochemical waste containing a high concentration of formaldehyde. Mills and Stack (1954) tested pure organic compounds, e.g. secondary and tertiary amines. McKinney, Tomlinson and Wilcox (1956) acclimated sludge to various aromatic substrates.

To keep the biological population active, it is necessary to return some of the settled sludge to the aeration chamber. The procedures used to recirculate sludge and to maintain the concentration of suspended solids constant in the aeration chamber vary. Logan and Heukelekian (1948) returned 25%, Morgan (1949) 35%, Kountz (1953) 50% and McKinney and Jeris (1955) 67% of the settled sludge. Weinberger (1949) maintained a constant concentration of suspended solids by removing excess sludge rather than by setting a fixed rate of sludge return. Strong and Hatfield (1954) and McKinney, Tomlinson and Wilcox (1956) recycled all of the secondary sludge by wasting only the supernatant liquor.

Use of a synthetic substrate.

One of the major problems in carrying out activated sludge research is the selection of a suitable substrate or "feed mixture". The problem resolves into the use of either a naturally-occurring sewage, or a synthetic substrate prepared in the laboratory.

Sewage is a heterogenous combination of organic compounds and biological life, the duplication of which is impossible. The disadvantages of using a naturally-occurring sewage as a substrate are:

a) That it is difficult to obtain and keep quantities of sewage of a uniform strength.

b) That the analyses required to establish the composition of sewage constitute a difficult task.

c) That the use of sewage would preclude the repetition of experiments, and verifying experiments.

d) That extreme fluctuations occur in the strength and composition of sewage from day to day.

e) That sterilization and storage of large volumes of sewage is difficult.

f) That heat treatment may alter the physical and chemical properties of sewage, e.g. loss of ammonia-nitrogen, precipitation of phosphate-phosphorus, and the production of "toxic" substances may take place.

Thus, if the experimental data were to be analyzed it would be difficult to determine the source of variation. Variations could be due to experimental errors, experimental parameters introduced, or to the substrate itself. Undoubtedly one of the chief reasons for the mass of contradictory data present in the literature has been the failure of some investigators to operate under comparable conditions, and the lack of appreciation and/or knowledge of the variables concerned.

The use of a synthetic substrate would correct most of the disadvantages of using a naturally-occurring sewage. The main criticism of using a synthetic substrate is that it is artificial. A synthetic substrate has the advantage that its composition is known, that its components can be separately prepared in desired concentrations, sterilized and diluted before use. Such a synthetic substrate should contain protein, fats, carbohydrates, ammonia-nitrogen, suspended solids,

inorganic salts, phosphorus and a buffer.

Synthetic substrates were used by a number of workers, Butterfield, Ruchhoft and McNamee (1937), Weinberger (1949), Garrett (1951), Smith (1952), Engelbrecht (1954), Greenberg, Klein and Kaufman (1955) and Pasveer (1955). These workers used peptone, starch, soap, skim milk or gelatin to provide the BOD* load. Inorganic salts added included $CaCl_2$, $MgSO_4$, NaCl and $Fe_2(SO_4)_3$. Weinberger (1949) used urea as a source of ammonia-nitrogen and Na_2HPO_4 to supply the phosphorus requirements. Smith (1952) used similar compounds in his studies. Greenberg, Klein and Kaufman (1955) used Na_2HPO_4, while Pasveer (1955) used urea and a potassium phosphate buffer. Symons and McKinney (1958) supplemented nitrogen in the form of $(NH_4)_2SO_4$, $NaNO_3$ and $NaNO_2$, while KH_2PO_4 was used to buffer the systems and to supply the phosphorus requirements. Gaudy, Engelbrecht and De Moss (1960) supplied NH_4Cl in combination with a 1 M potassium phosphate buffer.

Nutritional requirements of activated sludge.

Introductory remarks.

The biological synthesis of activated sludge depends on the nature and concentration of the carbonaceous materials that serve as a source of energy to the microorganisms. It also depends on a proper balance between nitrogen, phosphorus

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* 5-day biochemical oxygen demand at 20°C.

and the carbonaceous material. Small quantities of several other elements, such as potassium, calcium, sulphate and trace elements, are also required. These nutrients are normally present in natural waters in sufficient amounts to satisfy bacterial demand. It must be emphasized, that in industrial waste waters nitrogen and phosphorus are frequently deficient. Therefore these elements must be supplied as a nutrient supplement if optimum efficiency in purification is to be maintained.

Sawyer (1940a, 1941) showed that the rate of BOD removal in a sulphite waste liquor was increased by the addition of nitrogen and phosphorus. Domestic sewage normally contains nitrogen and phosphorus in excess, therefore, the addition of industrial wastes to sewer systems has long been a common practice. When admixture with domestic sewage is not feasible, the required need for nitrogen and phosphorus can be met by adding salts of inorganic nitrogen and phosphorus.

BOD:N:P ratios.

Early studies on the BOD test (Butterfield, Purdy and Thierault, 1931; Eldridge, 1933; Holderby and Lea, 1935; Lea and Nichols, 1936; and Lea, 1941) indicated the importance of nutrition in the biochemical oxidation of sewage. A BOD:N ratio of 16:1 and a BOD:P ratio of 40:1 were indicated by these workers to ensure that nitrogen and phosphorus were not limiting factors. Logan and Heukelekian (1948) and Rudolfs and Amberg (1953) maintained BOD:N and BOD:P ratios of 20:1

and 40:1, respectively. Sawyer, Frame and Wold (1955) used a BOD:N ratio of 20:1, while Amberg and Cormack (1957) maintained a BOD:P ratio of 60:1. The studies of Helmers et al. (1950, 1952) indicated an optimum BOD:N ratio of 17:1, and a BOD:P ratio of 100:1. These values agreed well with those reported by Sawyer (1940b, 1941).

Nitrogen requirements.

Sawyer (1940b) noted that sewages containing excessive amounts of nitrogen, produced sludges with a capacity for nitrification which increased as the concentration of the nitrogen increased. However, as the BOD:N ratio of the substrate reached a value of 16:1, or more, nitrification was stopped. Many industrial effluents, e.g. animal packing house and tannery wastes, contain nitrogen in excess of that required for stabilization by the microbial population of the activated sludge. Sawyer and Bradney (1945) encountered rising sludge in effluents containing large amounts of nitrates. Eckenfelder and O'Connor (1954) stated that when a nutritional supplement was required for biological processes, ammonia-nitrogen was used, since it was readily assimilable. They also stated that it was not advisable to add nitrates because the nitrates served as a secondary hydrogen acceptor. In secondary settling tanks the available dissolved oxygen may be depleted by denitrification processes, whereby nitrates are reduced to nitrogen gas and a floating sludge is produced.

Helmers et al. (1951, 1952) conducted laboratory

experiments on industrial wastes deficient in nitrogen and phosphorus. Experiments were performed to determine the amounts of additional nitrogen and phosphorus required for treatment of waste waters from cotton kiering, pulping of rope and brewing, by the activated sludge process. Mixtures of waste waters and 5% sewage were aerated for periods of 7 hr at temperatures of 10°, 20° and 30°C; the concentration of total solids was adjusted to 1.5 g/litre and varying amounts of inorganic salts of nitrogen and phosphorus were added. The BOD loading was about 70 lb/1000 cu ft of aeration capacity/day, except for cotton kiering waste treated at 10°C, when the load was about 36 lb/1000 cu ft. Bulking of sludge was controlled by chlorination. Nitrogen requirements were determined by measuring growth of sludge and by analyses of the waste waters and effluents. They came to the following conclusions:

a) Industrial wastes of the nature of those studied could be treated successfully in admixture with as little as 5% domestic sewage and with BOD loadings as high as 70 lb/1000 cu ft aeration capacity/day, if the nitrogen and phosphorus requirements were met.

b) Maximum nutritional requirements need not be supplied in order to achieve satisfactory treatments.

c) Nitrogen requirements, on the basis of BOD removal, were estimated to be 3, 4 and 3 lb/100 lb of 5-day BOD removed at 10°, 20° and 30°C, respectively.

d) Phosphorus requirements under similar conditions were 0.6,

0.7 and 0.5 lb/100 lb of 5-day BOD removed at 10°, 20° and 30°C, respectively.

e) A nitrogen deficiency reduced the rate of BOD removal, impaired the settling and dewatering characteristics of the sludge, and decreased the rate of sludge growth.

f) The nitrogen content of the dried activated sludge based on volatile matter was a good index of nitrogen deficiency. A value of less than 7% was indicative of a nitrogen deficiency. Similarly, a value of less than 1.2% of phosphorus was indicative of a deficiency of this element.

Kilgore and Sawyer (1953) showed that the nitrogen requirements of the microbes in the high-rate trickling filter was as great as, or possibly greater than, that of activated sludge. However, this did not hold for the phosphorus requirements, except in the case where rope and rag pulp liquor wastes were studied.

Hoover et al. (1951) determined the rate and extent of oxidation of skim milk, casein and lactose by activated sludge. In their experiments adequate amounts of nitrogen and phosphorus were supplied for sludge growth. When sufficient quantities of nitrogen were supplied, lactose was assimilated with an increase in sludge weight, equal to 43% of the added lactose. Lactose utilization was rapid under these conditions. If nitrogen was limited, the rate of lactose utilization was comparatively slow.

According to Porges, Jasewicz and Hoover (1956),

activated sludge oxidized stored material within the cells only after the concentration of nitrogen and phosphorus in solution became limiting. They also reported that an internal insoluble glycogen-like substance accumulated in their sludge. After the stored material within the cells had been consumed, true endogenous respiration started. The sludge mass consumed itself at a rate of about 1% of its own weight/hr. They formulated an empirical formula for their activated sludge mass to be of the form $C_5H_7O_2N$.

Symons and McKinney (1958) investigated the affect of various methods of nitrogen supplementation on the biochemistry of nitrogen in the synthesis of activated sludge. They developed an activated sludge using CH_3COONa as substrate. The nitrogen requirements were supplemented by using $(NH_4)_2SO_4$ as a source of NH_4^+, $NaNO_3$ as a source of NO_3^-, and $NaNO_2$ as a source of NO_2^-. To supply the necessary phosphorus and to buffer the systems, KH_2PO_4 was added to 1 g/litre. Each source of nitrogen was supplied at different levels, viz., 50.0, 37.5, 25.0 and 0.0 mg/litre as nitrogen, while the carbon source (CH_3COONa) was kept constant. Symons and McKinney (1958) recorded an increase in volatile aeration solids throughout the entire 35 days of treatment. In most of their treatments the nitrogen content of the sludge mass decreased throughout the test. The decrease was particularly evident in the systems in which 12.5 and 0.0 mg/litre nitrogen was added. All of the empirical formulas of the sludge mass deviated from the formula

$C_5H_7O_2N$, proposed by Porges, Jasewicz and Hoover (1956). A polysaccharide material slowly accumulated in the sludge mass throughout the test.

Symons and McKinney (1958) finally concluded that:

a) Batch-fed or conventional activated sludge systems should have sufficient nitrogen supplied to satisfy the high synthesis demand which occurs at the start of the aeration period. If this demand is not met, excess protoplasm would be destroyed during the aeration period.

b) Batch-fed or conventional activated sludge systems could not be operated without sludge wasting and without a gradual solids buildup, unless some solids escaped with the effluent.

c) All inorganic, nitrogenous radicles were available to the microorganisms as a source of nitrogen. Nitrate- and nitrite-nitrogen were less readily available than ammonia-nitrogen because of the extra energy required by the microorganisms for assimilation.

McKinney et al. (1958) discussed the role of nitrogen in the aerobic stabilization of organic wastes and investigated nitrogen supplementation in various forms. They studied six representative compounds of both aliphatic and aromatic types, such as: propylamine, propionitrile, nitropropane, aniline, benzonitrile and nitrobenzene. Activated sludges were acclimated to 500 mg/litre of each of the six organic compounds on a 24 hr fill-and-draw cycle (23 hr aeration, 1 hr sedimentation). After thorough acclimatization, the sludges were placed in Warburg respirometer flasks. Each of the six organic compounds

was added to determine its metabolic pathway. The following conclusions were drawn from their study.

a) Nitrogen fixation could supply nitrogen to a limited extent.

b) Oxygen derived from either nitrates or sulphates could be utilized to stabilize organic matter.

c) Metabolism of propylamine appeared to be by hydrolysis of the amino group followed by oxidation of propanol.

d) Metabolism of nitropropane was not determined because high volatility caused its loss from solution.

e) The four remaining compounds appeared to undergo hydrolysis followed by oxidation.

f) Nitrification could account for a major part of the oxygen uptake found in the Warburg studies on the metabolism of organic nitrogen.

g) Nitrogen balances were necessary to determine the fate of the nitrogen in the Warburg studies.

Morgan (1959) reported on the utilization of nitrogen-containing compounds for the biosynthesis of protein in activated sludge. He observed that urea-nitrogen appeared to be more readily available than nitrogen from even the simplest amino acid. Most of the amino acids added were oxidized in the citric acid cycle.

Experimental evidence (Helmers _et al_. 1951; Sawyer, 1956; McKinney _et al_., 1958; Symons and McKinney, 1958; Symons, McKinney and Hassis, 1960; and Symons _et al_., 1960) indicated that all inorganic nitrogen in both industrial wastes and

domestic sewage was available for sludge growth, but only a portion of the organic nitrogen was available. The amount of organic nitrogen that became available for bacterial assimilation varied widely, depending on the nature of the waste and the temperature of stabilization.

Phosphate requirements.

Phosphates may be occluded in sludges and slimes under alkaline conditions (Sawyer, 1956). Kilgore and Sawyer (1953) suggested that their trickling filter results were more accurate than their results obtained from activated sludge, because the pH of the activated sludge effluents produced, were in the range of 8 to 9, and that of the trickling filter effluents in the range of 7 to 8. They also stated that the phosphorus requirements of the microbes of the high-rate trickling filter was governed by the nature of the substrate, and to some extent by the amount of nitrogen available.

The effect of phosphorus on the characteristics of activated sludge was studied by Greenberg, Klein and Kaufman (1955). They observed the growth of sludges in a synthetic substrate containing varying amounts of phosphorus. The phosphorus concentrations of 0.0, 0.1, 0.8, 2.0 and 5.4 mg/litre were obtained by adding $Na_2HPO_4.2H_2O$. They came to the following conclusions:

a) Phosphorus deficiencies caused marked changes in the physical and chemical character of activated sludge solids. A tendency for phosphorus-deficient systems to produce

sludges having poor settling characteristics was most significant.

b) The development of an activated sludge did not occur in the complete absence of phosphorus. When phosphorus was present in sub-optimal concentrations, the growth of sludge solids was retarded and the rate of BOD removal reduced.

c) A deficiency of phosphorus resulted in reduced nitrogen utilization by the microorganisms.

d) At a BOD loading of 15.8 lb/day/1000 cu ft of aeration capacity and at a suspended solids concentration of 1.5 g/litre, the phosphorus requirement was 0.8 mg/litre, or 0.44 lb of phosphorus/100 lb of BOD removed.

Coe (1952) investigated the nutritional requirements of waste waters from several petroleum refinery units. He found that there was no significant correlation between BOD (or oxygen consumed) removal, and the utilization of nitrogen and phosphorus. Similarly, no significant correlation was found between sludge growth and the utilization of nitrogen and phosphorus. The ammonia-nitrogen and phosphate utilizations during treatment were approximately 19 mg/litre and 11 mg/litre, respectively, for an average removal of 1.1 g/litre BOD. He concluded that if these amounts of nitrogen and phosphorus were present in the waste water to be treated, no additional inorganic nutrients would be required.

Growth or sludge synthesis and calculation of nutritional requirements.

Sawyer (1956) stated that where the composition of the substrate (sewage) was uncertain, the practice has been to relate sludge growth to BOD. Sawyer (1941) in studies with sulphite waste liquor, established that the sludge weight increase was almost in direct proportion to the applied BOD of the waste. A yield of about 1 lb of dry sludge/lb of BOD removed was reported. Helmers et al. (1952) obtained uniform weight gain regardless of the substrate used. At BOD loadings of 70 lb/1000 cu ft/day, from 37 - 42 lb of new sludge was produced daily.

Heukelekian, Orford and Manganelli (1951) proposed the following formula for the calculation of sludge growth in domestic sewages:

$$A = 0.055S + 0.5B$$

where,

A = lb volatile solids accumulation/day,

B = lb volatile mixed liquor suspended solids, and

S = lb of BOD supplied/day.

Gellman and Heukelekian (1953) reported on studies with jute cook liquor, yeast waste, gum waste and boardmilk white water. They obtained a yield of approximately 0.5 lb of volatile solids in new sludge growth/lb of BOD applied. There was no apparent divergence from a straight line relationship over a wide range of BOD loadings.

Helmers et al. (1950, 1952) calculated the nutritional

requirements of activated sludge, using a theoretical balance equation. This equation was based on the assumption of an equilibrium between net gain and loss of nutrients. Hence, the sum of the nutritional elements present in the effluent together with those assimilated, should be equal to the nutritional elements supplied, thus,

$$I = E + \frac{Gn}{200} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

where,

I = the nutrient element in the influent feed mixture in mg/litre,

E = the nutrient element in the effluent in mg/litre,

G = the rate of sludge growth/day in mg/litre, and,

n = the percentage of nutrient element in the dry sludge (N used for percentage of nitrogen, and P for percentage of phosphorus).

The constants of equation (1) applied to their experimental conditions of three "feedings" per day and a 2:1 ratio of influent feed mixture to return sludge. The term $\frac{Gn}{200}$, or equivalently (I-E), represented the nutritional requirements of the activated sludge. The denominator 200, implied that the quantity of nutrient produced was greater than the quantity of nutrient supplied, or that the rate of sludge growth was too great by a factor of 2.

The nutritional requirements may be calculated from the growth and sludge analysis data, or from the analyses of feed

mixture and effluent. Both methods of calculation were used by Helmers et al. (1950, 1952). That the figures obtained by both methods approximated each other was one of the most important features of their work. The slight discrepancy was due to inherent errors in the methods of analyses and to small losses of nitrogen and sludge resulting in increased I_N-E_N values and decreased $\frac{GN}{200}$ values.

In calculating the amount of nutrient material actually required by the waste fraction in a waste-sewage mixture, Helmers et al. (1950, 1952) made deductions for the nutrient requirements of the sewage fraction. They suggested that after determining the nutritional requirements, variables such as the rate of BOD removal, effluent BOD, sludge volume index, and other sludge characteristics, should be compared with the available nitrogen and phosphorus. They believed that these criteria would help to calculate nitrogen and phosphorus requirements in activated sludge plant treatment.

Nitrogen and phosphorus requirements may also be computed from a material balance based on the maintenance of a minimum nitrogen and phosphorus content in the biological sludge produced by the system (Eckenfelder and O'Connor, 1954). For example, the calculation of nitrogen requirements may be based on the following data:

a) Approximately 60% of the BOD is assimilated by the sludge in the aeration period (Hoover et al., 1951; Placak and Ruchhoft, 1947).

b) 1 lb of biological volatile solids is equivalent to 1.4 lb of ultimate oxygen demand (UOD), (Placak and Ruchhoft, 1947).

c) A nitrogen content of 7% is necessary to maintain optimum efficiency of the process (Helmers et al., 1952).

An activated sludge plant was to be designed for an industry which would produce one million gallons of waste daily. Tests on the raw waste indicated an average BOD of 500 mg/litre and a total available nitrogen content of 10 mg/litre. Removal of 90% of the BOD was necessary to meet authorized receiving stream conditions. The nitrogen requirements can then be calculated as follows:

BOD removed	= 1 x 500 x 10 x 0.9
	= 4500 lb/day
Ultimate BOD removed	= 6750 lb/day
Biological volatile solids produced	$= \frac{6750 \times 0.6}{1.4}$
	= approx. 2900 lb/day
Total nitrogen required	= 0.07 x 2900
	= 203 lb/day
Nitrogen available in waste	= 1 x 10 x 10
	= 100 lb/day
Net nitrogen to be supplied	= 203 - 100
	= 103 lb/day.

(This is equivalent to approximately 4.5 lb of nitrogen/100 lb BOD removed by the system).

The phosphorus requirements may be computed in a similar manner.

Bulking of activated sludge.

Separation of the biological sludge from the treated waste is an essential part of the effective biological treatment of sewage. In the activated sludge process, the separation is accomplished by settling the sludge and allowing the cleared supernatant liquor to flow from the settling tank over a weir. Under certain conditions the sludge solids become "bulky", develop poor settling characteristics, and pass over the weir into the effluent, thus defeating to some degree the purposes of the treatment (Dondero, 1961).

A sheathed bacterium, Sphaerotilus has been isolated repeatedly from bulky activated sludge (Ruchhoft and Watkins, 1928; Smit, 1932, 1934; Lackey and Wattie, 1940; Littman, 1940; Tkachenko and Drublianez, 1959), but it is not the sole cause of bulking (Dondero, 1961). The conditions favouring the bulking of sludge by Sphaerotilus organisms have been studied with small-scale laboratory apparatus (Ingols and Heukelekian, 1939, 1940; Heukelekian and Ingols, 1940; Heukelekian, 1941; Ruchhoft and Kachmar, 1941).

Lackey and Wattie (1940) concluded that carbohydrate materials caused bulking in activated sludge. A strain of Sphaerotilus was isolated and grown in the presence of a number of substances but no single one was identified as a cause of excessive growth; most of the substances tested, which included glucose and disaccharides, are not normally found in sewage. They also concluded that some factor in plant operation might

be an important cause of bulking. The growth of this organism could be retarded only by chlorination.

Ingols and Heukelekian (1939, 1940) and Heukelekian and Ingols (1940) found that the principle causes of bulking of activated sludge were:

a) An excess of carbohydrate in relation to available nitrogen (C:N ratio greater than 8:1).

b) An excess of nutrients in relation to activated sludge solids concentration.

c) Insufficient aeration.

These conditions were believed to be more favourable to Sphaerotilus than to zoogleal forms, the predominant bacteria in normal activated sludge (Dondero, 1961). The strain of Sphaerotilus isolated by Littman (1940) did not grow well in sterile sewage unless a carbohydrate was added. He established that the volume of Sphaerotilus was approximately three times that of an equal weight of zoogleal sludge. This observation may provide an explanation of the poor settling qualities of Sphaerotilus-bulked sludge.

Ruchhoft and Kachmar (1941) concluded that Sphaerotilus was a delicate indicator of disturbances of the biological equilibrium of activated sludge, but not a primary cause of bulking. They concluded that Sphaerotilus was an aerobic, and not an anaerobic, organism. Stokes (1954) stated that there was no doubt that Sphaerotilus was an obligate aerobe, but that it was very difficult to establish beyond doubt, the aerobic nature of the organism. The marked preference of Sphaerotilus for

oxygen was indicated by its predominant surface growth in liquid media.

The substances promoting growth of Sphaerotilus are present in untreated sewage. This organism will only develop when raw sewage is diluted or treated biologically (Wuhrman, 1954). The nitrogen requirements of Sphaerotilus can be satisfied by ammonia, nitrates or amino acids, and sewage may contribute sufficient nitrogen and phosphorus to make up these deficiencies in spent sulphite liquor (Amberg and Cormack, 1960). Sphaerotilus grows best in pure culture in the pH range 5.5 to 10, with an optimum pH of about 7.

III. EXPERIMENTAL MATERIALS AND APPARATUS.

Apparatus.

Very little information is available on the nutritional requirements of microorganisms in trickling filters. The studies of Butterfield and Wattie (1941), Wattie (1942), Goldthorpe (1943) and Heukelekian (1945a,b,c,d) have demonstrated that qualitatively the predominant bacteria of standard trickling filter slimes are similar to those in the floc of activated sludge, and that the floc and slime are interchangeable in the activated sludge process. If the nutritional requirements for the activated sludge process and the high-rate trickling filter are similar under comparable conditions of temperature and loading, considerable time may be saved by making preliminary laboratory investigations on the activated sludge process. For this reason it was decided to use the activated sludge process to study the nitrogen and phosphorus requirements of a synthetic substrate.

The activated sludge unit used in this investigation was similar to the unit described by Symons, McKinney and Hassis (1960), and is shown in Figure I. The perspex unit had six compartments closed with a lid. Each compartment was provided with a gas outlet. This outlet facilitated the trapping of the escaping gas, and also provided a means for analyzing the gas to determine whether or not ammonia-nitrogen was stripped from solution. The unit was maintained at 20°C ± 1°C in an incubator. Air was supplied at a pressure of 10 psi through a glass diffuser (porosity 40 - 60 μ),

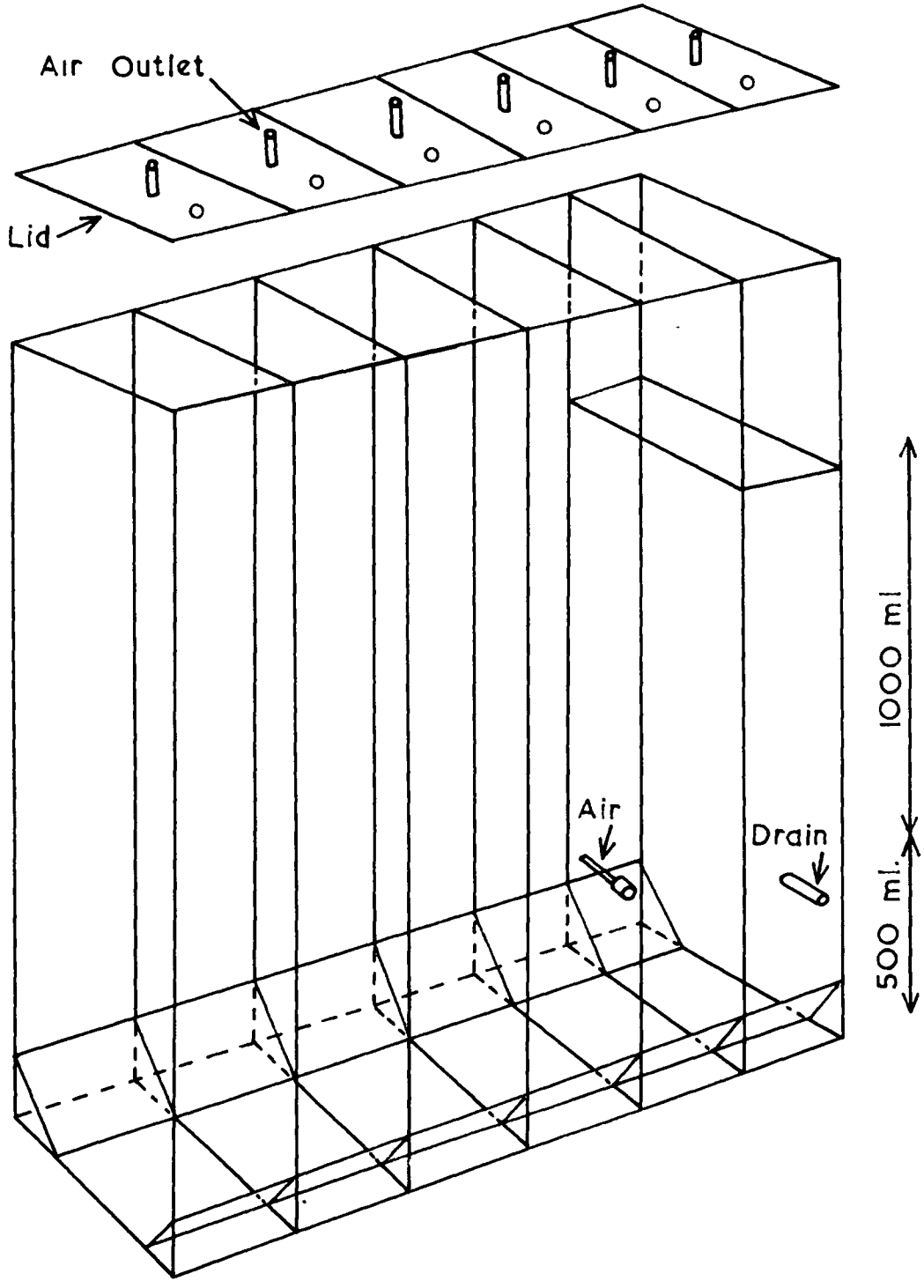

FIG 1. LABORATORY SCALE ACTIVATED SLUDGE PLANT

with a flow rate of 1.0 litre/min. The air flow was metered by calibrated flow meters connected into the airline of each unit. The quantity of air supplied was in excess of that usually provided in activated sludge aeration tanks (Haseltine, 1961).

The capacity of each compartment was 1.5 litres. At the end of the aeration period, aeration was stopped and the sludge allowed to settle. After 1 hr of sedimentation, 1 litre of the supernatant liquor was withdrawn. By adding 1 litre of substrate to the 0.5 litres of settled sludge, a sludge return of 33% by volume was obtained. To keep the sludge concentration constant a calculated amount of mixed liquor was withdrawn before aeration was stopped. For example, if the sludge concentration was to be kept at 1 g/litre and the concentration increased to 1.2 g/litre, the desired quantity of mixed liquor to be withdrawn, was calculated as follows:

$$\frac{1.2 - 1.0}{1.2} \times 1.5 = 0.25 \text{ litre of mixed liquor.}$$

The pilot plant was seeded with sewage from the Discovery Street sewer outlet into English Bay, Vancouver, and the sludge was developed as described by Symons, McKinney and Hassis (1960). The unit was operated for two months before any experiments were carried out.

Aeration studies of the pilot plant.

It is necessary to ensure that the mixed liquor in the aeration chamber contains an adequate concentration of dissolved oxygen in order to maintain aerobic conditions.

The concentration of dissolved oxygen in the mixed liquor is determined by the relative magnitude of the rates of supply and demand. Thus, the purification capacity of an activated sludge plant depends on the rate of oxygenation. Kessener and Ribbius (1934) advanced the concept of "oxygenation capacity" (O.C.), defined as the amount of oxygen dissolved/m^3 of water/hr at 760 mm Hg and 10^oC, when the oxygen content of the water is zero. Pasveer (1953) proposed the following formula to account for the different rates of diffusion of oxygen in water:

$$\text{Oxygenation capacity (O.C.)} = KC_s\sqrt{\frac{k_{10}}{k_{t_1}}} \text{ g } O_2/m^3 \text{of water/hr} \quad ..(1)$$

where,

k_{10} = rate of diffusion of O_2 in H_2O at 10^oC,

k_{t_1} = " " " " " " " " " temperature t_1,

K = overall absorption coefficient, and

C_s = oxygen saturation in H_2O at 10^oC and 760 mm Hg.

The O.C. is directly proportional to C_s and thus depends on the temperature of the water and the barometric pressure.

Determination of the value of K.

If re-aeration was the only cause of changes in C (concentration of dissolved oxygen at time t), then K could be determined from an experiment in which the rate of change of concentration was found. This rate may be determined by reducing the dissolved oxygen content of the water physically

or chemically. The course of re-aeration could then be examined periodically by sampling the water and analyzing the samples for their oxygen content, e.g. by the method of Winkler, or some modification of it (Standard Methods, 1960).

During the course of re-aeration, the rate of change of concentration is proportional to the oxygen deficit, and is given by the following formula:

$$\frac{dC}{dt} = K(C_s - C) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

where,

C = the concentration at time t, and

C_s = the saturation concentration.

The constant K is known as the overall absorption coefficient and measures the rate of change in concentration of dissolved oxygen when the saturation deficit, $(C_s - C)$ is unity (Gameson, Truesdale and Downing, 1955). K is generally expressed in reciprocal hr.

K may be obtained by plotting the natural logarithm of the oxygen deficit, $\log_e(C_s - C)$, against time. However, K may also be calculated from the equation:

$$\log \frac{C_s - C_1}{C_s - C_2} = K(t_2 - t_1) \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (3)$$

where,

C_1 and C_2 = the oxygen concentration at time t_1 and t_2, respectively.

The value of K depends on the volume of water, V, and the area of exposed surface, A. The ratio V/A is referred to as the aeration depth. The transfer or exit coefficient, F, is defined by the relation:

$$F = \frac{KV}{A} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (4)$$

This coefficient measures the rate of change of dissolved oxygen in a unit depth of water when the saturation deficit is unity, and is expressed in cm/hr.

Morgan and Bewtra (1960) drew attention to the need for a standard method for evaluating the oxygen absorption characteristics and recommended that appropriate studies be carried out with clear tap water at 20°C and at a dissolved oxygen concentration of zero.

To determine the value of K, the following experiment was carried out: the dissolved oxygen concentration of the tap water in the aeration chamber (20°C) was reduced to almost zero by the addition of Na_2SO_3 solution and using cobalt as a catalyst. An initial sample was taken for dissolved oxygen (D.O.) analysis, and aeration was started. After a given time interval aeration was stopped and the dissolved oxygen content of the water was determined. These values are reported in Table 1.

Figure 2 was plotted from these values and K was found to be 0.42/min, or 25.2/hr. The transfer or exit coefficient F, was calculated to be 600 cm/hr (equation 4).

The values obtained for K and F indicated that the system was well-aerated (Downing and Truesdale, 1956).

Table 1.

Increase in the dissolved oxygen concentration with aeration.

Aeration time in mins	Dissolved oxygen concentration (mg/litre)
1.0	3.3
2.0	5.3
3.0	6.5
4.0	7.5
5.0	8.1
6.0	8.4

Figure 3 was plotted from the values presented in Table 1 to show the rate of re-aeration of the water in the activated sludge unit. The time required to saturate the water with oxygen from a dissolved oxygen concentration of zero was in excess of 9 min. A figure of 10.8 min was obtained when calculated from the following formula:

$$T = \frac{2.303}{K} \log \frac{C_s}{C_s - C} \quad \ldots\ldots\ldots\ldots\ldots \quad (5)$$

where,

C_s = 9.2 mg/litre at a water temperature of 20.0°C and at a barometric pressure of 760 mm Hg.

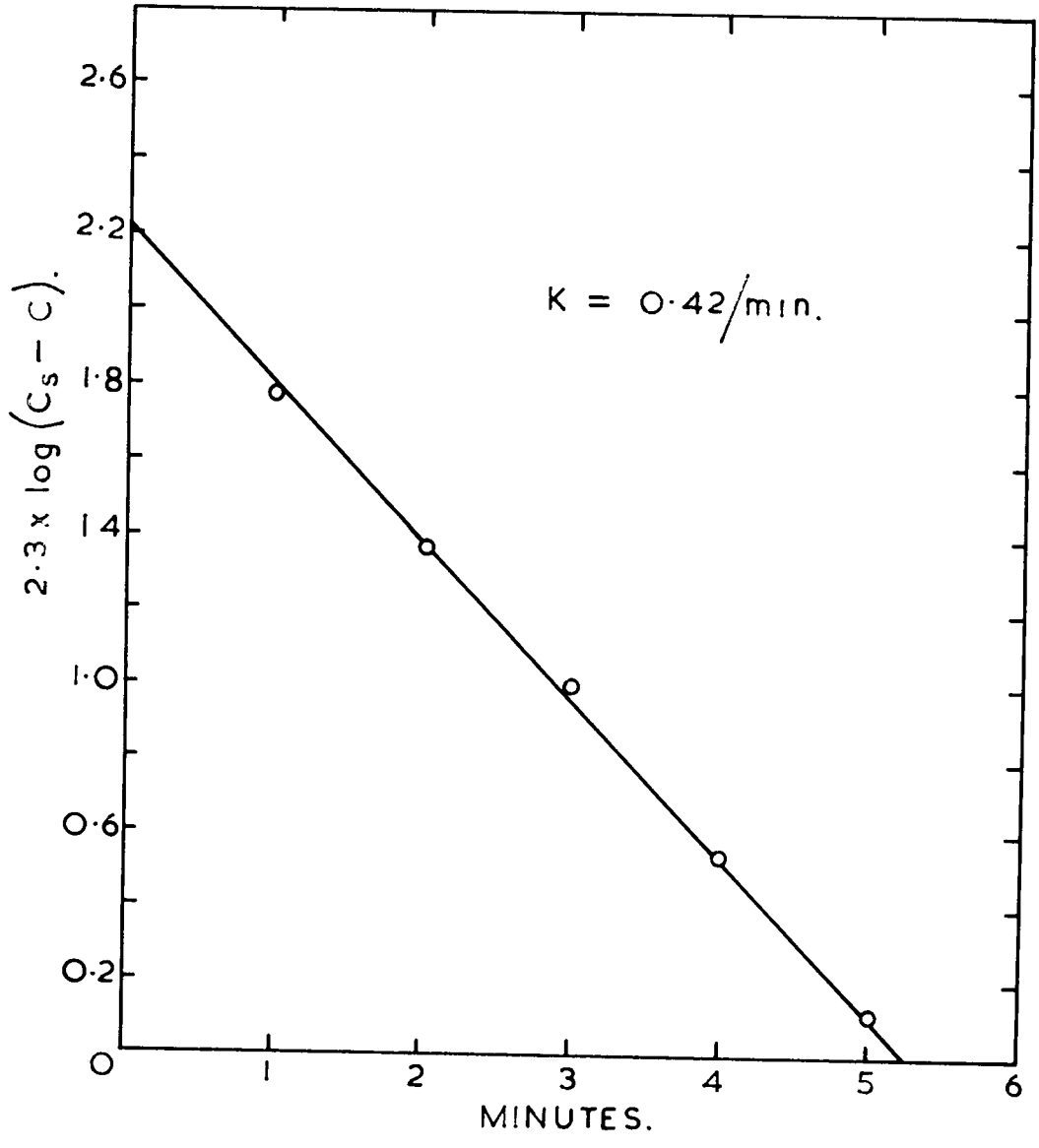

FIG 2. PLOT OF OXYGEN SATURATION DEFICIT ($C_s - C$) TO OBTAIN K.

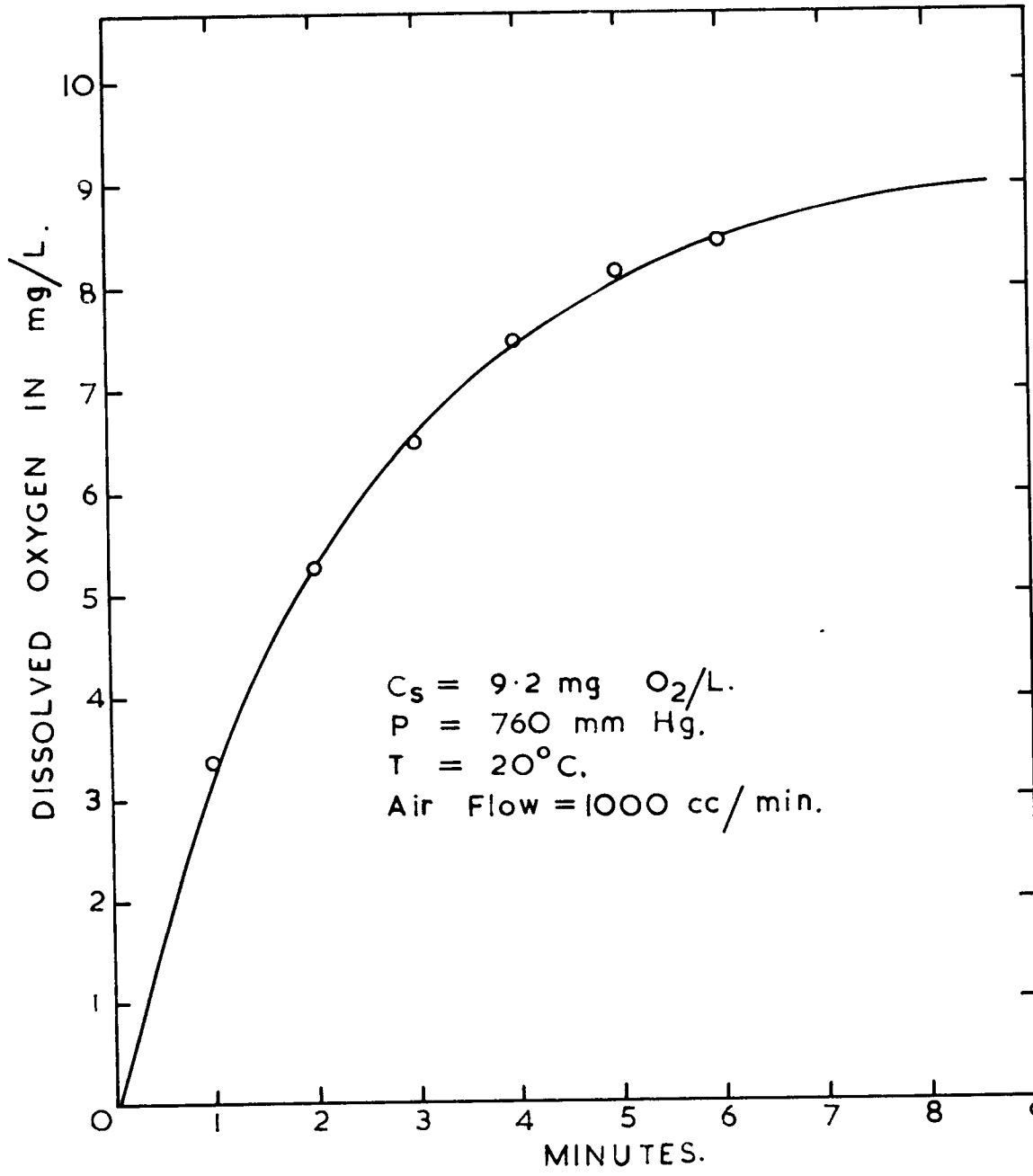

FIG 3. RATE OF OXYGENATION OF PURE WATER IN THE ACTIVATED SLUDGE UNIT.

The oxygenation capacity of the aeration chamber at 20°C was calculated from equation (1):

$$O.C. = K.C_s \sqrt{\frac{k_{10}}{k_{20}}} \text{ g } O_2/m^3/hr.$$

$$= 25.2 \times 9.2 \times \sqrt{\frac{1.45}{1.45 \times 1.45}}$$

$$= 25.2 \times 9.2 \times \sqrt{\frac{1}{1.45}}$$

$$= 192.5 \text{ g } O_2/m^3/hr \text{ at } 20^\circ C.$$

where,

$K = 25.2$

$C_s = 9.2$ at 20°C

$k_{10} = 1.45 \times 10^{-5}$ cm^2/sec

$k_{20} = 1.45k_{10}$.

Synthetic substrate.

The substrate developed for this study had the following composition:

Nutrient broth	100	mg/litre
Starch	400	"
Castile soap	200	"
Casamino acids	100	"
Ca^{++} as $CaCl_2.2H_2O$	5	"
Mg^{++} as $MgSO_4.7H_2O$	5	"
K^+ as KCl	5	"

$NaHCO_3$	400 mg/litre
Fullers earth	30 "
Cellulose	5 "

Nutrient broth and casamino acids are commonly used as a source of protein (Difco Manual, 1953). Castile soap simulated the grease and soaps commonly found in sewage. Carbohydrates were supplied by the soluble starch. The suspended solids were supplied by diatomaceous earth (Fullers earth). To provide additional trace elements Vancouver tap water was used as a solvent rather than distilled water. The average analysis of the tap water is listed in Table 2.

Triplicate BOD determinations on each of three separate batches of synthetic substrate, seeded with activated sludge, yielded an average first order rate constant of 0.12/day and a calculated ultimate oxygen demand of 1130 mg/litre. The average lag period was 1 day. The rate constant for settled sewage seed was 0.30/day and a calculated ultimate oxygen demand of 920 mg/litre. The rate constant of 0.12/day compares favourably with that normally obtained in naturally-occurring sewage. Figure 4 illustrates the course of a typical BOD reaction of the synthetic substrate after inoculation with settled sewage and activated sludge mixed liquor. The value of the rate constant, K, was obtained by the method of Sheehy (1960).

The synthetic substrate had the following average analysis (54 observations), the 95% confidence intervals for the means are also shown:

Table 2.

Average chemical and physical analyses of water supplies of the Greater Vancouver water district*.

	Capilano intake	Seymour intake	Coquitlam intake
Appearance	Clear	Clear	Clear
Odour	None	None	None
Colour	<10	<10	<10
Turbidity (on silica scale)	<5	<5	<5
pH	6.4	6.8	6.3
Total solids	21.7	27.5	18.8
Total fixed solids	13.8	19.1	11.1
Total volatile solids	7.9	8.4	7.7
Phenolphthalein alkalinity (as $CaCO_3$)	nil	nil	nil
Total alkalinity (as $CaCO_3$)	3.3	5.3	2.9
Total hardness (as $CaCO_3$)	4.6	6.3	3.5
Cl'	0.7	0.5	0.6
SO_4''	1.8	2.3	1.8
F'	<0.01	<0.01	<0.01
Cu	<0.03	<0.03	<0.03
Fe (total)	0.14	0.16	0.11
SiO_2	3.0	3.0	3.0
Ammonia-N	0.017	0.007	0.01
Nitrate-N	0.2	0.1	0.1
Nitrite-N	<0.002	<0.002	<0.002

Note: (1) $<$ = less than,

(2) The above figures represent the average of a number of analyses throughout the year, and are reported in mg/litre

* The values were kindly supplied by the Greater Vancouver water district.

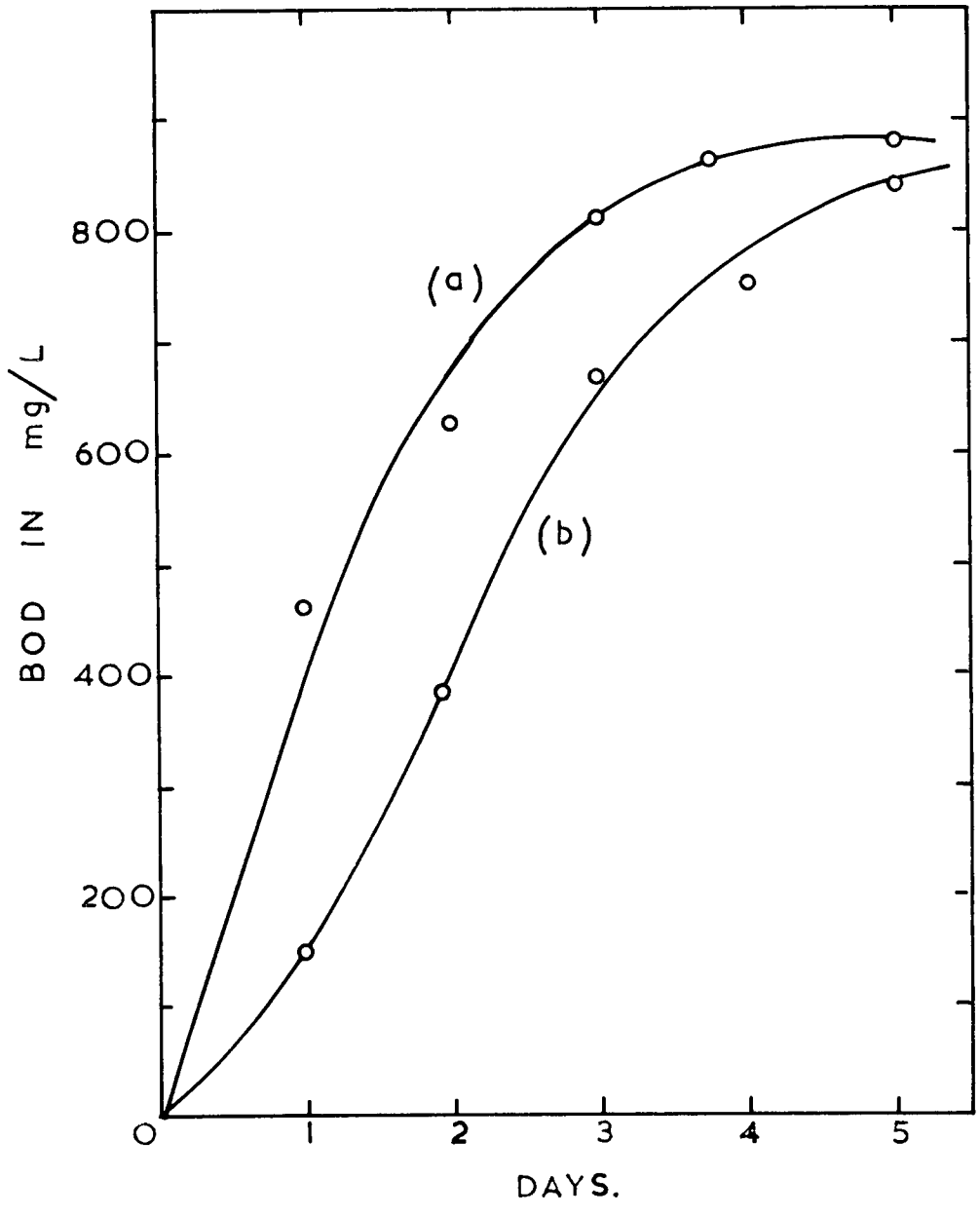

FIG 4. PROGRESSION OF THE SYNTHETIC SEWAGE BOD WHEN INOCULATED WITH SETTLED SEWAGE (a) OR ACTIVED SLUDGE MIXED LIQUOR (b).

BOD	840.0 ± 21.0 mg/litre
COD	1230.0 ± 30.0 "
Alkalinity as $CaCO_3$	289.0 ± 3.0 "
Total phosphorus	2.0 ± 0.1 "
Total nitrogen (organic)	22.6 ± 1.3 "
NH_3 - N	nil
NO_2' - N	nil
NO_3' - N	nil
pH	8.8 ± 0.2

Additional nitrogen and phosphorus were added as $(NH_4)_2SO_4$ and K_2HPO_4, respectively. The calculated ultimate oxygen demand of 1130 mg/litre (mixed liquor seed) approximated the COD value of 1230 mg/litre. Therefore, it was decided to use the mixed liquor seed instead of settled sewage seed.

IV. ANALYTICAL METHODS.

Chemical methods of analysis.

The analyses of the substrate, effluents and sludges for BOD, NO_2^-, organic nitrogen, pH, alkalinity, volatile solids at 600°C, sludge concentration by weight and sludge density index were made in accordance with Standard Methods (1960). The other analyses were carried out as follows. All results are reported in mg/litre, unless otherwise stated.

Ammonia-nitrogen.

Ammonia-nitrogen was determined by the Conway microdiffusion technique (1957). Modified Conway units were used (Öbrink, 1955). A 2% (W/V) boric acid solution was prepared by dissolving the acid in a 0.005% (V/V) solution of a non-ionic detergent*.

Samples of the effluents and substrates were analyzed in triplicate. A 45% (W/V) K_2CO_3 solution in 0.025% (V/V) of non-ionic detergent was used to distill ammonia into the boric acid solution. To each sample of 3 ml, 1 ml of the K_2CO_3 solution was added. An additional 2.5 ml of the K_2CO_3 solution was added to the closing chamber. The centre chamber contained 1 ml of the boric acid solution. The prepared units were incubated at 30°C for 24 hr and the distilled ammonia-nitrogen titrated with 0.005 N H_2SO_4.

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* Tergitol non-ionic NPX, manufactured by Union Carbide Chemicals Company.

Chemical oxygen demand.

The modified COD method of Symons, McKinney and Hassis (1960) was used.

Phosphate-phosphorus.

Soluble orthophosphate.

The ascorbic acid method as described by Murphy and Riley (1958) was used, with some modification. The colour-developing reagent was prepared as follows:

Solution A. 15 g ammonium molybdate was dissolved in 1 litre of 3 N H_2SO_4.

Solution B. Fresh 10% (W/V) solutions of ascorbic acid were prepared at monthly intervals and refrigerated (Chen, Toribara and Warner, 1956).

To 200 ml of solution (A) in a 250 ml standard flask, 5.3 ml of solution (B) was added, and the contents diluted to 250 ml; the colour-developing reagent was prepared immediately prior to use. The blue colour was allowed to develop for 24 hr at room temperature. The intensity of the colour was measured at 827 mμ in a spectrophotometer.

Total phosphate-phosphorus.

The total phosphorus of the sludge was determined according to the ammonium-molybdo-vanado method as described in the Official Methods of Analysis of the Association of Official Agricultural Chemists (1960). The sludge was

digested with concd HNO_3 in a Kjeldahl flask until colourless, and then reduced to a small volume. The contents were filtered through a Whatman No. 42 filter paper into a 50 ml standard flask, diluted to approximately 25 ml, 10 ml of the colour-developing reagent added, and the volume made up to 50 ml with distilled water. The colour was allowed to develop for at least half an hour and then measured at 400 mμ.

Nitrate-nitrogen.

Nitrate-nitrogen was determined colorimetrically according to the salicylate method of Müller and Wideman (1955).

Nucleic acids.

The nucleic acids were isolated from the sludge as described by Schneider (1957).

A 25 mg sample of sludge mixed liquor was centrifuged at 10,000 x g for 10 min, the supernatant liquor decanted and 5 ml cold 10% (W/V) trichloroacetic acid (TCA) added. The suspension was well stirred, centrifuged, and the supernatant liquor discarded. The sludge was washed once more with 5 ml cold 10% TCA, and finally resuspended in 10 ml of 5% cold TCA. The tubes, containing the suspension, were now heated for 15 min at 90°C with occasional stirring, then cooled and centrifuged. The supernatant liquor was poured into a 50 ml standard flask. The precipitate was again washed with 2.5 ml 5% TCA and the supernatant liquor collected in the same standard flask.

The volume in the flask was then made up to 50 ml with distilled water. A blank was prepared in a similar manner.

The absorbance of the solutions was measured at 260 mμ and 280 mμ, using the blank to set the spectrophotometer. The concentration of nucleic acids was read off from the nomograph prepared by Warburg and Christian (1941). The concentration of nucleic acids is in arbitrary units and does not specifically indicate deoxyribonucleic acid (DNA) or ribonucleic acid (RNA).

Carbohydrate.

The carbohydrate content of the sludge, expressed as glucose, was determined as outlined by Hassid and Abraham (1957).

A 10 mg sample of sludge mixed liquor was centrifuged at 10,000 x g for 10 min, the supernatant liquor removed and 5 ml 30% (W/V) KOH added to the sediment. The tubes, containing the suspension, were heated in a boiling waterbath for 20 - 30 min, then cooled and the contents transferred to a 50 ml standard flask and made up to volume.

A 5 ml aliquot containing 15 - 150 μg of carbohydrate material, expressed as glucose, was pipetted into test tubes and cooled in ice. To these tubes were added 10 ml 0.2% (W/V) anthrone in 95% (V/V) H_2SO_4, and the contents mixed well. The tubes were transferred to a boiling waterbath and heated for exactly 15 min. The tubes were then immediately cooled in ice water and allowed to reach room temperature. A standard

containing 100 μg glucose and a blank containing distilled water were prepared simultaneously. The absorbance was measured at 620 mμ.

Estimation of microbial population of the sludges.

Microfauna.

Protozoan counts were made with a haemocytometer. Counts included all the protozoa present in 0.9 cubic mm of sludge, spread over 9 square divisions of the haemocytometer. The number of protozoa present was calculated per mg of volatile sludge. The number of counts to be made on each sludge was determined according to the formula of Steel and Torrie (1960). It was found that 12 separate counts of the protozoa present in each sludge would be sufficient to detect reliable differences of 10% in the protozoal population.

Microflora.

The sludge from each treatment was diluted 1:100 with a buffered dilution water (Standard Methods, 1960) and dispersed in a Waring blendor for 30 sec. The dispersed sludge was plated on the following media according to Standard Methods (1960):

Nutrient agar	- bacterial population
Sabouraud dextrose agar	- yeasts and moulds
Glycerol-glucose agar	- actinomycetes
Selective acid agar	- moulds.

The plates were incubated for one week at 20°C and then

counted. The results are reported per mg of volatile sludge. The composition of the media used is given in the appendix.

Respiration studies.

The rate of oxygen uptake of activated sludge was measured with a Warburg manometer.

The sludge was prepared by removing 25 ml of mixed liquor and centrifuging at 3500 x g for 10 min. The supernatant liquor was discarded. The sludge was given two consecutive washings by resuspension in M/30 Tris* buffer (pH = 7.9). The sludge was then resuspended and restored to its original volume with M/30 Tris buffer. From the well-mixed suspension (mechanically stirred) 2 ml portions were transferred to the reaction flasks. The centre vial contained 0.2 ml 20% (W/V) KOH plus folded filter paper and 0.5 ml glucose (50 μM) was placed in the side arm. The measurements were conducted at 20°C. After 1 hr of oxygen uptake measurements ("endogenous") the glucose was tipped in and the measurements continued for another hour. The rates of glucose utilization by the differently treated sludges were then calculated. All the determinations were carried out in duplicate and the respiration rates reported in μl O_2/hr/mg volatile sludge.

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* Tris-hydroxy-amino-methane.

V. EXPERIMENTAL PROCEDURES.

Preparation of synthetic substrate.

The composition of the substrate used, was as outlined on p. 34. The substrate was prepared in a concentrated form (X 200). Preparation of the substrate involved the mixing and dilution of eight separate solutions. The reasons for adopting this method of preparation were as follows:

a) Some of the components, when prepared in concentrated form, formed a precipitate.

b) It was desirable to vary the concentration of nitrogen and phosphorus.

c) The components providing the BOD load had to be sterilized if they were to be stored for any length of time.

The separate solutions were prepared as follows:

1) The nutrient broth*, starch* and casamino acids* were prepared together, dispensed as a concentrate in 5 ml quantities into test tubes, and sterilized by autoclaving for 15 min at 15 psi.

2) The soap was prepared separately, dispensed in 5 ml quantities into test tubes, and sterilized, as above.

3) $CaCl_2$ + KCl.

4) $MgSO_4$.

5) $NaHCO_3$.

6) Fullers earth + cellulose.

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* Manufactured by Difco.

7) K_2HPO_4.

8) $(NH_4)_2SO_4$.

Components (3) to (8) were not sterilized, but kept in reagent bottles and the required amounts pipetted daily. The PO_4''' and NH_4^+ solutions were refrigerated and the required amounts pipetted daily.

The pH was not controlled to within very close limits as no buffer was available to accomplish such a control. Tris was used for two months and a very narrow pH range could be maintained. No degradation of Tris was observed during the two month period, as indicated by COD and Kjeldahl nitrogen determinations of the effluent. At the time that Tris was used, no analytical technique was available to determine Tris in solution. Two analytical techniques (Rosen, 1961; Linn and Roberts, 1961) have since become available to determine Tris in solution. Tris contains nitrogen as an amino group and the possibility that some microorganisms might degrade Tris was always present.

On two occasions sludge was inoculated into the following base medium:

K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 "
NaCl	0.1 "
$MnSO_4.4H_2O$	0.001 g
$Na_2MoO_4.2H_2O$	0.001 "
$FeCl_3.6H_2O$	0.001 "
Tap water	856 ml

This base medium was dispensed into test tubes in 8.7 ml amounts, and sterilized. Tris buffer, containing 10.0 mg nitrogen/ml, a 10% (W/V) glucose solution, and a $(NH_4)_2SO_4$ solution containing 10.0 mg nitrogen/ml, were sterilized separately by autoclaving. The following combinations were prepared:

Medium A. Base medium + 0.4 ml Tris + 1 ml glucose.

Medium B. " " + 0.4 ml $(NH_4)_2SO_4$ + 1 ml glucose.

Medium C. " " + 0.2 ml $(NH_4)_2SO_4$ + 0.8 ml Tris + 1 ml glucose.

Medium D. " " + 1.4 ml Tris.

A 1:100 dilution was prepared from the sludge and inoculated in triplicate into media A, B, C and D. The tubes were incubated at 20°C for 1 week, the results are given in Table 3.

Table 3.

Growth of activated sludge microorganisms in a medium containing Tris and $(NH_4)_2SO_4$ as sources of nitrogen after 48 hr incubation.

Treatment	Growth	Final pH
Medium A - inoculated	-	7.9
" " - not inoculated	-	7.9
" B - inoculated	++	4.7
" " - not inoculated	-	7.0
" C - inoculated	+++	5.9
" " - not inoculated	-	7.9
" D - inoculated	-	7.9
" " - not inoculated	-	7.9

Note: + indicates growth,
- " no growth.

From these results it was concluded that the organisms could not utilize Tris as a source of carbon and/or nitrogen after two months of treatment with Tris. The higher pH of Medium C was due to the buffering action of Tris. Tris had no suppressing effect on growth when $(NH_4)_2SO_4$ was utilized as a source of nitrogen.

To avoid the risk of adaptation of the sludge organisms to Tris as a substrate, it was decided to discontinue the use of Tris as a buffer. Mahler (1961) pointed out that Tris cannot be considered chemically inert or unreactive in a wide variety of enzymatic reactions. He emphasized that its possible role and influence should be examined critically in each individual case. However, in this case no disadvantages were observed over the two month period of its use.

Analytical control of the pilot plant.

In each treatment procedure a unit was supplied with synthetically prepared substrate for 32 days, in which nitrogen and phosphorus were present in the required concentrations. Every 4th day the effluents were filtered through Whatman No. 1 filter paper and immediately analyzed chemically. The samples were refrigerated until analysis was completed, usually within 24 hr. The substrate was similarly analyzed once a week.

The sludge concentration by weight and the sludge density index were determined every alternate day. The sludge density index was calculated from the volume of sludge settling after 30 min in a 50 ml graduated cylinder. The solids concentration was

reduced to the desired level by withdrawing a calculated volume of mixed liquor before aeration was stopped. The volatile solids content was determined simultaneously. An accurate record of sludge growth was kept for each treatment.

The following analyses and observations were made during the course of an experiment.

Substrate and effluent.

Biochemical oxygen demand (BOD).

Chemical oxygen demand (COD).

Nitrogen as NH_3, NO_2', NO_3' and organic nitrogen.

Phosphorus as PO_4'''.

pH.

Alkalinity expressed as $CaCO_3$.

Mixed liquor and sludge.

Nitrogen content of the volatile solids.

Phosphorus content of the volatile solids.

Volatile solids content.

Sludge concentration by weight.

Sludge density index (SDI).

Sludge growth.

Respiration rate of the sludge.

Estimation of bacterial population.

Estimation of protozoal population.

Statistical design of the experiment.

To study the effect of nitrogen, phosphorus and loading simultaneously, a 3x3x2 partially confounded, factorial design was

used. The design and analysis of the experiment were carried out according to the methods of Yates (1937) and Cochran and Cox (1957).

Nitrogen was supplied at three levels, phosphorus at three levels and the sludge concentration by weight at two levels. The design gave a total of 18 treatments. Where a treatment is given as 221, it is understood to mean $N_2P_2S_1$, i.e. nitrogen, phosphorus and sludge concentration at their highest levels. Similarly, treatment 010 would indicate nitrogen at its lowest level, phosphorus at its intermediate level and sludge concentration at its lowest level.

The nitrogen-phosphorus (NP) and the nitrogen-phosphorus-sludge concentration (NPS) interactions were partially confounded. The design, in two replications, was as outlined in Table 4. The order of each set of six treatments in a block, was randomized by means of the tables of random permutations, as given by Cochran and Cox (1957).

The sludge from treatment 221 (block Ia) was used to prepare sludge for block Ib at the end of the treatment period. Treatment 221 may be regarded as a treatment control, because nitrogen and phosphorus were supplied in excess of the nutritional requirements of the microorganisms. Therefore, it was assumed that the constitution of the biological population of that sludge did not change drastically and could be used as an inoculum for further sludge production. Similarly, the sludge of treatment 220 (block Ib) was used to prepare sludge for block Ic of replication I. In this way, the same sludge was carried through the experiment.

Table 4.

Statistical design of a 3x3x2, partially confounded, factorial experiment. Two replications in blocks of six units were used.

Replication I			Replication II		
Block No.			Block No.		
a	b	c	a	b	c
200	000	100	000	100	200
010	110	210	110	210	010
120	220	020	220	020	120
001	101	201	201	001	101
111	211	011	011	111	211
221	021	121	121	221	021

Sludge from the sewage aeration plant, North Shore, Vancouver, was used to prepare sludge for replication II. The same method of sludge preparation was followed to prepare sludge for blocks IIb and IIc.

The sludge for treatment in any specific block was prepared as follows: after the removal of 1 litre of mixed liquor of either treatment 221 or 220, at the completion of a treatment period, the remaining 0.5 litre was fed daily with the synthetic substrate. Nitrogen and phosphorus were supplied in excess of the nutritional requirements of the microorganisms, until the sludge concentration rose to approximately 4 g/litre. The sludge was

then divided into six equal portions and introduced into each unit. Nutrients were supplied in identical concentrations (excess) until the sludge concentration in all the units had risen to about 3 g/litre. The sludge concentration in each unit was then reduced to the required concentration for that treatment, and treatment of the sludges commenced. Nitrification was evident in all the units at the commencement of the treatment of each block.

The levels or concentrations of nitrogen, phosphorus and sludge concentration used in the present investigation, were as follows:

Treatment level	Nitrogen in mg/litre	Phosphorus in mg/litre	Sludge concentration in mg/litre
0	22.6	2.0	1000
1	37.6	6.5	2000
2	60.0	13.8	-

Calculation of nutritional requirements and nutrient balances.

Helmers *et al*. (1950, 1951) showed that the nutritional requirements of a sludge may be computed from differences in the concentrations of available nutrients, (see p. 19 for a discussion of their work). For the purposes of the present investigation the term $\frac{Gn}{200}$, as used by Helmers *et al*. (1950, 1951), was replaced either by N for the percentage of nitrogen, or by P for the percentage of phosphorus. The new term GN (i.e. for nitrogen) gave the quantity of nitrogen produced by the sludge directly

in mg/day.

The calculation of the nutrient balances as reported in Tables 8 and 9, will be demonstrated for the data of treatment 110. Treatment 110 was supplied with 37.6 mg nitrogen/day. The effluent contained 0.3 mg nitrogen/litre (Table 5). Average sludge growth during the 32 days of treatment was 477 mg/day. The nitrogen content of the sludge decreased from 10.2% to 7.6%, with an average nitrogen content of 8.9% (Table 6 and Figure 9).

Hence,

$$I_N = 37.6 \text{ mg nitrogen /day},$$

$$E_N = 0.3 \text{ mg nitrogen/day},$$

$$G = 477 \text{ mg/day},$$

$$N = 8.9\%.$$

For a nutrient balance $I_N - E_N$ must be equal to GN.

Thus,

$$I_N - E_N = 37.6 - 0.3$$

$$= 37.3 \text{ mg nitrogen/day},$$

and,

$$GN = \frac{477 \times 8.9}{100}$$

$$= 42.5 \text{ mg nitrogen/day}.$$

Therefore, the sludge consumed 37.3 mg nitrogen/day to maintain a sludge growth rate of 477 mg/day. Hence, the quantity of nitrogen (GN) removed from the system, exceeded the quantity of nitrogen consumed by 5.2 mg nitrogen/day. The nitrogen content of the sludge was progressively decreased by this procedure. This

calculated decrease in nitrogen was also demonstrated by the decrease in the nitrogen content of the sludge with time, as determined from Figure 9.

The quantity of nitrogen lost from the system may also be calculated as follows: if the sludge did not lose nitrogen, the nitrogen content of the sludge would have remained at the initial value of 10.2%. However, the sludge had an average nitrogen content of 8.9%. Thus the nitrogen content of the sludge decreased by 1.3%, which amounted to $\frac{1.3 \times 477}{100}$, or 6.2 mg nitrogen/day.

The nutrient balance ($I_N - E_N = GN$) indicated that the system lost 5.2 mg nitrogen/day. The latter calculation indicated a loss of 6.2 mg nitrogen/day. Therefore, a daily negative balance of 1.0 mg nitrogen was obtained. The other nutrient balances were calculated similarly, and are reported in Tables 8 and 9.

Figures 5 - 22 were obtained by drawing smooth curves through the points. Hence, the calculated average nitrogen and phosphorus values, as reported in Table 6, were influenced by the position of these curves. Small variations in the quantity of nitrogen utilized (i.e. $I_N - E_N$) would also have an influence on the difference between these two values.

VI. RESULTS AND DISCUSSIONS.

Effluent and sludge characteristics.

The average qualities of the effluents and the sludge characteristics for each treatment, during the 32 days of treatment at 20°C, are given in Tables 5 and 6. The gas leaving the units after aeration contained no ammonia-nitrogen. The effluents contained no ammonia-nitrogen and the nitrite-nitrogen content did not exceed 2.1 mg/litre. Nitrification was evident only in those treatments that received optimum or excess nitrogen. The alkalinity and pH decreased in all cases where nitrification occurred. The phosphate-phosphorus content of the effluents did not exceed 4.3 mg/litre. The chemical quality of the effluents remained satisfactory at all times. BOD removal was always in excess of 95% (Table 5). The BOD loadings were 56 and 28 lb BOD/100 lb sludge solids/day for the lower and higher sludge concentrations, respectively. The efficiency of BOD removal agrees with the recommendations of Haseltine (1956), who indicated that for loadings of 56 and 28 lb BOD/100 lb sludge solids/day, an efficiency of at least 90% could be expected. No change in the efficiency of BOD removal could be detected either by BOD or COD measurements during the entire treatment period of 32 days.

The sludge growth rate and the percentage of volatile matter was fairly constant, but the SDI varied slightly more (Table 6). The sludge growth rate (cell synthesis) corrected to a volatile basis, varied from 338 mg to 437 mg/day. Sawyer (1941,

1956), Heukelekian, Orford and Manganelli (1951), Helmers et al. (1952) and Gellman and Heukelekian (1953) indicated that a sludge growth rate of 420 mg/day could be expected. No decline in growth rate was observed during the 32 days of treatment. The average values as listed in Table 6, do not reveal the changes that occurred in the sludge during the 32 days of treatment. The nitrogen and phosphorus content of the sludges decreased markedly in the treatments where the BOD:N and BOD:P ratios were wide. Therefore, Figures 5 - 22 were plotted illustrating the decrease in the nitrogen and phosphorus content of the sludges, and the changes in SDI and growth rate during the 32 days of treatment.

The quantities of nitrogen and phosphorus consumed (I_N-E_N and I_P-E_P) were calculated from the data in Table 5, and are reported in Table 7. From Table 7 the optimum BOD:N and BOD:P ratios were found to be 23:1 and 168:1, respectively. For these ratios, the activated sludge maintained a nitrogen content of more than 7% and a phosphorus content of more than 1.4%, respectively. The maximum BOD:N and BOD:P ratios were 19:1 and 81:1, respectively. For the latter ratios the sludges maintained a nitrogen content of about 10% and a phosphorus content of about 2.2%. Sawyer (1956) indicated that the optimum BOD:N and BOD:P ratios were in the order of 32:1 and 150:1, and the maximum BOD:N and BOD:P ratios 17:1 and 90:1, respectively. The values obtained in the present study agree with the values reported in literature.

The nitrogen and phosphorus balances were calculated from Tables 6 and 7, as outlined on p. 53, and reported in Tables 8 and 9.

Table 5.

Average analyses of effluent from differently treated units during 32 days at 20°C.

Treatment	BOD	COD	pH	Alkalinity as mg/litre $CaCO_3$	N as NH_3	N as NO_2'	N as NO_3'	N as Organic N	P as PO_4'''
000	6.5	61	8.5	268	nil	nil	nil	nil	nil
010	5.8	49	8.5	283	"	"	"	"	3.5
020	4.9	59	8.4	279	"	"	"	1.1	4.3
100	4.1	54	8.5	205	"	1.9	1.2	0.8	nil
110	3.7	52	8.5	241	"	nil	nil	0.3	"
120	5.1	44	8.4	246	"	"	"	0.1	4.1
200	5.2	57	7.9	162	"	1.1	9.8	3.0	nil
210	4.0	72	7.7	100	"	2.1	15.7	2.0	1.9
220	3.6	59	8.0	173	"	2.0	5.6	3.1	3.0
001	6.1	44	8.5	282	"	nil	nil	0.1	nil
011	4.5	64	8.3	264	"	"	"	0.9	1.9
021	4.2	54	8.5	290	"	"	"	nil	3.2
101	4.4	57	8.3	227	"	"	"	"	nil
111	3.8	44	8.4	237	"	"	"	"	2.0
121	4.8	62	8.3	219	"	"	"	2.0	2.8
201	5.2	65	7.7	65	"	1.0	15.5	nil	nil
211	4.7	73	7.7	163	"	0.5	16.3	2.4	"
221	4.1	46	8.0	175	"	0.1	3.5	4.6	3.4

Note: 1) BOD = 5-day biochemical oxygen demand at 20°C in mg/litre.
2) COD = chemical oxygen demand in mg/litre.
3) All the other values are expressed in mg/litre.

Table 6.

Average sludge characteristics after 32 days of treatment at 20°C.

Treatment	Sludge concentration by weight in mg	Sludge growth per day in mg	Volatile matter at 600°C (%)	Sludge density index	% N	% P
000	1143	455	89	0.9	6.9	1.0
010	1184	498	87	0.4	6.9	1.4
020	1116	465	86	2.0	6.8	2.0
100	1082	481	88	1.2	8.1	1.4
110	1161	477	89	1.0	8.9	1.6
120	1189	471	86	0.5	9.0	2.1
200	1180	497	89	1.0	9.5	1.1
210	1146	424	88	1.8	9.6	1.6
220	1164	484	86	1.3	10.1	2.2
001	2137	528	87	2.1	7.3	1.2
011	2098	392	86	1.8	7.4	1.7
021	2047	443	88	1.5	7.4	2.2
101	2090	438	90	0.7	9.2	1.2
111	2227	466	87	1.7	9.4	1.4
121	2048	480	84	1.8	8.7	2.2
201	2063	427	88	1.8	9.8	1.3
211	2117	433	87	0.7	9.6	1.7
221	2130	515	85	1.7	10.2	2.1

Table 7.

Nitrogen and phosphorus consumed by sludges when treated with different concentrations of nitrogen and phosphorus.

Treatment	I_N	E_N	I_N-E_N	I_P	E_P	I_P-E_P
000	22.6	nil	22.6	2.0	nil	2.0
010	22.6	"	22.6	6.5	3.5	3.0
020	22.6	1.1	21.5	13.8	4.3	9.5
100	37.6	3.9	33.7	2.0	nil	2.0
110	37.6	0.3	37.3	6.5	"	6.5
120	37.6	0.1	37.5	13.8	4.1	9.7
200	60.0	13.9	46.1	2.0	nil	2.0
210	60.0	19.8	40.2	6.5	1.9	4.6
220	60.0	10.7	49.3	13.8	3.0	10.8
001	22.6	0.1	22.5	2.0	nil	2.0
011	22.6	0.9	21.7	6.5	1.9	4.6
021	22.6	nil	22.6	13.8	3.2	10.6
101	37.6	"	37.6	2.0	nil	2.0
111	37.6	"	37.6	6.5	2.0	4.5
121	37.6	2.0	35.6	13.8	2.8	11.0
201	60.0	16.5	43.5	2.0	nil	2.0
211	60.0	19.2	40.8	6.5	"	6.5
221	60.0	8.2	51.8	13.8	3.4	10.4

Note: I_N = mg nitrogen supplied to each treatment.
I_P = mg phosphorus " " " " .
E_N = mg nitrogen present in effluent.
E_P = mg phosphorus " " " .

Table 8.

Calculated nitrogen balances for sludges after 32 days of treatment at 20°C. These values were calculated from the quantity of nitrogen consumed (I_N-E_N) and the quantity of nitrogen produced by the sludge (GN). All values are reported as mg N/day.

Treatment	Nitrogen produced (GN)	Nitrogen consumed (I_N-E_N)	Excess nitrogen produced GN-(I_N-E_N)	Nitrogen lost by sludge	Nitrogen unaccounted for
000	31.4	22.6	8.8	15.0	-6.2
010	34.4	22.6	11.8	16.4	-4.6
020	31.6	21.5	10.1	15.8	-5.7
100	39.0	33.7	5.3	10.1	-4.8
110	42.5	37.3	5.2	6.2	-1.0
120	42.4	37.5	4.9	5.7	-0.8
200	47.2	46.1	1.1	3.5	-2.4
210	40.7	40.2	0.5	2.5	-2.5
220	48.9	49.3	-0.4	0.5	-0.9
001	42.8	22.5	20.3	15.3	+5.0
011	29.0	21.7	7.3	11.0	-3.7
021	32.8	22.6	10.2	12.4	-2.2
101	40.3	37.6	2.7	4.4	-1.7
111	43.8	37.6	6.2	3.7	+2.5
121	41.8	35.6	6.2	7.2	-1.0
201	41.8	43.5	-1.7	1.7	-3.4
211	41.6	40.8	0.8	2.6	-1.8
221	52.5	51.8	0.7	0.0	+0.7

Table 9.

Calculated phosphorus balances for sludges after 32 days treatment at 20°C. These values were calculated from the quantity of phosphorus consumed (I_P-E_P) and the quantity of phosphorus produced by the sludge (GP). All values are reported as mg P/day.

Treatment	Phosphorus produced (GP)	Phosphorus consumed (I_P-E_P)	Excess phosphorus produced GP-(I_P-E_P)	Phosphorus lost by sludge	Phosphorus unaccounted for
000	4.6	2.0	2.6	5.5	-2.9
010	7.0	3.0	4.0	4.0	0.0
020	9.3	9.5	-0.2	0.9	-1.1
100	6.7	2.0	4.7	3.8	+0.9
110	7.6	6.5	1.1	2.9	-1.8
120	9.9	9.7	0.2	0.5	-0.3
200	5.5	2.0	3.5	5.5	-2.0
210	6.8	4.6	2.2	2.5	-0.3
220	10.6	10.8	-0.2	0.0	-0.2
001	6.3	2.0	4.3	5.3	-1.0
011	6.7	4.6	2.1	2.0	+0.1
021	9.7	10.6	-0.9	0.0	+0.9
101	5.3	2.0	3.3	4.4	-1.1
111	6.5	4.5	2.0	3.7	-1.7
121	10.6	11.0	-0.4	0.0	-0.4
201	5.6	2.0	3.6	3.8	-0.2
211	7.4	6.5	0.9	2.2	-1.3
221	10.8	10.4	0.4	0.5	-0.1

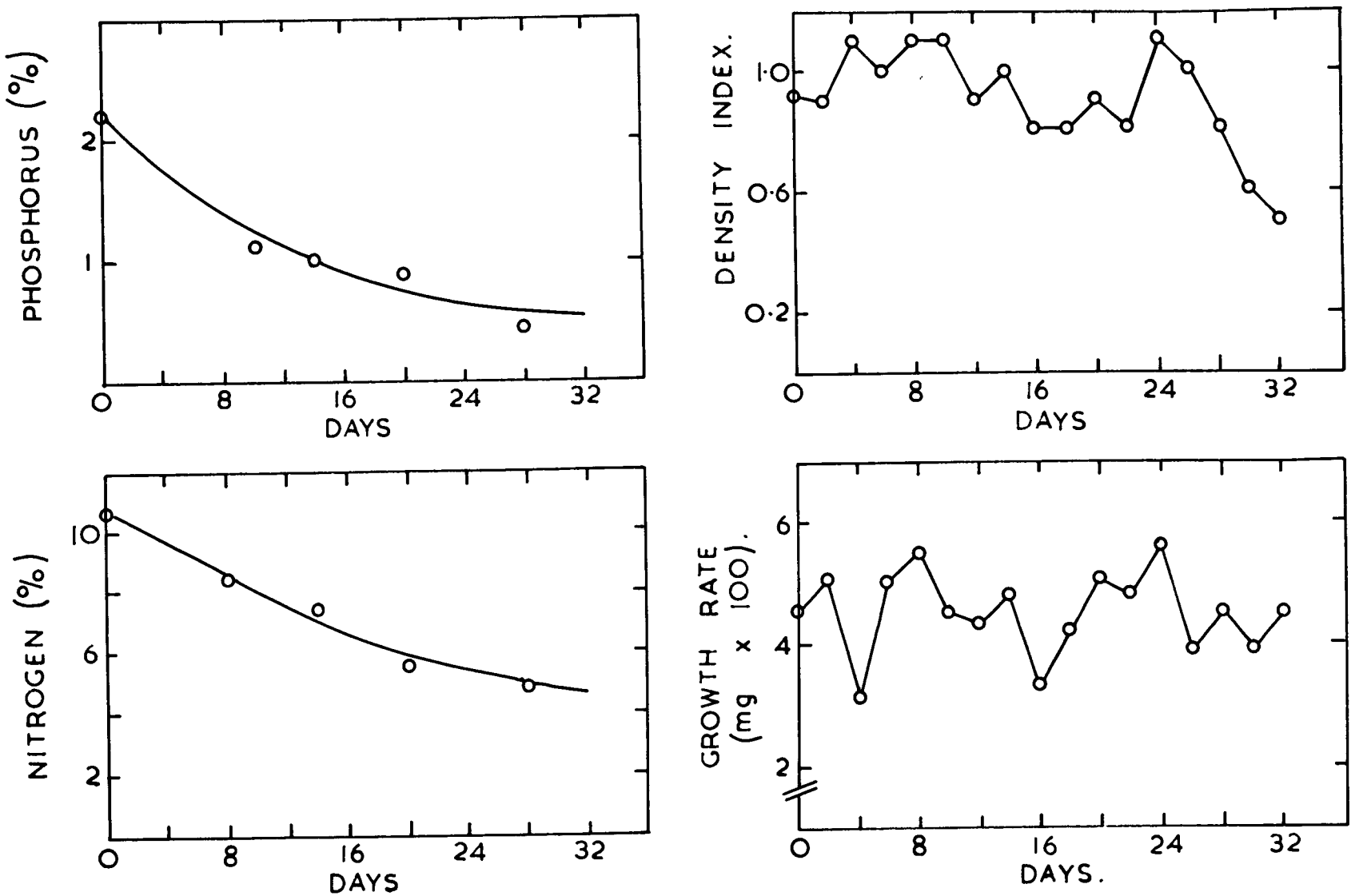

FIG 5. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT OOO.

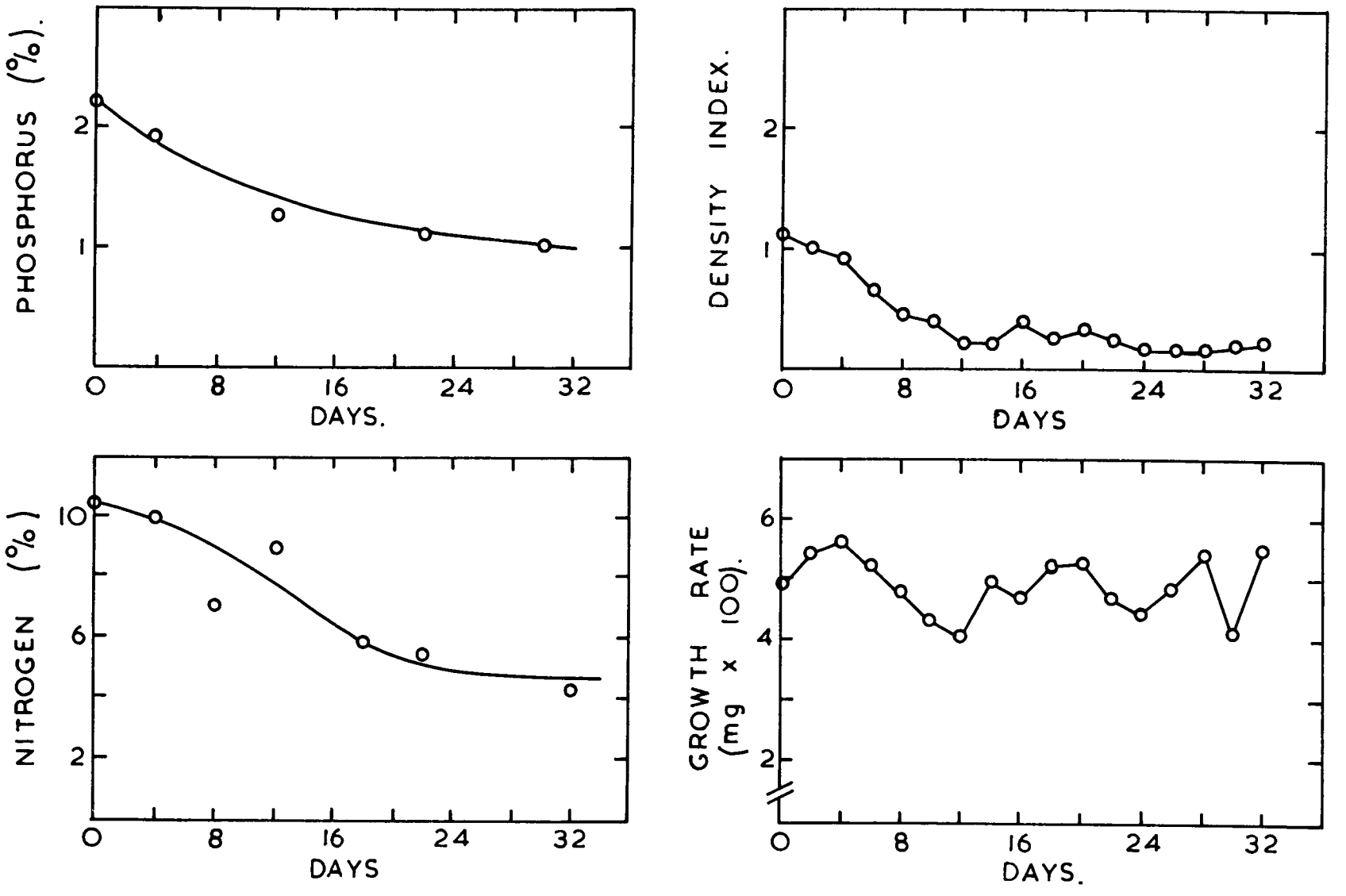

FIG 6. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT OIO.

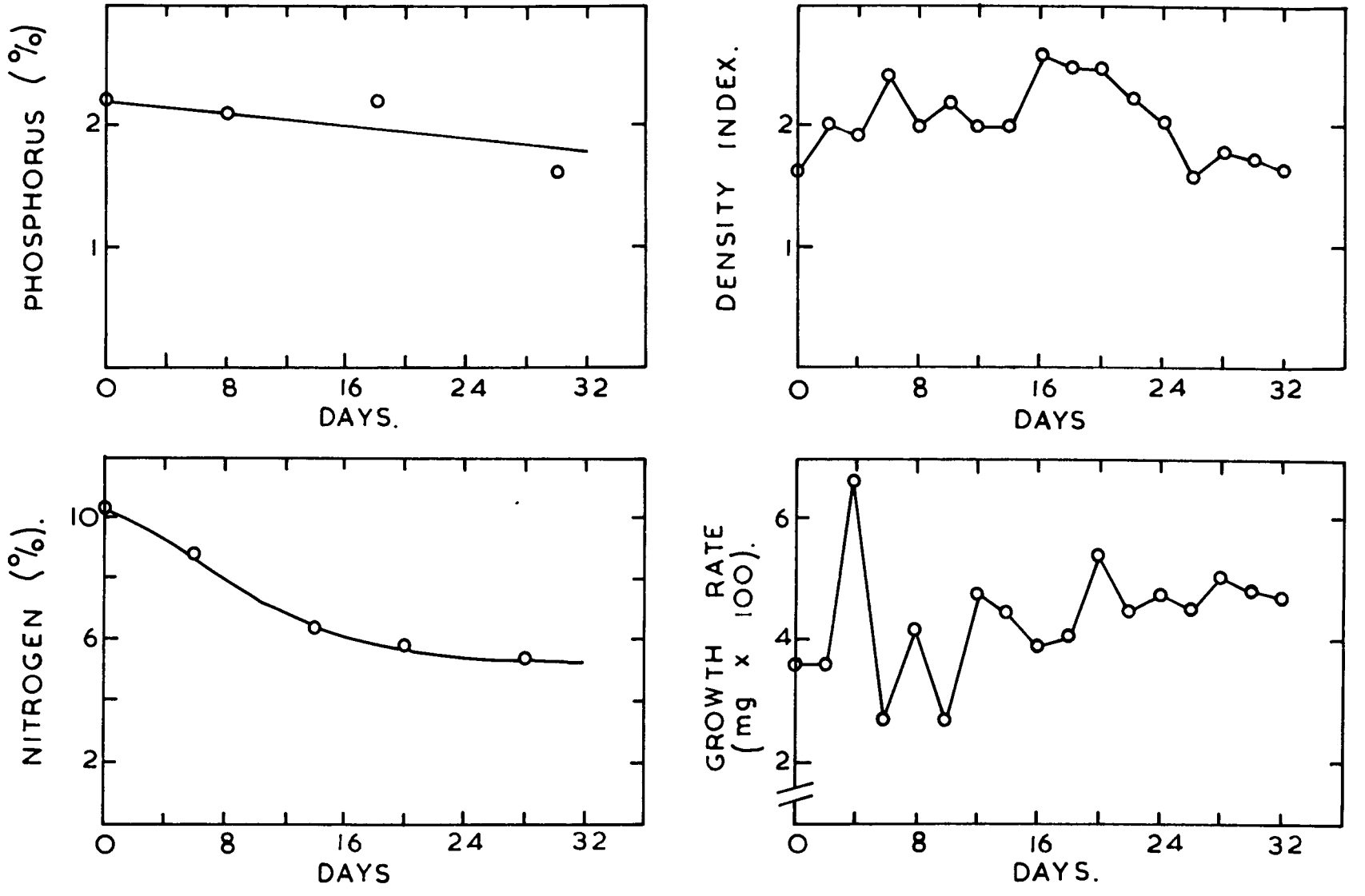

FIG 7. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT O2O.

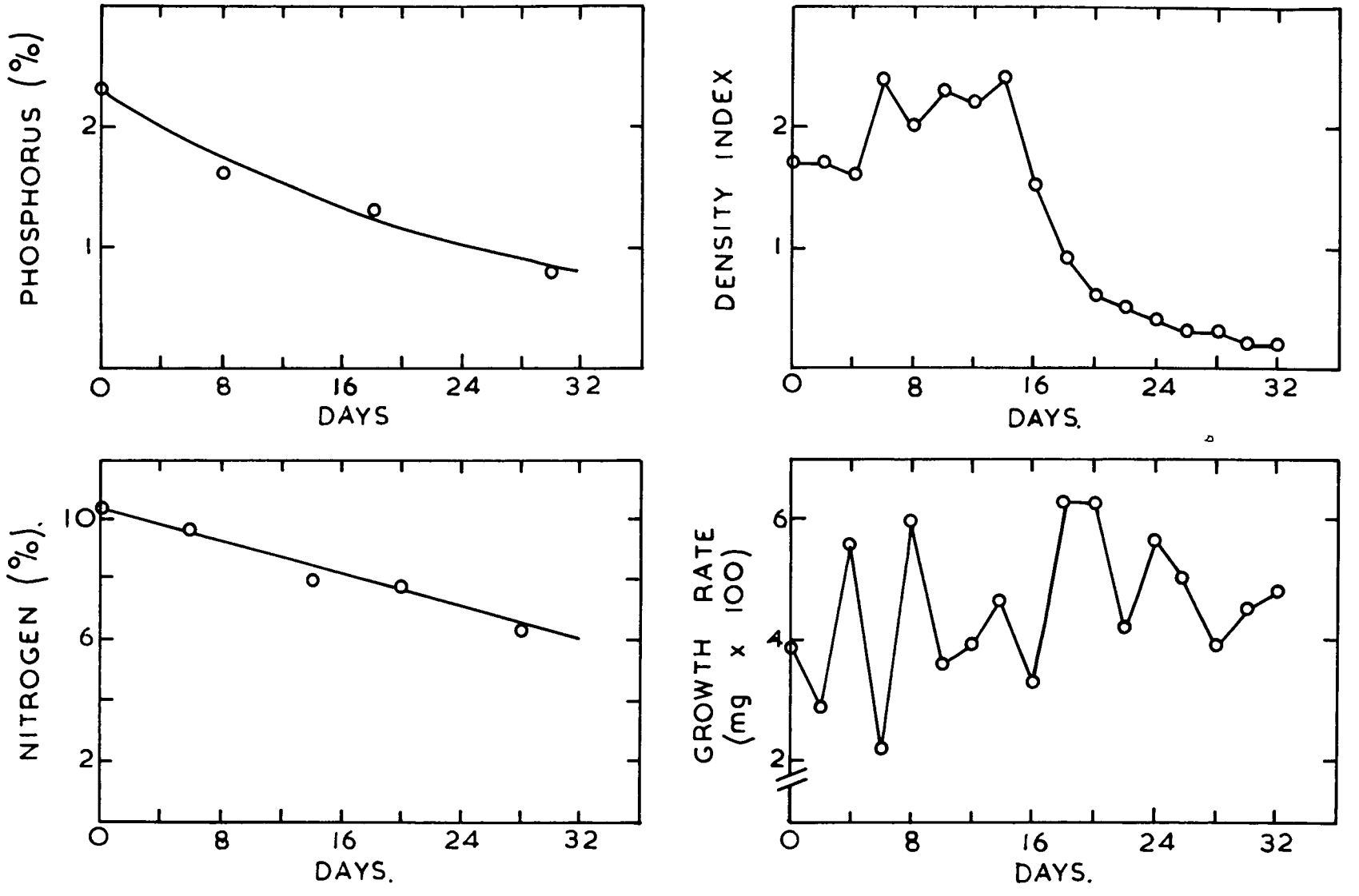

FIG 8. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 100.

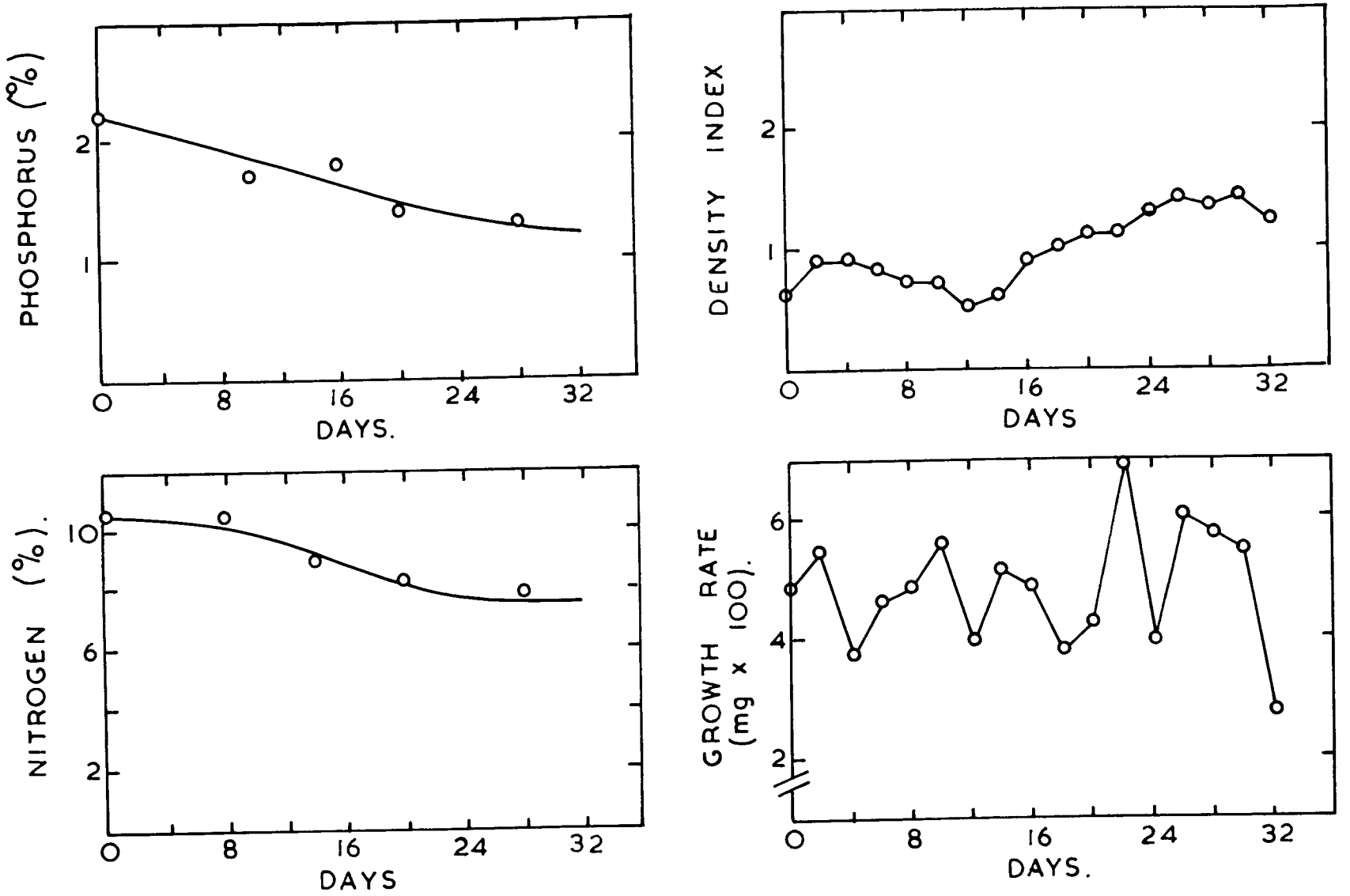

FIG 9. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 110.

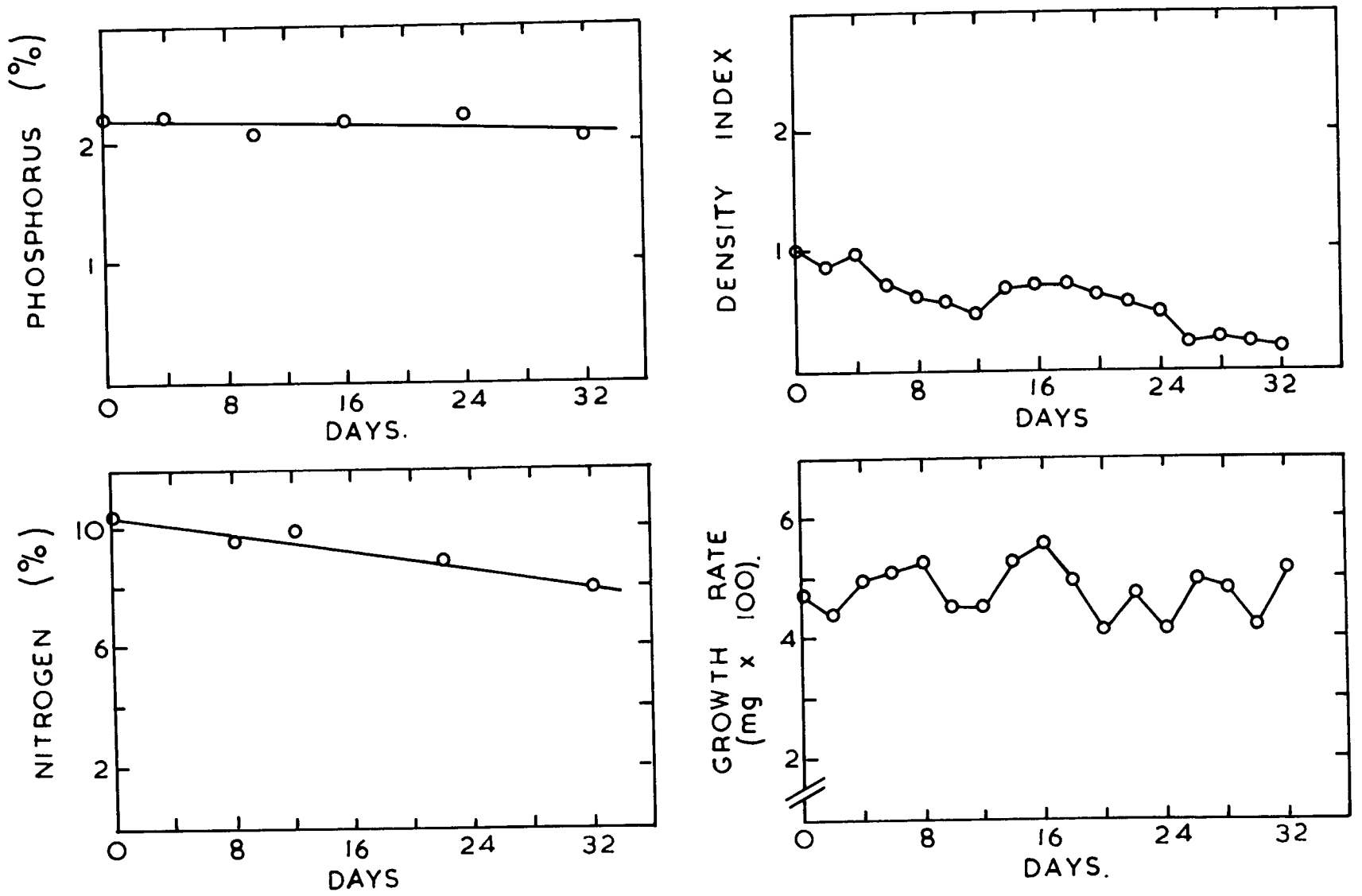

FIG 10. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE FOR TREATMENT 120.

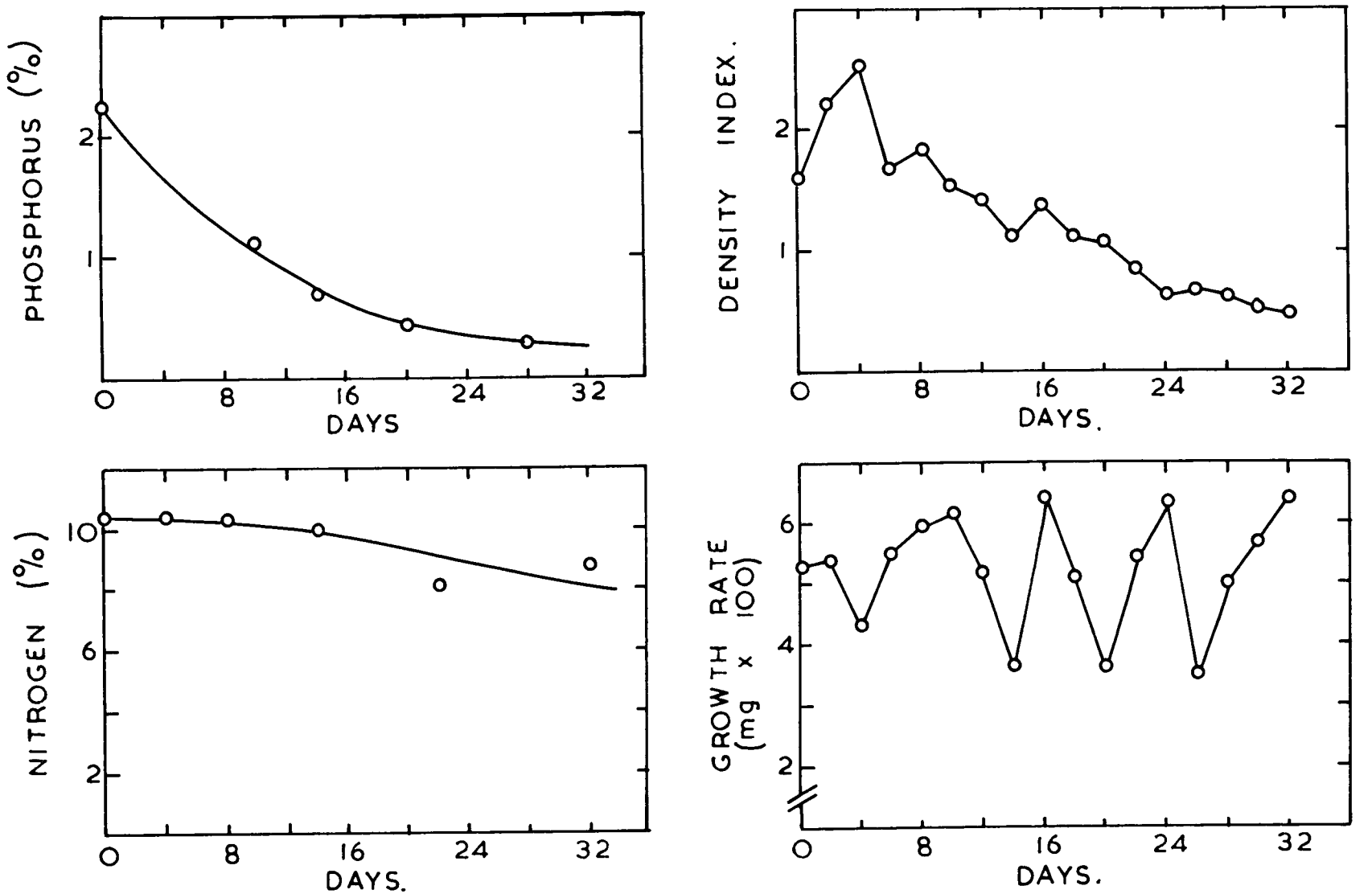

FIG 11. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 200.

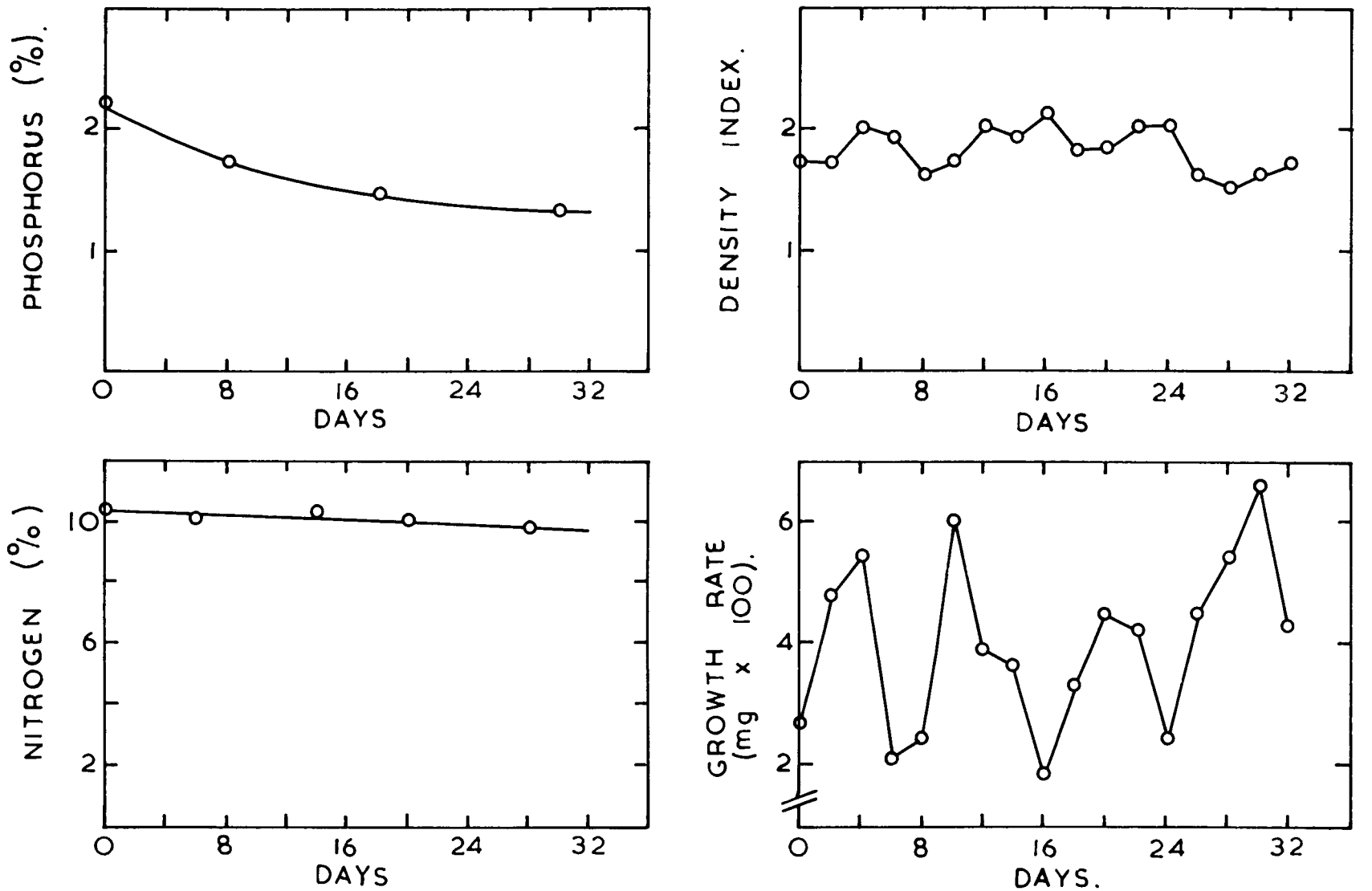

FIG 12 NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 210.

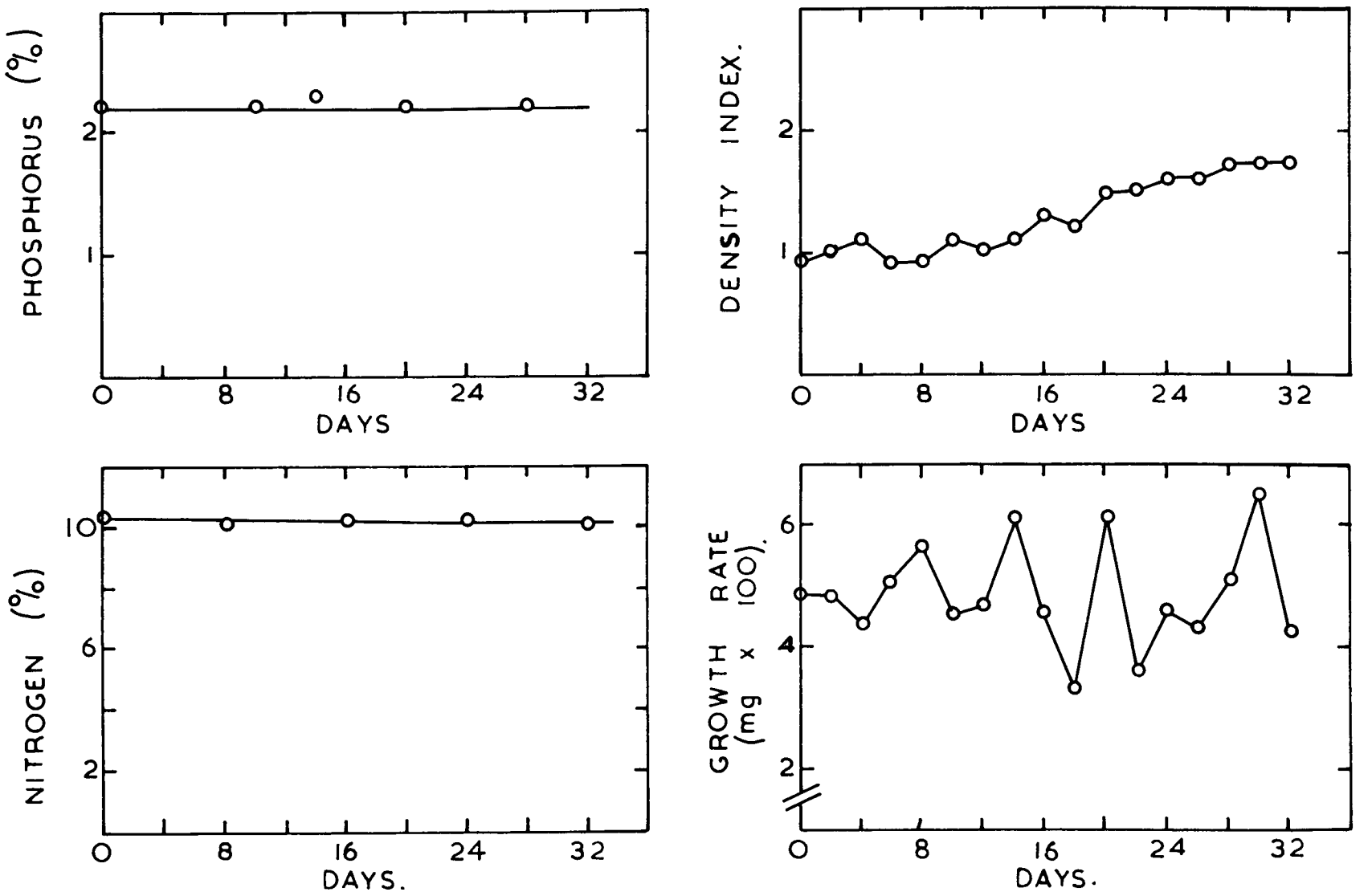

FIG 13. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 220.

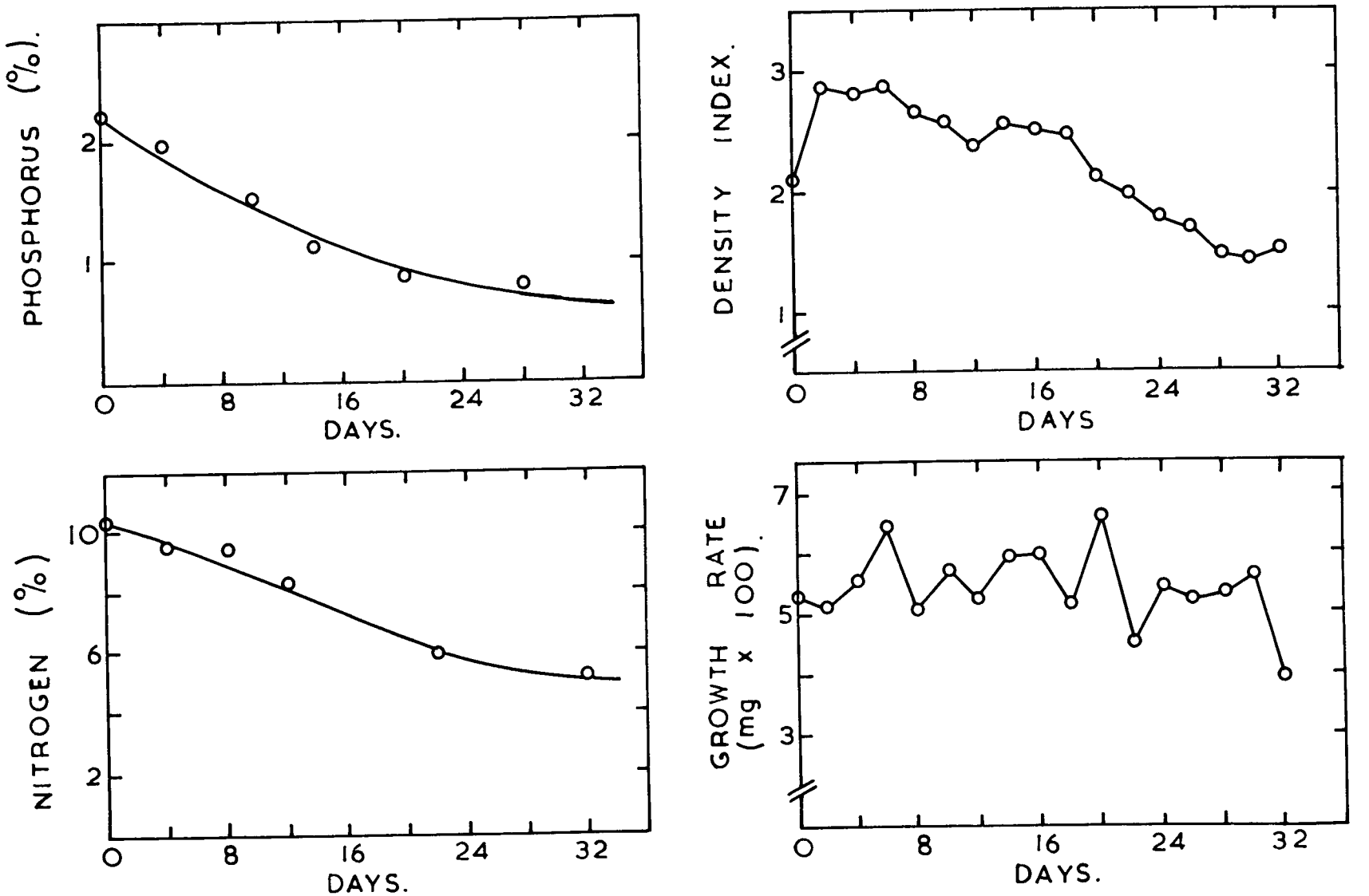

FIG 14. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT OOI.

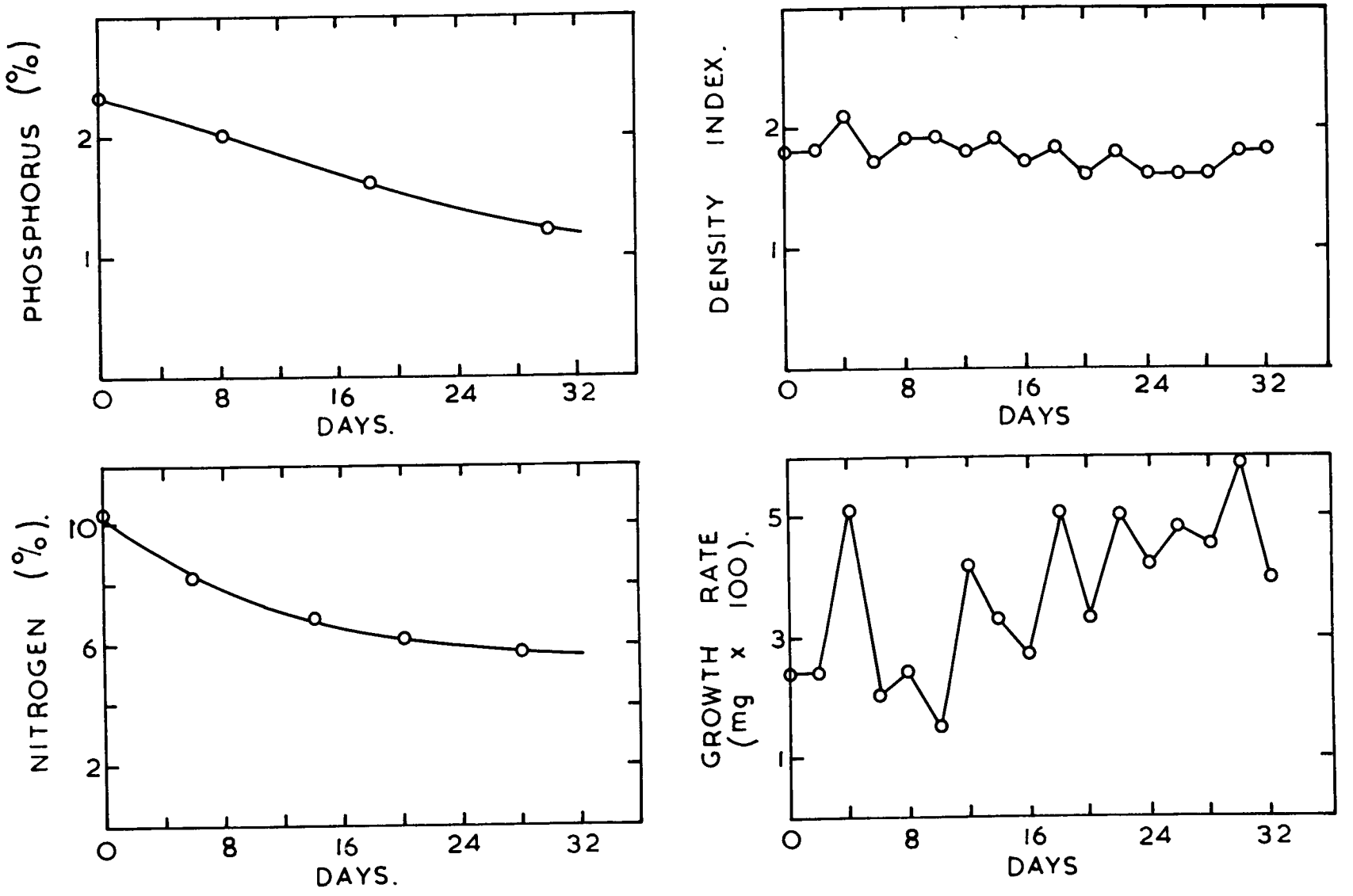

FIG 15 NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT O11.

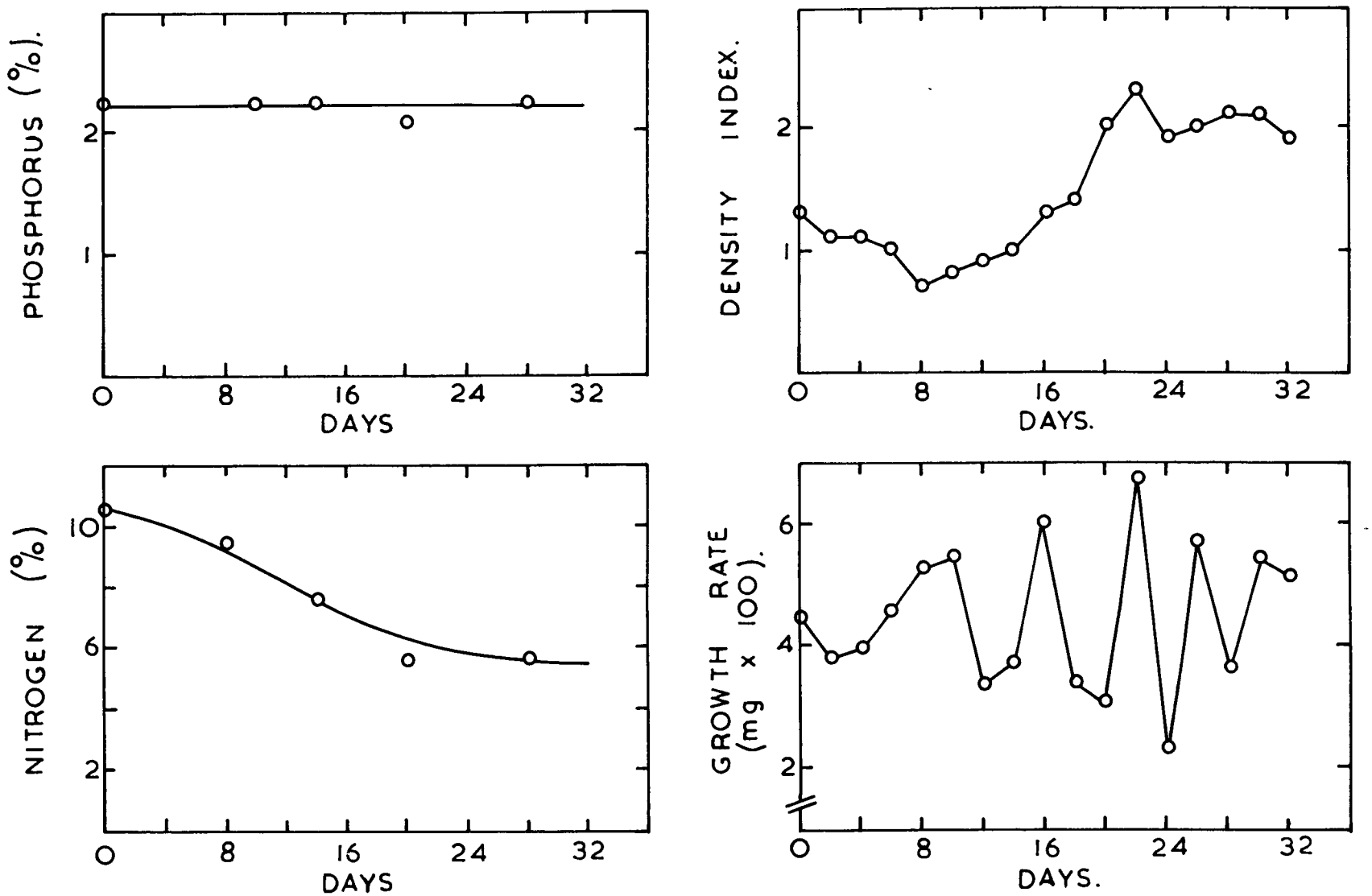

FIG 16. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT O21

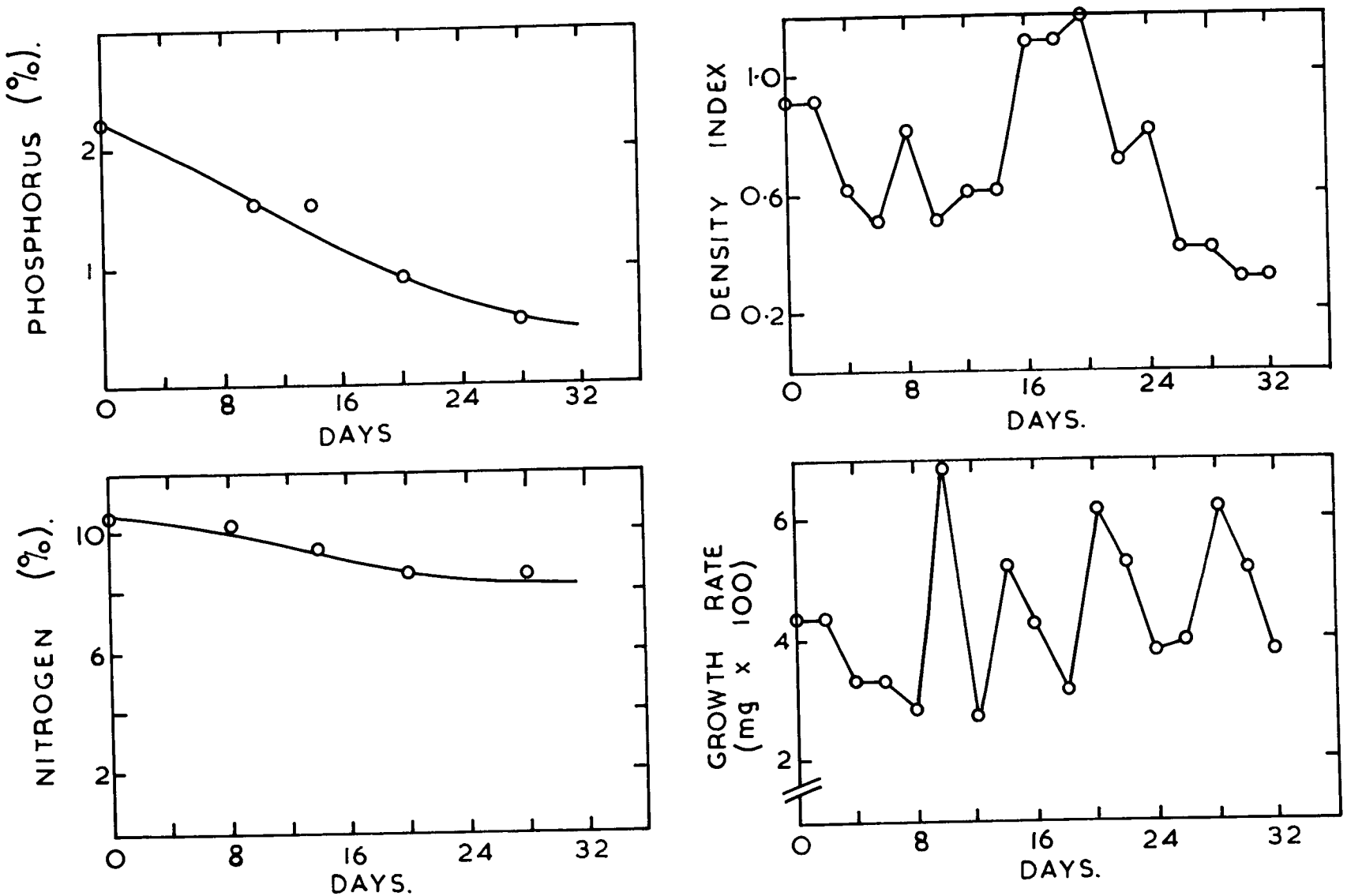

FIG 17. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 101.

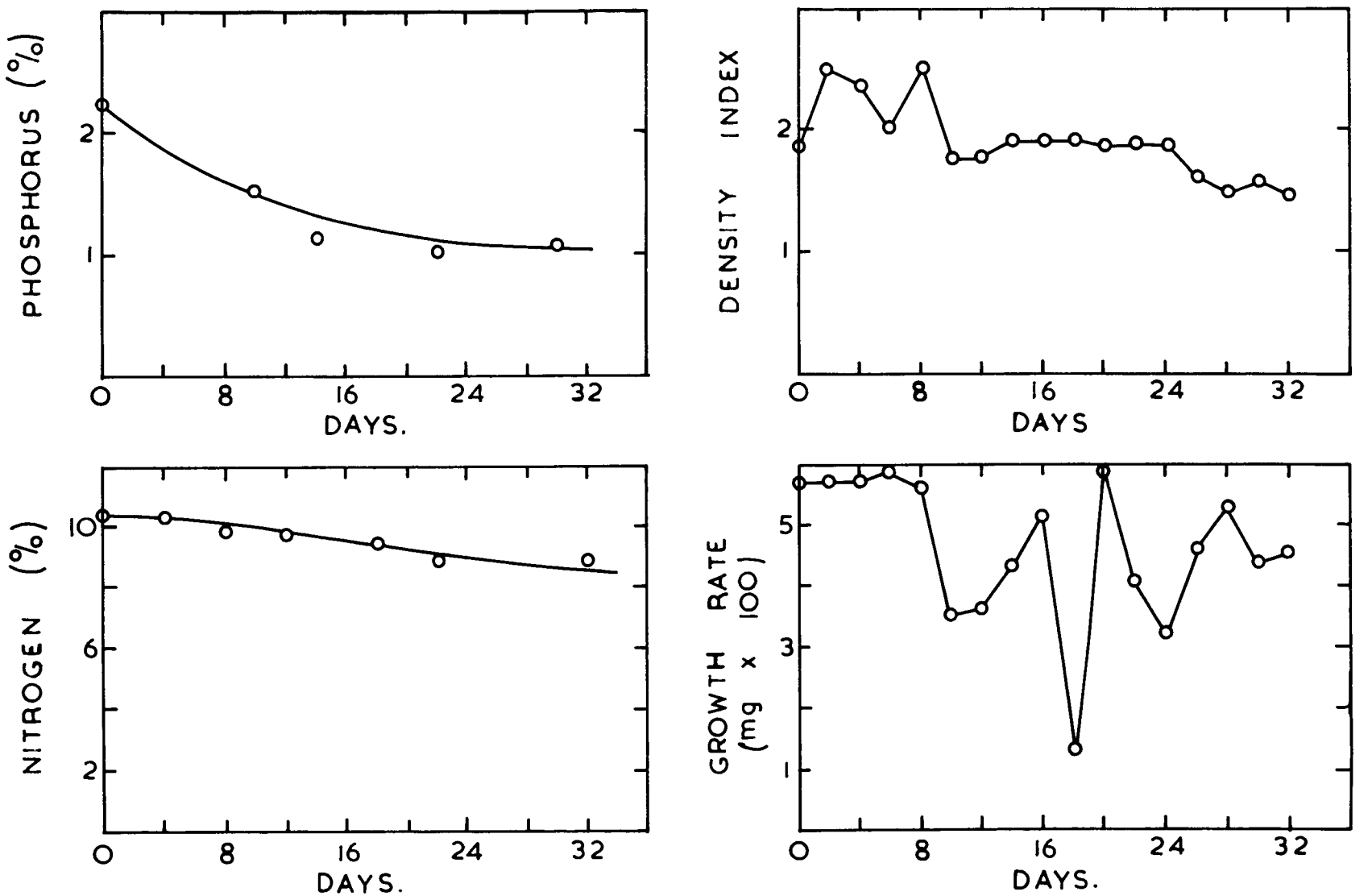

FIG 18. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT III.

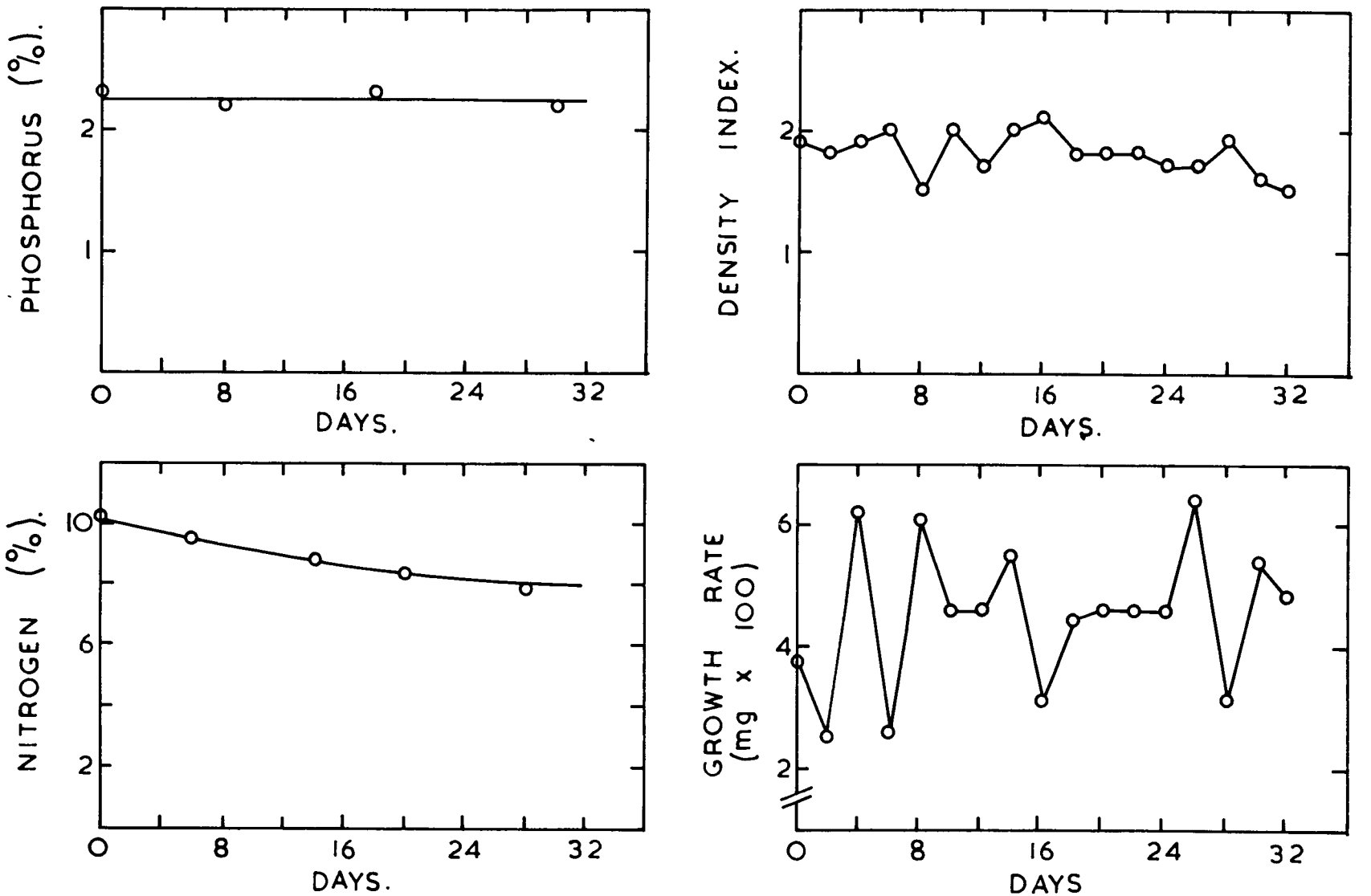

FIG 19. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 121.

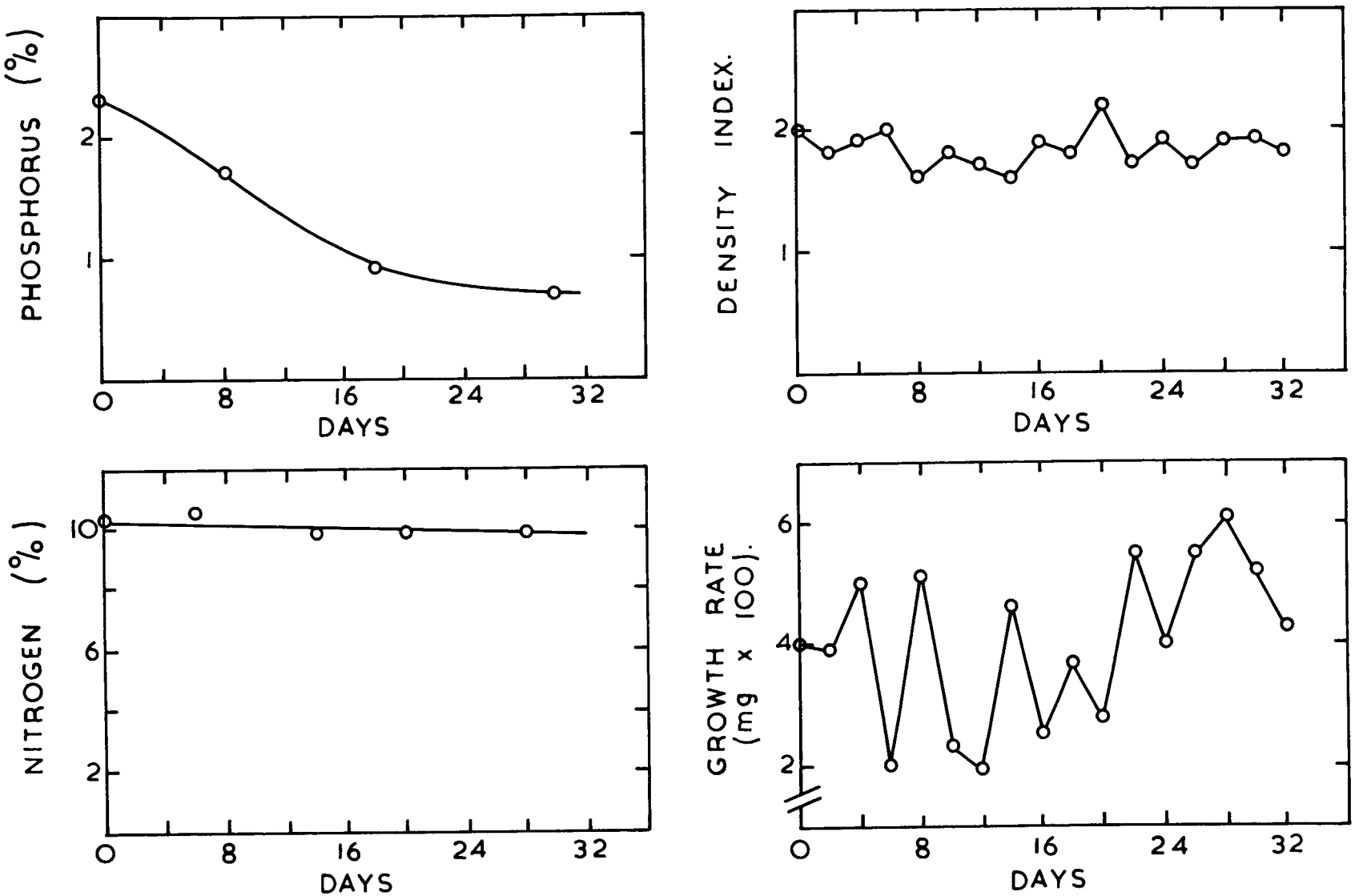

FIG 20. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 201

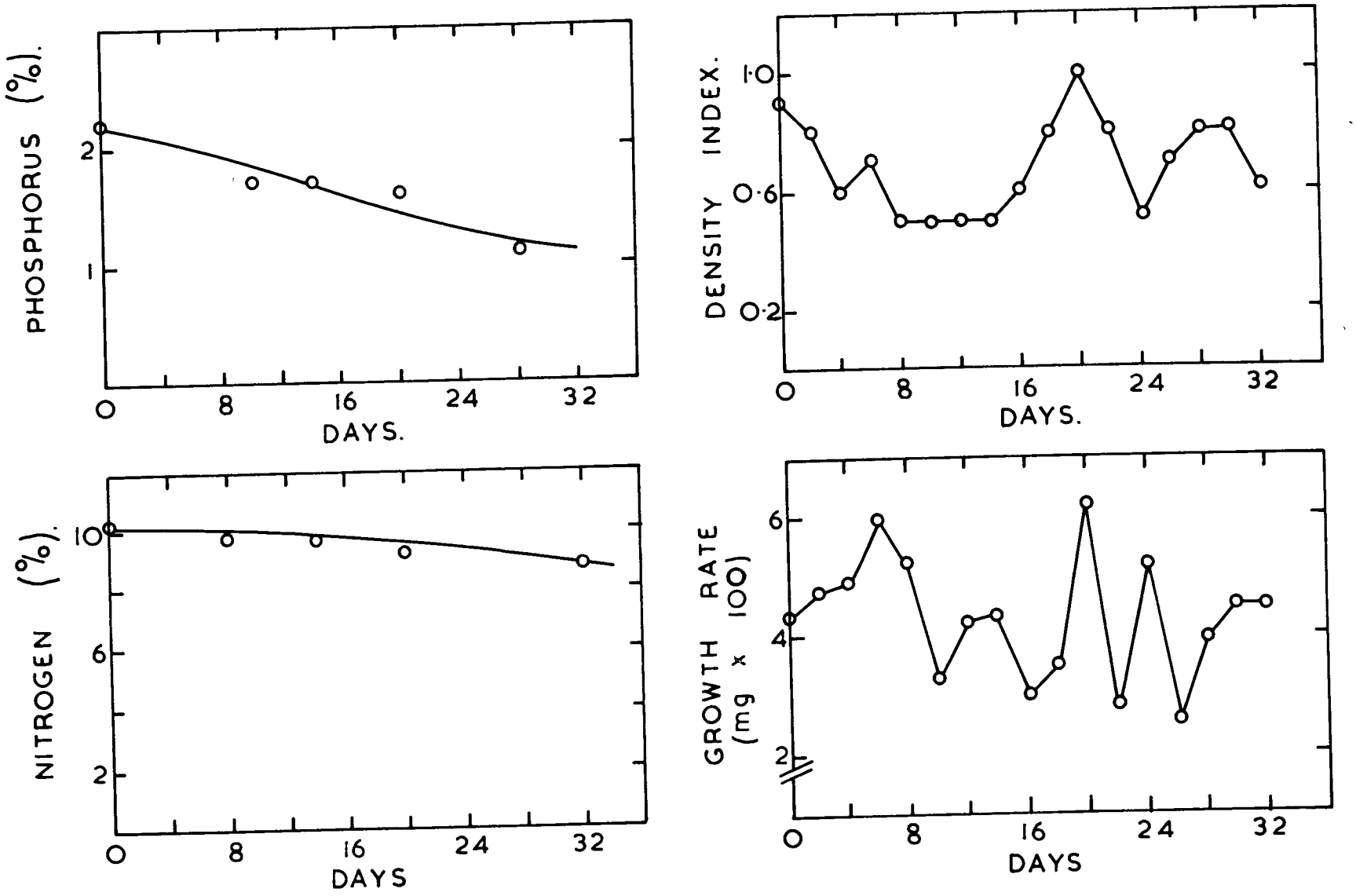

FIG 21. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 211.

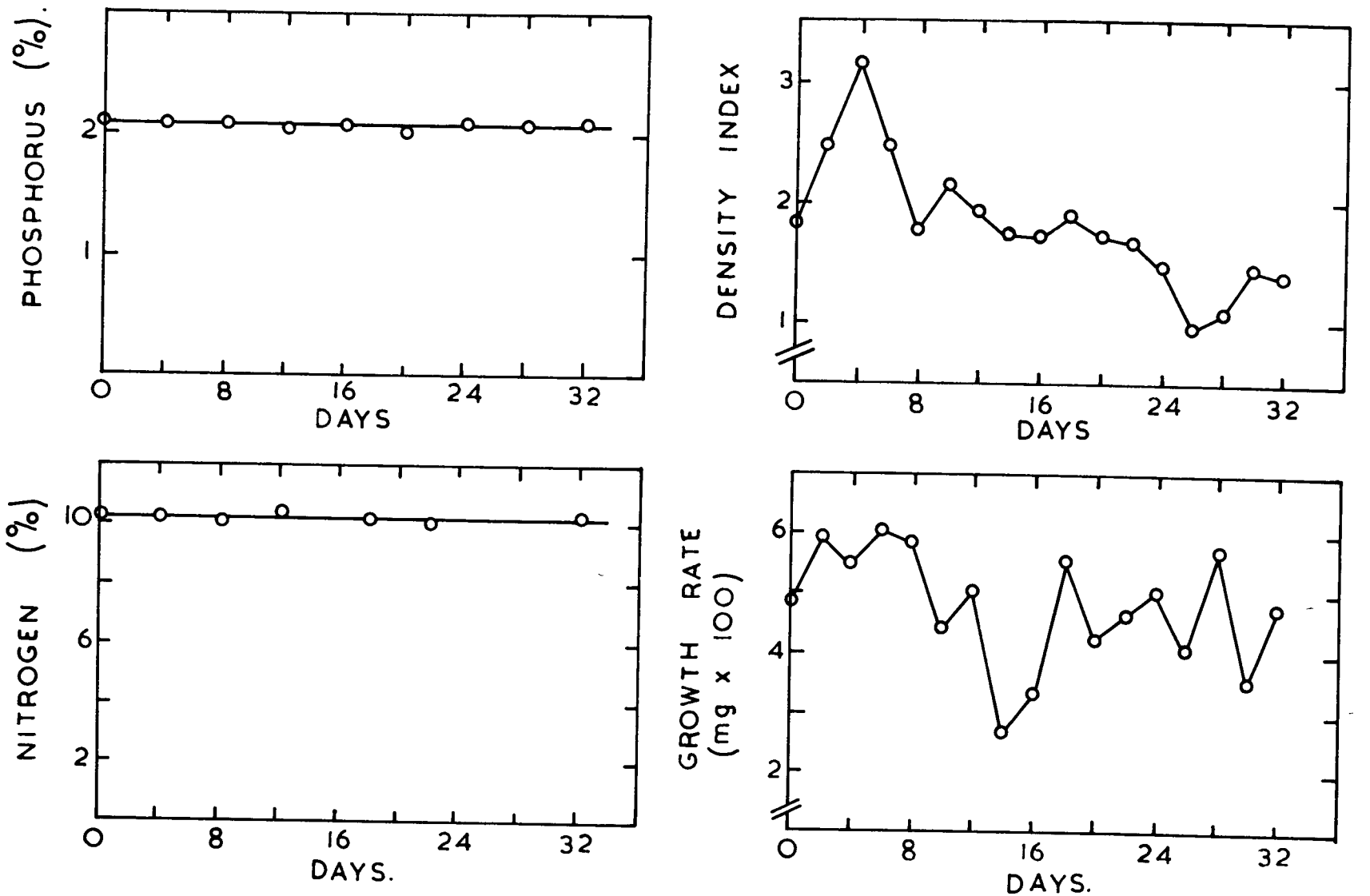

FIG 22. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 221.

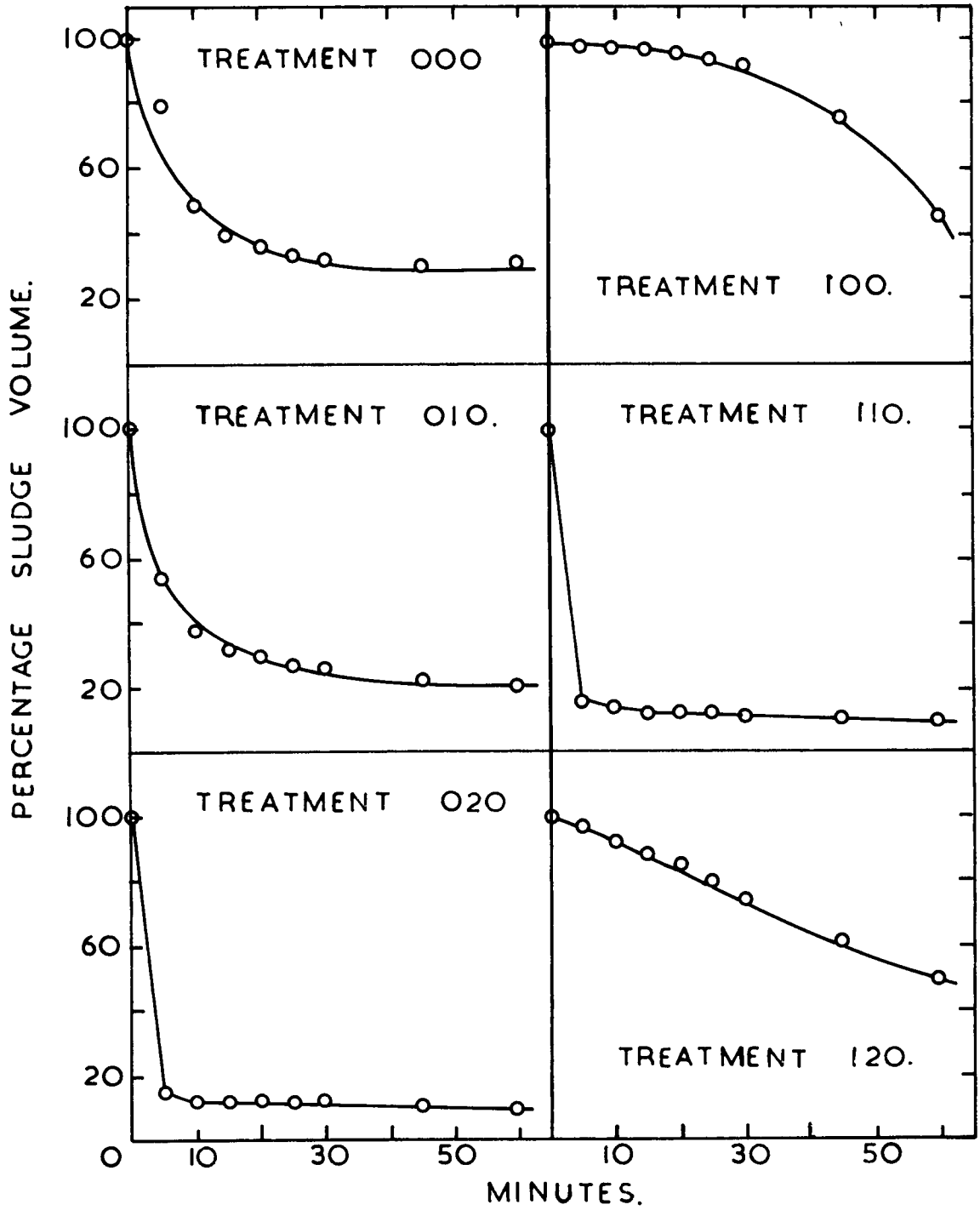

FIG 23. SETTLING RATE OF SLUDGES AFTER 32 DAYS TREATMENT AT 20°C.

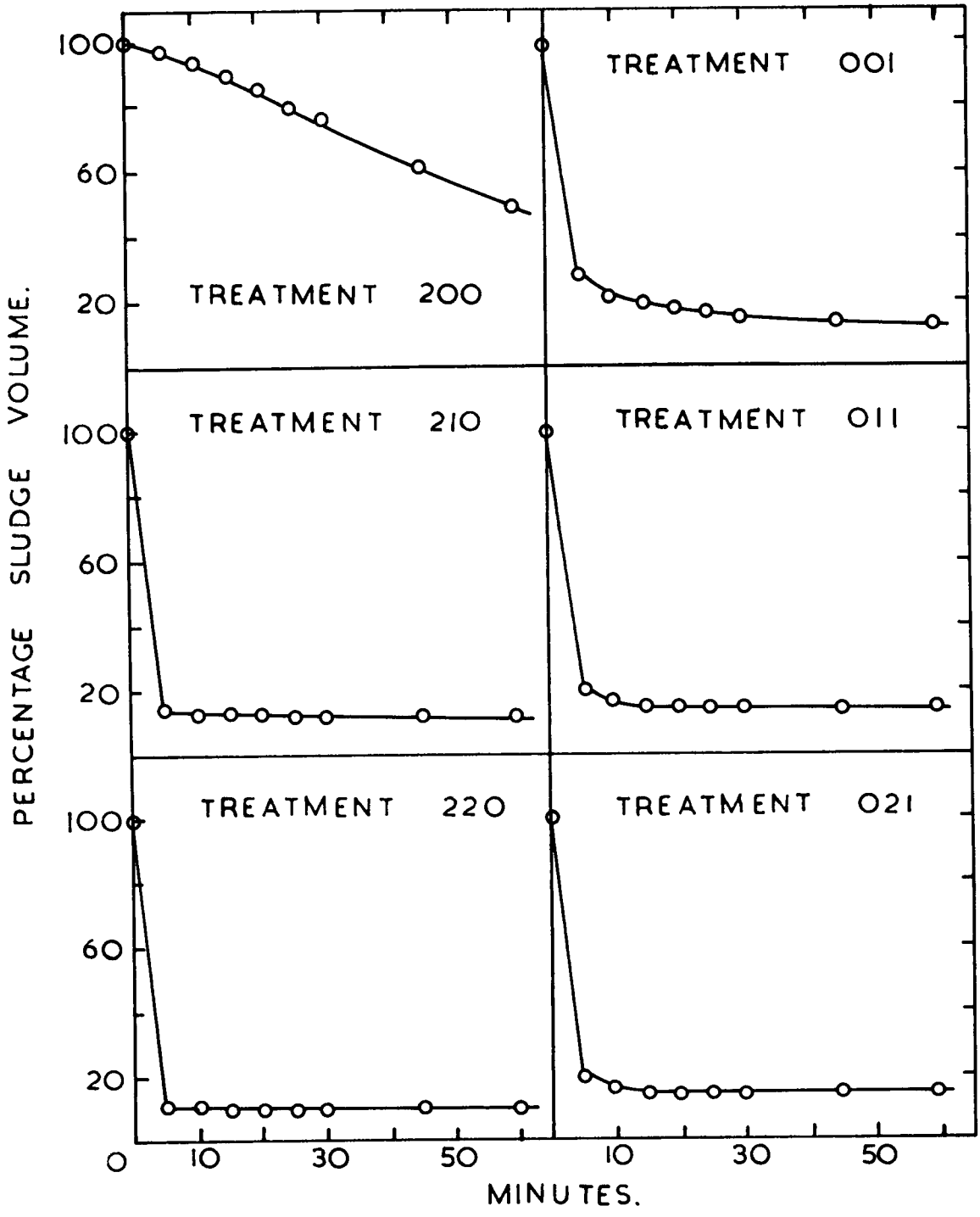

FIG 24. SETTLING RATE OF SLUDGES AFTER 32 DAYS TREATMENT AT 20°C.

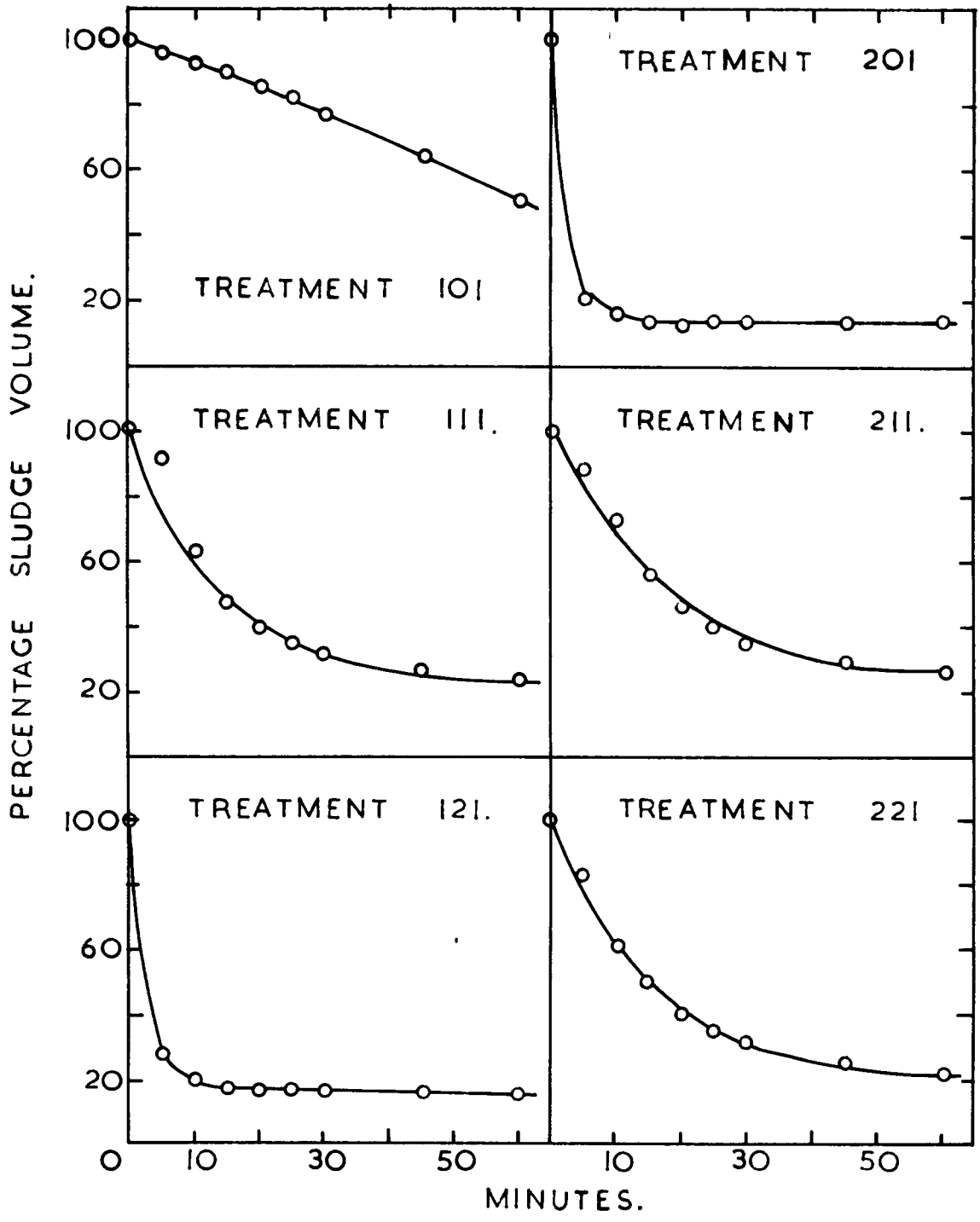

FIG 25. SETTLING RATE OF SLUDGES AFTER 32 DAYS TREATMENT AT 20°C.

The quantities of nitrogen unaccounted for varied from a negative value of 6.2 mg to a positive value of 5.0 mg (Table 8). The quantities of phosphorus unaccounted for varied from a negative value of 2.9 mg to a positive value of 0.9 mg (Table 9). The reasons for the discrepancies in the nitrogen and phosphorus balances were discussed on p. 54.

Figures 23, 24 and 25 show the rates of settling of the sludges after 32 days of treatment at 20°C. Some of the sludges (treatments 100, 120, 200 and 101) settled very slowly and their SDI decreased to low values (Table 6).

Microflora.

The microflora of the sludges of replication I was estimated at the end of a treatment period as described on p. 43. The results are listed in Table 10. The Sabouraud medium gave substantially higher counts than the selective acid medium. This was probably due to the difference in pH. The pH of the Sabouraud medium was 5.8 as compared to 3.8 for the selective acid medium. Little evidence of actinomycetes was observed on the glycerol-glucose agar. Counts obtained on this medium were largely due to bacteria. Nutrient agar supported the highest number of microorganisms.

No marked differences in the number of bacteria present in the sludges could be detected either by the standard plate or direct microscopic counts. The standard plate count is not satisfactory for estimating a heterogenous population because there is no universal medium which permits development of all types of microflora. The sludges showed mainly Gram-negative rods and a few

Table 10.

Average microbial counts on four different media after sludge treatment for 32 days at 20°C.

Treatment	Nutrient agar 1.	Glycerol-glucose agar 2.	Sabouraud dextrose agar 3.	Selective acid agar (pH=3.8) 4.
000	1.4×10^7	1.0×10^6	19.4×10^5	27.0×10^3
010	15.2 "	9.0 "	36.0 "	6.0 "
020	22.8 "	10.6 "	35.0 "	81.0 "
100	11.4 "	2.8 "	17.2 "	1.4 "
110	1.5 "	0.8 "	18.4 "	1.5 "
120	14.0 "	5.8 "	5.0 "	7.5 "
200	10.1 "	6.4 "	13.0 "	2.0 "
210	35.0 "	21.4 "	24.1 "	100.0 "
220	2.2 "	1.5 "	45.0 "	2.0 "
001	12.6 "	7.3 "	25.0 "	4.0 "
011	21.4 "	6.4 "	20.5 "	49.0 "
021	0.6 "	1.0 "	10.0 "	0.6 "
101	1.2 "	0.8 "	39.0 "	54.0 "
111	5.1 "	2.1 "	30.0 "	8.6 "
121	10.4 "	5.8 "	10.4 "	27.0 "
201	6.4 "	1.4 "	11.4 "	3.8 "
211	0.6 "	1.0 "	30.0 "	15.0 "
221	11.7 "	8.7 "	25.0 "	14.0 "

Note: The counts are expressed as counts/mg volatile sludge, incubated for 1 week at 20°C.

1. Mainly bacteria.
2. Actinomycetes and bacteria.
3. Yeasts and moulds.
4. Mainly fungi.

cocci. The direct microscopic count gave numbers about 100 times greater than those obtained by the standard plate count. However, it was difficult to estimate the number of bacteria present in a sheath of Sphaerotilus by means of the direct microscopic count, and no distinction between dead and live bacteria was made by this method.

Little could be concluded from the fact that fungi were present in the sludge (Table 10). Their numbers did not seem to show any general trend, for example, control treatments 220 and 221 yielded relatively few fungi. Treatment 210 yielded the largest number of fungi, (100×10^3/mg volatile sludge), but its equivalent treatment at the higher sludge level (211), yielded only 15.0×10^3 fungi/mg volatile sludge. The number of fungal colonies developing on the plates may have been larger than the actual number of fungal organisms present in the sludge. The number of colonies could have been increased considerably by the treatment the sludges received before plating (i.e. by Waring blendor dispersion).

Standard Methods (1960) and Allen (1944) recommended the use of nutrient agar for the bacteriological examination of sewages and sludges. The higher counts obtained on nutrient agar (Table 10), substantiated the fact that nutrient agar was the most suitable plating medium for the sludges produced in the present study.

Microfauna.

The protozoal population of the sludges was estimated at the completion of a treatment period. No attempt was made to estimate the relative abundance of the different species; only total numbers are given. Table 11 indicates the occurrence of different

Table 11.

Average protozoal population of the sludges after 32 days of treatment at 20°C.

Treatment	Number of Protozoa present	Epistylis	Euplotes	Vorticella	Suctoria	Colpoda	Amoeba	Dileptus	Peranema	flagellates	ciliates
000	2920	-	+	+	-	-	+	+	+	-	+
010	1480	+	-	-	-	+	+	-	-	+	+
020	53000	+	-	+	-	-	-	+	-	-	+
100	36263	-	+	-	+	-	-	-	-	+	-
110	2580	-	+	+	-	+	+	-	-	-	+
120	4430	+	-	+	+	-	+	+	-	+	-
200	4850	+	+	-	-	-	-	-	-	+	-
210	2994	-	+	-	-	-	-	+	-	-	+
220	1750	-	-	+	-	+	+	+	+	-	+
001	4560	-	-	+	+	+	-	+	-	+	+
011	4072	+	-	+	+	-	+	-	-	-	+
021	4760	+	+	-	+	-	-	-	-	-	+
101	2390	-	-	+	-	+	-	-	-	-	+
111	1650	+	+	+	-	-	-	-	-	+	-
121	7858	-	-	+	+	-	+	+	-	-	-
201	11184	-	+	-	+	+	-	+	-	-	+
211	4375	-	+	+	-	-	-	+	+	-	+
221	3140	+	+	+	+	-	+	-	-	+	-

Note: + indicates the organism was present.
- " " " " not present.

species, as well as the total numbers of protozoa present. Throughout the experiment it was observed that the larger, stalked ciliates, Vorticella and Epistylis, were most abundant. Suctoria and a very small ciliated protozoan followed these two organisms in frequency. The substantially higher numbers of protozoa recorded for treatments 020, 100 and 201 were due to an outburst of a small ciliated protozoan on the day of counting. The sludges were observed microscopically once a week, and similar outbursts were frequently noted.

No marked difference in the protozoal populations of the different sludges were found. Most of the protozoal species were bacterial feeders. The influence of supplying different BOD:N and BOD:P ratios was therefore not reflected in the protozoal populations.

Respiration studies.

The respiration rate of the washed cells and the stimulation of the respiration rate by glucose for both replications, is given in Table 12. The respiration rates of the washed sludges were small in all instances, but addition of glucose had a marked effect on these rates. The largest response to glucose was observed in control treatments 220 and 221, and the smallest response in treatments 000 and 001. Although no additional nitrogen or phosphorus was supplied in treatments 000 and 001, the BOD and COD removal remained in excess of 95% for all four treatments (Table 5). Hence it was concluded that in spite of the respiration rate being much lower in the sludges of treatments 000 and 001, the metabolic activity was still large enough to utilize all of the BOD supplied

Table 12.

Average stimulation of the respiration rate of washed sludges in µl O_2/mg volatile sludge/hr at 20°C by the addition of 50 µM glucose.

Treatment	Average respiration rate of washed sludge	Average stimulation of respiration rate of sludges by glucose
000	3.0	5.8
010	2.8	6.1
020	2.3	8.8
100	1.5	7.2
110	1.9	23.1
120	2.3	18.0
200	3.2	6.2
210	1.3	20.0
220	2.5	32.7
001	2.6	7.1
011	1.9	12.9
021	1.3	9.8
101	2.5	11.3
111	3.0	19.1
121	3.0	26.2
201	2.9	10.6
211	3.0	31.8
221	2.8	30.4

during 24 hr.

Glucose was selected because it could be readily utilized and contained no nitrogen and phosphorus. The stimulation of the respiration rate by the glucose was calculated from the difference between the respiration rate when glucose was added, and the respiration rate of the washed sludge.

However, the question arose whether or not to subtract the "endogenous" respiration. This problem has been the subject of numerous pure culture studies in the microbiological field (Umbreit, Burris and Stauffer, 1957).

It was possible that the utilization of an initially present endogenous substrate may have been completely stopped in the presence of an exogenous carbon source, retarded or accelerated. Endogenous respiration is extremely difficult to study, as it may also depend on the type of organisms present. However, in a heterogenous population such as activated sludge, a subtraction for endogenous respiration seemed advisable. The respiration rate of the washed sludge was not a true endogenous respiration, as determinable in pure culture work. It was impossible to separate the viable cells from the dead material and to remove all nutrients that may have been adsorbed and enmeshed in the sludge. The respiration rate of the washed sludge was small (Table 12) and was subtracted from the respiration rate after glucose was added.

VII. STATISTICAL EVALUATION OF THE EXPERIMENT.

The values for the stimulation of the respiration rate of the sludges by glucose (Table 13), formed the basis for the statistical evaluation of the experiment. In the statistical design used, the nitrogen-phosphorus (NP) interaction and the second order interaction, nitrogen-phosphorus-sludge concentration (NPS), were partially confounded. Therefore, the NP and NPS interactions were adjusted for block effects, as outlined by Cochran and Cox (1957).

The analysis of variance, as presented in Table 14, led to the following conclusions:

1. The main effects of nitrogen and phosphorus on the metabolic activity of the sludges were significant at the 99% level.
2. The main effect of sludge concentration on the metabolic activity of the sludges was significant at the 95% level.
3. A significant interaction existed between nitrogen and phosphorus at the 99% level.
4. No significant interaction was observed between nitrogen and sludge concentration by weight, or phosphorus and sludge concentration by weight.

The NP interaction, adjusted for block effects, is clearly demonstrated in Figure 26. The values, from which Figure 26 was plotted, are given in Table 15.

The significant NP interaction implies that the differences among responses to nitrogen varied with the level of phosphorus, where the responses were measured over both levels of sludge

Table 13.

Stimulation of the respiration rate of differently treated sludges after 32 days of treatment at 20°C. Two replications in blocks of six units were used.

Replication I.

Block Ia		Block Ib		Block Ic	
Treat-ment	Resp. rate	Treat-ment	Resp. rate	Treat-ment	Resp. rate
100	7.6	200	6.2	000	6.0
210	12.9	010	4.1	110	25.6
020	9.3	120	18.2	220	35.1
201	11.2	001	6.0	101	12.0
011	13.1	111	19.1	211	33.3
121	28.7	221	29.1	021	10.0

Replication II.

Block IIa		Block IIb		Block IIc	
200	6.1	000	5.6	100	6.8
010	8.1	110	20.6	210	27.0
120	17.8	220	30.3	020	8.2
101	10.5	201	10.0	001	8.2
211	30.3	011	12.6	111	19.0
021	9.5	121	23.7	221	31.5

Table 14.

Analysis of variance showing subdivision of treatment sum of squares in a 3x3x2 partially confounded experiment. Two replicates were used.

Source	df	SS	MSS	F
Nitrogen (N)	2	1141.50	570.75	30.70 **
Phosphorus (P)	2	1154.30	577.15	31.05 **
Sludge concentration (S)	1	108.20	108.20	5.82 *
NP	4	348.10	87.02	4.67 **
NS	2	6.40	3.20	0.17
PS	2	10.00	5.00	0.27
NPS	4	126.20	31.55	1.70
Replication	1	0.09	0.09	-
Error	17	315.21	18.54	-
Total	35	3210.00		

Note: ** indicates significance at the 99% level.
* " " " " 95% " .

Table 15.

NP two-way table adjusted for block effects and measured over both levels of sludge concentration.

Phosphorus	Nitrogen		
	N_0	N_1	N_2
P_0	29.5	35.8	31.0
P_1	43.1	77.5	105.2
P_2	28.2	96.4	127.0

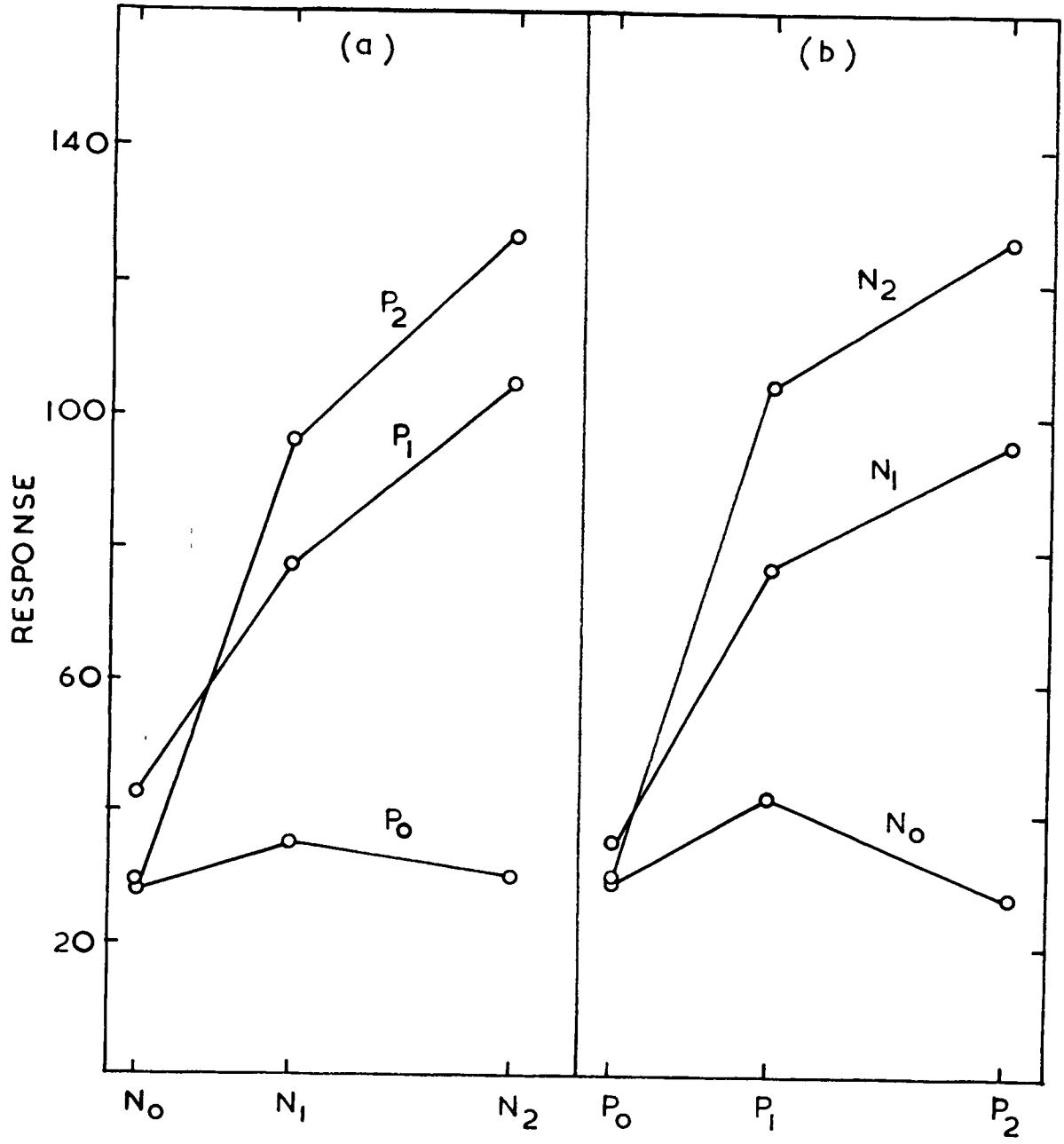

FIG 26. NITROGEN-PHOSPHORUS INTERACTION, ADJUSTED FOR BLOCK EFFECTS.

concentration (Figure 26a). Alternatively, the differences among responses to phosphorus varied with the level of nitrogen, where the responses were again measured over both levels of sludge concentration (Figure 26b). Examination of the differences in responses reveals the following:

1. The response to nitrogen within level P_0 was not significant.
2. The response to nitrogen within level P_1 was significant at the 99% level.
3. The response to nitrogen within level P_2 was significant at the 99% level.
4. The response to phosphorus within level N_0 was not significant.
5. The response to phosphorus within level N_1 from P_0 to P_1, was significant at the 99% level, whereas the response from P_1 to P_2 was significant at the 95% level.
6. The response to phosphorus within level N_2 from P_0 to P_1 was significant at the 99% level, whereas the response from P_1 to P_2 was significant at the 95% level.

The rate of response to nitrogen decreased with increasing concentrations of nitrogen for all three levels of phosphorus (Figure 26a). Similarly, the rate of response to phosphorus decreased with increasing concentrations of phosphorus over all three levels of nitrogen (Figure 26b).

The significant NP interaction was further investigated as follows: the values for the quantity of nitrogen and phosphorus consumed (I_N-E_N and I_P-E_P), as given in Table 7, may be written in the following form (Table 16).

Table 16.

Calculation of the average quantities of nitrogen and phosphorus consumed for each applied level of nitrogen and phosphorus.

mg of nitrogen consumed			mg of phosphorus consumed			
N_0	N_1	N_2	P_0	P_1	P_2	
22.6	33.7	46.1	2.0	3.0	9.5	
22.6	37.3	40.2	2.0	6.5	9.7	
21.5	37.5	49.3	2.0	4.6	10.8	
22.5	37.6	43.5	2.0	4.6	10.6	
21.7	37.6	40.8	2.0	4.5	11.0	
22.6	35.6	51.8	2.0	6.5	10.4	
22.3	36.6	43.6	2.0	5.0	10.3	Average

The N_0 level indicates the quantity of nitrogen consumed when 22.6 mg nitrogen was supplied daily. Similarly, the P_1 level indicates the quantity of phosphorus consumed when 6.5 mg phosphorus was supplied daily. The other average quantities of nitrogen and phosphorus consumed were obtained in a similar manner.

The calculations and discussions to follow are based on the values obtained for replication I. All units of the factorial experiment are involved in measuring any one main effect or interaction. Therefore, it must be kept in mind that although the values for only one replication were used, the effects of the actual levels of nitrogen and phosphorus applied, were measured at the six levels of the other factors in each replication.

By plotting the average values for the quantities of

nitrogen and phosphorus consumed as obtained in Table 16, a curvilinear relationship was indicated. A logarithmic transformation of the quantity of phosphorus consumed gave a straight line relationship between the quantities of nitrogen and phosphorus consumed. However, the same straight line relationship could not be obtained by plotting the logarithm of the quantity of nitrogen consumed, against linear increments of the quantity of phosphorus consumed. Thus a dependent variable was indicated and in this case it was phosphorus. The regression line for the quantities of nitrogen and phosphorus consumed, together with the 95% confidence intervals, are shown in Figure 27. The correlation coefficient was almost equal to 1 and therefore only one line was plotted. The regression coefficient was significant at the 95% level.

For a true nitrogen consumption of 20 mg/day, the true phosphorus consumption lies between log P = -0.35 to log P = 0.75, or equivalently, 0.5 mg to 5.6 mg phosphorus. Similarly, for a nitrogen consumption of 40 mg/day, the true phosphorus consumption lies between 2.5 mg to 11.2 mg/day with a 95% probability (Figure 27). For a true phosphorus consumption of 2.5 mg/day, the nitrogen consumption lies between 15 mg to 40 mg/day.

The above reasoning applied only to the average values and not to the individual values as outlined in Table 16. A similar type of graph was plotted for the individual values of Table 7, and is given in Figure 28. Figure 28 is markedly different from Figure 27. The components of the joint relationship of the two variables, the regression of phosphorus with nitrogen and the

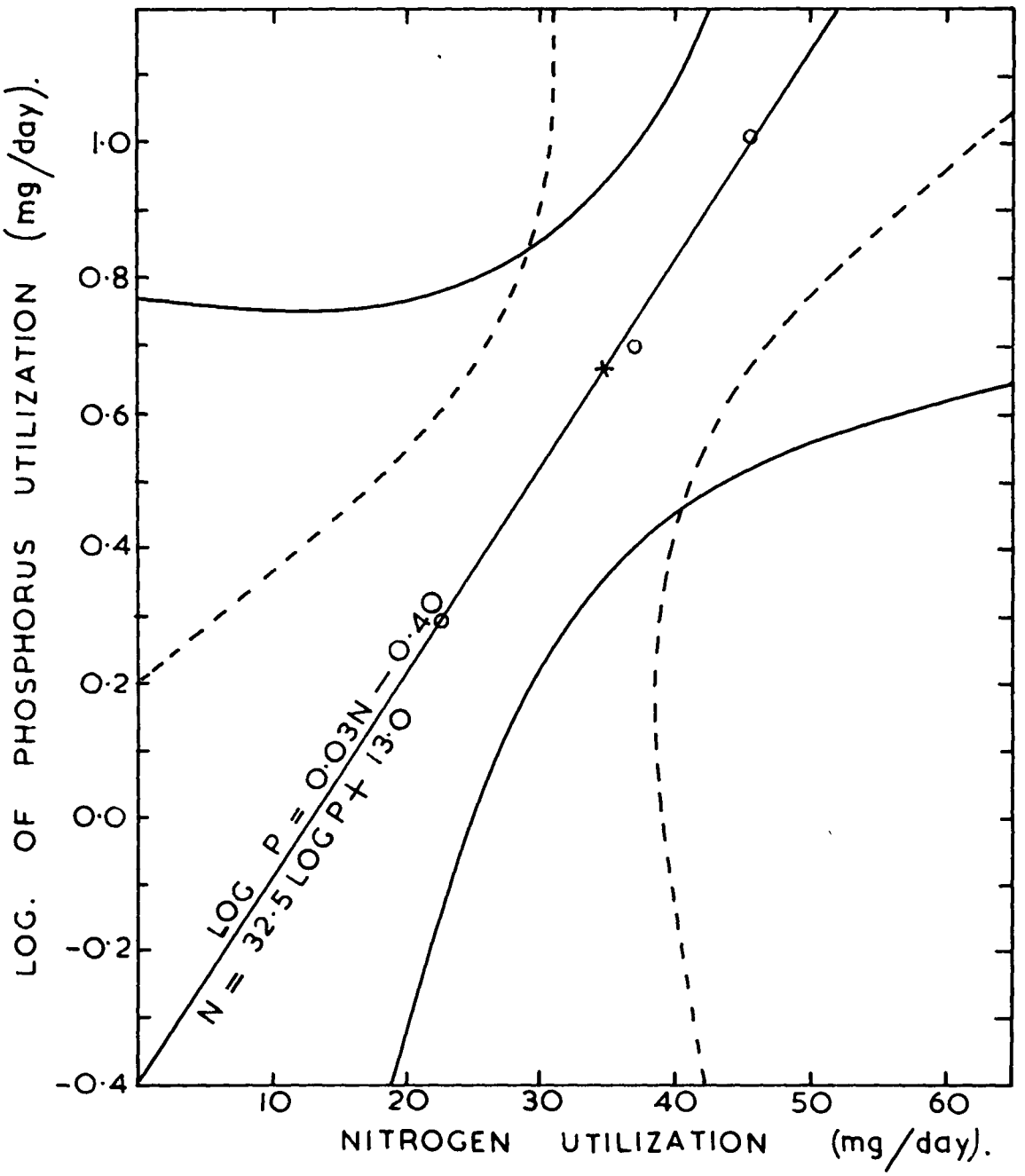

FIG 27 REGRESSION OF PHOSPHORUS UTILIZATION WITH NITROGEN UTILIZATION THE REGRESSION LINE WAS PLOTTED FOR THE AVERAGE VALUES FROM TABLE 16. THE 95 % CONFIDENCE INTERVALS FOR THE NITROGEN-PHOSPHORUS (------) AND PHOSPHORUS-NITROGEN (——) REGRESSION ARE ALSO SHOWN.

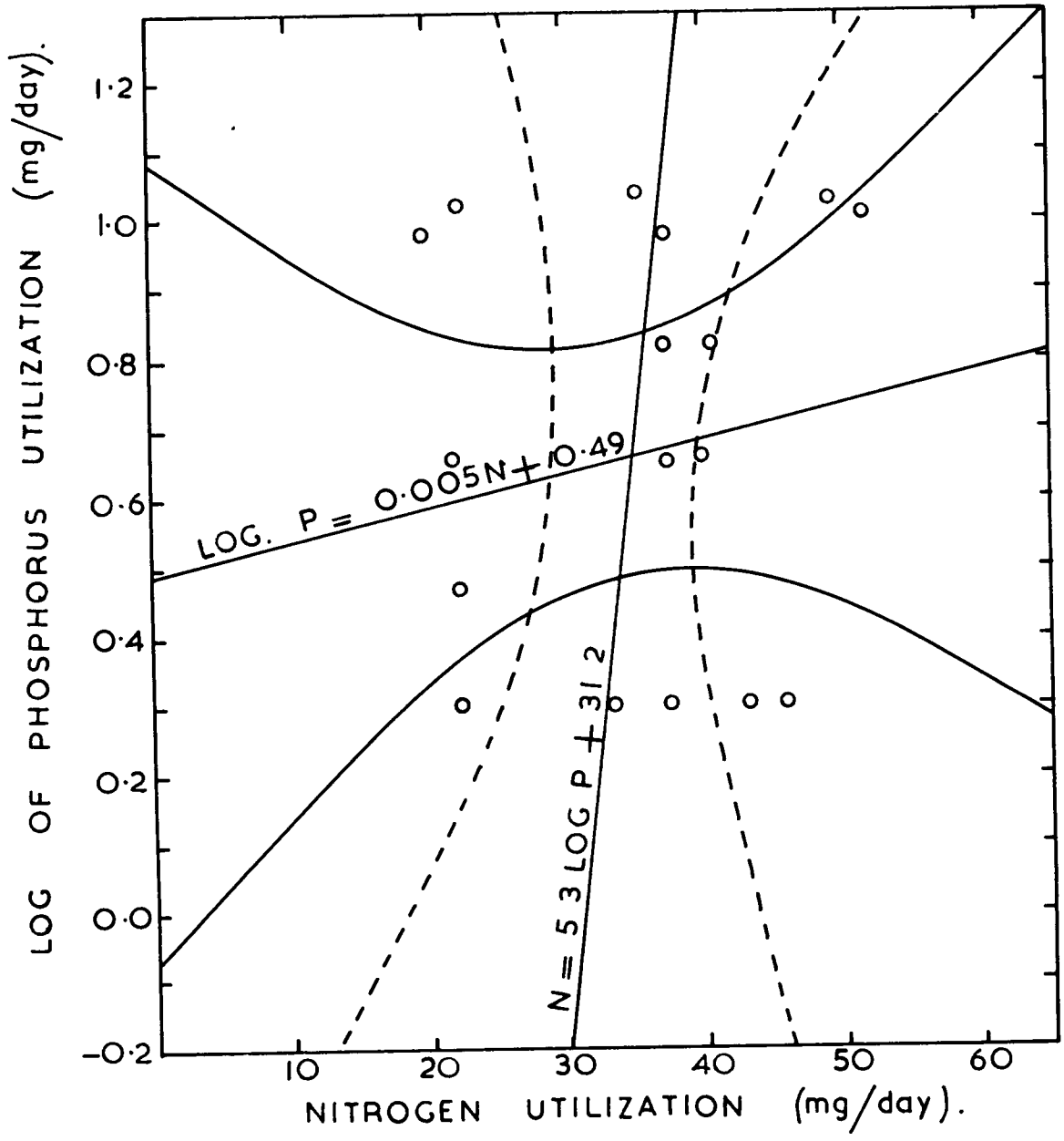

FIG 28. REGRESSION OF PHOSPHORUS UTILIZATION WITH NITROGEN UTILIZATION THE REGRESSION LINE WAS PLOTTED FOR THE INDIVIDUAL VALUES FROM TABLE 7 THE 95 % CONFIDENCE INTERVALS FOR THE NITROGEN-PHOSPHORUS (-----) AND PHOSPHORUS-NITROGEN (———) REGRESSION ARE ALSO SHOWN.

regression of nitrogen with phosphorus, were separated. The regression coefficient for the regression of phosphorus with nitrogen was significant at the 99% level. However, the regression coefficient for the regression of nitrogen with phosphorus was not significant. The correlation coefficient was not significant. These observations substantiate the results from the analysis of variance (Table 14), where a significant nitrogen-phosphorus interaction was indicated. Thus the interaction resolved into a dependence of the quantity of phosphorus consumed on the quantity of nitrogen consumed, also, the quantity of phosphorus consumed increased logarithmically with the quantity of nitrogen consumed.

A casual inspection of Figure 28 gave the impression that the laws of chance did not hold true. The 95% confidence limits imply that only 1 out of 20 observations should fall outside the confidence limits shown. However, 6 of the 18 values fell outside the limits. This observation may be explained by the fact that in some cases either the quantity of phosphorus, or the quantity of nitrogen supplied, were limiting, i.e. levels P_0 or N_0 were limiting. Of the values where nitrogen and phosphorus were either optimum, or in excess, only 1 value fell outside the limits. Thus where no limitations or restrictions were imposed on the values, the laws of chance held true. Therefore it may be concluded that Figure 28, and the conclusions drawn from it, were valid.

An experiment to determine the effect of adding nitrogen and phosphorus to a nutrient deficient sludge (treatment 001) was carried out as follows: after 32 days of treatment sludge was removed from treatment 001 and centrifuged at 10,000 x g for 15 min.

The sludge was resuspended in the synthetic substrate containing no additional nitrogen or phosphorus. To a Warburg flask was added 3 ml of the sludge suspension, 0.5 ml of a solution containing nitrogen, and 0.5 ml of a solution containing phosphorus. The ratio of BOD:sludge concentration:nitrogen:phosphorus was identical to that in the unit receiving treatment. The 18 treatments were now replicated on a smaller scale in the Warburg reaction flask. In this case the sludge under study was the nutrient deficient sludge. The experiment was designed as a randomized complete block experiment and two replications were done. The respiration rates were measured at 30°C over 1 hr. The results are listed in Table 17.

No significant increase in the respiration rate due to increased quantities of nitrogen and phosphorus was observed (Table 17). No benefit from the increased quantities of nitrogen and phosphorus could be determined for the short term experiments.

Table 17.

Respiration rate of a nutrient deficient sludge when treated with different ratios of BOD:N:P. The respiration rates were measured at 30°C and are reported in µl O_2/mg sludge/hr.

Treatment	Respiration rate	
	Replication I	Replication II
000	16	23
010	22	31
020	22	21
100	23	22
110	23	23
120	25	22
200	24	23
210	24	21
220	24	25
001	21	21
011	21	22
021	22	22
101	23	24
111	24	24
121	24	25
201	23	23
211	23	23
221	25	26

VIII. INFLUENCE OF NUTRITION ON THE BULKING OF ACTIVATED SLUDGE.

Bulking of activated sludge is the phenomenon whereby the sludge solids (floc) become bulky, develop poor settling characteristics and pass over the weirs into the clarified effluent.

From Figures 23, 24 and 25 it can be seen that the settling rates of the sludges of treatments 100, 120, 200 and 101 were slow. These sludges had a tendency to bulk, and their sludge density index (SDI) dropped to low values (Table 6). However, in spite of the SDI of treatments 000 and 010 being low, i.e. 0.50 and 0.25, the settling rates in these two sludges were still not slow. It is possible that the sludge of treatment 000 in replication I would have bulked in a few days after the experiment was completed, because the decrease in SDI was rather rapid at that time (Figure 5). The sludge of treatment 000 in replication II bulked and attained a SDI of 0.25 after 32 days of treatment. The tendency to bulk was confirmed for treatments 100 and 101 in replication II. The sludges of treatments 000 and 001 also bulked. The sludges of treatments 120 and 200 did not bulk in replication II. The results are summarized in Table 18.

The question arose whether bulking was due to:

a) the growth of a particular microorganism, or

b) the accumulation of polysaccharide material in the sludges, or

c) the action of both factors (a) and (b).

After 32 days of treatment the polysaccharide content of

Table 18.

Occurrence of bulking in differently treated sludges after 32 days at 20°C.

Treatment	Occurrence of bulking in	
	Replication I	Replication II
000	-?	+
010	-	-
020	-	-
100	+	+
110	-	-
120	+	-
200	+	-
210	-	-
220	-	-
001	-	+
011	-	-
021	-	-
101	+	+
111	-	-
121	-	-
201	-	-
211	-	-
221	-	-

Note: + indicates that bulking occurred.
- " " " did not occur.

the sludges, expressed as glucose, were determined together with their final nitrogen and nucleic acid contents. The results are listed in Table 19.

The total nucleic acid (NA) content of the sludges varied (Table 19). The sludges of wide BOD:N and BOD:P ratios had the lowest NA content (average value of 1.7%). The sludges of narrow ratios had a NA content of about 5.8%. A significant correlation existed between the nitrogen and nucleic acid contents of the sludges. The correlation coefficient, r, was equal to 0.88, with n = 18. The regression line between these two variables and the 95% confidence belt are shown in Figure 29.

The NA and nitrogen determinations were chemical determinations and did not distinguish between living and dead material. Therefore, neither of these determinations could be successfully used as an estimation of the living matter present. For this purpose the NA and nitrogen contents of the sludges should be correlated with biological studies such as the metabolic activities of the sludges, measured by their respiration rates. No correlation was found between the direct microscopic bacterial counts and the NA contents of the sludges.

An accumulation of carbohydrate material was observed in the sludges of wide BOD:N and BOD:P ratios (Table 19). The sludges of treatments 000 and 001 contained 27.8% and 14.7% carbohydrate material, expressed as glucose, whereas treatments 220 and 221 contained only 8.2% and 9.2%, respectively. The accumulation of carbohydrate material was also observed by Porges, Jasewicz and Hoover (1956) and Symons and McKinney (1958). The sludges of the

Table 19.

Average percentages of nitrogen, nucleic acids and carbohydrates expressed as glucose, in sludges after 32 days treatment. The determinations were carried out on the dried sludges (105°C).

Treatment	Average nitrogen (%)	Average nucleic acid (%)	Average carbohydrate as glucose (%)	Average calculated protein (%Nx6.25)
000	4.27	1.54	27.8	26.7
010	4.69	2.04	38.0	29.3
020	4.61	2.85	28.0	28.8
100	6.12	2.90	21.6	38.3
110	6.94	4.25	19.6	43.3
120	8.07	5.07	16.7	50.5
200	8.44	4.70	22.5	52.7
210	9.24	5.75	10.7	57.7
220	9.42	5.75	8.2	58.8
001	4.37	1.87	14.7	27.3
011	4.26	2.62	11.5	26.6
021	4.26	2.35	14.5	26.6
101	8.32	4.66	15.2	52.0
111	8.25	4.95	12.6	51.6
121	7.84	4.65	7.1	49.0
201	9.35	5.00	11.1	58.4
211	8.67	5.25	8.2	54.2
221	10.00	5.78	9.2	62.5

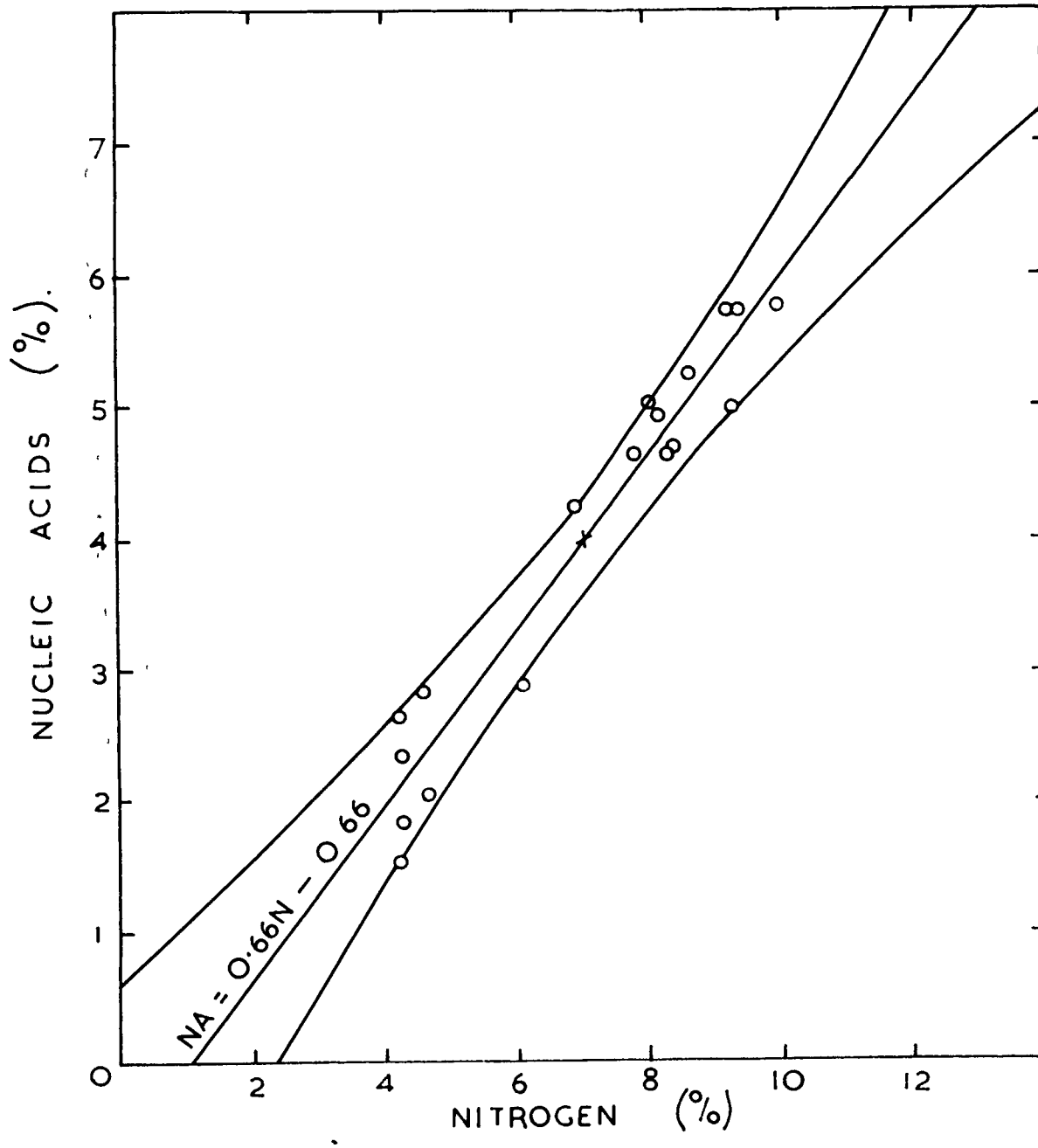

FIG 29 THE CORRELATION BETWEEN THE NITROGEN AND NUCLEIC ACID CONTENT OF THE SLUDGES THE 95% CONFIDENCE BELT IS ALSO SHOWN

lower sludge concentration accumulated more carbohydrate material than the sludges of the higher sludge concentration.

The differences in the amount of carbohydrate material accumulated, became smaller as the nitrogen and phosphorus contents of the sludges increased, i.e. the concentration of carbohydrate material for treatment 220 was 8.2% and that of treatment 221, 9.2%. No marked accumulation of polysaccharide material occurred in the sludges that bulked.

The carbohydrate content of the sludges apparently did not influence the occurrence of bulking. Therefore, an attempt was made to establish whether or not a microorganism was responsible for bulking. Microscopic study of the bulking sludges revealed the presence of a sheathed bacterium in large numbers. This bacterium, which resembled Sphaerotilus morphologically, was isolated and purified. The isolation medium used, casamino acids + glycerol + yeast extract (CGY), was that suggested by Dondero (1961) and the composition of the medium is given in the appendix. The isolated organism grew profusely in this medium and developed a thick pellicle at the surface of the broth. Large colonies developed within 24 hr at 30°C on the solid CGY medium. The colonies were smooth, with frimbriate edges, resembling the smooth colony of Sphaerotilus, demonstrated by Stokes (1954).

Long, filamentous, rods could easily be seen when the isolated bacterium was stained with crystal violet, and examined under oil-immersion. The morphology of the isolate was identical to that of Sphaerotilus, described by Stokes (1954). The general

morphology of the isolate (broth culture, 48 hr) can be seen in Plates I and II. The staining characteristic of these cells was Gram-negative.

The following two experiments were carried out to determine the actual cause of bulking of activated sludge.

Experiment A.

A non-bulking sludge (treatment 221) was divided into six equal portions (approximately 500 mg of sludge in each case). Four of the six portions were inoculated with 150 mg of a bulking sludge (treatment 001). The six portions (a, b, c, d, e and f) were then treated as summarized below:

Portions of sludge (code)	Treatment	Nutrients
a	Not inoculated	+
b	" "	-
c	Inoculated	+
d	"	+
e	"	-
f	"	-

Note: + indicates that nitrogen and phosphorus were added as for treatment 221.

- indicates that no additional nitrogen and phosphorus were added as for treatment 001.

The treatment cycle (23 hr aeration, 1 hr sedimentation) was continued as usual, and the SDI determined daily. The results are summarized in Table 20.

Table 20.

Changes in the sludge density index of an activated sludge (treatment 221) when inoculated with a bulking sludge (treatment 001) and treated with a synthetic substrate containing excess and limiting quantities of nitrogen and phosphorus.

Sludge code	Sludge density after:						Occurrence of bulking
	2 days	4 days	6 days	8 days	10 days	12 days	
a	1.80	1.75	1.72	1.70	1.65	1.60	-
b	1.57	1.57	1.55	1.50	1.42	1.38	-
c	1.55	1.48	1.38	1.38	1.38	1.38	-
d	1.78	1.70	1.48	1.25	1.00	0.82	-
e	1.48	1.32	0.78	0.38	0.20	0.20	+
f	1.32	0.98	0.45	0.20	0.20	0.20	+

Note: + indicates that bulking occurred.
- " " " did not occur.

Within one week bulking occurred in sludges e and f. Sludges c and d showed no signs of bulking during the time that the experiment was conducted. The SDI of the uninoculated sludge (b) decreased during this time, but not as markedly as in sludges e and f, and bulking was not evident after 12 days. It may be concluded from experiment (A) that the original bulking sludge contained some agent that could cause bulking in a non-bulking sludge, but only when the BOD:N and BOD:P ratios were wide.

Experiment B.

To establish if a pure culture of *Sphaerotilus sp.* could produce bulking, a similar experiment to that described under (A) was conducted, but this time inoculating the non-bulking sludge with the isolated, pure culture of *Sphaerotilus sp.* The results are listed in Table 21.

Bulking occurred in sludges e and f. Sludges c and d showed no signs of bulking. Plate III demonstrates the differences in the sludge volume after settling for 1 hr. Sludges e and f occupied a volume of about 58% of the total volume of 50 ml, and had a fluffy appearance. Sludges a, b, c and d occupied from 10% to 12% of the total volume, and the supernatant liquor was crystal clear. Bulking occurred in the same treatments as in experiment (A).

It may be concluded from experiments (A) and (B) that the bulking sludge contained an agent which caused bulking in a non-bulking sludge. The same effect was brought about by

Table 21.

Changes in the sludge density index of a non-bulking activated sludge when inoculated with a pure culture of Sphaerotilus sp. and treated with a synthetic substrate containing excess and limiting quantities of nitrogen and phosphorus.

Sludge code	Sludge density after:						Occurrence of bulking
	2 days	4 days	6 days	8 days	10 days	12 days	
a	1.50	1.60	1.40	1.43	1.34	1.54	-
b	1.45	1.30	1.42	1.54	1.43	1.67	-
c	1.50	1.45	1.50	1.43	1.54	1.67	-
d	1.45	1.50	1.40	1.50	1.30	1.50	-
e	1.45	1.40	1.00	0.65	0.36	0.38	+
f	1.40	1.25	1.00	0.59	0.33	0.37	+

Note: + indicates that bulking occurred.
- " " " did not occur.

inoculating a non-bulking sludge with a pure culture of a filamentous, sheathed bacterium, Sphaerotilus sp. The bulking conditions occurred only in the treatments where the BOD:N and BOD:P ratios were wide. Bulking could not be produced where these ratios were narrow. This observation is confirmed by the results presented in Table 18, i.e. bulking occurred repeatedly in the treatments where either nitrogen or phosphorus, or both, were limiting.

The differences in sludge texture between a bulking and a non-bulking sludge can be seen in Plates IV to XI. The bulking sludge had a marked fluffy appearance and was usually lighter in colour. Microscopic examination showed that the flocs of a bulking sludge were small in size and had a marked filamentous appearance (Plates V, VII, IX, X and XI). The bulking sludges contained large numbers of Sphaerotilus organisms. The flocs of the bulking sludges settled very slowly (Plate III, sludges e and f).

The flocs of the non-bulking sludges were larger in size, less filamentous and contained relatively few Sphaerotilus organisms (Plates IV, VI and VIII). The flocs of the non-bulking sludges settled rapidly (Plate III, sludges a, b, c and d).

A further experiment was conducted to determine if the settling rate of the bulking sludge could be improved by the addition of excess nitrogen and phosphorus. Excess nitrogen and phosphorus were added to the treatments that bulked (sludges e and f). No improvement in the settling characteristics of the bulking sludges was observed in a 5 day period.

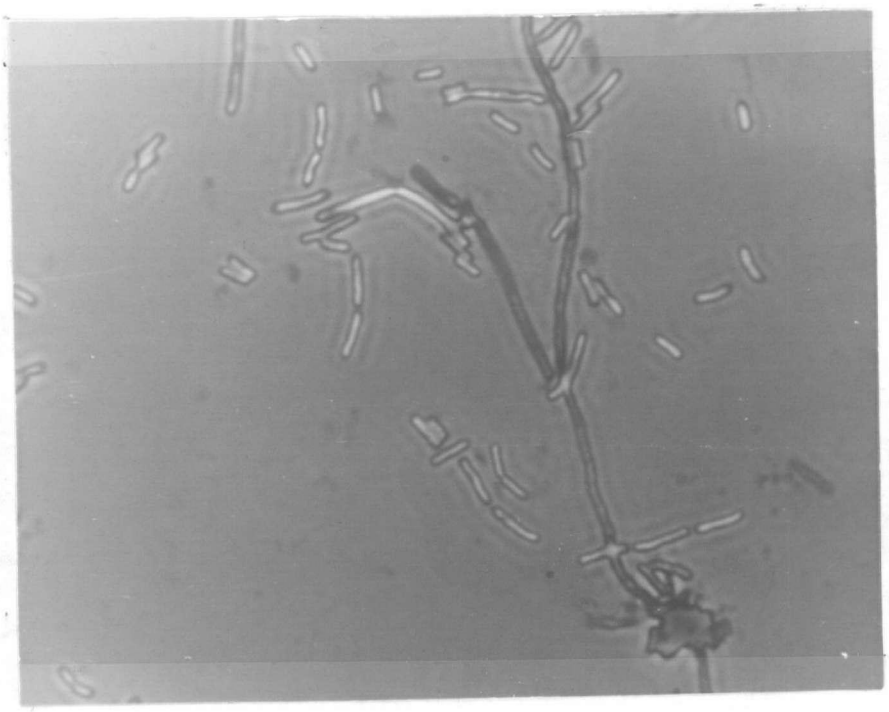

Plate I.

Pure culture of isolated *Sphaerotilus sp.*
(48 hr broth culture). Phase contrast x 2000.
A single thread of the bacterium extends across
the entire microscopic field.

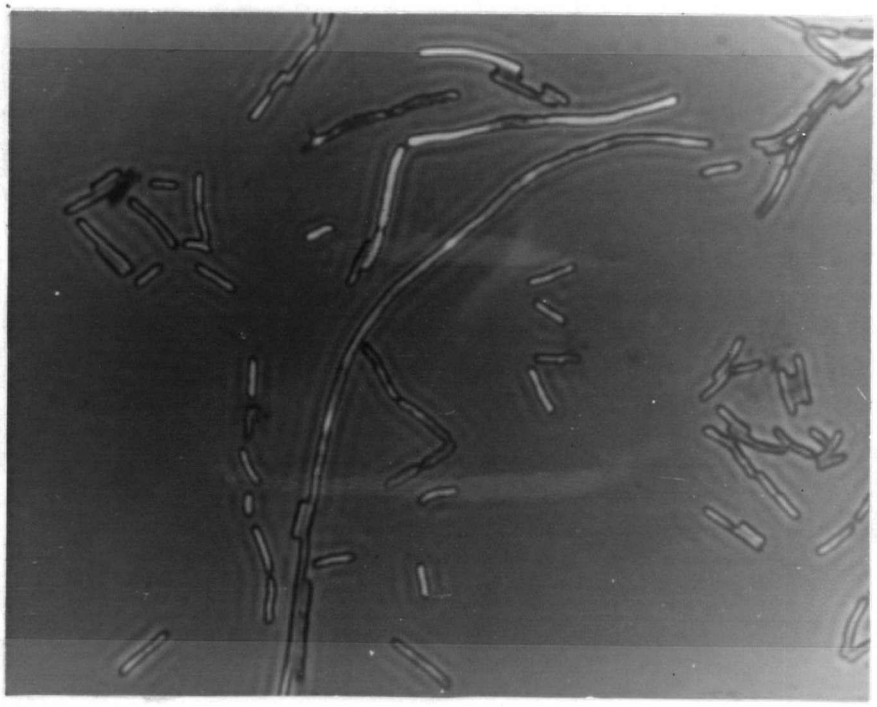

Plate II.

Pure culture of isolated *Sphaerotilus sp.*
(48 hr broth culture). Phase contrast x 2000.
The typical habit of growth in
long threads can be seen.

Plate III.

Changes in the volume of sludge settled after 1 hr, when a non-bulking sludge was inoculated with a pure culture of *Sphaerotilus sp.* and treated for 12 days as follows:

Measuring cylinder	Treatment	Nutrients
a	Not inoculated	+
b	" "	-
c	Inoculated	+
d	"	+
e	"	-
f	"	-

Note: + indicates that nitrogen and phosphorus were added as for treatment 221.
- indicates that no additional nitrogen and phosphorus were added as for treatment 001.

The changes in SDI with time are given in Table 21.

To face Plate III, p. 115.

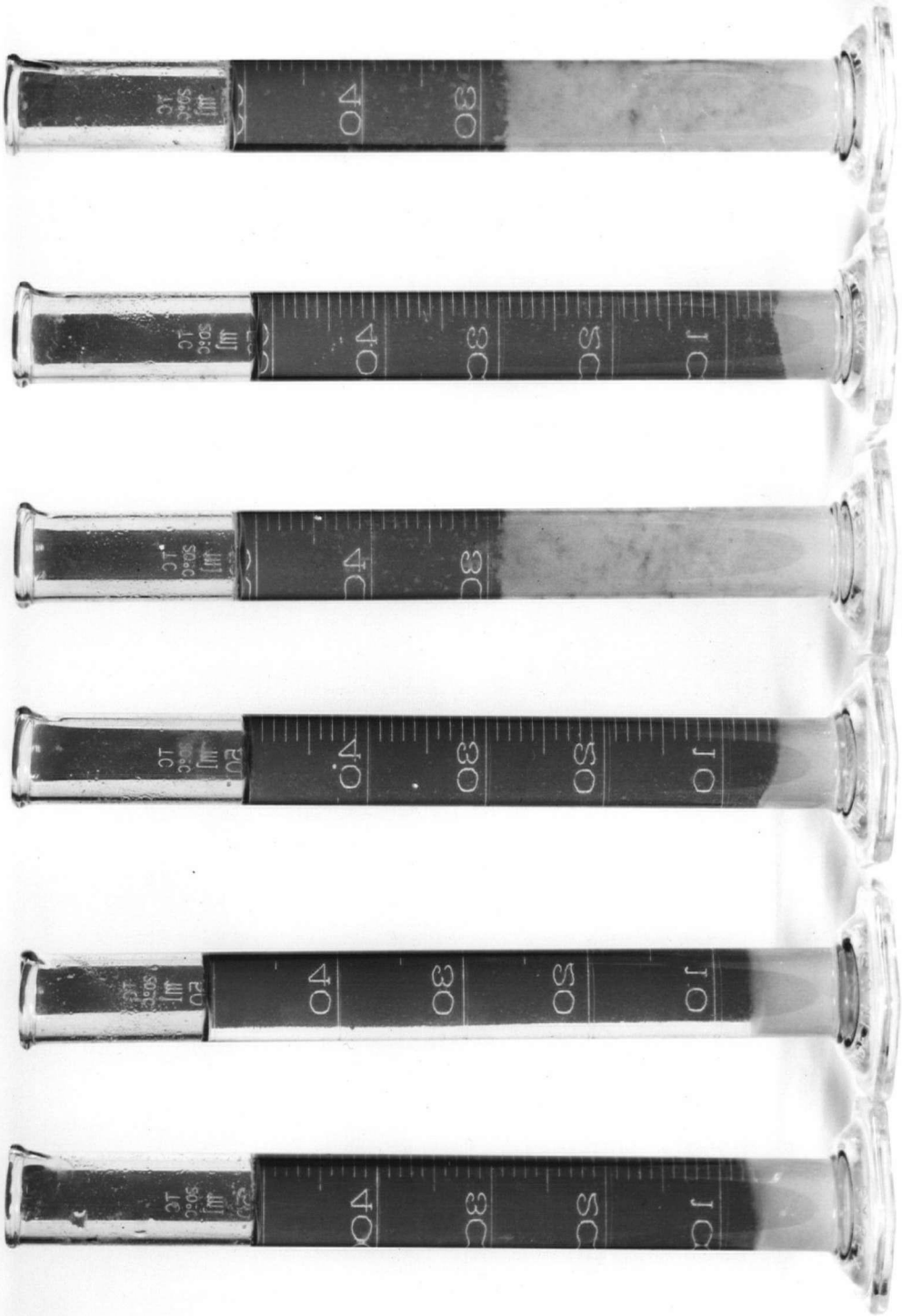

Plate III.

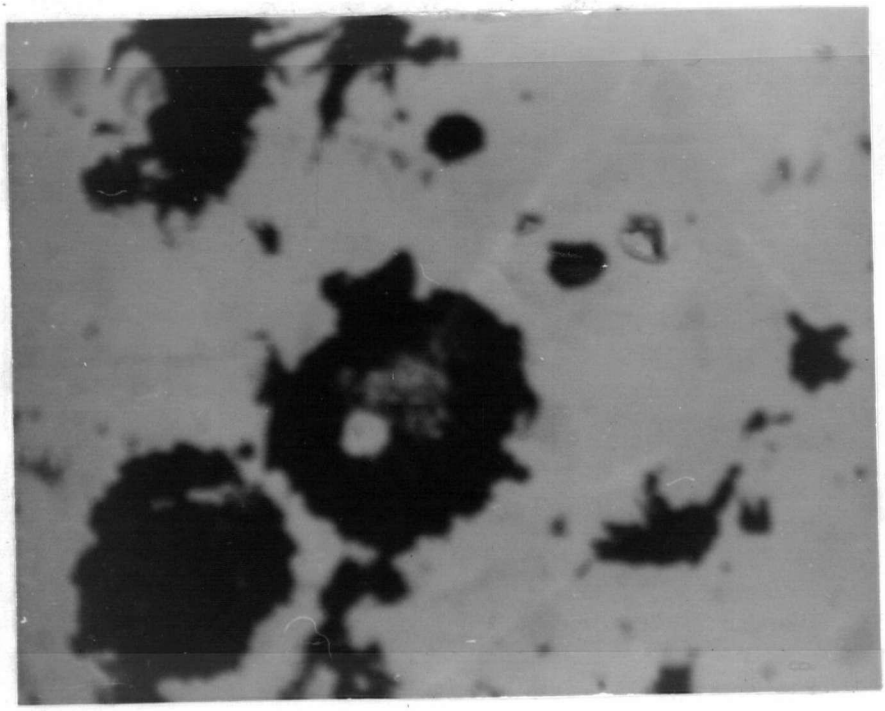

Plate IV.

Texture of a typical non-bulking sludge x 50.
The sludge was air dried and then
stained with crystal violet.

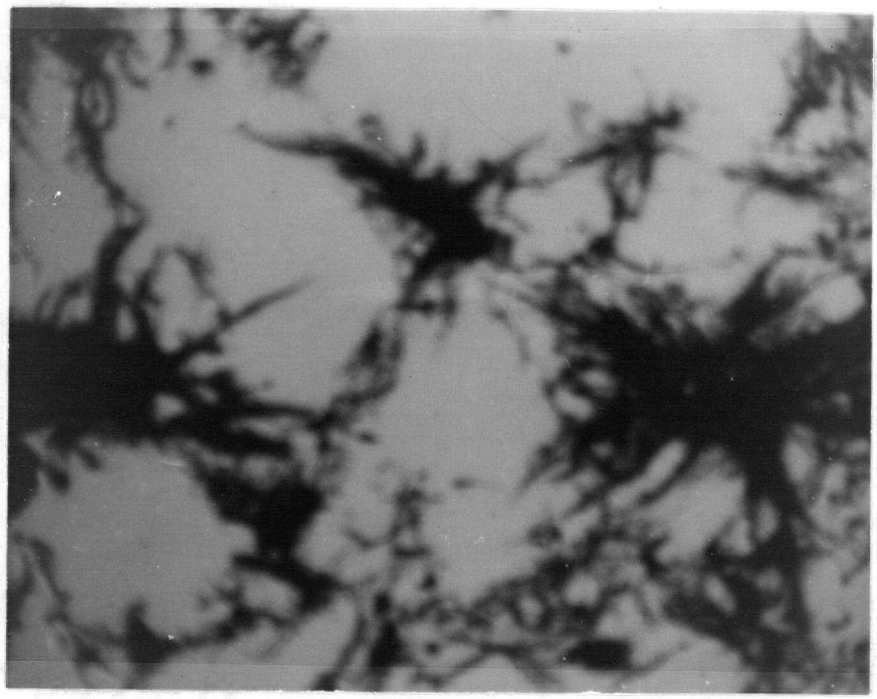

Plate V.

Texture of a typical bulking sludge x 50. The sludge was prepared as for Plate IV.
The overall filamentous appearance of the bulking sludge is evident (compare Plate IV). The overall size of the floc particles is much smaller than that of the non-bulking sludges.

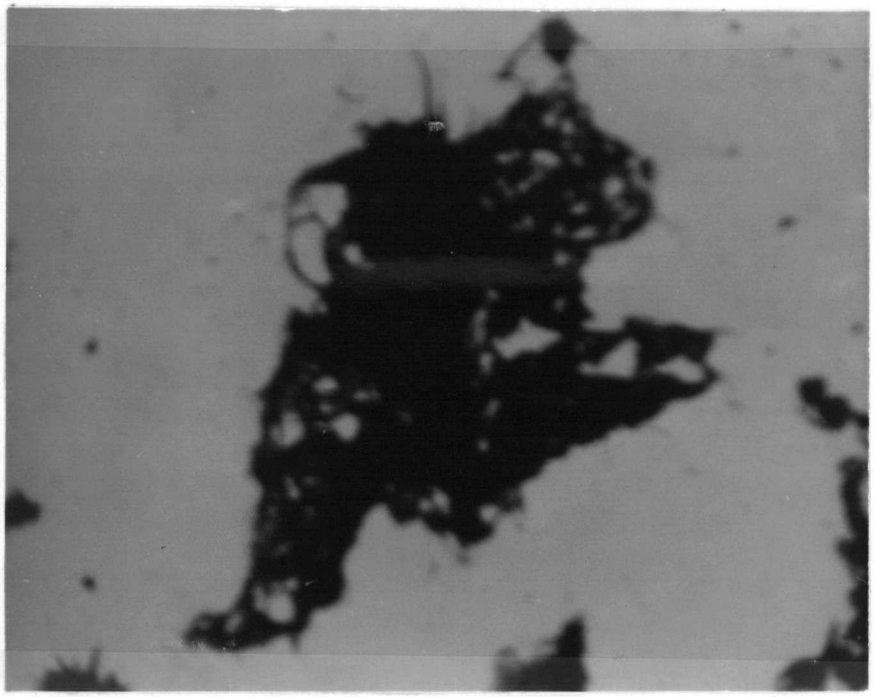

Plate VI.

A non-bulking sludge under higher magnification (x 1000). The presence of a few filaments can be seen.

Plate VII.

A bulking sludge under higher magnification (x 1000). The filamentous appearance of the sludge is evident.

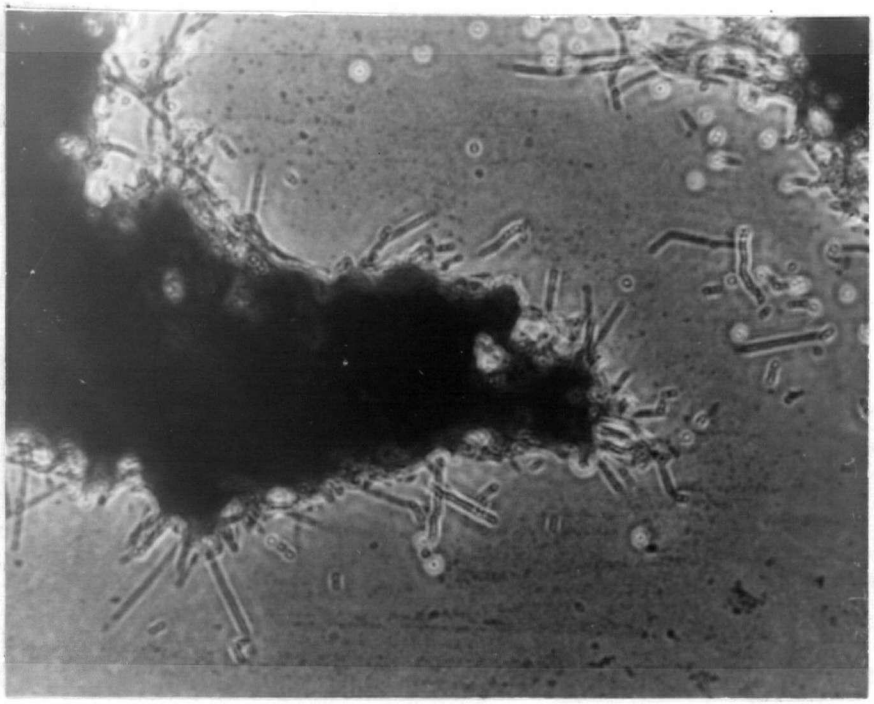

Plate VIII.

A non-bulking sludge (phase contrast x 2000). The presence of a filamentous organism is evident but in smaller numbers than in a bulking sludge (Plate IX).

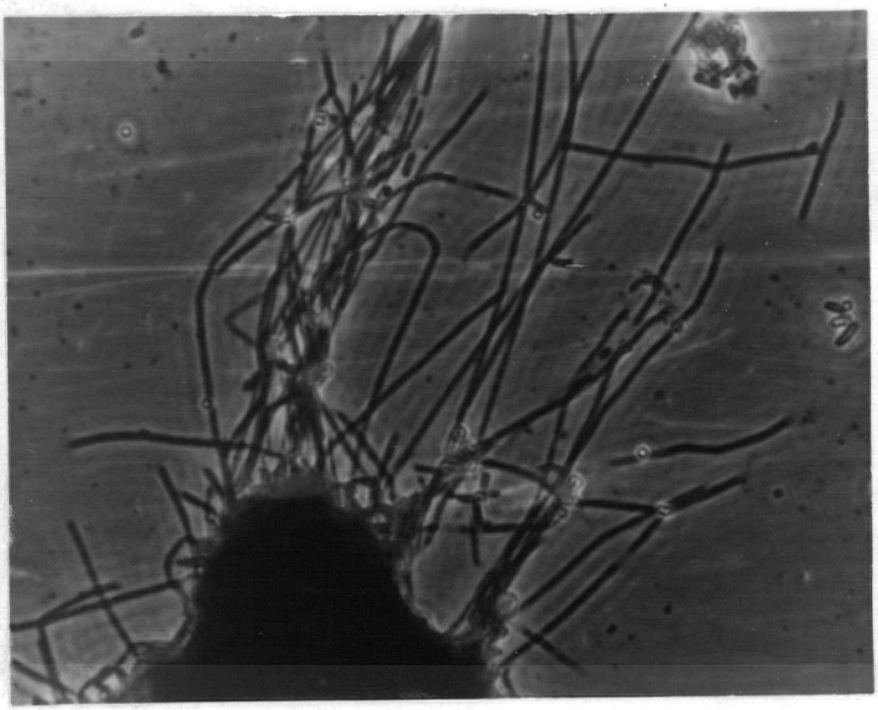

Plate IX.

A bulking sludge (phase contrast x 2000). The overall presence of long threads of the sheathed bacterium *Sphaerotilus* is evident. Some empty spaces can be seen in the sheaths. Note the increase in length of the threads as contrasted to the length of the threads in a non-bulking sludge (Plate VIII).

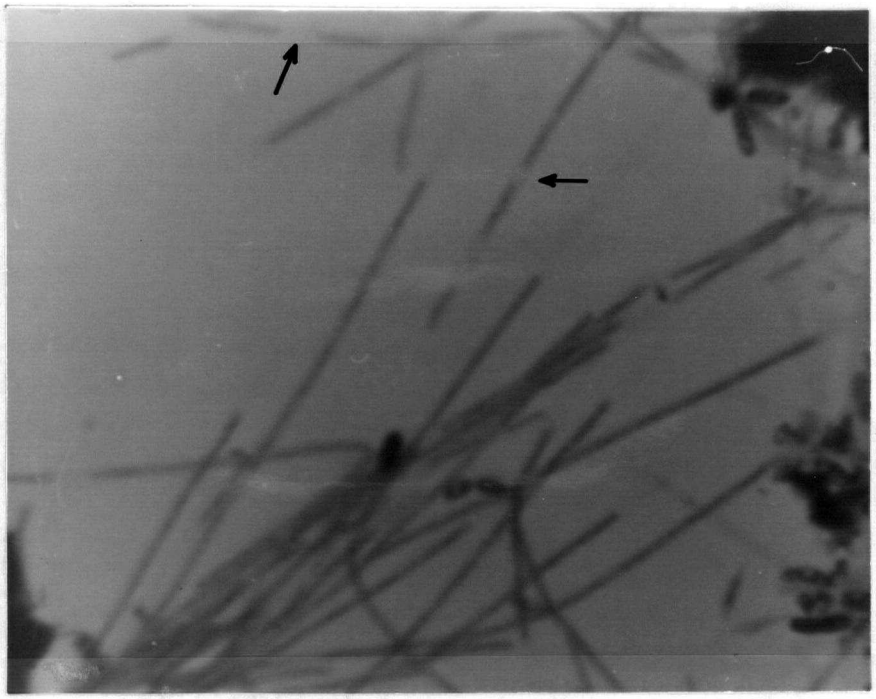

Plate X.

A bulking sludge (x 2000).
Empty spaces in the sheaths are indicated.

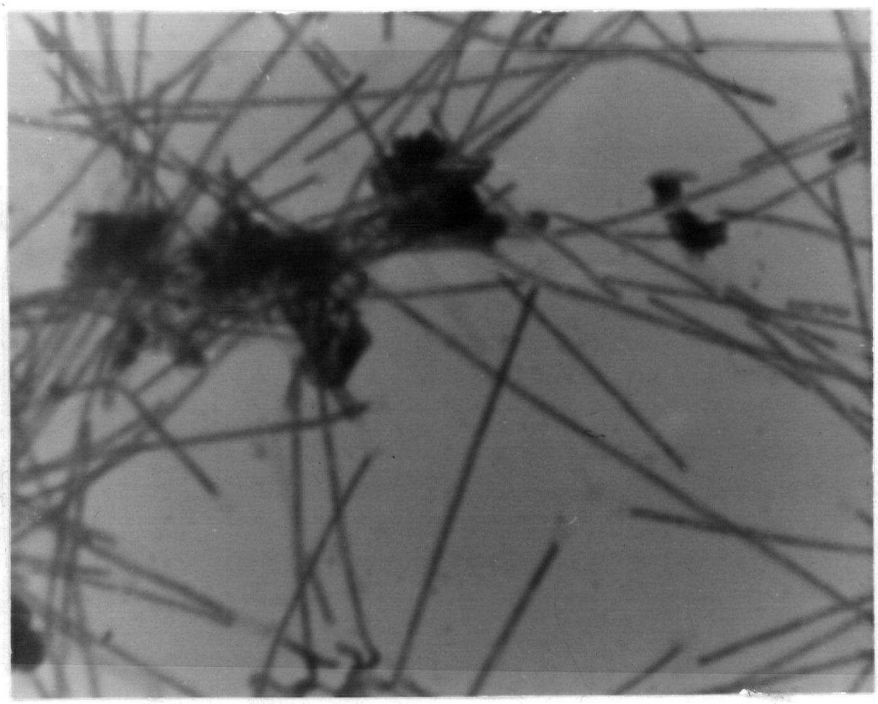

Plate XI.

A bulking sludge (x 2000). The small particles of sludge are completely enmeshed by the filamentous growth of *Sphaerotilus*.

The rate of settling of the sludges was not increased by the addition of electrolytes. A 0.1 N solution was prepared of each of the following electrolytes: $AlCl_3$, $CaCl_2$, HCl and NaCl. To twelve measuring cylinders, each containing 50 ml of sludge, was added 1, 2 or 5 ml of each electrolyte separately. The rates of settling were then observed and compared to a control to which no electrolyte had been added. No differences in the settling rates were noted.

Bulking is a common phenomenon and Sphaerotilus organisms have frequently been isolated from bulking sludge (Dondero, 1961). Ingols and Heukelekian (1939) and Heukelekian and Ingols (1940) suggested that the principle causes of bulking of activated sludge were:

a) An excess of carbohydrate material in relation to nitrogen in the substrate (C:N ratio greater than 8:1).

b) An excess of nutrients in relation to activated sludge solids concentration.

c) Insufficient air.

Factor (b) may be ruled out in the present study. The BOD load was not excessive as indicated by the constant rate of BOD removal. The BOD load was also constant with respect to the concentration of sludge solids.

Factor (c) may also be ruled out, because the quantity of air supplied to the units was constant and greater than the amount normally supplied in the activated sludge process (p. 27). The sludges that bulked did not receive less air than those that

did not bulk.

Bulking occurred only in the treatments where the BOD:N and BOD:P ratios were wide (Table 18 and Figures 23 - 25). Bulking was most severe when the synthetic substrate had a BOD:N ratio of 37:1, and a BOD:P ratio of 420:1. Sphaerotilus organisms were present in all sludges, but their growth was favoured by wide BOD:N and BOD:P ratios of the synthetic substrate. Hence, it was concluded from these experiments that bulking was caused by wide BOD:N and BOD:P ratios of the substrate, and by the presence of Sphaerotilus organisms.

IX. SUMMARY AND CONCLUSIONS.

A 3x3x2 partially confounded factorial design was used in the present investigation to study the effect of nitrogen and phosphorus on the nutrition of the microorganisms in, and the settling characteristics of, activated sludge. Nitrogen and phosphorus were each supplied in three different concentrations. The sludge concentration was maintained constant at two different levels. The experiment was replicated twice. A synthetic substrate of highly reproducible composition (BOD = 840 $\pm$ 21 mg/litre, n = 54), was used in this study.

BOD removal was always in excess of 95%, but the nitrogen and phosphorus content of the sludges decreased as the quantity of available nitrogen and phosphorus per unit of BOD removed, decreased.

A marked difference in respiration rates between the sludges was observed when glucose was provided as substrate in respirometric studies. The sludges of narrow BOD:N and BOD:P ratios respired approximately 5 times more rapidly than the sludges of wide BOD:N and BOD:P ratios. However, the aeration period (23 hr) was long enough for the sludges with lower respiration rates to achieve complete BOD removal.

An accumulation of carbohydrate material was observed in the sludges of wide BOD:N and BOD:P ratios. The sludges of wide ratios (treatments 000 and 001) contained 27.8% and 14.7% carbohydrate material expressed as glucose, whereas sludges of narrow ratios (treatments 220 and 221) obtained only 8.2% and 9.2%,

respectively. The differences in the amount of carbohydrate material accumulated, became smaller as the nitrogen and phosphorus contents of the sludges increased.

No marked differences in the compositions of either the protozoal or bacterial populations were observed. The total nucleic acid content of the sludges varied. The sludges of wide BOD:N and BOD:P ratios had the lowest nucleic acid content (1.7%), whilst the sludges of narrow BOD:N and BOD:P ratios had the highest nucleic acid content (5.8%). A significant correlation existed between the nucleic acid and the nitrogen contents of the sludges.

A highly significant interaction existed between the quantities of nitrogen and phosphorus consumed by the activated sludge microorganisms. The quantity of phosphorus consumed increased logarithmically with, and depended on, the quantity of nitrogen consumed.

Impairment of the settling characteristics, or bulking, occurred only in treatments where the BOD:N and BOD:P ratios of the substrate were wide and the filamentous bacterium Sphaerotilus was present. Bulking was most severe when the BOD:N and BOD:P ratios of the substrate were greater than 37:1 and 420:1, respectively. Sphaerotilus was present in all sludges, but its growth was favoured by wide BOD:N and BOD:P ratios of the substrate.

The following conclusions were drawn from the present study:

1. To produce a biological growth or an activated sludge with maximum nitrogen and phosphorus contents, the BOD:N and BOD:P ratios of the substrate should not be wider than 19:1 and 81:1,

respectively.

2. To produce a biological growth or an activated sludge with at least 7% nitrogen and 1.4% phosphorus, the BOD:N and BOD:P ratios of the substrate should not be wider than 23:1 and 168:1, respectively.

3. A significant interaction existed between the quantities of nitrogen and phosphorus consumed by the activated sludge microorganisms.

4. The phosphorus requirements of the activated sludge microorganisms increased logarithmically with, and depended on, the quantities of nitrogen consumed.

5. Bulking of activated sludge occurred only in those sludges where the BOD:N and BOD:P ratios were wider than 22:1 and 168:1, respectively. Bulking was most severe when the BOD:N and BOD:P ratios were 37:1 and 420:1, respectively.

6. Sphaerotilus organisms were present in all sludges, but their development was favoured by sludges of wide BOD:N and BOD:P ratios.

7. Bulking did not occur in systems in which the BOD:N and BOD:P ratios were narrower than 19:1 and 81:1, respectively, even if the sludges were inoculated with a pure culture of Sphaerotilus sp.

8. Bulking of sludge was due to the combined effects of wide BOD:N and BOD:P ratios, which, in turn, favoured the growth of Sphaerotilus organisms.

X. APPENDIX.

The media used to estimate the microflora of the sludges were as follows: the nutrient agar and Sabouraud dextrose agar were prepared from Difco dehydrated media (Difco Manual, 1953). The glycerol-glucose agar had the following composition (Waksman, 1950):

Glucose	0.2 g
Glycerol	10.0 "
Sodium aspariginate	1.0 "
K_2HPO_4	1.0 "
Agar	15.0 "

Tap water to 1000 ml and pH adjusted to 7.0.

The selective acid agar had the following composition:

Dextrose	10.0 g
Peptone	5.0 "
K_2HPO_4	1.0 "
$MgSO_4 \cdot 7H_2O$	0.5 "
Agar	20.0 "

Tap water to 1000 ml.

The pH of the sterilized medium was adjusted with sterile 0.01 N H_2SO_4 to pH 3.8 immediately prior to use.

The medium used for the isolation of Sphaerotilus from the activated sludge was as follows (Dondero, 1961):

Casamino acids	5.0 g
Glycerol	10.0 "

Yeast extract	1.0 g
H_2O	1000 ml

and for a solid medium 15 g/litre agar.

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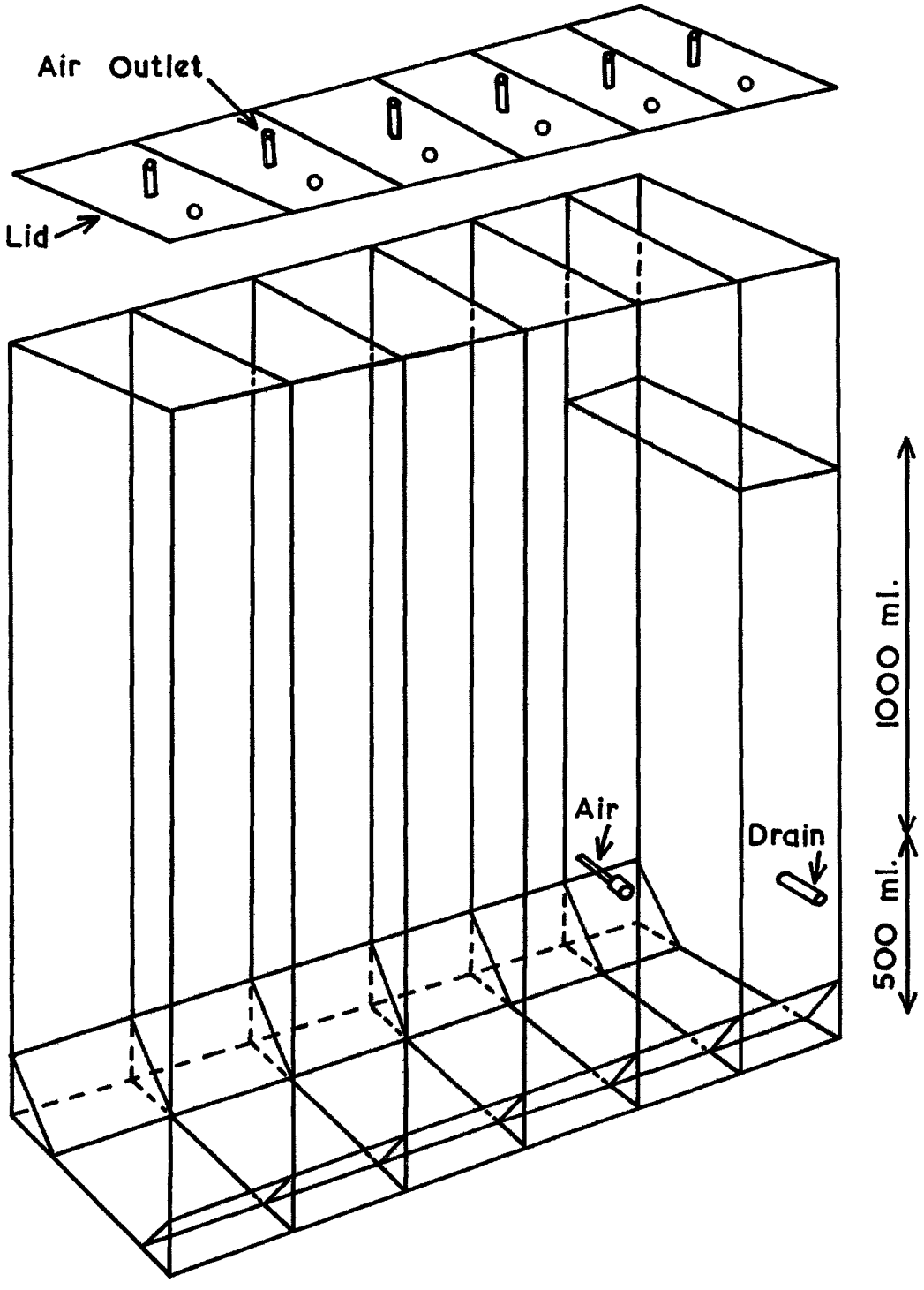

FIG 1. LABORATORY SCALE ACTIVATED SLUDGE PLANT.

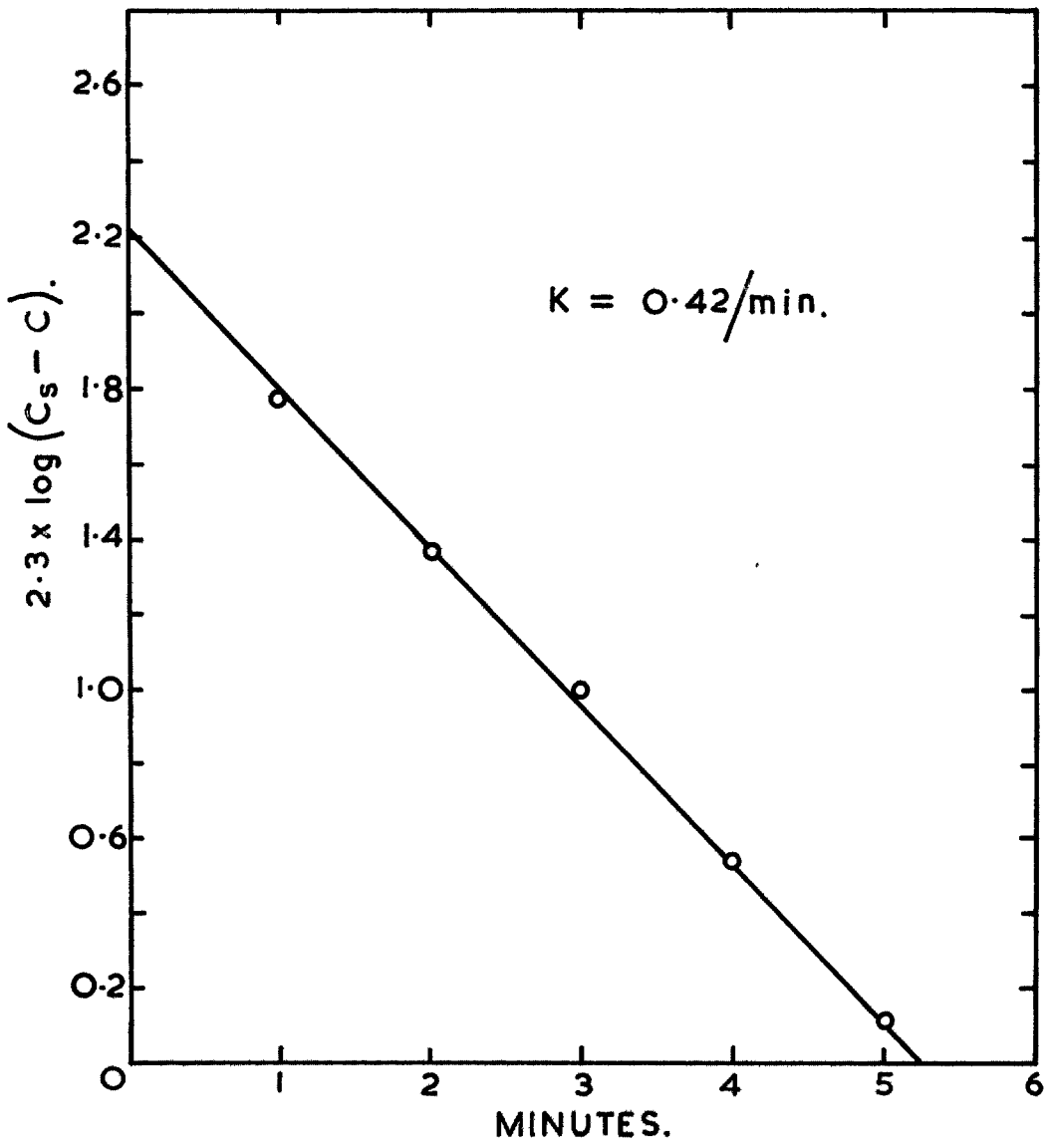

FIG 2. PLOT OF OXYGEN SATURATION DEFICIT $(C_s - C)$ TO OBTAIN K.

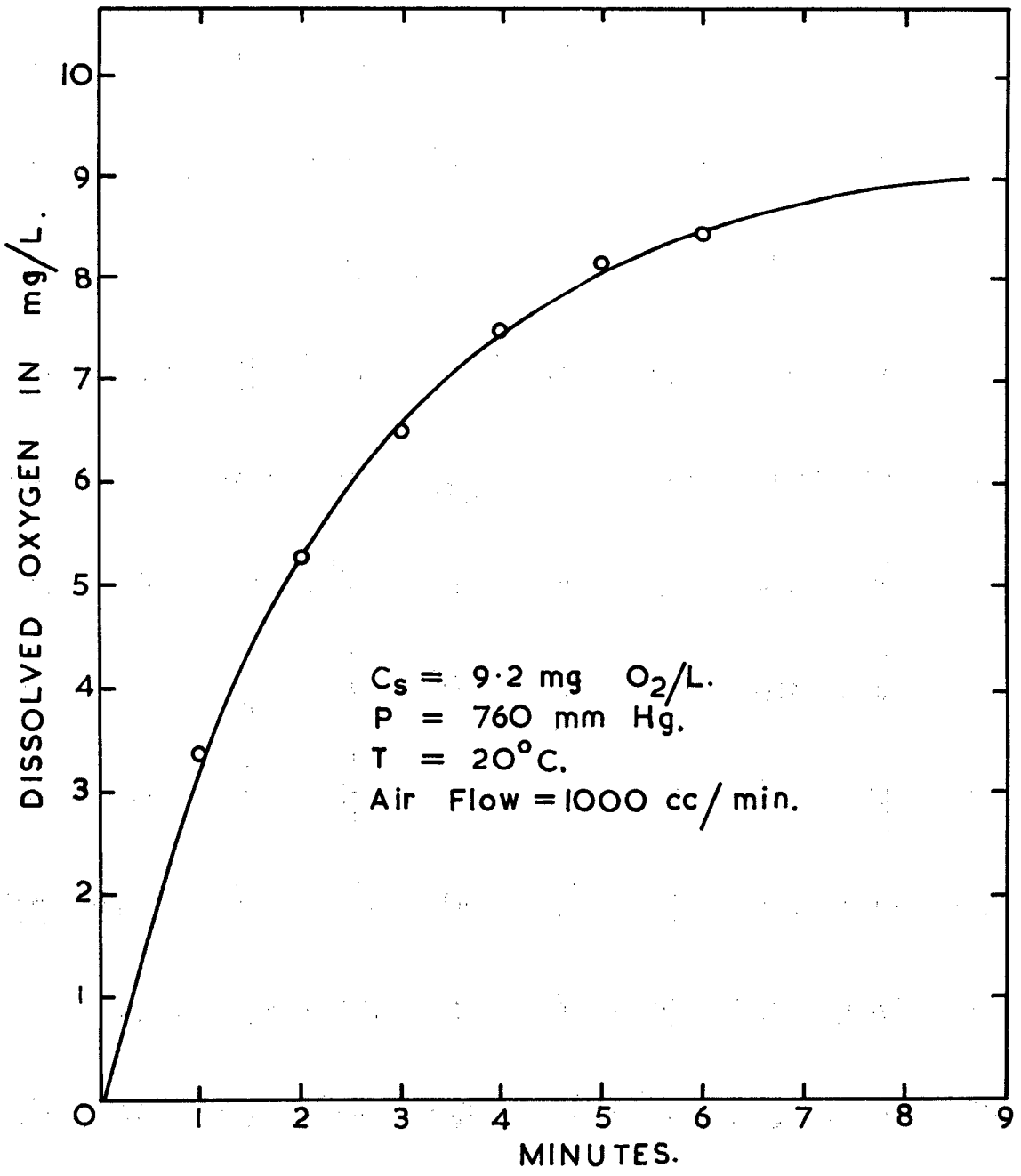

FIG 3. RATE OF OXYGENATION OF PURE WATER IN THE ACTIVATED SLUDGE UNIT.

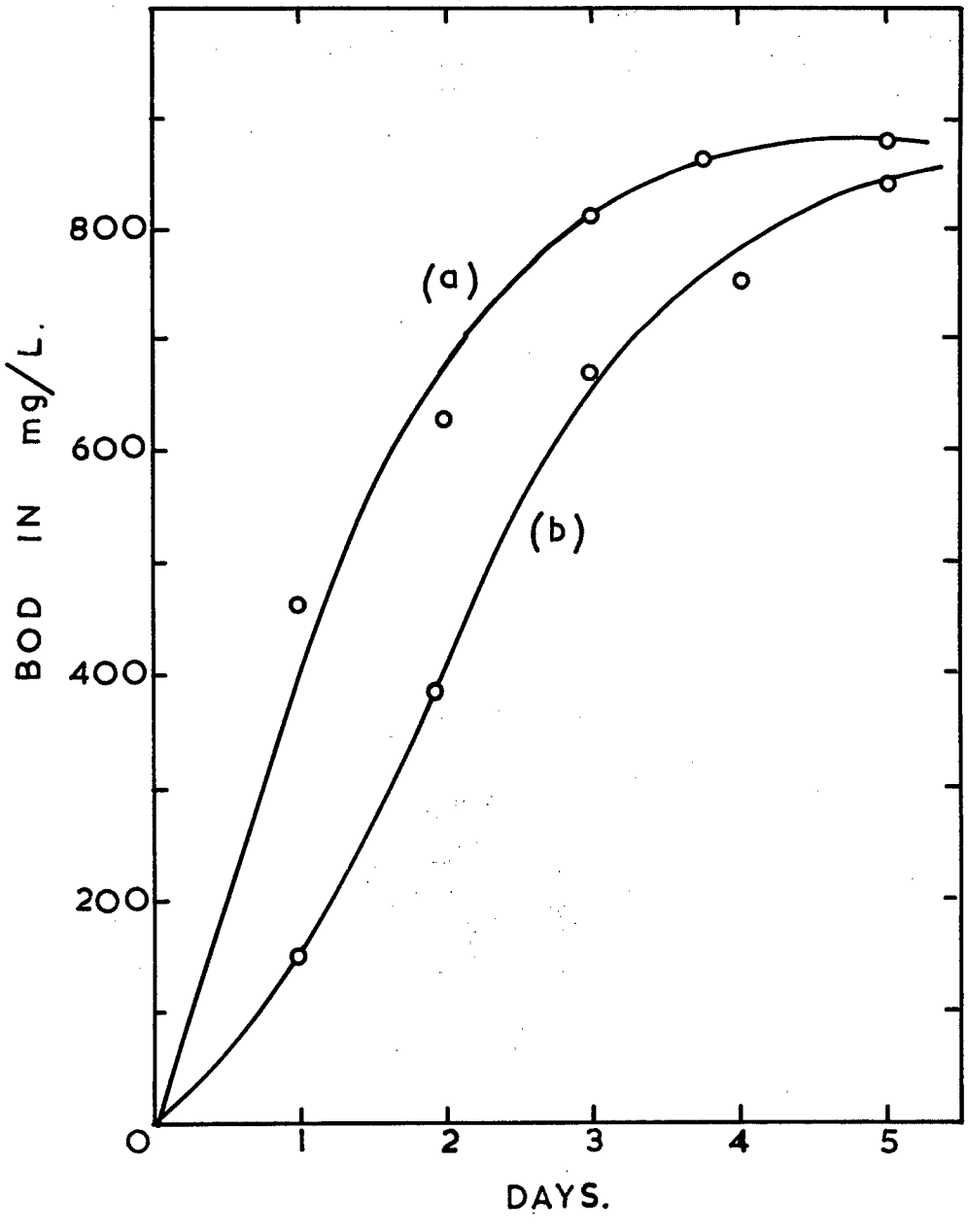

FIG 4. PROGRESSION OF THE SYNTHETIC SEWAGE BOD WHEN INOCULATED WITH SETTLED SEWAGE (a) OR ACTIVED SLUDGE MIXED LIQUOR (b).

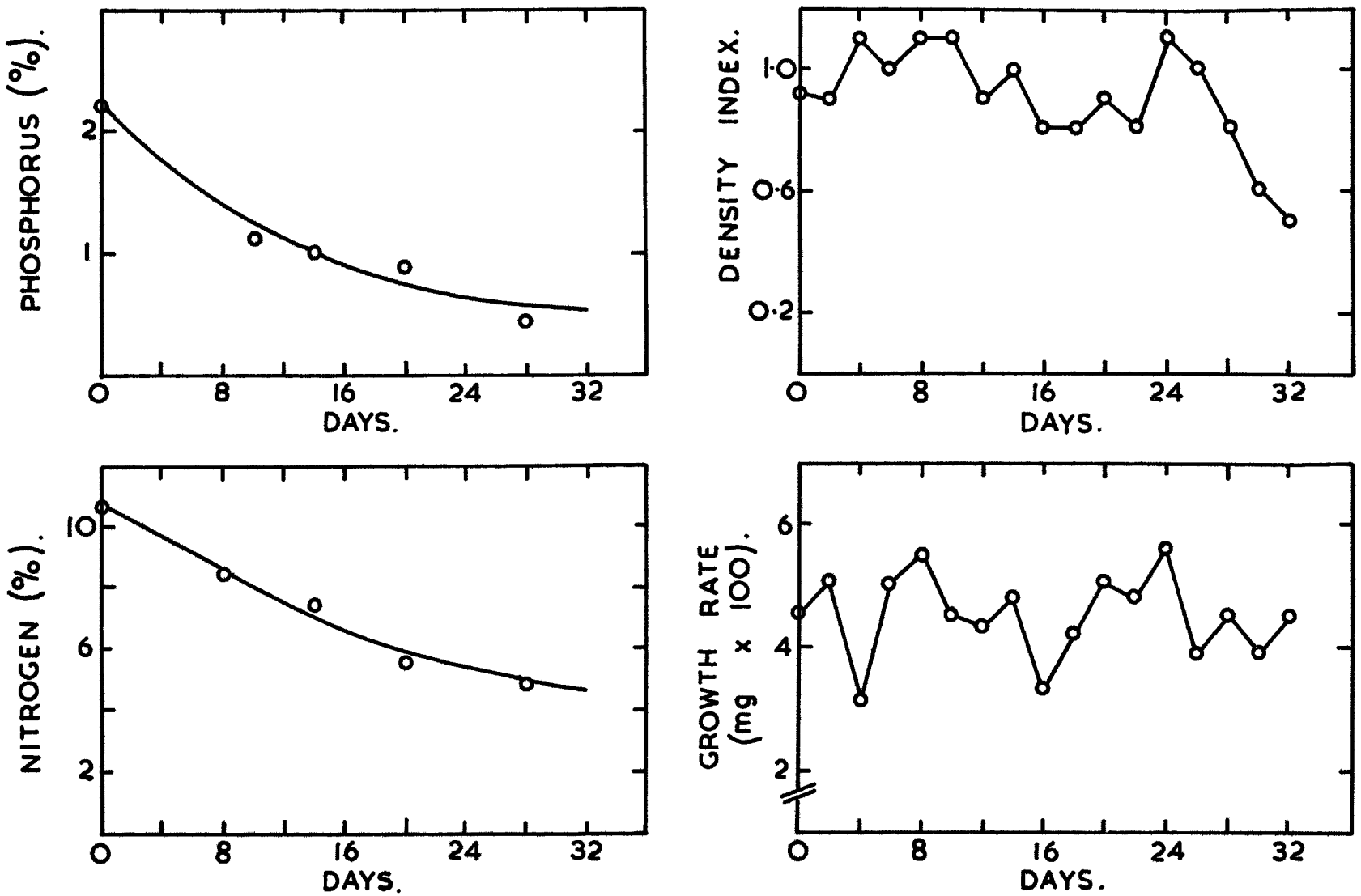

FIG 5. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT OOO.

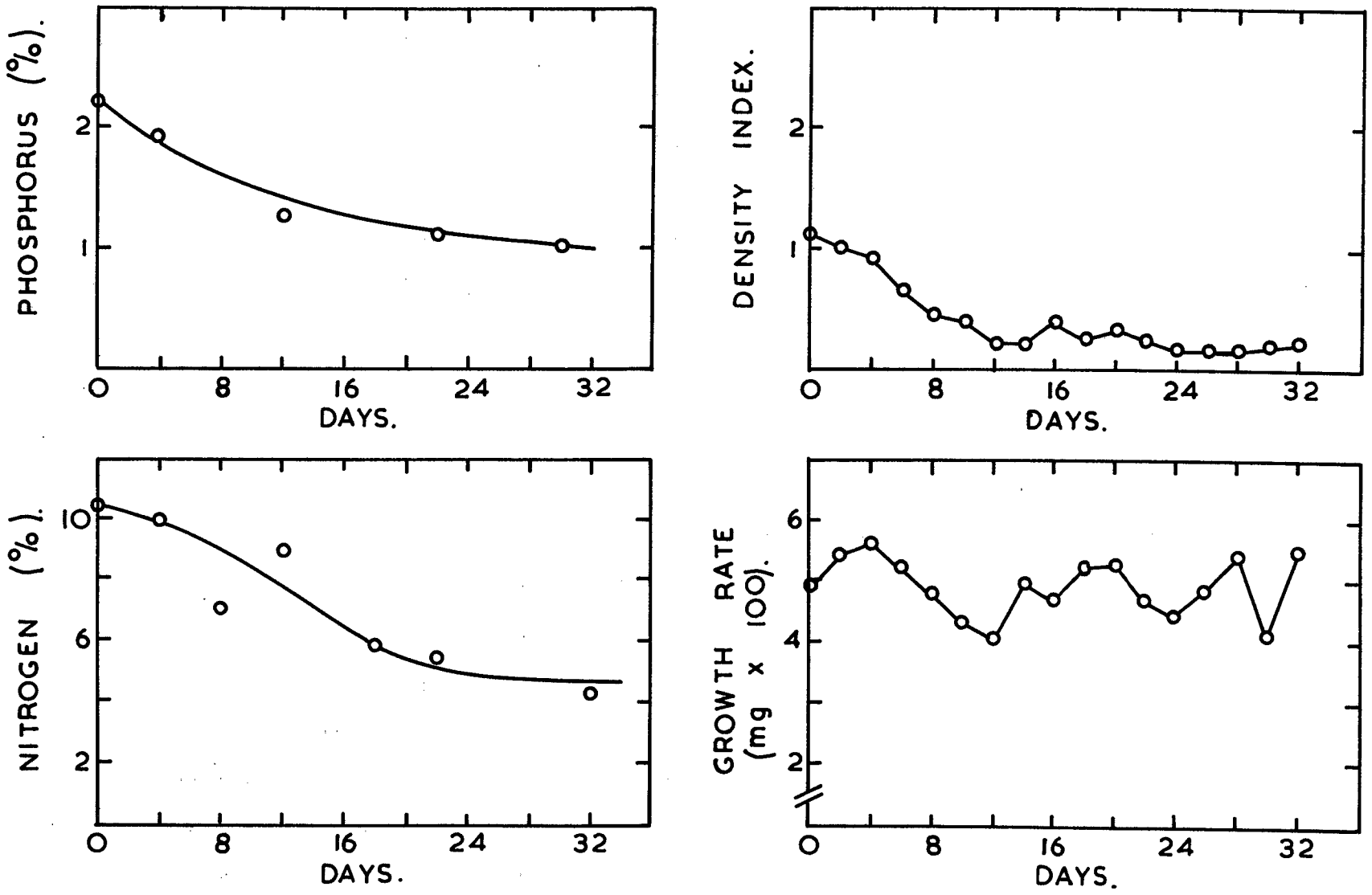

FIG 6. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT OIO.

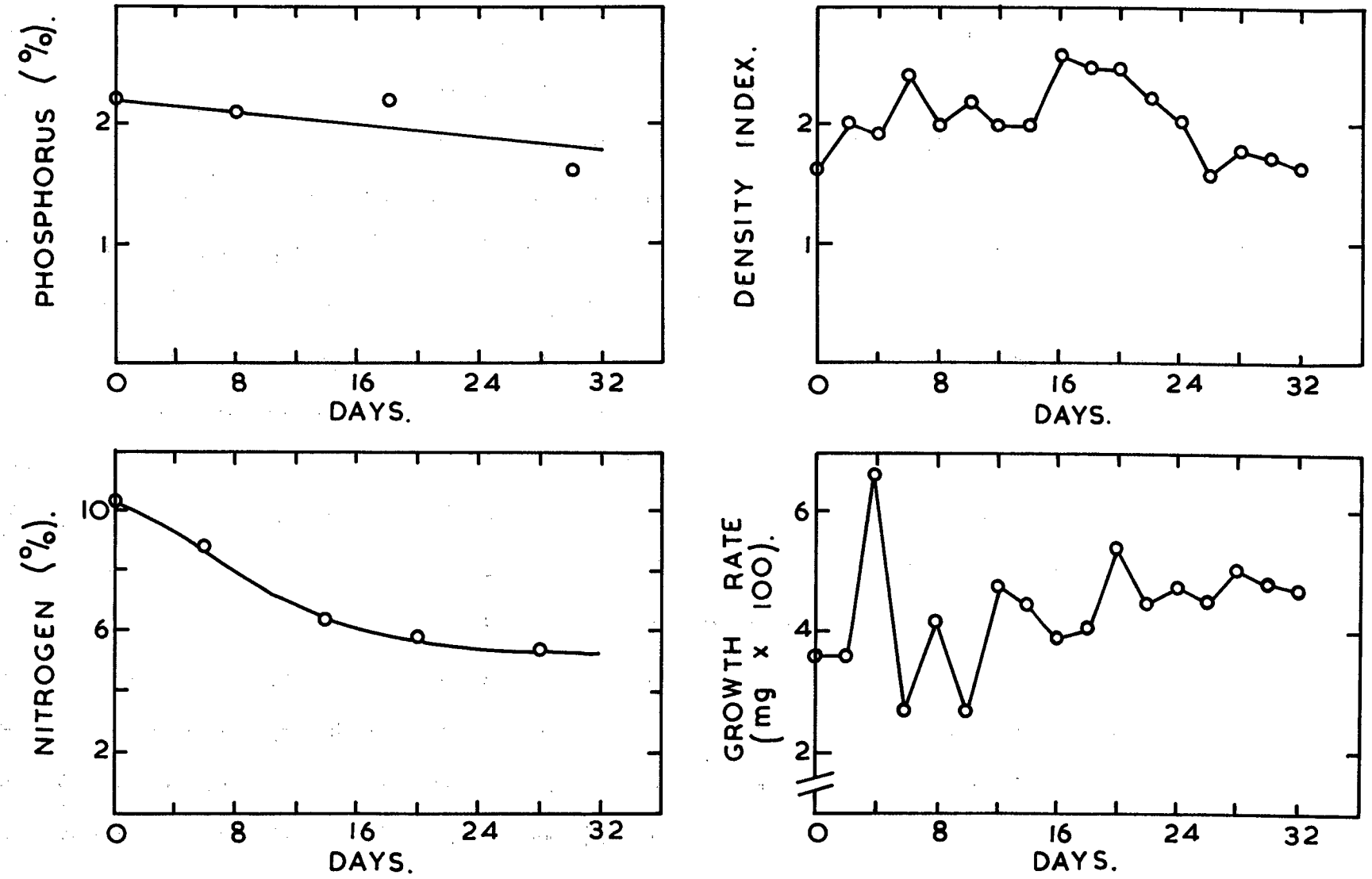

FIG 7. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT O2O.

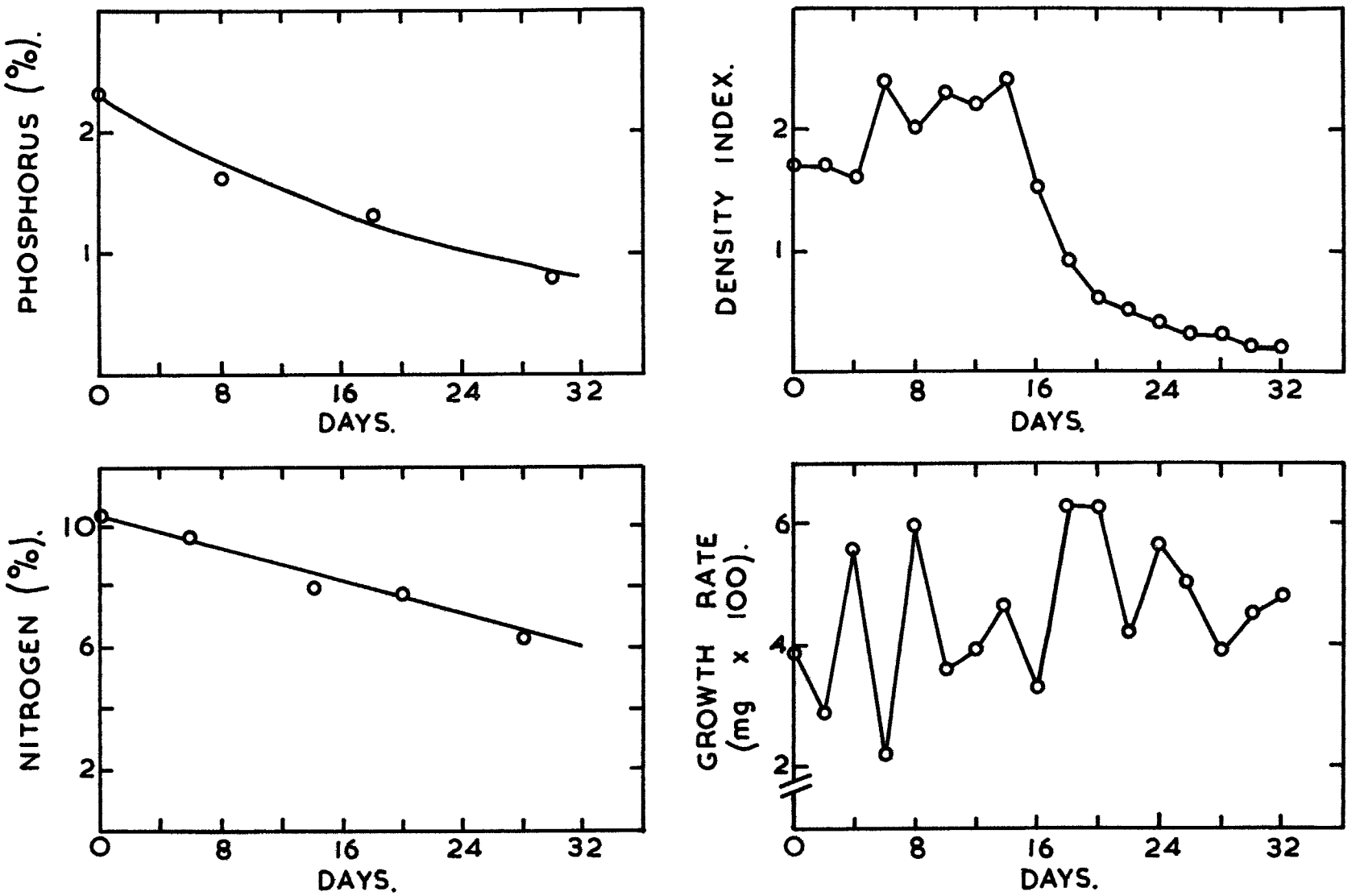

FIG 8. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 100.

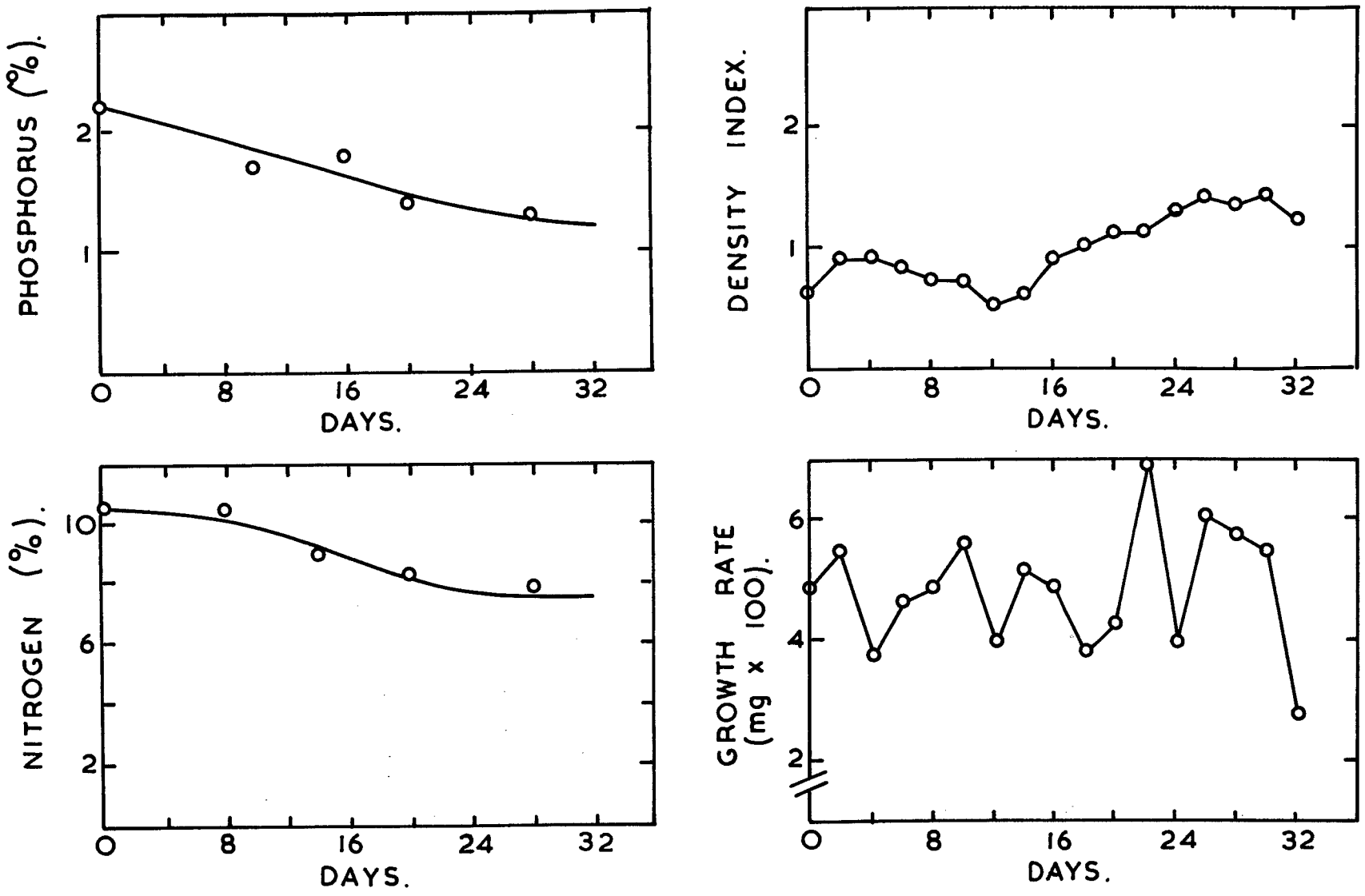

FIG 9. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 110.

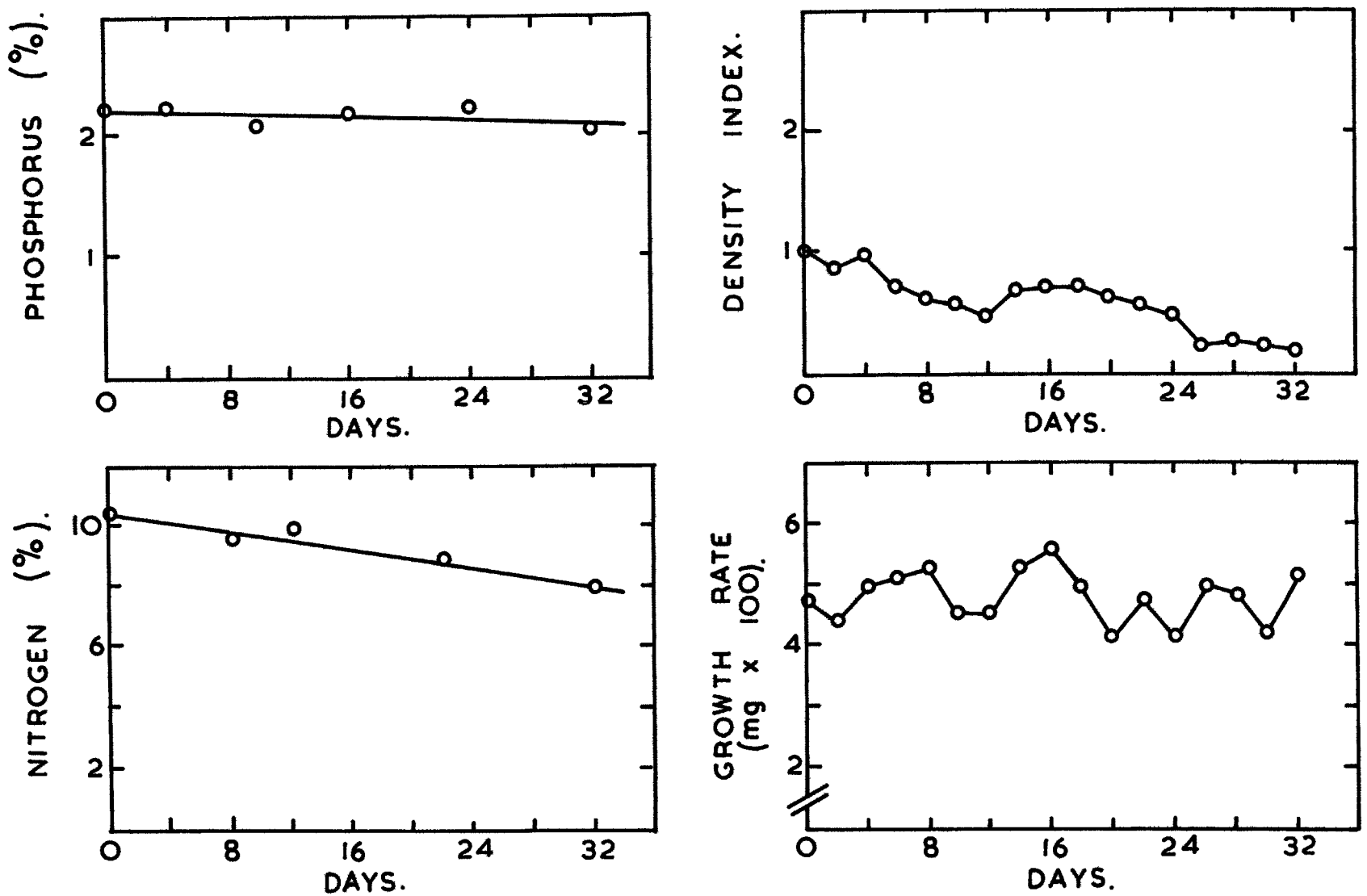

FIG 10. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE FOR TREATMENT 120.

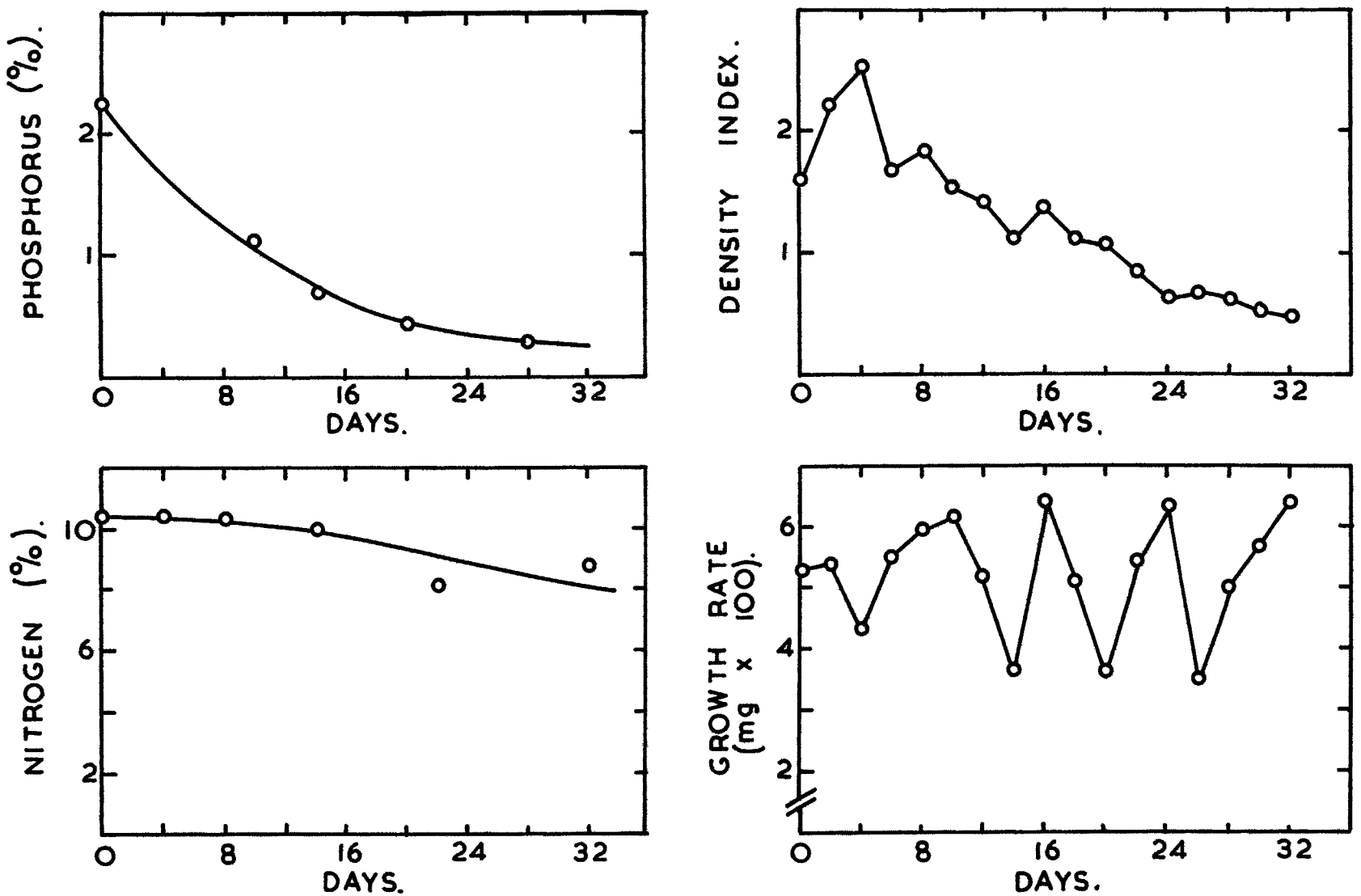

FIG 11. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 200.

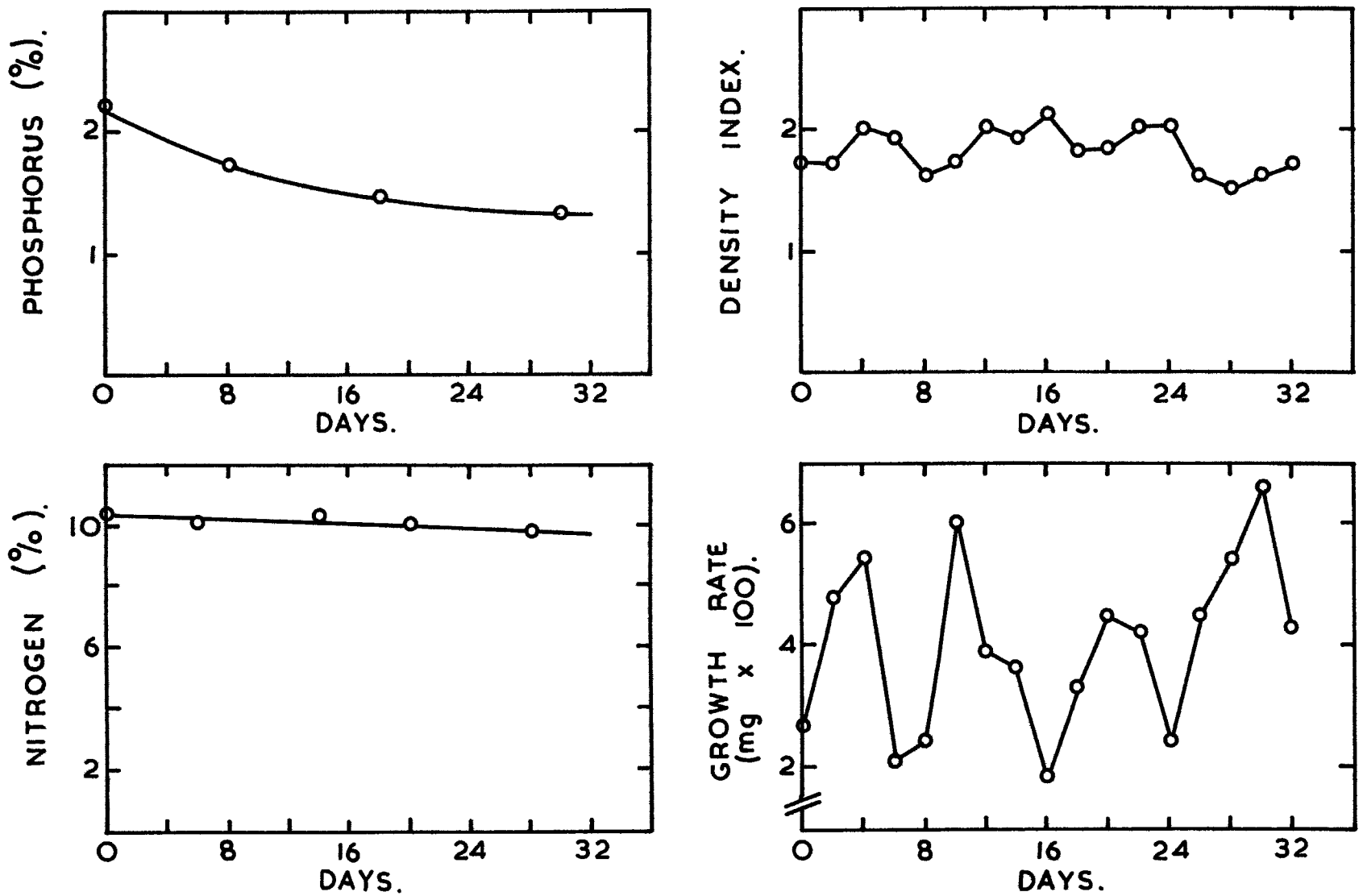

FIG 12. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 210.

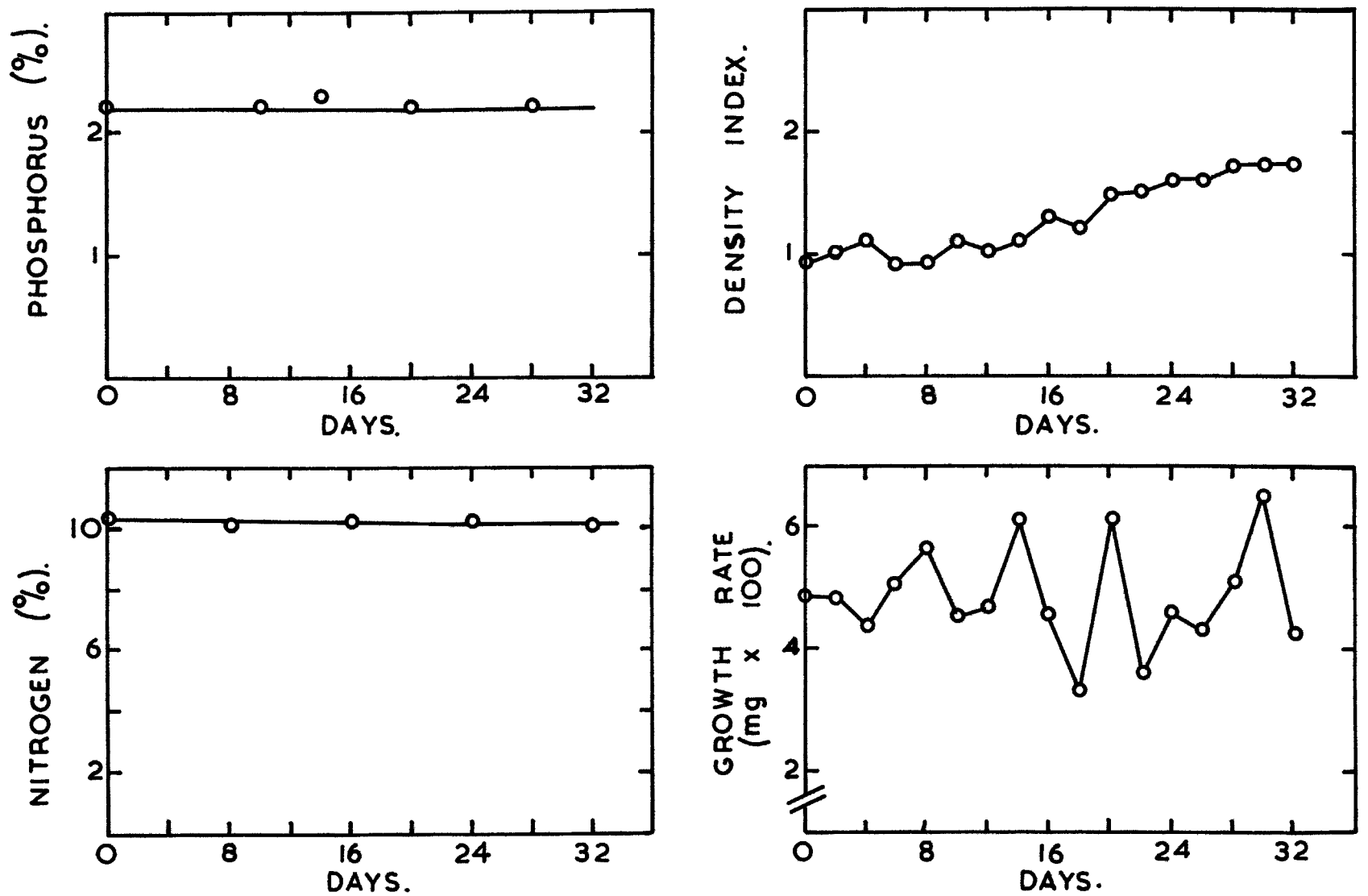

FIG 13. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 22O.

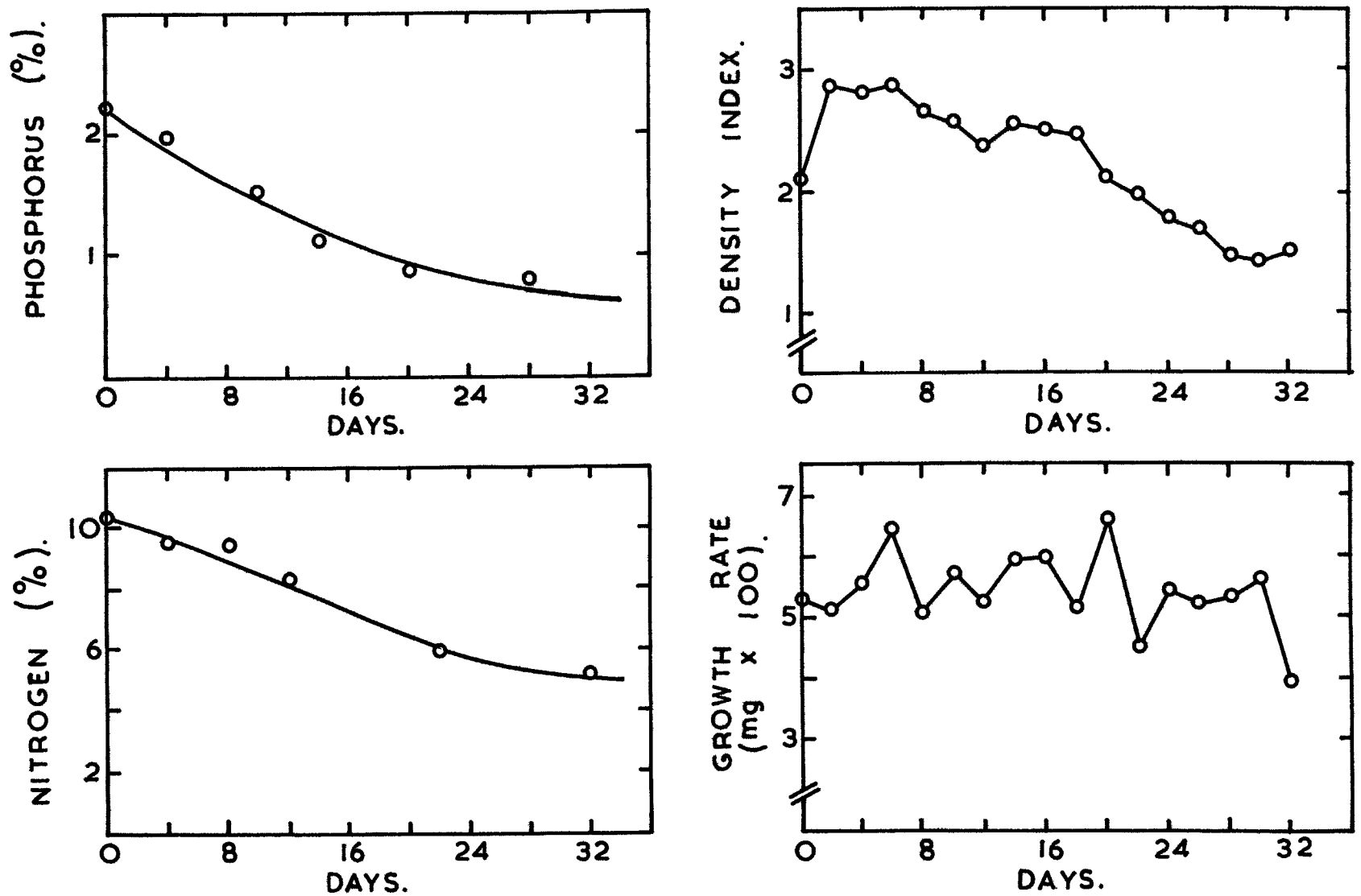

FIG 14. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 001.

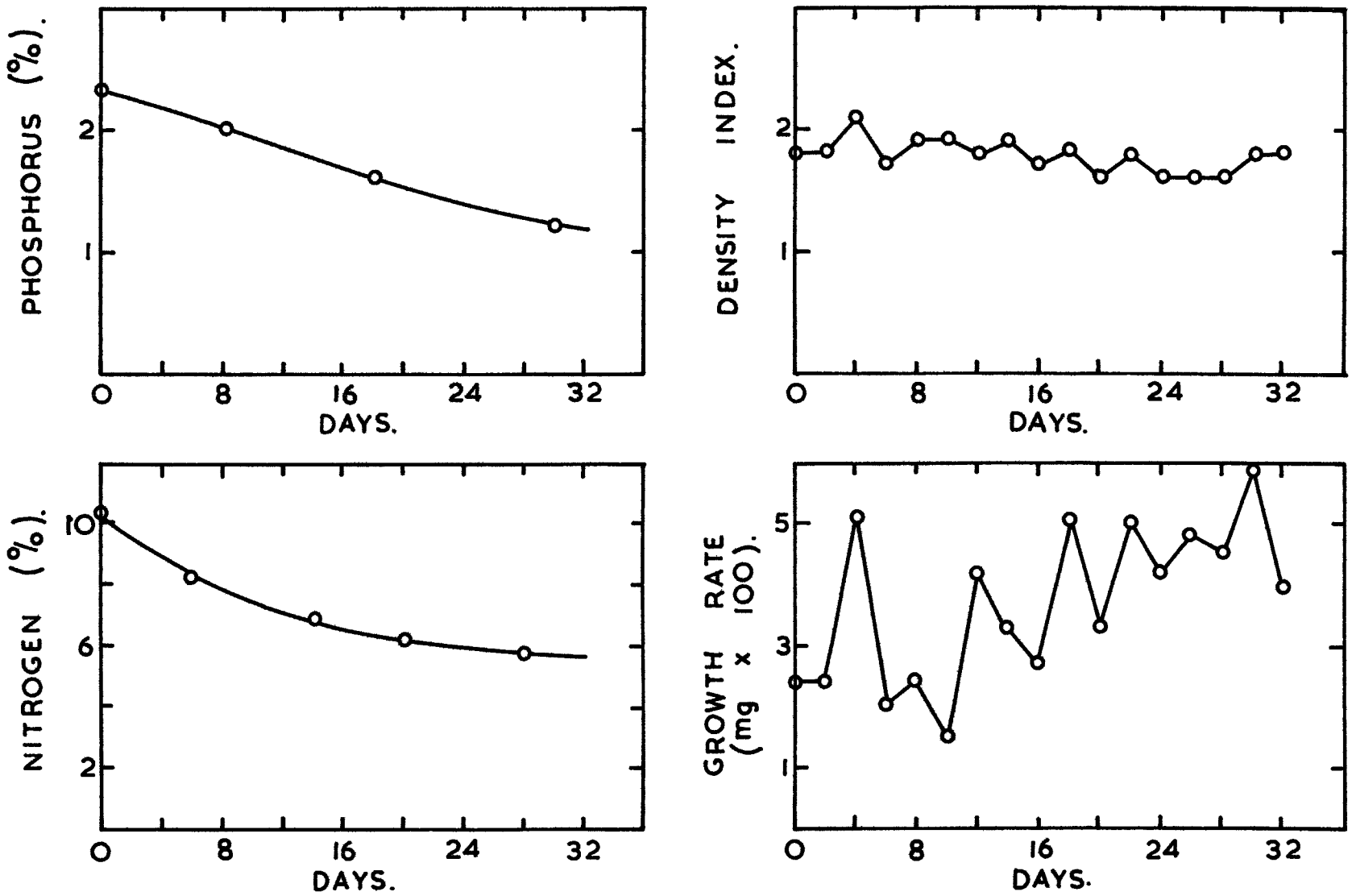

FIG 15. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT O11.

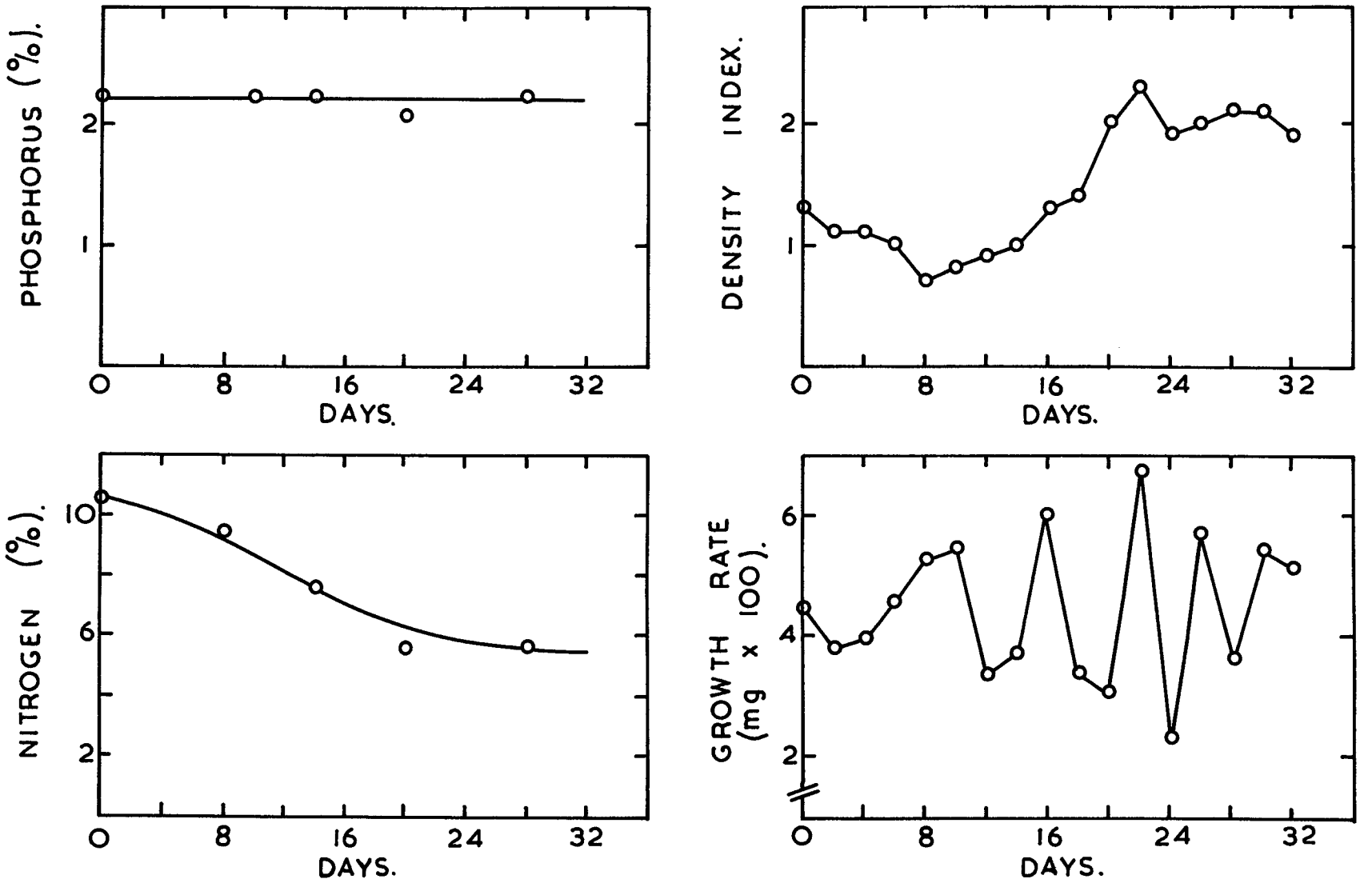

FIG 16. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT O21.

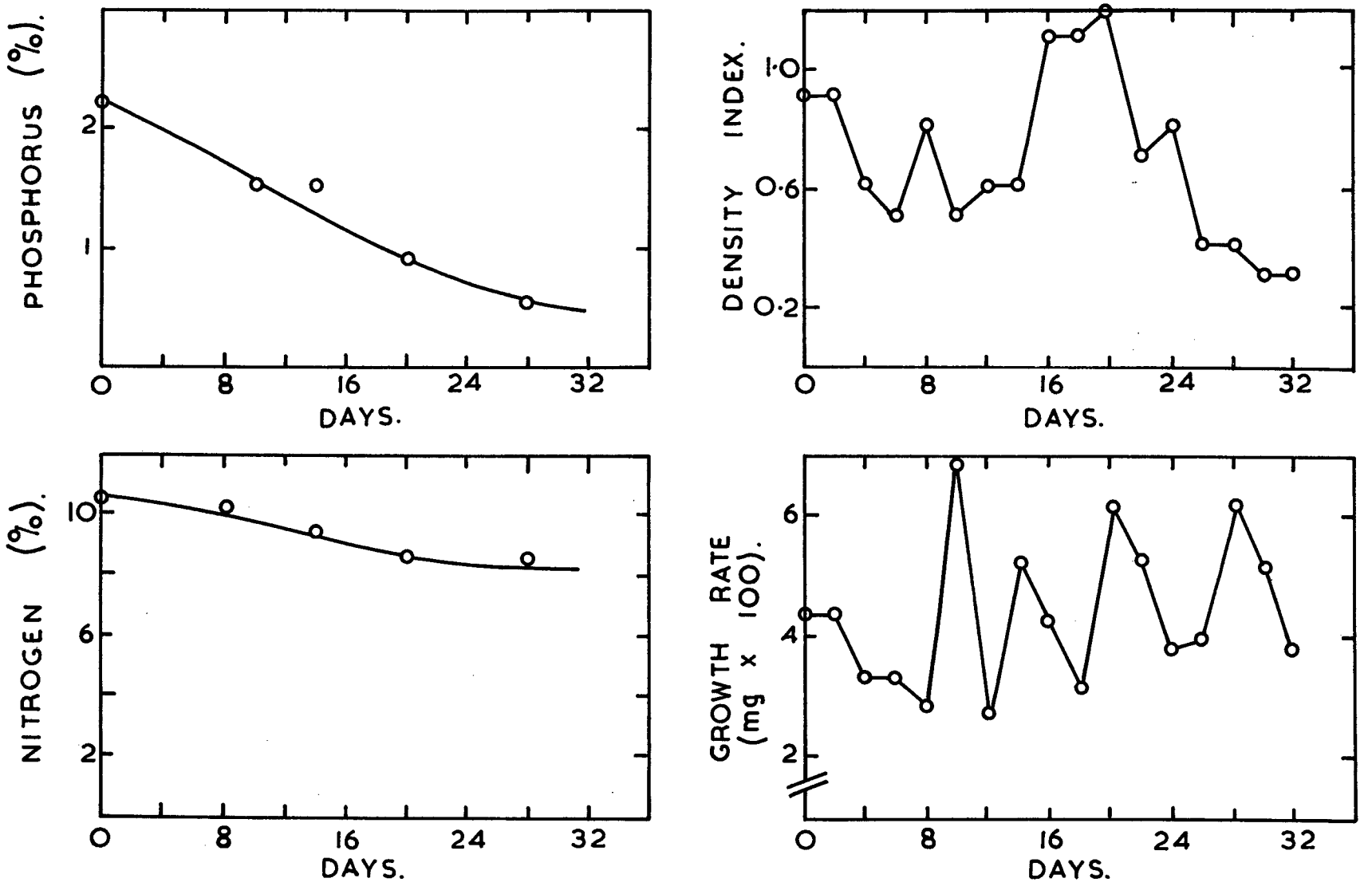

FIG 17. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 101.

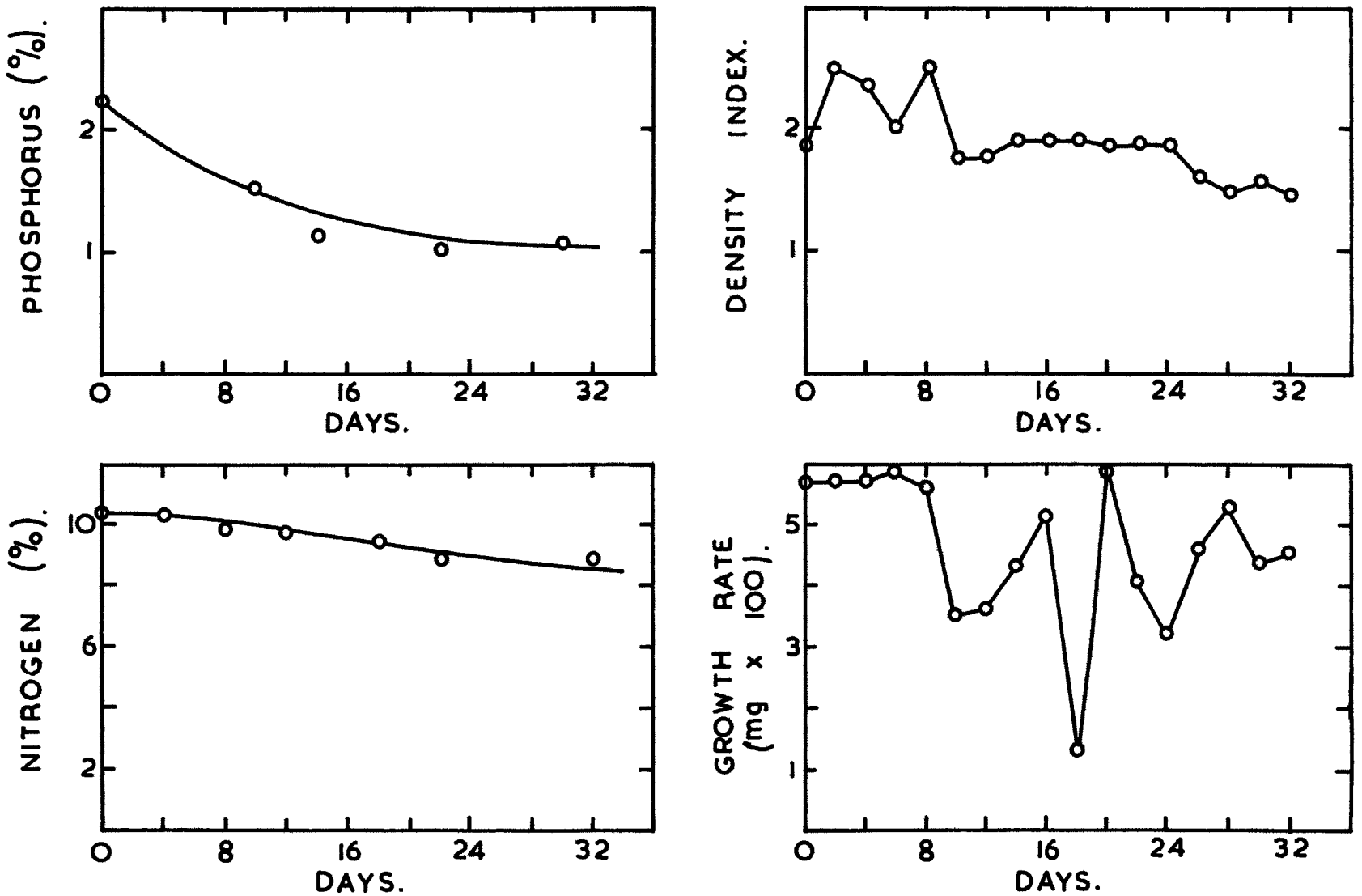

FIG 18. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT III.

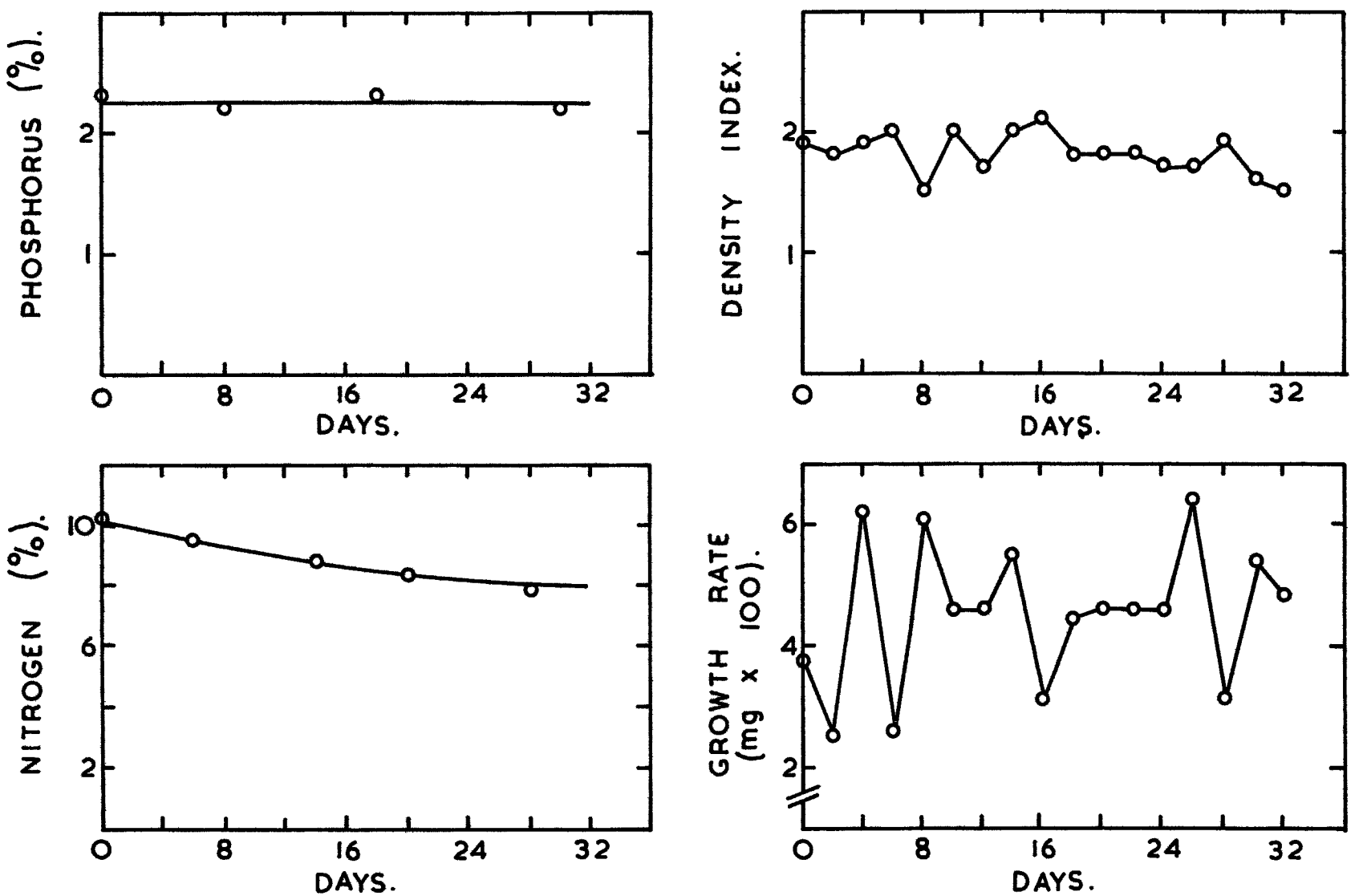

FIG 19. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 121.

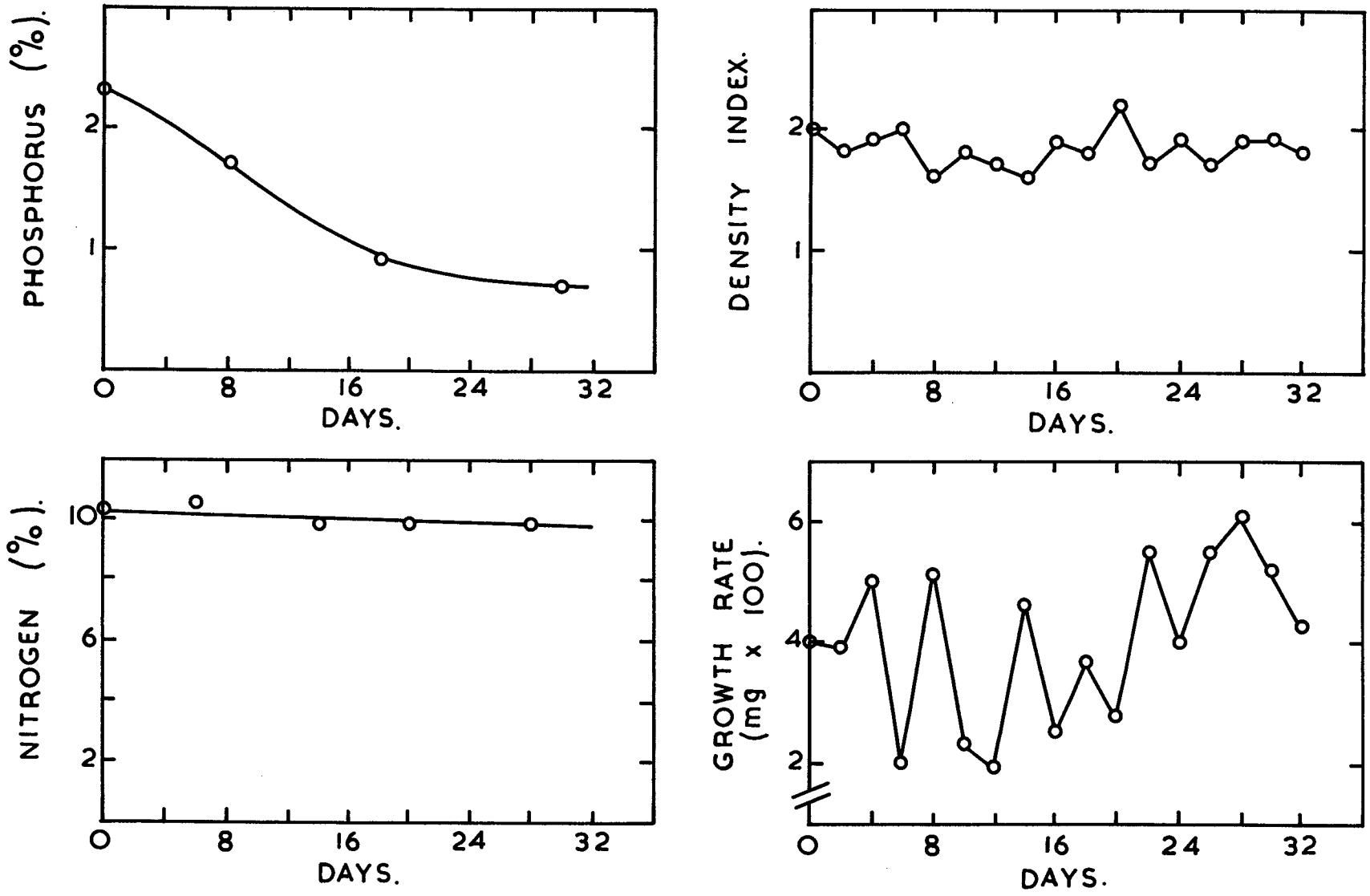

FIG 20. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 201.

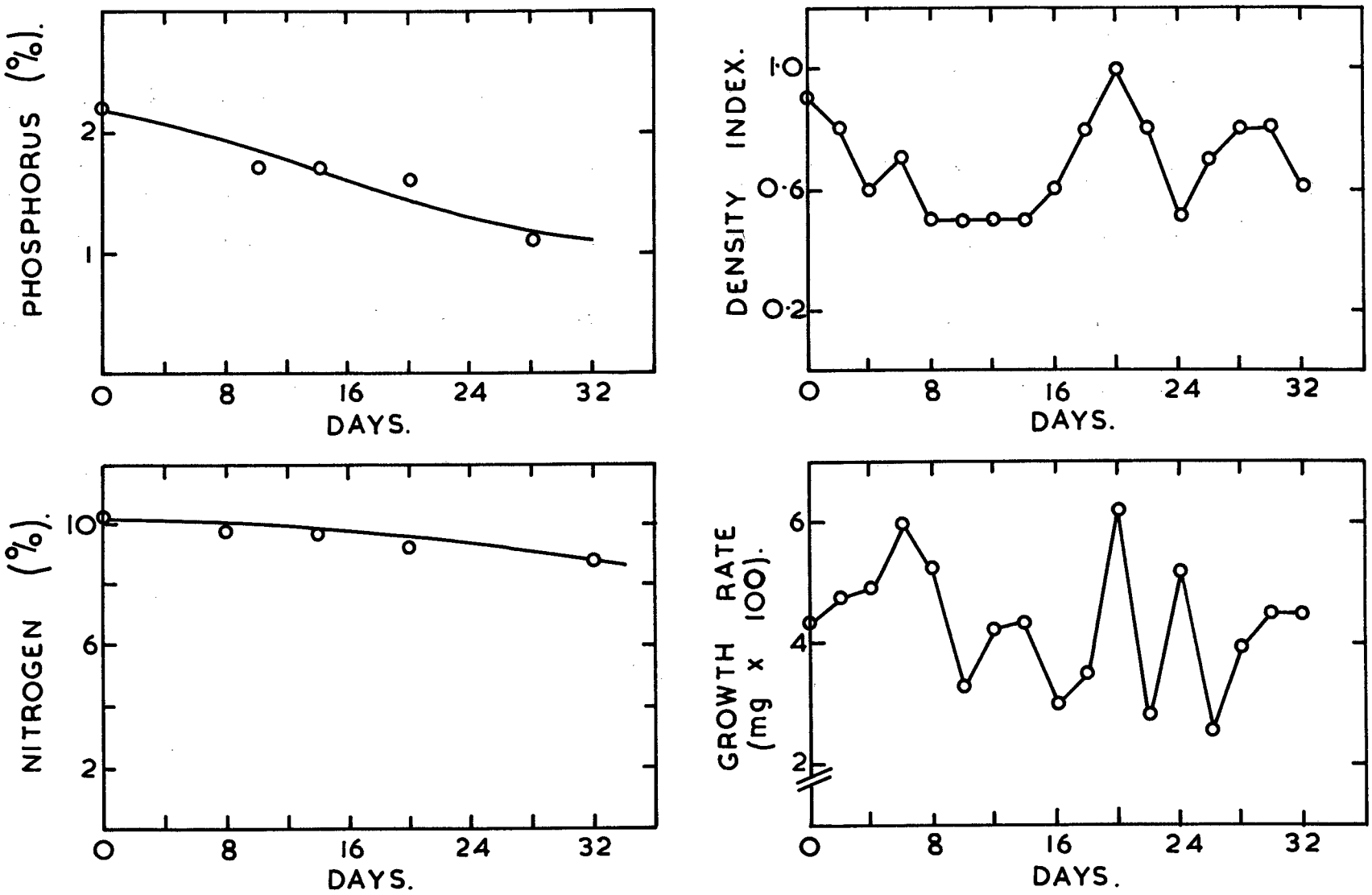

FIG 21. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 211.

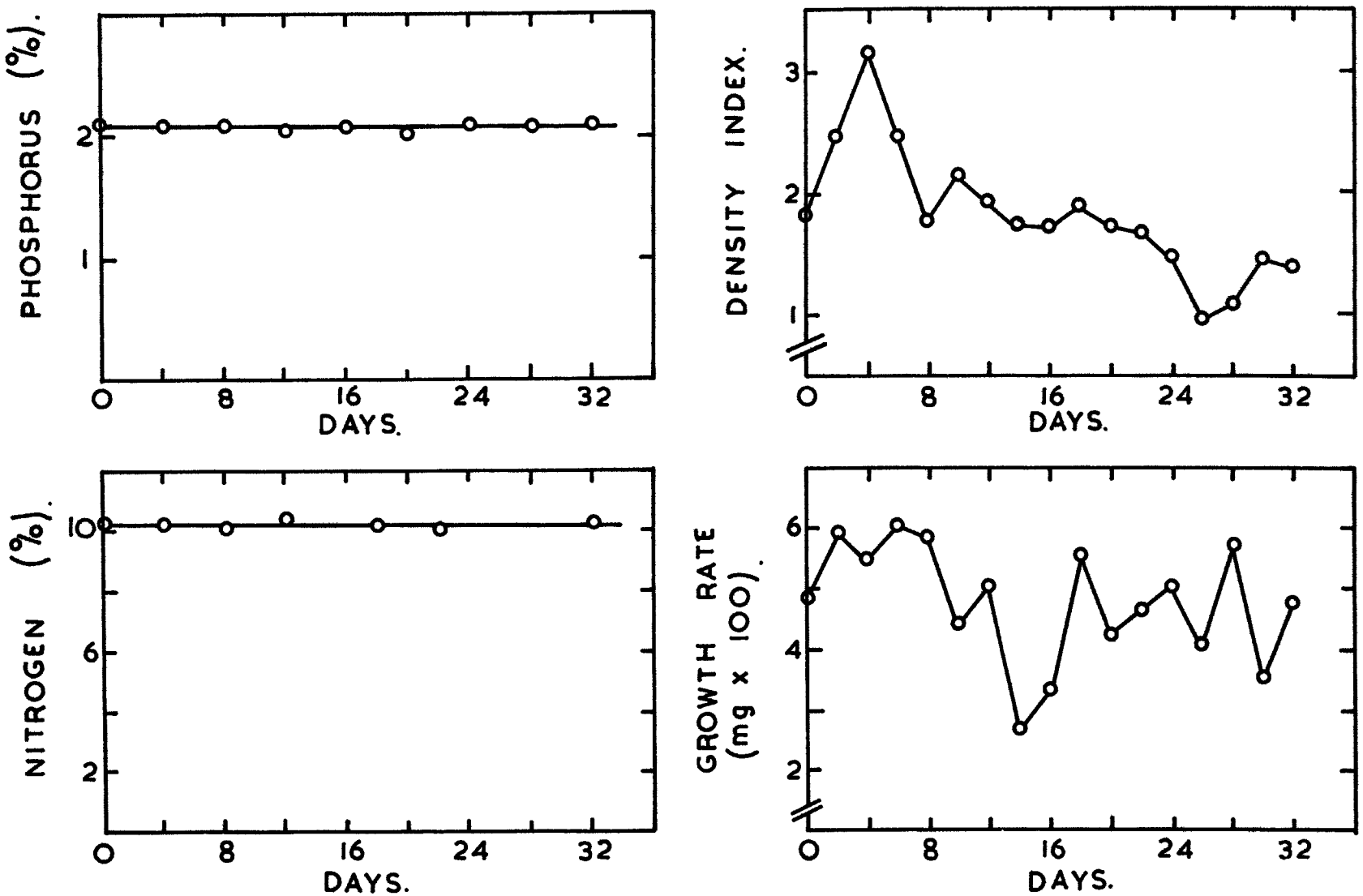

FIG 22. NITROGEN AND PHOSPHORUS CONTENT, GROWTH RATE AND DENSITY INDEX OF SLUDGE, FOR TREATMENT 221.

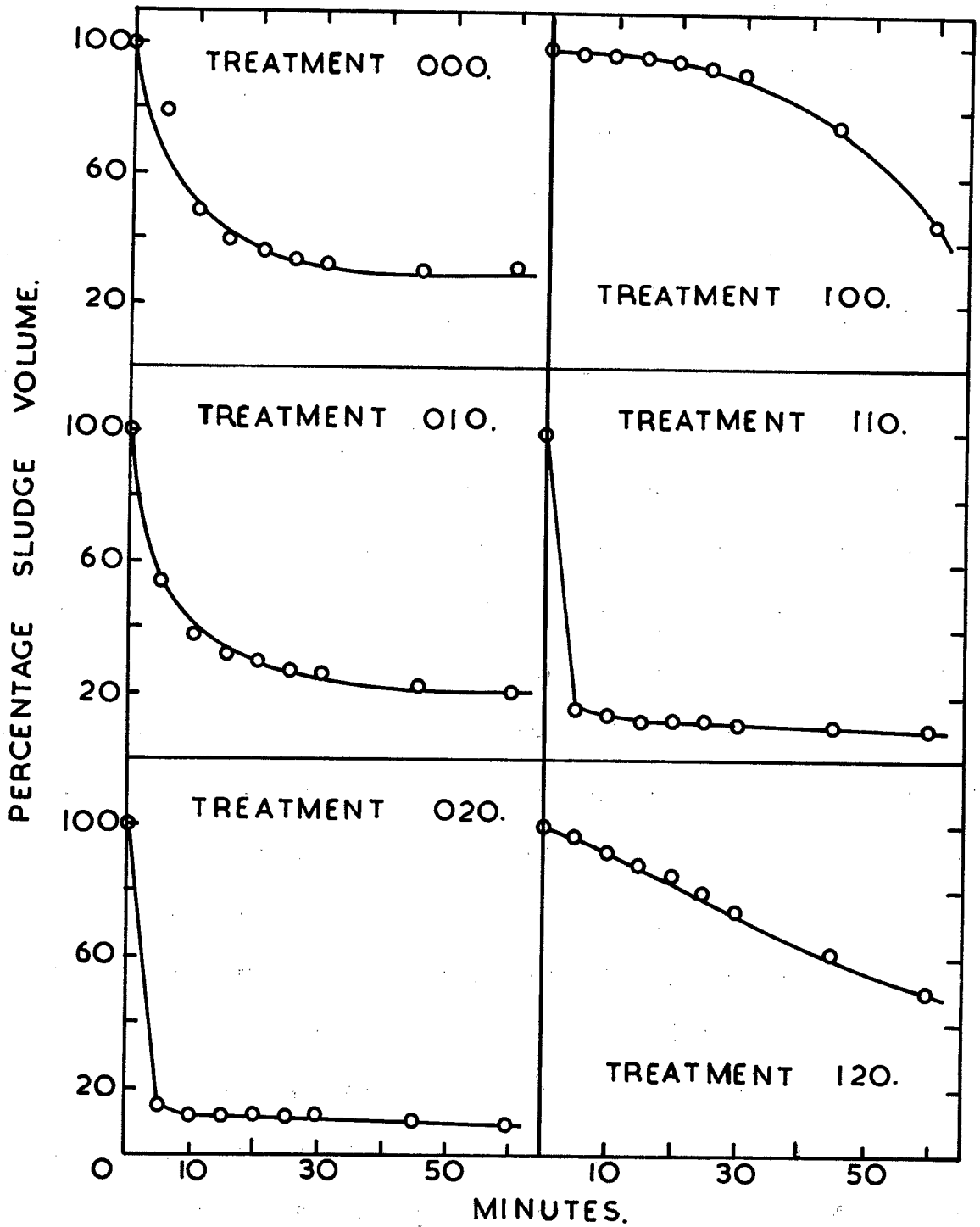

FIG 23. SETTLING RATE OF SLUDGES AFTER 32 DAYS TREATMENT AT 20°C.

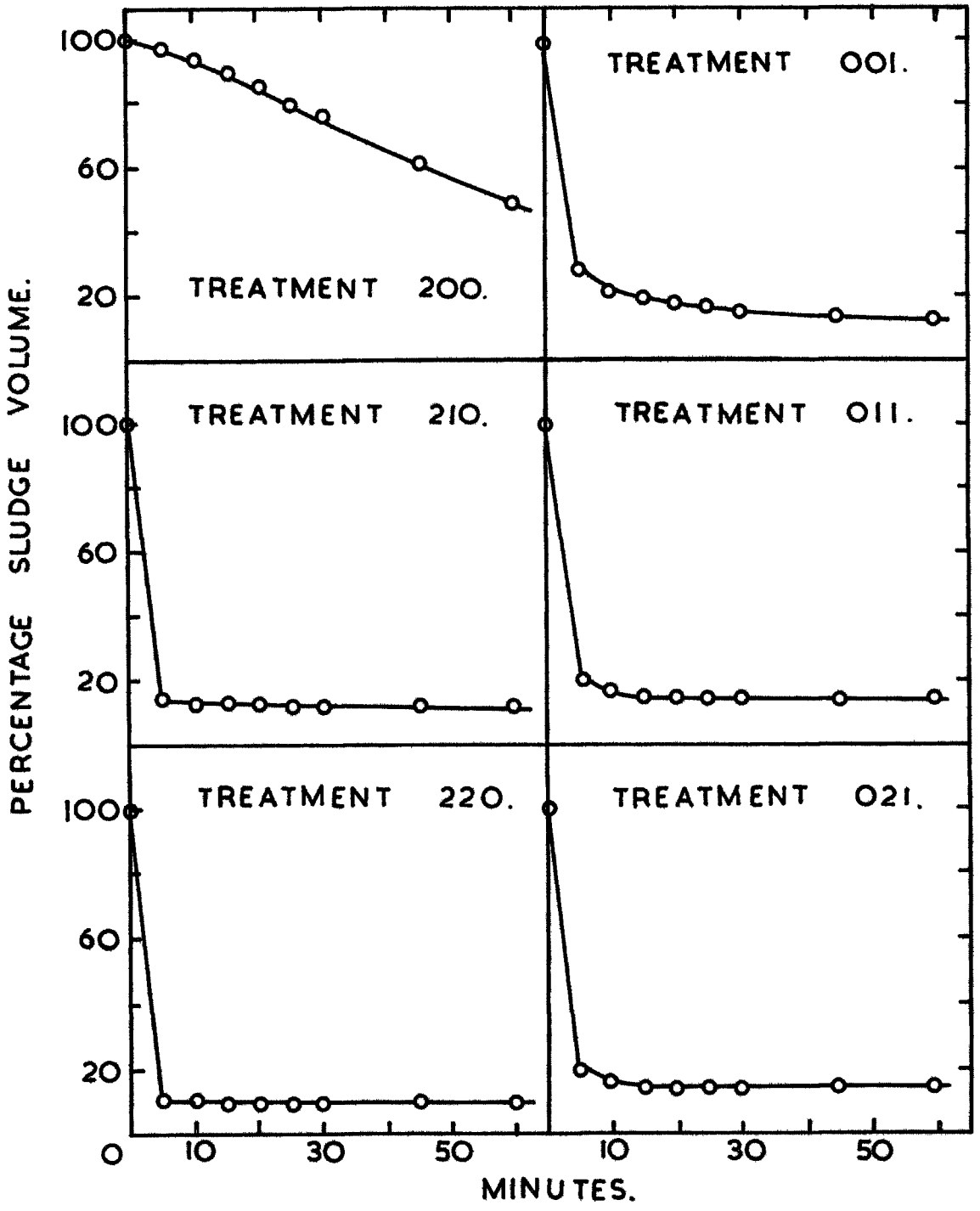

FIG 24. SETTLING RATE OF SLUDGES AFTER 32 DAYS TREATMENT AT 20°C.

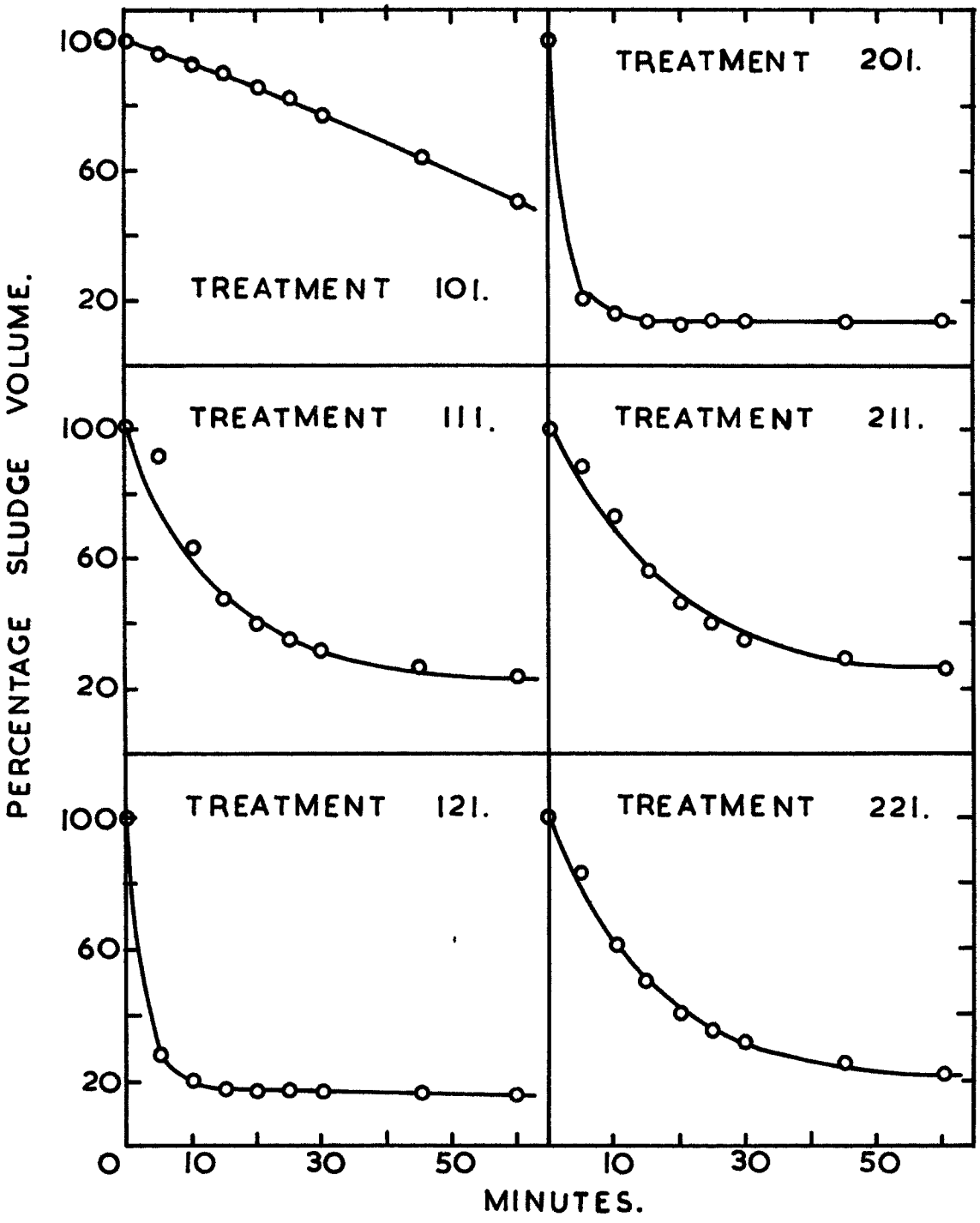

FIG 25. SETTLING RATE OF SLUDGES AFTER 32 DAYS TREATMENT AT 20°C.

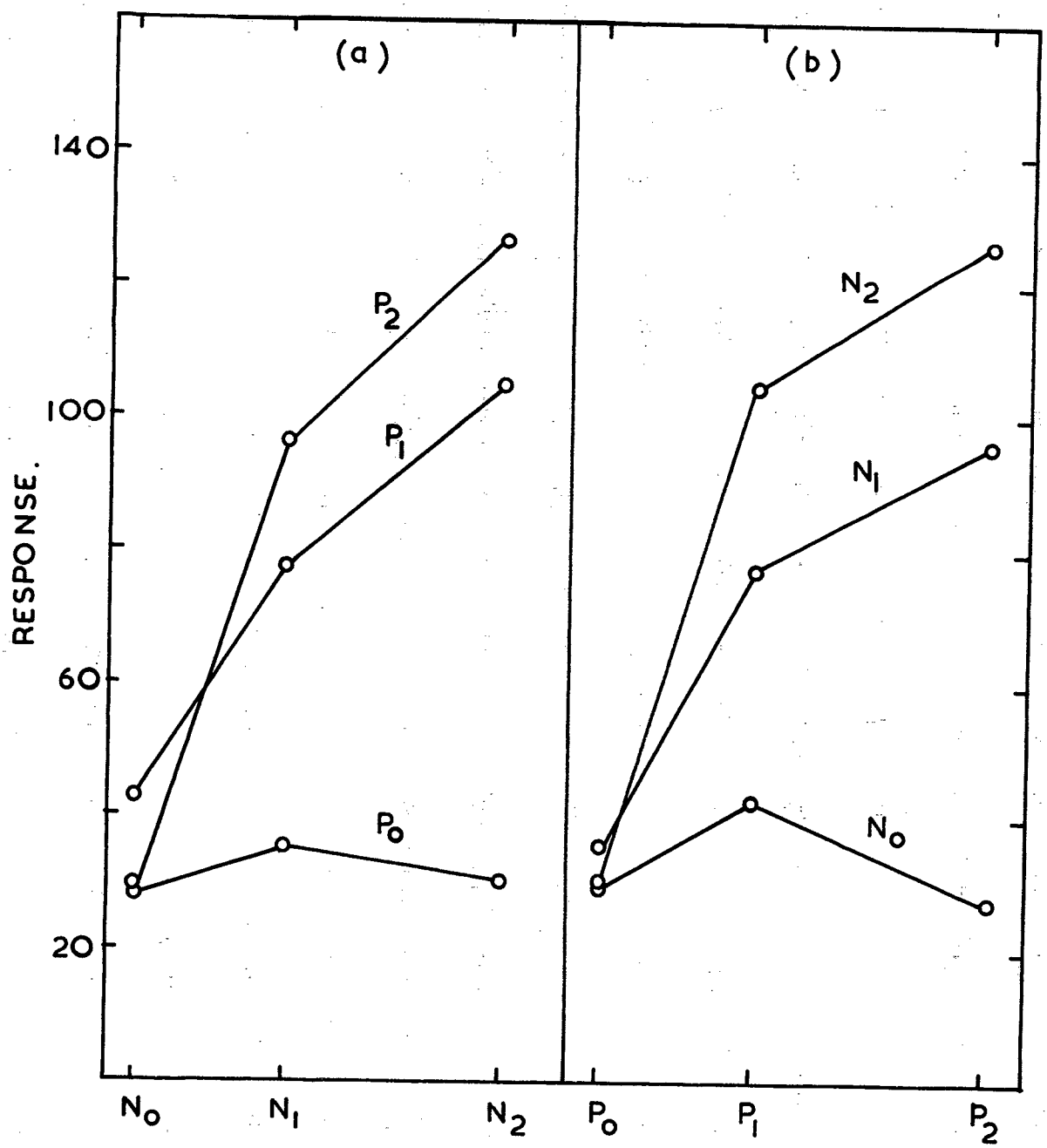

FIG 26. NITROGEN-PHOSPHORUS INTERACTION, ADJUSTED FOR BLOCK EFFECTS.

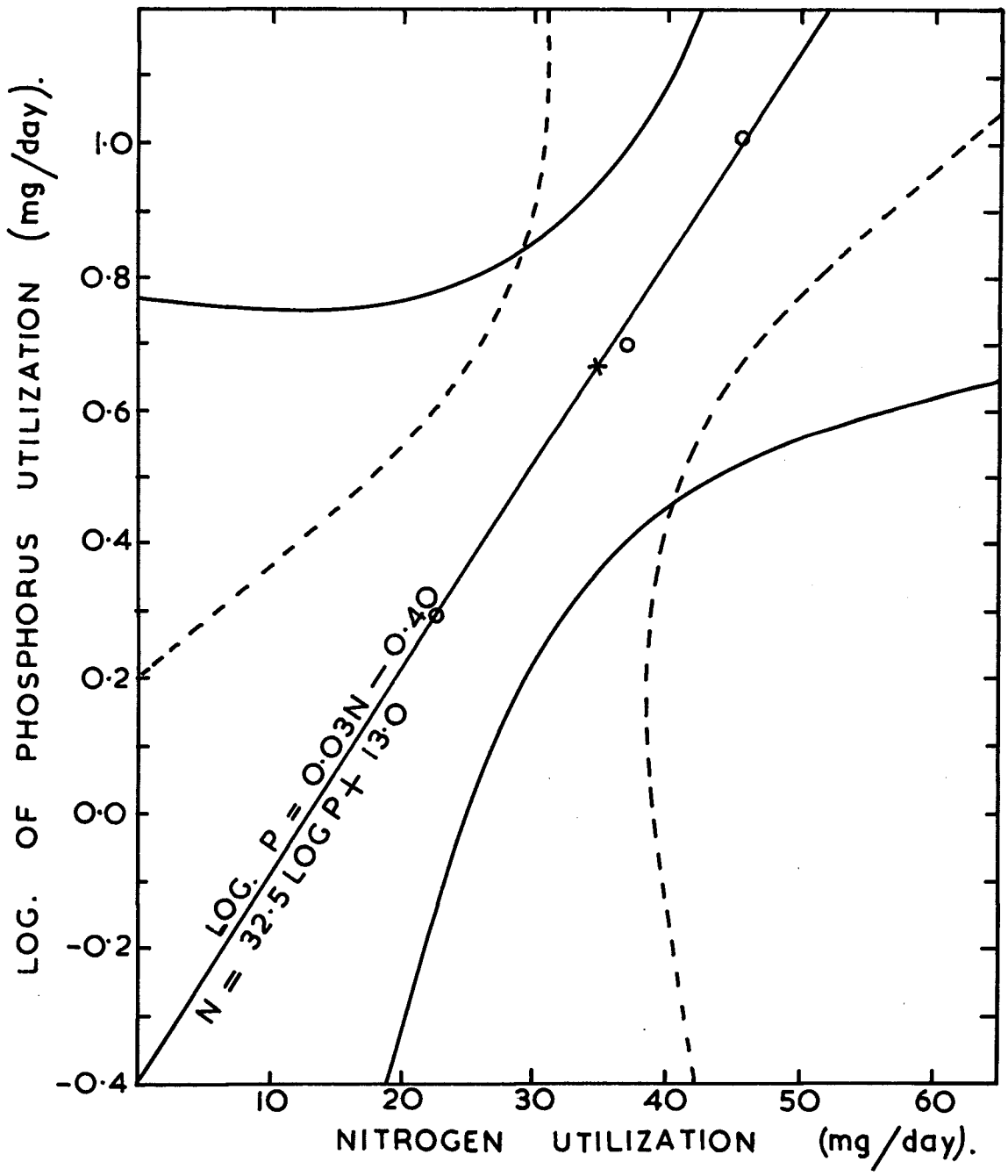

FIG 27. REGRESSION OF PHOSPHORUS UTILIZATION WITH NITROGEN UTILIZATION. THE REGRESSION LINE WAS PLOTTED FOR THE AVERAGE VALUES FROM TABLE . THE 95 % CONFIDENCE INTERVALS FOR THE NITROGEN-PHOSPHORUS (------) AND PHOSPHORUS-NITROGEN (——) REGRESSION ARE ALSO SHOWN.

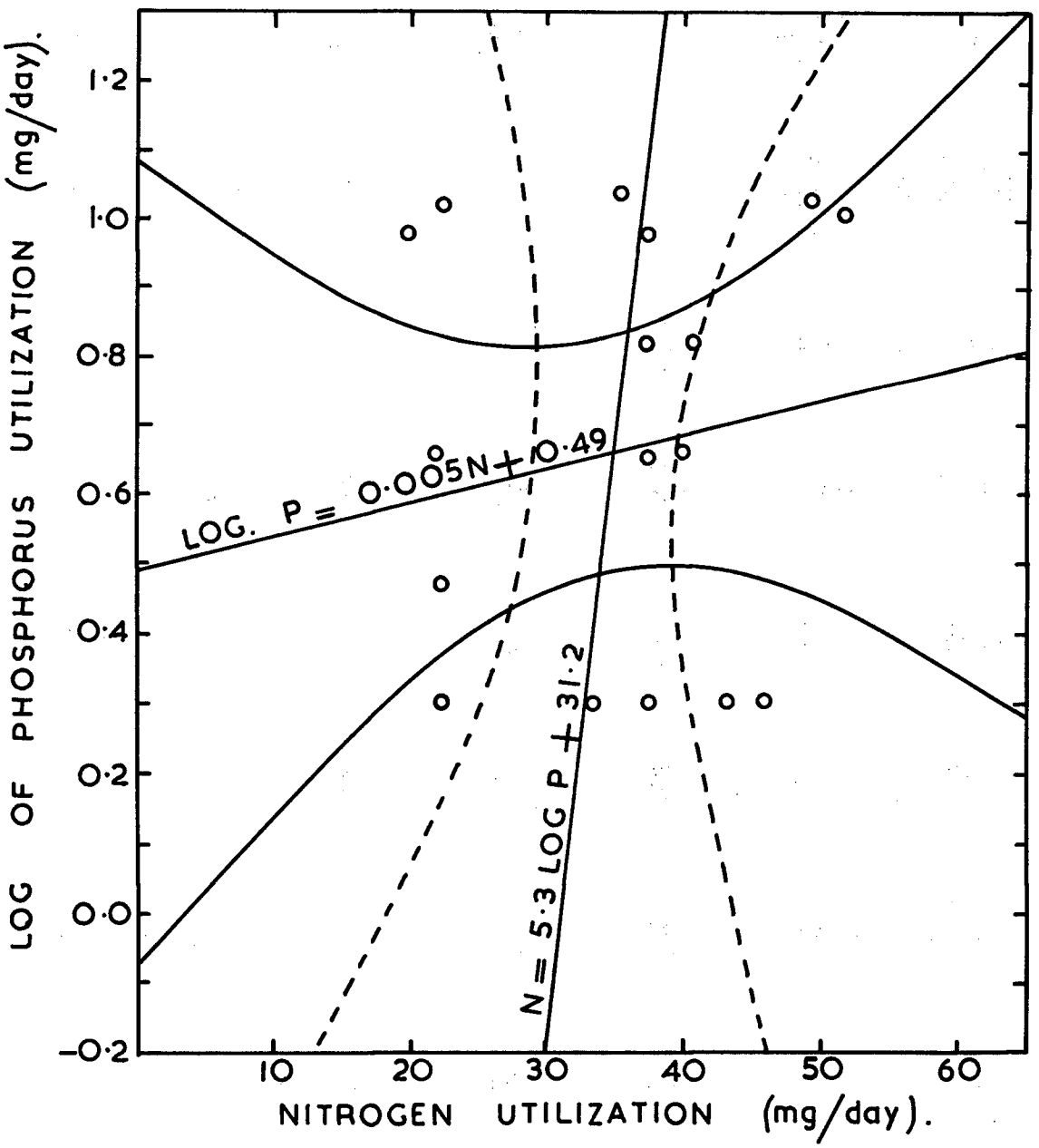

FIG 28. REGRESSION OF PHOSPHORUS UTILIZATION WITH NITROGEN UTILIZATION. THE REGRESSION LINE WAS PLOTTED FOR THE INDIVIDUAL VALUES FROM TABLE . THE 95% CONFIDENCE INTERVALS FOR THE NITROGEN-PHOSPHORUS (-----) AND PHOSPHORUS-NITROGEN (———) REGRESSION ARE ALSO SHOWN.

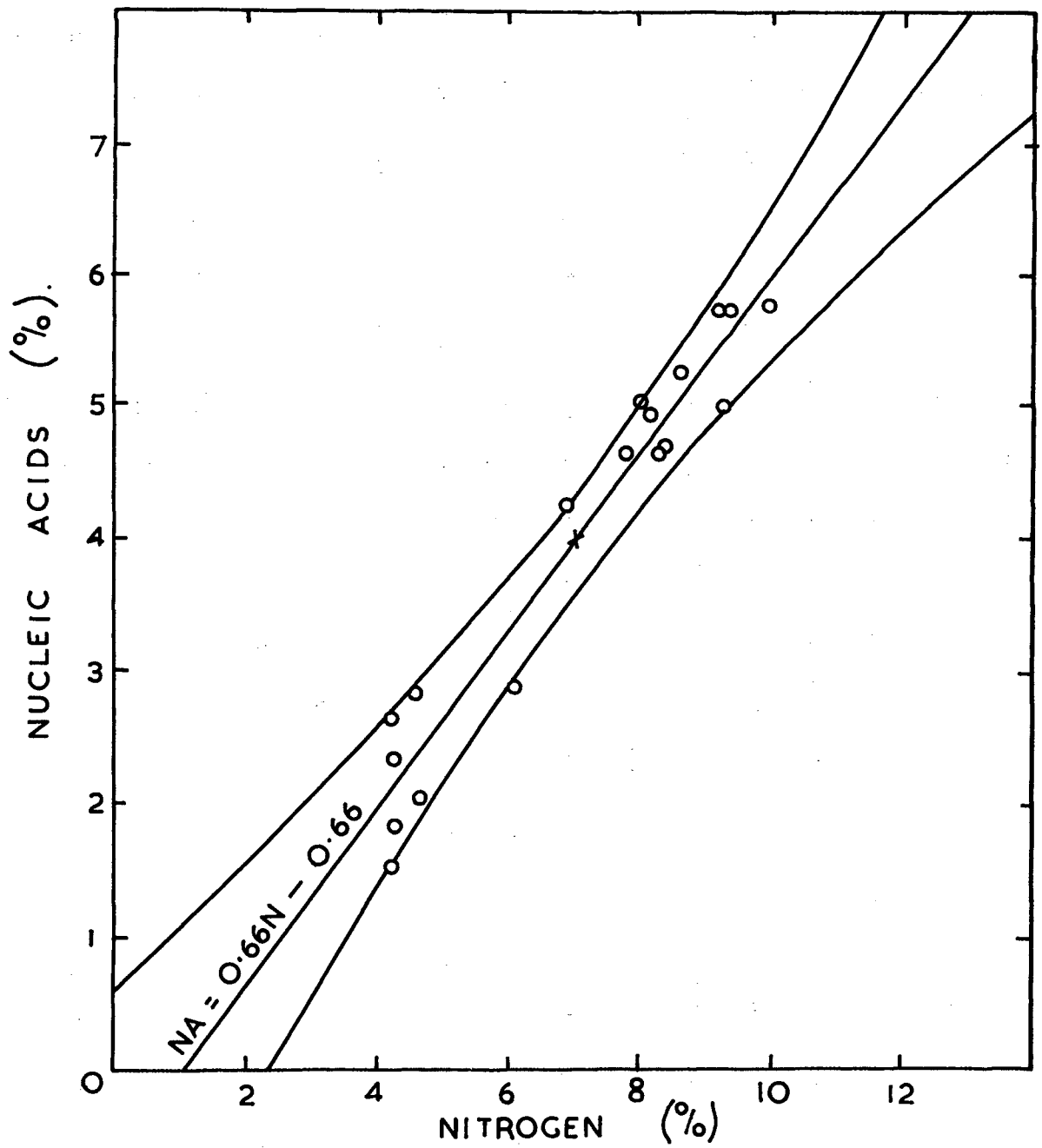

FIG 29 THE CORRELATION BETWEEN THE NITROGEN AND NUCLEIC ACID CONTENT OF THE SLUDGES. THE 95% CONFIDENCE BELT IS ALSO SHOWN.