STUDIES ON THE EFFECTS OF SOME HERBICIDES ON SOIL NITRIFICATION

BY

ABIODUN CRAIG 'DEBONA

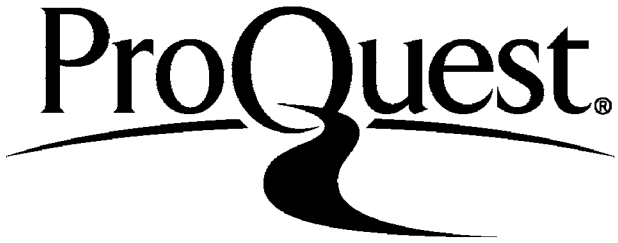
A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE FACULTY OF SCIENCE IN THE UNIVERSITY OF LONDON.

August 1967,

Bedford College,

Regent's Park,

London, N.W.1



ACKNOWLEDGEMENTS

I wish to thank Professor L.J. Audus, my supervisor, for helpful criticism during the preparation of this thesis, and the Ministry of Overseas Development, London, for the award of a SCAAP Fellowship from November 1964 to September 1967.

My thanks are also due to Mr. J.C. Funnel for technical assistance and to Dr. J. Prebble of the Biochemistry Department for the use of the fermentor.

Thanks also to Plant Protection Ltd., Shell Research, May and Baker, Fisons, Dow Chemicals and Monsanto Chemical Co. for the gift of herbicides; to Dr. Nutman, Rothamsted Experimental Station, for the gift of Nitrosomonas cultures; and to the Professor of Microbiology at Hamburg University for the gift of Nitrobacter cultures.

ABSTRACT

An investigation was carried out into the effects of Bromoxynil, Chloroflurazole, Chlorothamid, Dichlobenil, Diquat, Endothal, Ioxynil, Paraquat, Picloram, Propanil and 2,3,6-TBA on soil nitrification.

~~Methods employed were~~ The perfusion technique was employed for studies on herbicide effects on the nitrification process in soil previously enriched with nitrifiers, and also on their effects on the enrichment growth of nitrifiers in a soil continuously treated with the herbicides.

The results obtained with these methods were compared with those from nitrite production and utilization by pure cultures of Nitrosomonas sp and Nitrobacter sp respectively, and with measurement of oxygen uptake by these organisms and enriched soil, by conventional Warburg techniques for these herbicides.

The results indicate that there were differential effects of the herbicides on the growth and metabolism of the nitrifying organisms; the nitrification process was affected at a lower herbicide concentration than the growth rate. The evidence suggests that there was an adaptation of the nitrifying organisms during growth, i.e. a selective proliferation of a tolerant strain.

Ioxynil, propanil, chloroflurazole and bromoxynil were the most toxic while dichlobenil, paraquat and endothal were the least toxic of the herbicides. Endothal in the

soil perfusion experiments, and also in the pure cultures, stimulated nitrification, while paraquat was more inhibiting in the pure cultures than in the perfuser experiments.

CONTENTS

GENERAL INTRODUCTION

The term herbicide, as used in agriculture, applies to a heterogeneous group of chemicals with the property of eradicating all vegetation or of selectively killing weeds without seriously injuring surrounding crops. Although certain chemicals were known to be harmful to plants, the implication that they could be used as weed killers was not fully realized until the late 19th Century when Bonnet in 1897 (quoted by Zaki, 1965) reported that Bordeaux mixture sprayed over vines selectively killed the charlock growing beneath them. Copper sulphate was found to be selective against charlock and other copper compounds were tried as herbicides, together with sulphuric acid, various arsenic compounds, phenolic compounds and many other organic and inorganic compounds. These compounds and general plant poisons are usually applied in large quantities.

A new era in weed control was opened by the introduction of substituted benzoic and phenoxyacetic acids (Zimmerman et al, 1942). Concurrently and independently there were references to 2,4-D and 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) (Hamner and Tuckey, 1944), 2,4-D and 2-methyl-4-chloroacetic acid (MCPA) (Slade, Templeman and Sexton, 1945; Nutman, Thornton and Quastel, 1945; Blackman, 1945), as selective herbicides. These auxin type herbicides were effective at very low rates of application and affected

particular plant enzyme systems.

Since the turn of this decade, the number of known herbicides has increased steadily and at present well over 100 are produced commercially. It is not untimely to mention here, that a good proportion of the herbicides now in use are not of the auxin type.

Contact and translocated herbicides are primarily directed at the foliage and enter the plants through leaves and stems; inevitably some may and do drop on to the soil. Residual herbicides by contrast are applied directly to the soil where they persist for varying lengths of time, depending on their volatility, chemical nature and the prevailing environmental conditions--nature of the soil, rainfall, etc.

When agricultural soils are treated with herbicides for the purpose of weed control, whatever mode of application is adopted (soil-applied for pre-emergence and foliage-applied for post-emergence weed control), the chemical eventually arrives at the soil surface. Once the herbicide is in the soil system, one of ~~three~~ two things may happen: a) it may be degraded by biological or chemical means; b) it may be immobilized. Before breakdown or immobilization, it may or may not have some effect on the soil microbial system.

Soil micro-organisms play an essential role in the

maintenance of soil fertility. There is a great (but unacknowledged) dependence on natural systems for regeneration, and on maintenance of soil fertility in developing countries, since owing to ignorance and the low per capita incomes of the peasants in these areas, they use (if any at all) very little amounts of artificial fertilizers. The world soil system, therefore, turns out or is able to turn out several tons of nitrate per acre (the most heavily used mineral) per annum. Of all the natural systems that produce nitrogen in forms available to plants (eg. fixation by root nodules of leguminous plants--Rhizobium spp, Clostridium spp; fixation by aerobic bacteria--Azotobacter spp and nitrification by Nitrosococcus spp, Nitrosomonas spp and Nitrobacter spp), by far the most important is nitrification. It is essential to ensure that the large-scale use of herbicides would not detrimentally affect the nitrification of the soil. It should be pointed out though, that too high a rate of nitrification would lead to a leaching out of nitrates from the soil.

Nitrification is the process whereby the ammonium ion is converted to nitrate and takes place in all fertile soils. It is usually supposed in soils of more-or-less neutral reaction at least, to be mediated by two types of organisms working sequentially--Nitrosomonas (which oxidises the ammonium ion to nitrite) and Nitrobacter (which

oxidises the nitrite so formed to nitrate).

These two genera constitute the classic group of 'nitrifying organisms,' and it is on these two genera that attention will be focused in this work. It should be remembered, however, that some nitrification, i.e. conversion of ammonia to nitrate, may be carried out by other organisms (Schmidt, 1954; Eylar and Schmidt, 1959), although it is generally assumed that the contribution of the 'non-classic' nitrifiers to the total nitrification in soil is quantitatively small. Field and laboratory studies have demonstrated various degrees of growth and or respiratory inhibition of bacteria and or fungi; extensive reviews on general microbiological effects of herbicides have been written (Audus, 1960; Fletcher, 1960). The effects vary greatly with the chemicals and dosage and the organisms concerned. Considerable work has been carried out on herbicidal influences on metabolism of various soil microflora but less information is available for the responses of the nitrifying bacteria in particular.

Almost all commercial herbicides were first developed empirically and studies of their modes of action were undertaken only after their economic benefits were recognized. The results of these investigations are of considerable interest to biology and agriculture as they furnish new insights into the physiological processes of the plants etc. and at the same time provide a rational

approach to the development of new chemicals to satisfy specific needs.

There are two main ways in which nitrification, and various inhibitors of the process, may be studied. One is to study the process as it actually occurs in the soil, and for this type of study, the technique that has proved to be biochemically the most useful and adaptable is the 'soil perfusion' technique (Audus, 1946; Quastel & Scholefield, 1951). A solution of the ammonium salts is perfused and reperfused through a column of soil that is kept in an aerated condition by air dragged through the column by the perfusing solution. Small samples of the solution are taken from time to time and analysed for ammonium ions, nitrite and nitrate; these analyses show the course of the nitrification as it is proceeding in the soil column. Inhibitors of the process are followed by comparing the rate of nitrification in a soil perfused with a solution of ammonium salts containing inhibitor with the rate in a soil percolated with a similar solution free of inhibitor (or herbicide). If the soil is one that has not recently carried out any appreciable nitrification (i.e. 'fresh' soil), there is likely to be a lag period before any ammonium is nitrified, because the nitrifying population is small; later, small amounts of nitrite appear with the build up of the population of Nitrosomonas. The appearance of this

nitrite stimulates the growth of Nitrobacter with consequent oxidation of the nitrite to nitrate. Thereafter, the oxidation of ammonium yields nitrate alone, because the Nitrobacter population can usually oxidise the nitrite as fast as it is formed. When an appreciable population of nitrifiers has been built up in this way, in a soil, and the soil is re-perfused with fresh solutions of substrata, oxidation of the ammonium to nitrate takes place without any lag period and usually at a linear rate, since the nitrifying population are as great as the soil will bear; such soils are said to be 'enriched' (Lees and Quastel, 1946; Lees, 1949; Quastel and Scholefield, 1951; Quastel, 1955). It is also possible to remove the soil from the perfuser after enrichment and follow nitrification in it by transferring samples to a Warburg vessel and observing oxygen uptakes in the presence of ammonium ions, with or without the addition of any inhibitor under investigation (Quastel and Scholefield, 1951; Hale, Hulcher and Chappell, 1958). The advantage of the technique in the studies of inhibitors of nitrification is that it permits direct analyses of the metabolic processes in an undisturbed soil column; inhibitions take place under conditions approximating those obtainable in the field, and it is thus of considerable practical value. The perfuser is well suited to the kinetic studies of

microbiological transformations in the soil because it allows for repeated samplings without physically disturbing the soil column. On the other hand, there is no guarantee that any substance X, which is found to inhibit nitrification in a soil perfuser, will necessarily inhibit nitrification as studied in pure cultures of the nitrifying organisms, since there is always the possibility that X is changed into some other compound Y in the soil by the activities of various soil organisms, and that Y, not X, is the actual inhibitor; on the other hand it gives an idea of what may happen in practical agriculture.

Studies of inhibitions of nitrification in pure cultures of Nitrosomonas and Nitrobacter are free of this drawback, but have the disadvantage that they are tedious to carry out. The organisms are slow to grow in pure culture and difficult to culture continuously under strictly autotrophic conditions.

The experiments described in this thesis were carried out to determine the effects of some of the herbicides (so far not studied) listed in Table I, on nitrification in soil under laboratory conditions, on the growth of the nitrifying bacteria in pure cultures, and on the oxidation of ammonium by enriched soil and Nitrosomonas, and of nitrite by Nitrobacter. Two aspects of herbicidal effects on soil nitrification were studied--both of these were based on the assumption that 'nitrification during

soil perfusion is due to the metabolism of proliferating organisms' and therefore, 'granting no interfering factors, the amount of nitrate appearing in a perfusate should increase in a logarithmic manner with respect to time' (Quastel and Scholefield, 1951). In the first instance, the herbicide was perfused (with ammonium sulphate solution) through non-enriched soil in order to determine, by following the kinetics of enrichment, the effects on the growth of the organisms, nitrification being measured by total NO_3 production. When the organisms were grown in pure cultures, nitrification was measured by the appearance in, or disappearance of, nitrite from the medium. The respiratory experiments were conducted using standard Warburg apparatus. The uptake of oxygen during the oxidation of ammonium and nitrite was followed in the presence and absence of the herbicides to see how, and whether or not, respiratory metabolism of the nitrifying organisms were affected by the herbicides.

At the time of conducting these experiments, there were no records of the effects of the herbicides used on nitrification and some came onto the market during the period of these experiments.

THE EFFECT OF HERBICIDES ON THE NITRIFICATION PROCESS IN THE SOIL.

INTRODUCTION

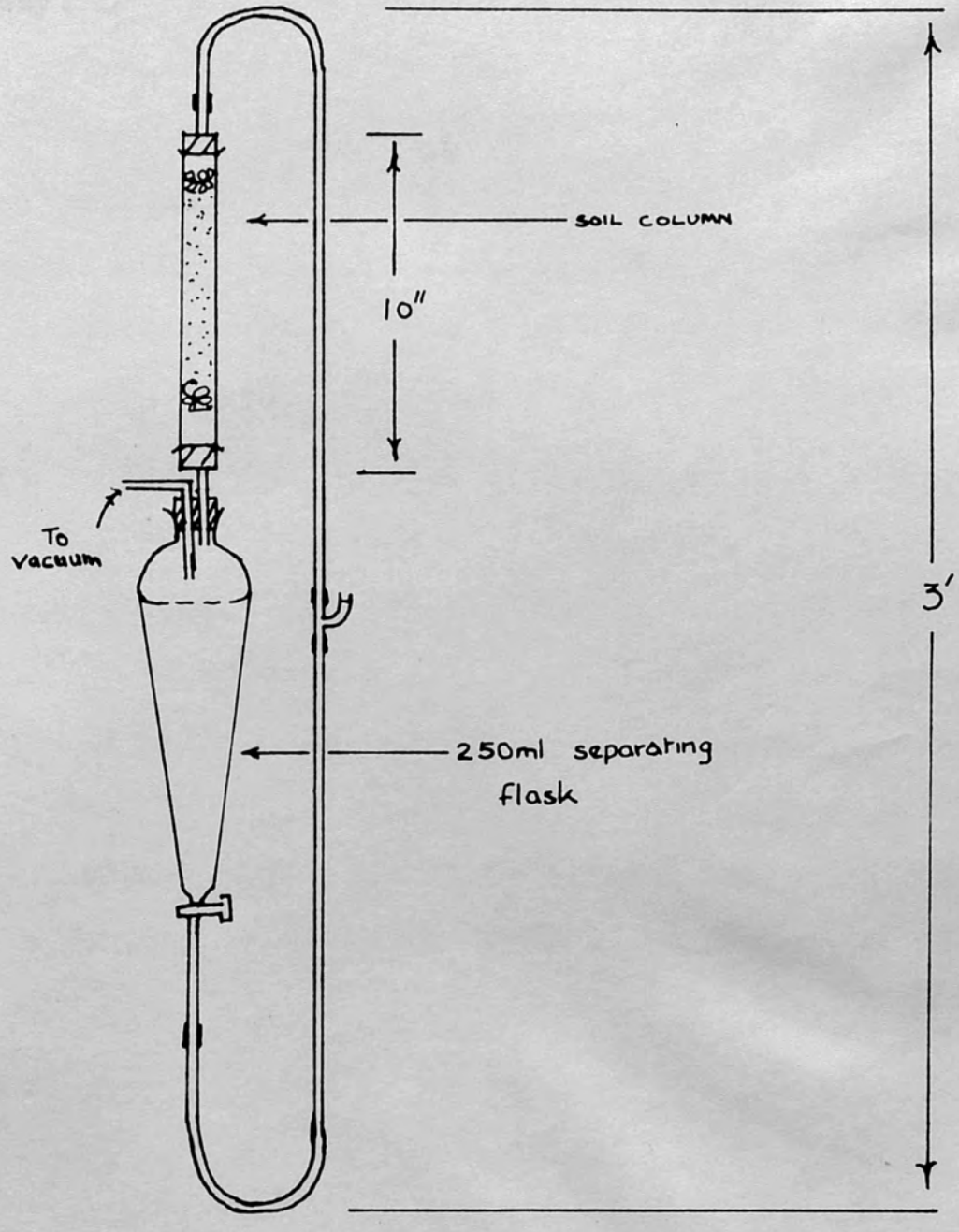
Lees and Quastel (1946 a,b) have established that the course of nitrate formation which is "autocatalytic" in fresh air dried soil, becomes linear and shows no 'lag', when such soils are enriched with nitrifying bacteria by preliminary perfusion of the soil with ammonium salts. Quastel and Scholefield (1951) stated that 'such a soil brings about a relatively rapid rate of nitrification, that this rate remains constant during subsequent perfusions of the soil with ammonium salts, the soil thus behaving like an enzyme-system'. The effects of herbicides or other 'inhibitors' on such a system will show in the steepness of the gradient or the slope of the lines produced, depending on whether the observed effects are inhibitory or stimulatory. The few available reports on the effects of herbicides on soil nitrification were restricted to a study of the effects of these chemicals on total nitrification and did not consider the effects on the process itself. It was therefore decided quite early in the programme of the present studies to include a study of the effects of these herbicides on the process of soil nitrification as it occurs in an enriched soil.

MATERIALS AND METHODS

Preparation of soil samples.

The loam soil used throughout these experiments was obtained from the top 3" layer of a fallow plot at the Botany Department Gardens, Bedford College, Regent's Park, London, N.W.1, England. The soil was initially passed through a ½"-mesh riddle (this served the purpose of removing large stones and debris) and then air-dried under laboratory conditions. That portion of the air-dried soil which passed through a 4-mm mesh but was retained by a 2-mm mesh screen was used. (It was earlier found that the inclusion of particles smaller than 2 mm led to a blocking up of the air-pores between the soil crumbs, and so stopping the percolation of water through the soil columns in the perfusers.) 50 gms of soil prepared as described above was placed in a perfusion apparatus similar to that described by Audus, 1946, but slightly modified. (Plate I) A series of 17 such apparatuses was set up in a manifold, each connected by a tap to the vacuum supply. The soil-column tubes were wrapped in black polythene, and the whole set-up was covered to half its length with a sheet of black polythene - the general idea was to prevent growth of algae in the tubes, which was largely achieved. Ideally the experiments should be set up in a dark room,

PLATE 1



THE PERFUSER

but lack of space prevented this. However, it is not thought that this affected the results of the experiment in any way.

Preparation of Reagents

All solutions and chemicals used were of 'reagent grade' stock.

Ammonium sulphate $(NH_4)_2SO_4$ solution (0.0062 M)

Copper sulphate ($CuSO_4$) solution (0.02 M)

Hydrogen peroxide (H_2O_2) (20 vols.)

Phenol disulphonic acid Reagent was prepared as stated in Snell and Snell (1936) Colorimetric Methods of Analysis, using appropriate amounts of the chemicals. The Reagent was heated for 2 hours continuously, on a water bath during preparation.

Ammonium hydroxide was usually kept in the refrigerator and used as necessary.

Bedford College garden-soil contained about 31 mg NO_3-N per ml.

Herbicides: The list of herbicides used and their sources of supply is given in Table I.

TABLE I

Common and chemical names of the herbicides tested and sources of supply.

P = Pure
F = Formulated

Common name or other designation		Chemical name	Source
Bromoxynil	P	3,5Dibromo-4-hydroxybenzonitrile	May & Baker
Chlorflurazole (NC 3363)	P	4,5-Dichloro-2-trifluoromethyl benzimidazole	Fisons
Chlorothamid (Prefix)	P	2,6-Dichlorothiobenzamide	Shell
Dichlobenil (Casoron)	P	2,6-Dichlorobenzonitrile	Shell
Diquat	P	1,1-Ethylene-2,2-dipyridylium dibromide	ICI (plant protection)
Endothal	F	3,6-Endoxohexahydrophthalic acid (sodium salt)	Niagara Chemicals Division
Ioxynil (ACP 62-177)	P	3,5-Diiodo-4-hydroxybenzonitrile	May & Baker
Monuron	P	3-(p-chlorophenyl)-1,1-dimethyl urea	du Pont
Paraquat	P	1,1-Dimethyl-4,4-dipyridylium dichloride	ICI (plant protection
Pichloram (Tordon)	F	4-Amino-3,5,6-trichloropicolinic acid	Dow Chemicals
Propanil (Rogue, Stam F-34)	F	3,4-Dichloropropionanilide	Monsanto Chemical Company
2,3,6-TBA	P	2,3,6-Trichlorobenzoic acid	ICI
(NC 2265)	P	4,5,6,7-Tetrachloro-2-trifluoro-methyl benzimidazole	Fisons

MATERIALS AND METHODS

50 gms of fresh soil prepared as described was perfused with ammonium sulphate solution only for periods of 20-25 days. Thereafter, the perfusing fluid was drained off and replaced with fresh solution at 20-day intervals over a period of two months; the soil was leached of nitrites and nitrates with distilled water over 24 hours before replacement with fresh solution. This served the purpose of washing out from the soil any nitrates and nitrites that were held therein, for their presence in large amounts suppresses nitrification. At the end of this period, a stage was reached when the rate of conversion of ammonium to nitrate remained fairly constant, and the soil was then assumed to be enriched. Soil columns prepared in the manner described above were washed with distilled water for periods of 3-4 days, the water in the separating funnel was replaced daily with a fresh supply, and tested for the presence of any residual NO_3^-. After the third washing, there was usually very little NO_3^- left and the columns were deemed ready for use. Such soils were perfused with the appropriate concentrations of the different herbicides (0, 50, 100, 500 and 1000 ppm active ingredient in 200 ml ammonium sulphate solution), and the NO_3-N content determined daily, over a ten-day period.

1 ml samples of the perfusate were taken daily throughout the experimental period and placed in centrifuge tubes to which spatula-end amounts of about 5 mgms of a 1:2::$Ca(OH)_2$:$MgCO_3$ mixture and 1 ml of a 0.02 m copper sulphate solution were added, shaken vigorously and left overnight. The tubes were then centrifuged and 1 ml of supernatants placed in 25 ml volumetric flasks, to which 0.2 ml of hydrogen peroxide solution was later added, and taken to dryness at 110°C in an electric oven. The flasks were allowed to cool and 1 ml of the phenol-disulphonic acid reagent was added, swirled round, and left to stand for 30 minutes; thereafter, 10 ml of distilled water was added together with enough ammonium hydroxide to produce a yellowish colour (usually about 3 mls), and the flasks were left to cool in a fume cupboard. Enough ammonium hydroxide was added to bring the volume up to the 25 ml mark and the flasks were then vigorously shaken. The intensity of the yellow colour so obtained was measured in an EEL colorimeter with blue filters, and the nitrate-nigrogen content was obtained from the readings by means of a KNO_3- standard curve.

Random tests were carried out on perfusates to determine whether there was any nitrite accumulation. The general level of nitrite detected was very low and usually of the same amounts in both the controls and in

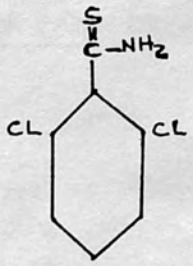
the test-perfusates. This means that the effects observed (at least at high herbicide concentrations, where no nitrates were obtained) were presumably on the first (i.e. Nitrosomonas) stage of the process.

RESULTS

When enriched soils were perfused with ammonium sulphate solution no lag was obtained (Figs. 1-11), and nitrification proceeded at constant rates. Treatments of enriched soil with herbicide affected the nitrification process *per se* (Table II). Increase in herbicide concentration was usually accompanied by a corresponding decrease in the nitrification rate. The nitrification rates at various concentrations of the different herbicides, expressed as percentages of the controls, were calculated and plotted against log. of herbicide concentration (Figs. 12 and 13). Nitrification in Propanil (Fig. 13) ceased at 56 ppm, while this happened in Bromoxynil at 133 ppm, Chlorothamid at 500 ppm (Fig. 13), 178 ppm in Ioxynil, 500 ppm in Chloroflurazole (Fig. 12), and Picloran at 566 ppm (Fig. 12). Endothal (Fig. 12) stimulated the rate of nitrification (this was obtained on two occasions--Figs. 3 and 3A and the broken lines in Fig. 12)--an increase in herbicide concentration led to an increase in nitrification rate.

FIG 1

THE EFFECT OF CHLOROTHAMID ON THE NITRIFICATION PROCESS WHEN ENRICHED SOIL IS PERFUSED WITH AMMONIUM SULPHATE SOLUTION



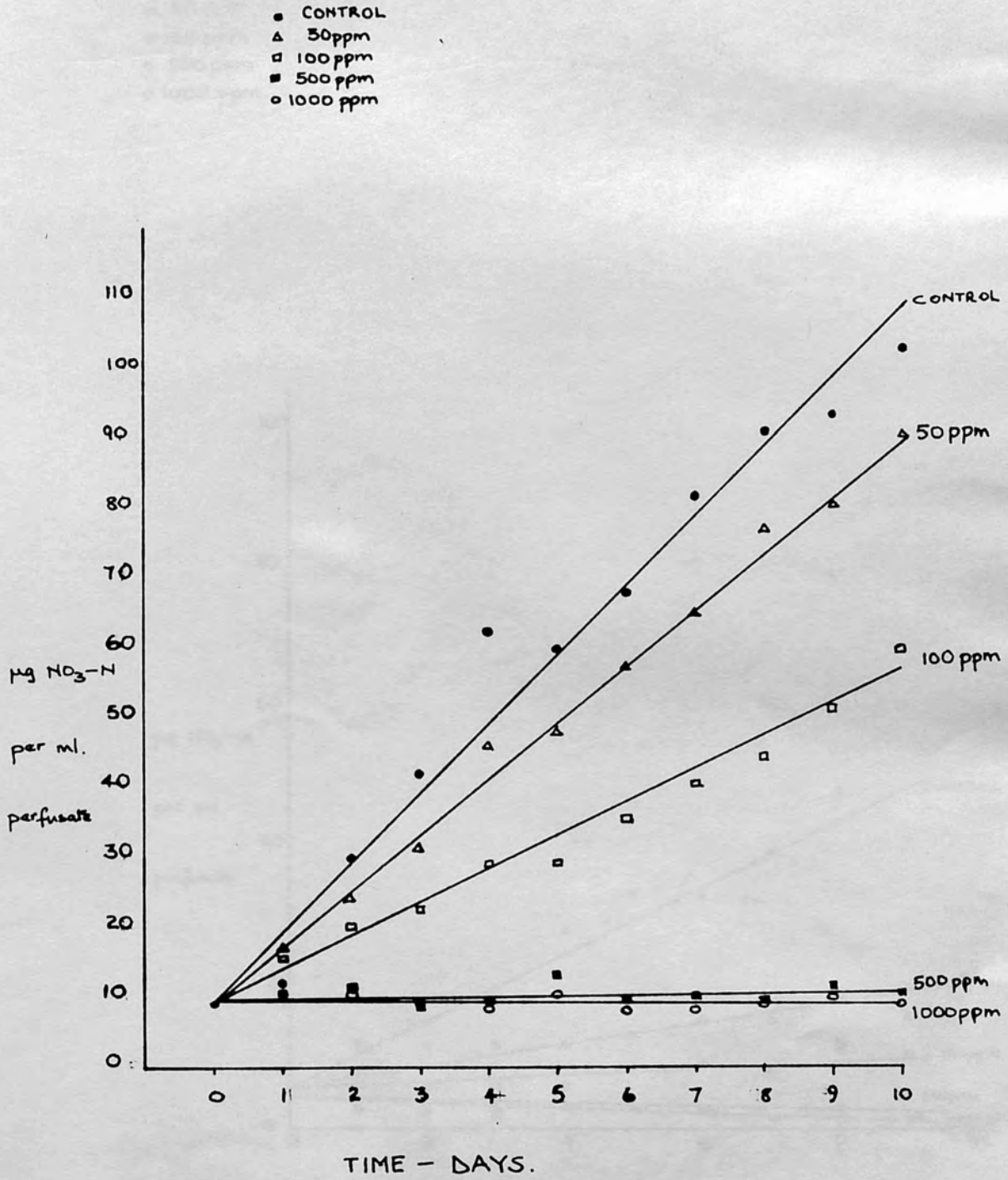
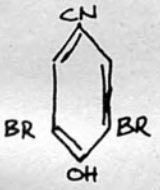


FIG 2

THE EFFECT OF BROMOXYNIL ON THE NITRIFICATION PROCESS

WHEN ENRICHED SOIL IS PERFUSED WITH

AMMONIUM SULPHATE SOLUTION



- ● CONTROL
- △ 50 ppm
- □ 100 ppm
- ■ 500 ppm
- ○ 1000 ppm

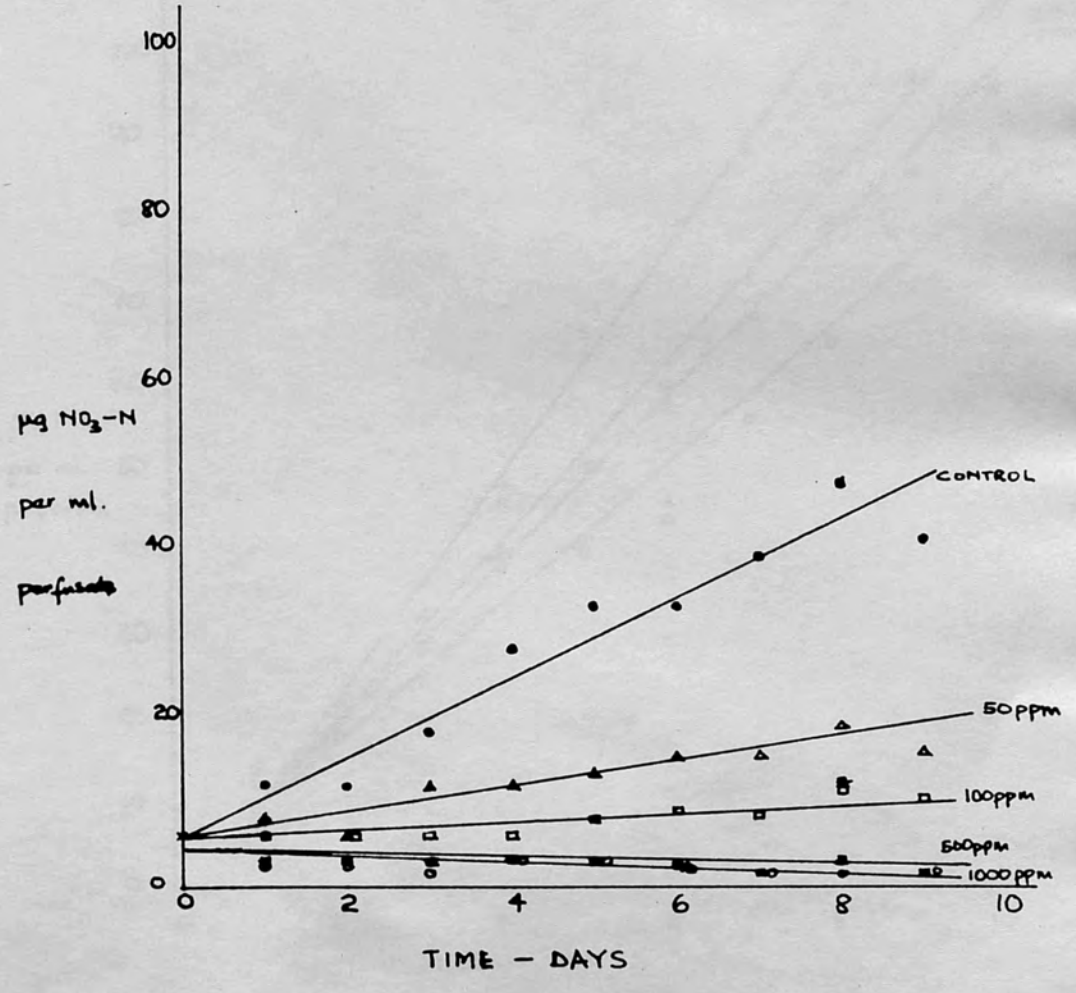


FIG 3

THE EFFECT OF ENDOTHAL ON THE NITRIFICATION PROCESS WHEN ENRICHED SOIL IS PERFUSED WITH AMMONIUM SULPHATE SOLUTION

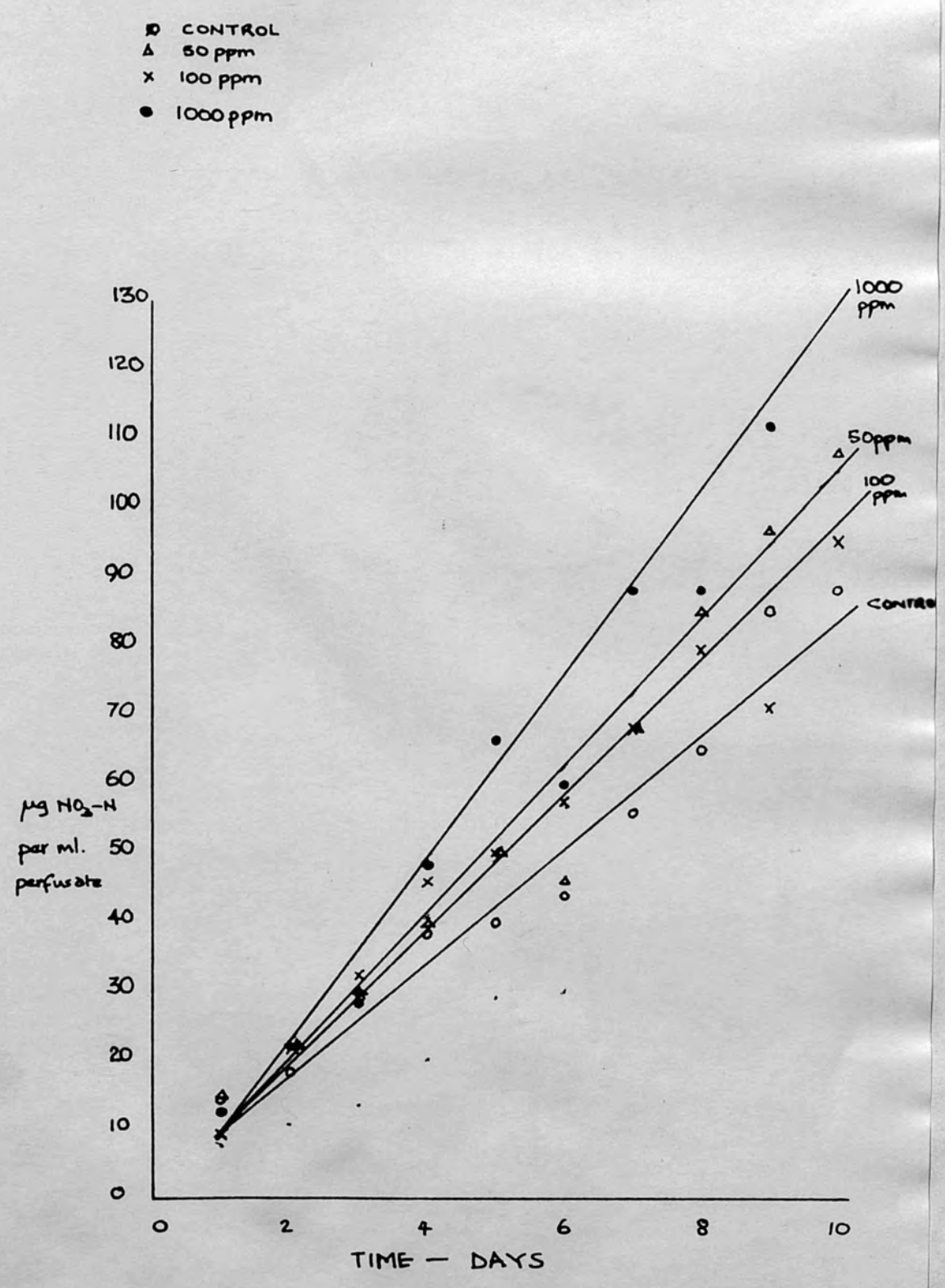


FIG 3A

THE EFFECT OF ENDOTHAL ON THE NITRIFICATION PROCESS WHEN ENRICHED SOIL WAS PERFUSED WITH AMMONIUM SULPHATE SOLUTION

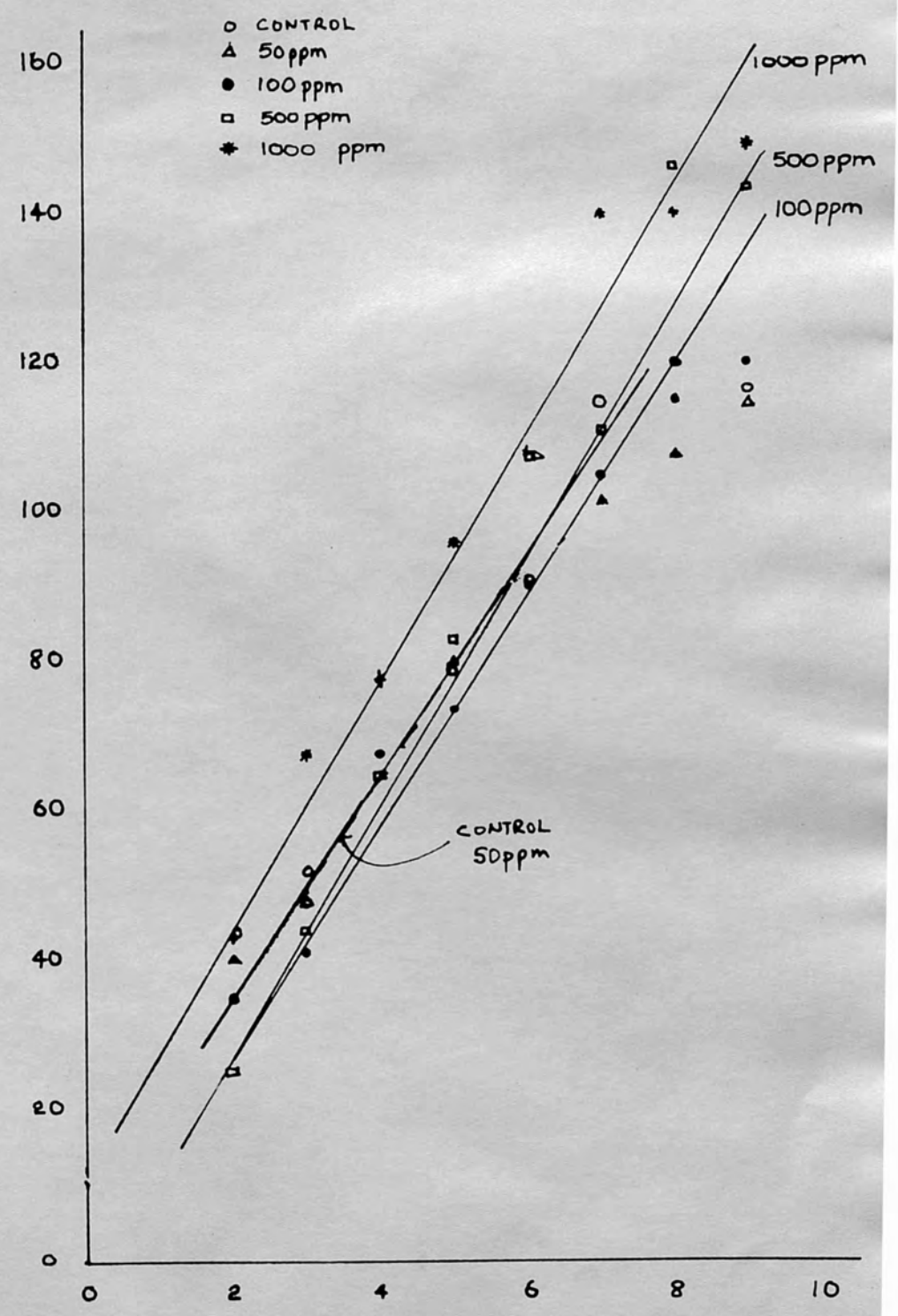


FIG 4

THE EFFECT OF PICLORAM ON THE NITRIFICATION PROCESS WHEN ENRICHED SOIL IS PERFUSED WITH AMMONIUM SULPHATE SOLUTION.

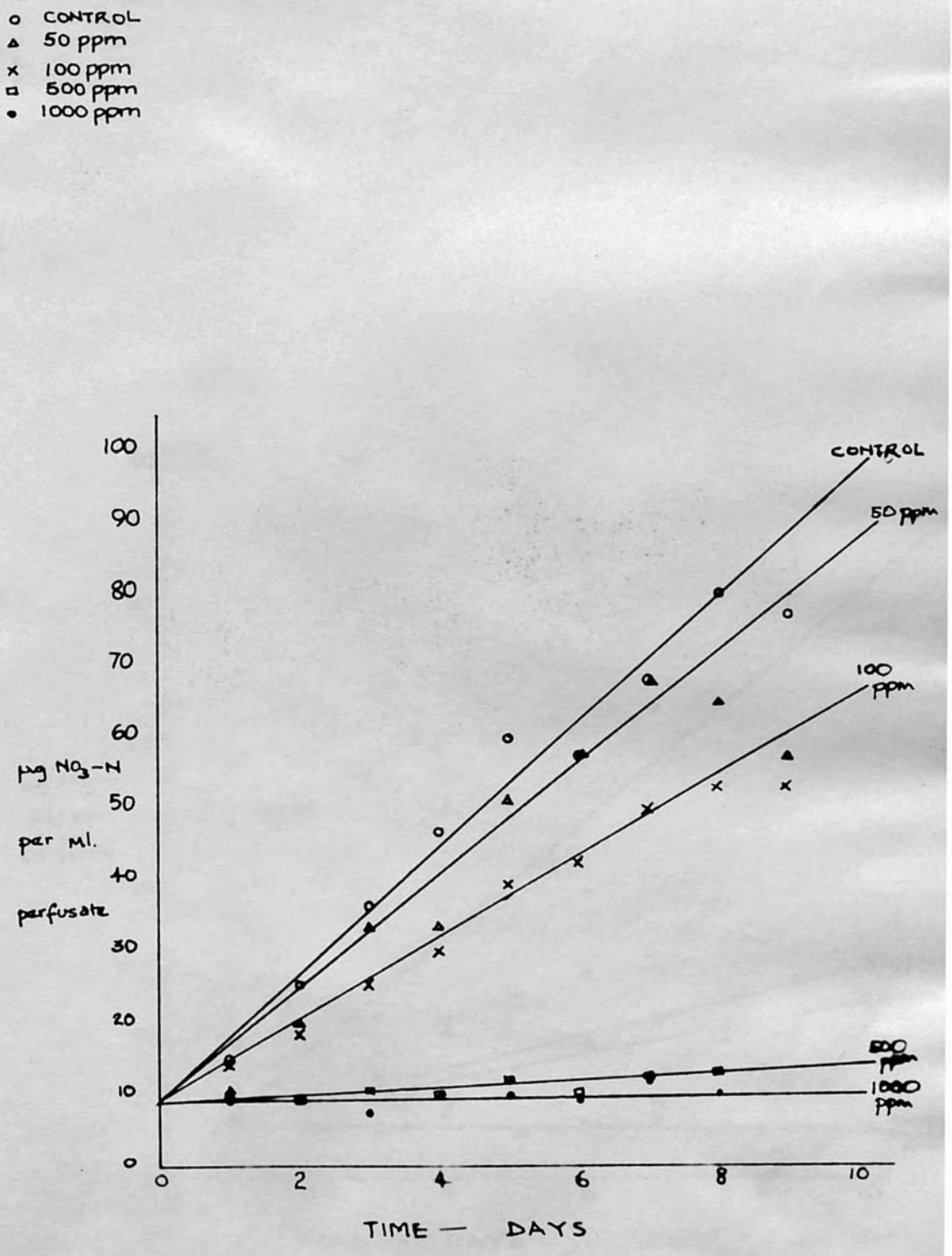


FIG 5

THE EFFECT OF CHLOROFLURAZOLE ON THE NITRIFICATION PROCESS WHEN ENRICHED SOIL IS PERFUSED WITH AMMONIUM SULPHATE SOLUTION.

● CONTROL
▲ 50 ppm
□ 100 ppm
■ 500 ppm
○ 1000 ppm

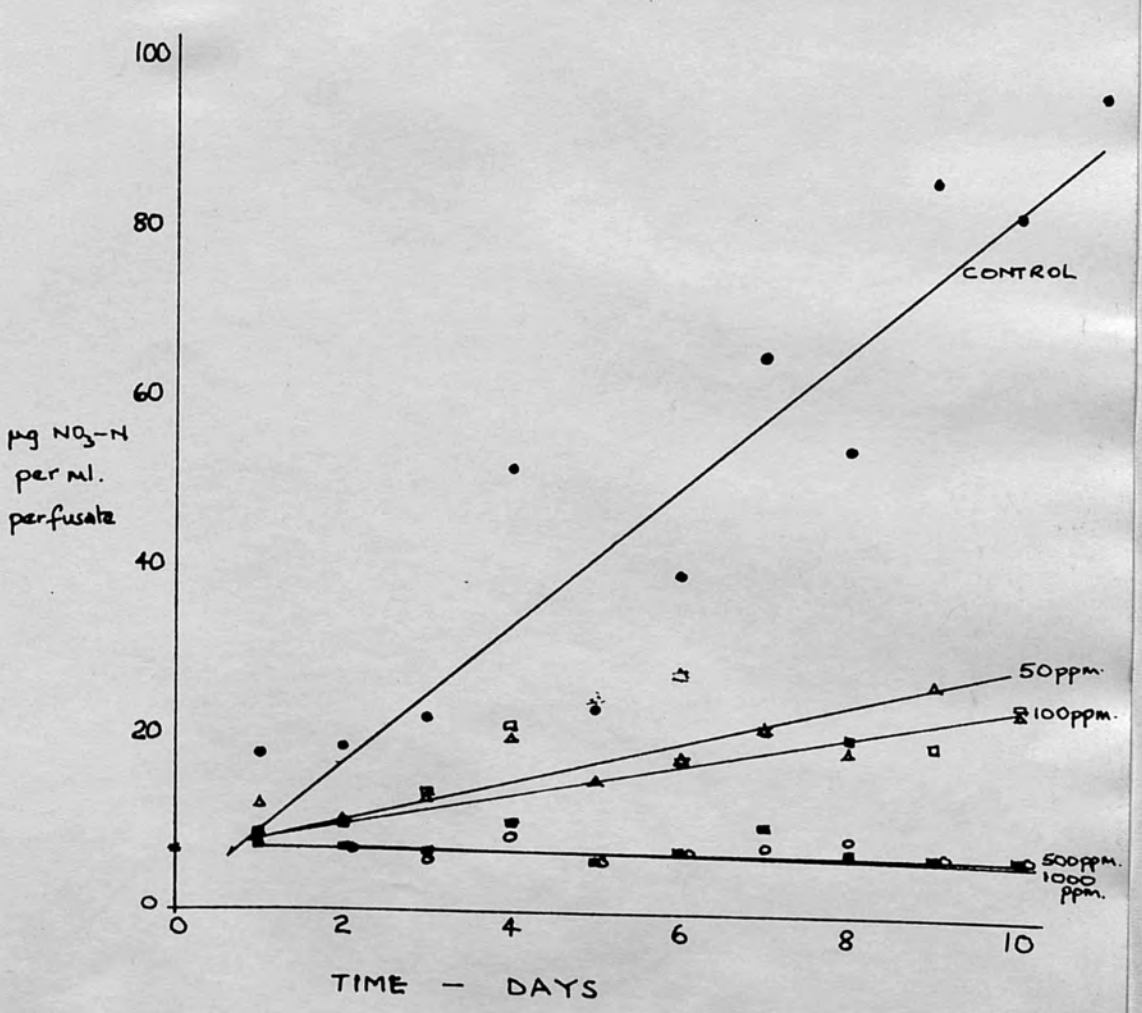
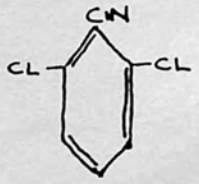


FIG 6

THE EFFECT OF DICHLOBENIL ON THE NITRIFICATION PROCESS WHEN ENRICHED SOIL IS PERFUSED WITH AMMONIUM SULPHATE SOLUTION.



● CONTROL
△ 50 ppm
■ 500 ppm
○ 1000 ppm

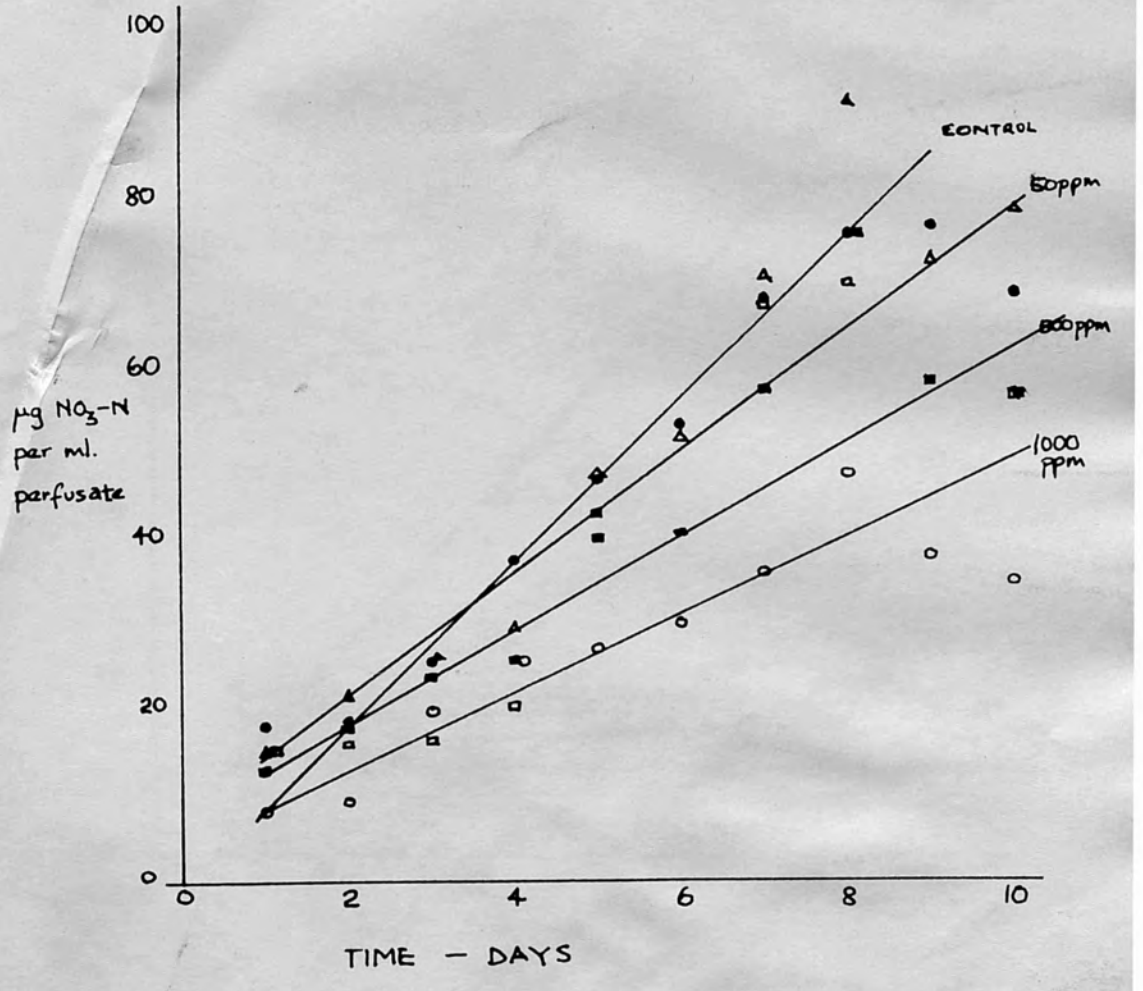
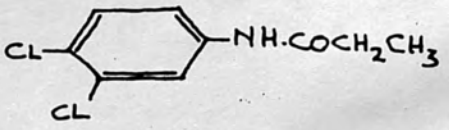


FIG 7

The EFFECT OF PROPANIL ON THE NITRIFICATION PROCESS WHEN ENRICHED SOIL IS PERFUSED WITH AMMONIUM SULPHATE SOLUTION.



- ● CONTROL
- △ 50 ppm
- □ 100 ppm
- ■ 500 ppm
- ○ 1000 ppm

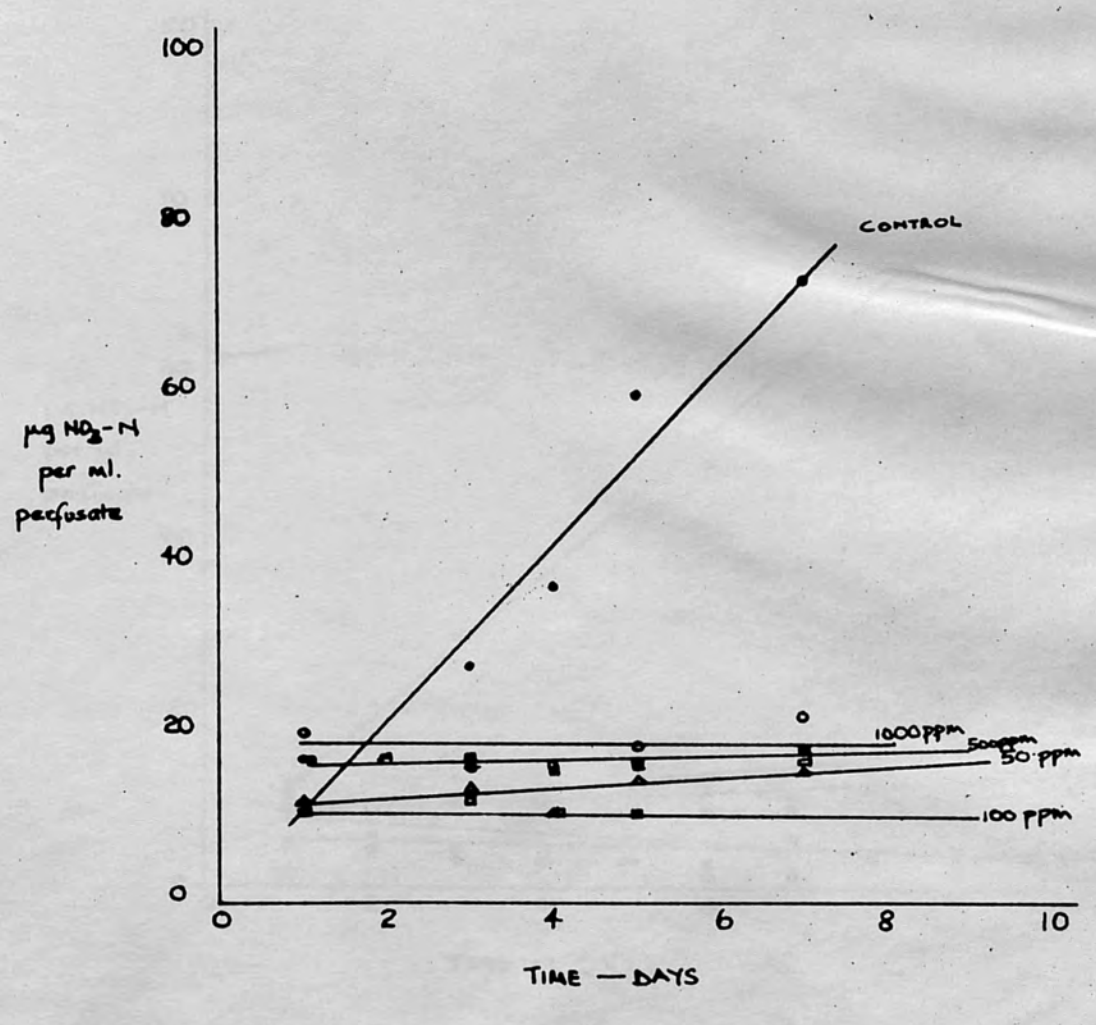
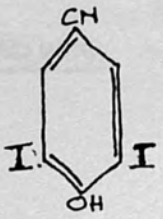


FIG 8

THE EFFECT OF IOXYNIL ON THE NITRIFICATION PROCESS WHEN ENRICHED SOIL IS PERFUSED WITH AMMONIUM SULPHATE SOLUTION.



- CONTROL
- △ 50 ppm
- □ 100 ppm
- ■ 500 ppm
- ○ 1000 ppm

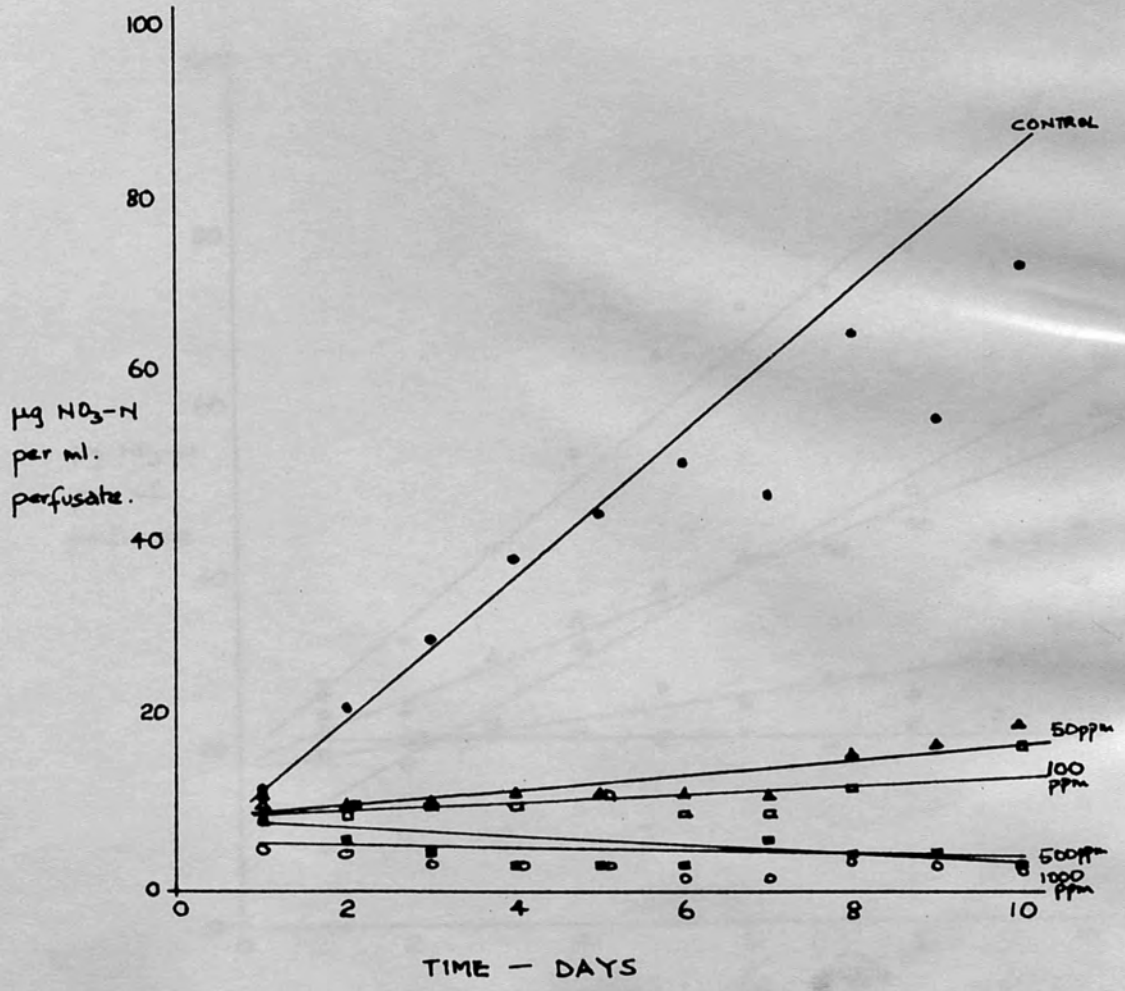
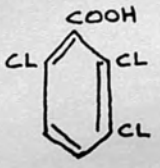


FIG 9

THE EFFECT OF 2,3,6-TBA ON THE NITRIFICATION PROCESS WHEN ENRICHED SOIL IS PERFUSED WITH AMMONIUM SULPHATE SOLUTION.



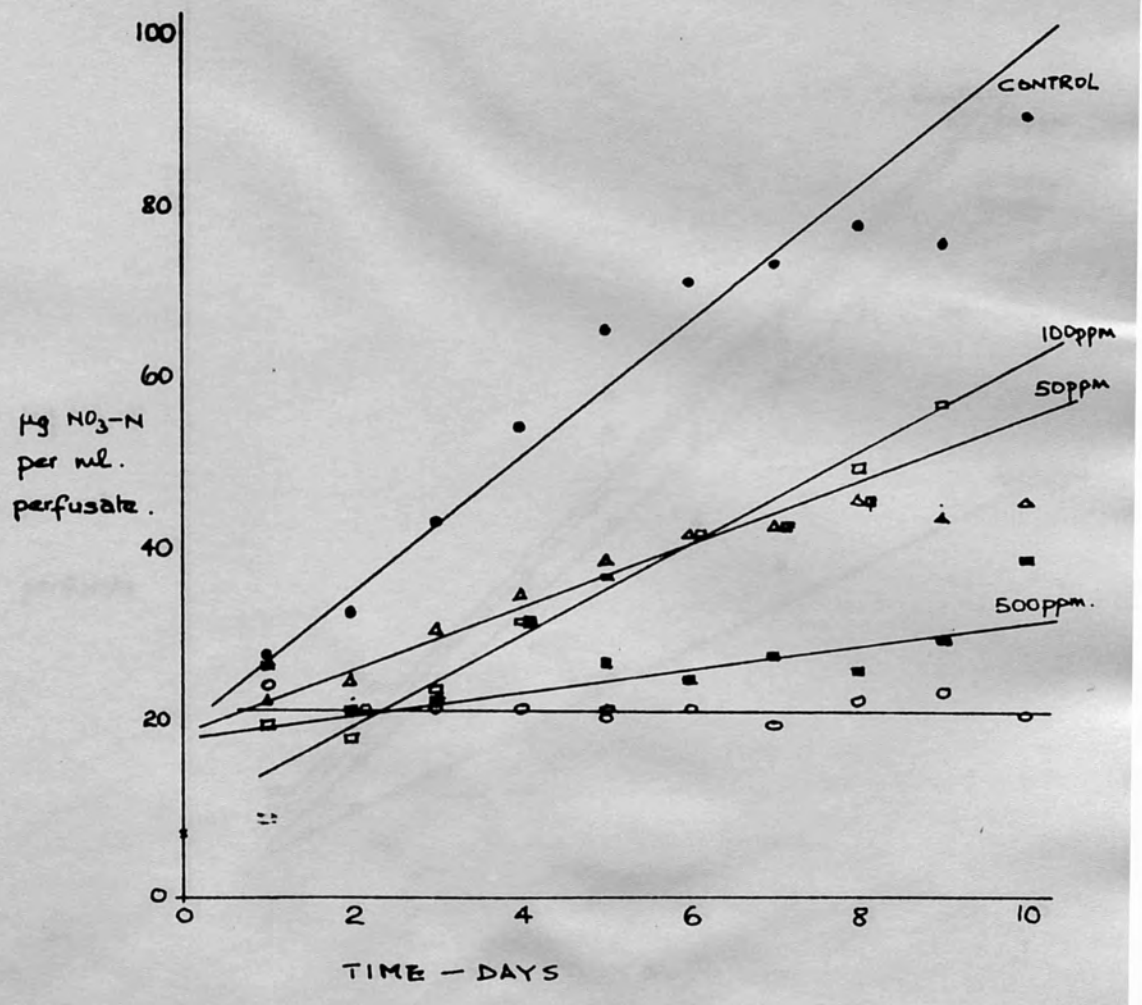
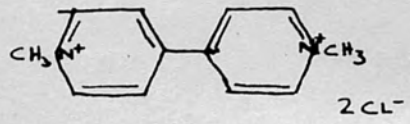


FIG 10

THE EFFECT OF PARAQUAT ON THE NITRIFICATION PROCESS



- ● CONTROL
- △ 50 ppm
- □ 100 ppm
- ■ 500 ppm
- ○ 1000 ppm

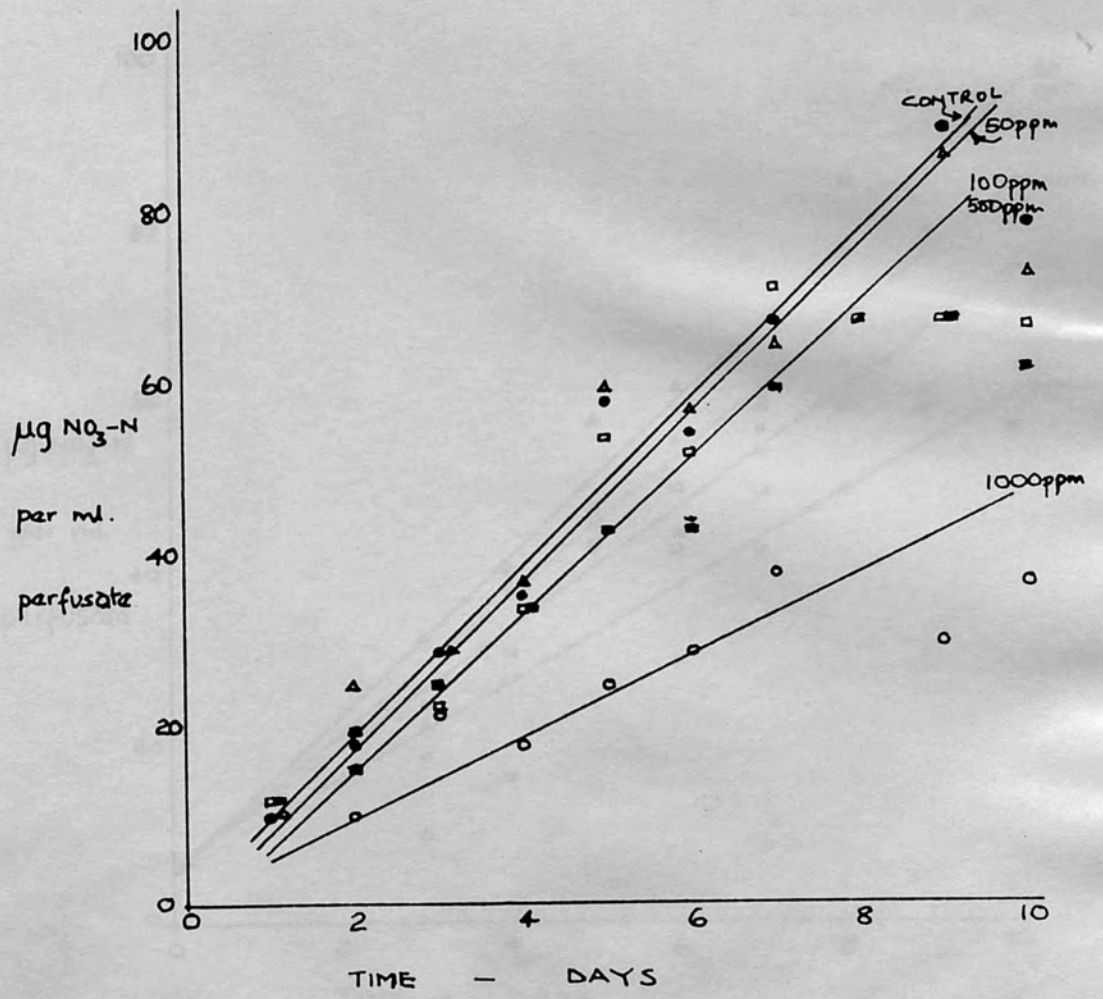


FIG 11

THE EFFECT OF DIQUAT ON THE NITRIFICATION PROCESS

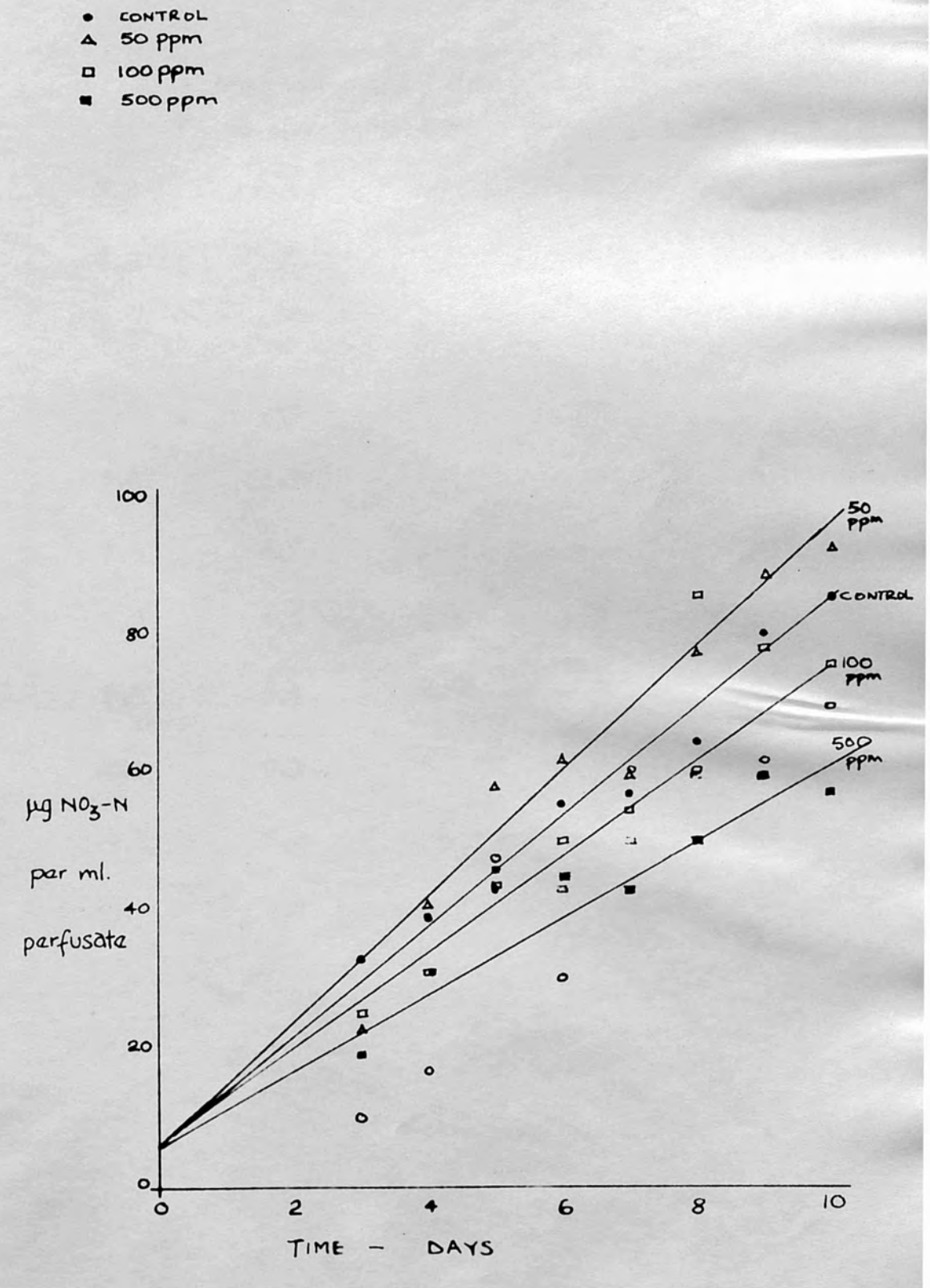


TABLE II

The nitrification rates when enriched soil was perfused with ammonium sulphate solution in the presence of the herbicides listed and at the concentrations indicated.

Herbicides	Nitrification rates at the concentrations indicated (μg NO_3-N / ml / day).				
	0	50 ppm	100 ppm	500 ppm	1000 ppm
Chloroflurazole	8.2	2.4	1.9	0	0
Endothal	8.2	10.6	9.8	-	13.2
Picloram	8.9	7.9	5.8	0.5	0
Diquat	8.0	9.2	7.0	5.6	-
Paraquat	9.9	9.9	9.1	9.1	4.8
2,3,6TBA	7.9	2.7	5.3	0.8	0
Bromoxynil	4.8	1.6	0.4	0	0
Ioxynil	8.3	0.9	0.44	0	0
Propanil	10.5	0.5	0	0	0
Dichlobenil	9.8	7.2	-	5.6	4.7
Chlorothamid	9.9	8.0	4.7	0	0

FIG 12.

% OF CONTROL NITRIFICATION RATES PLOTTED AGAINST LOG. CONCENTRATION OF HERBICIDES.

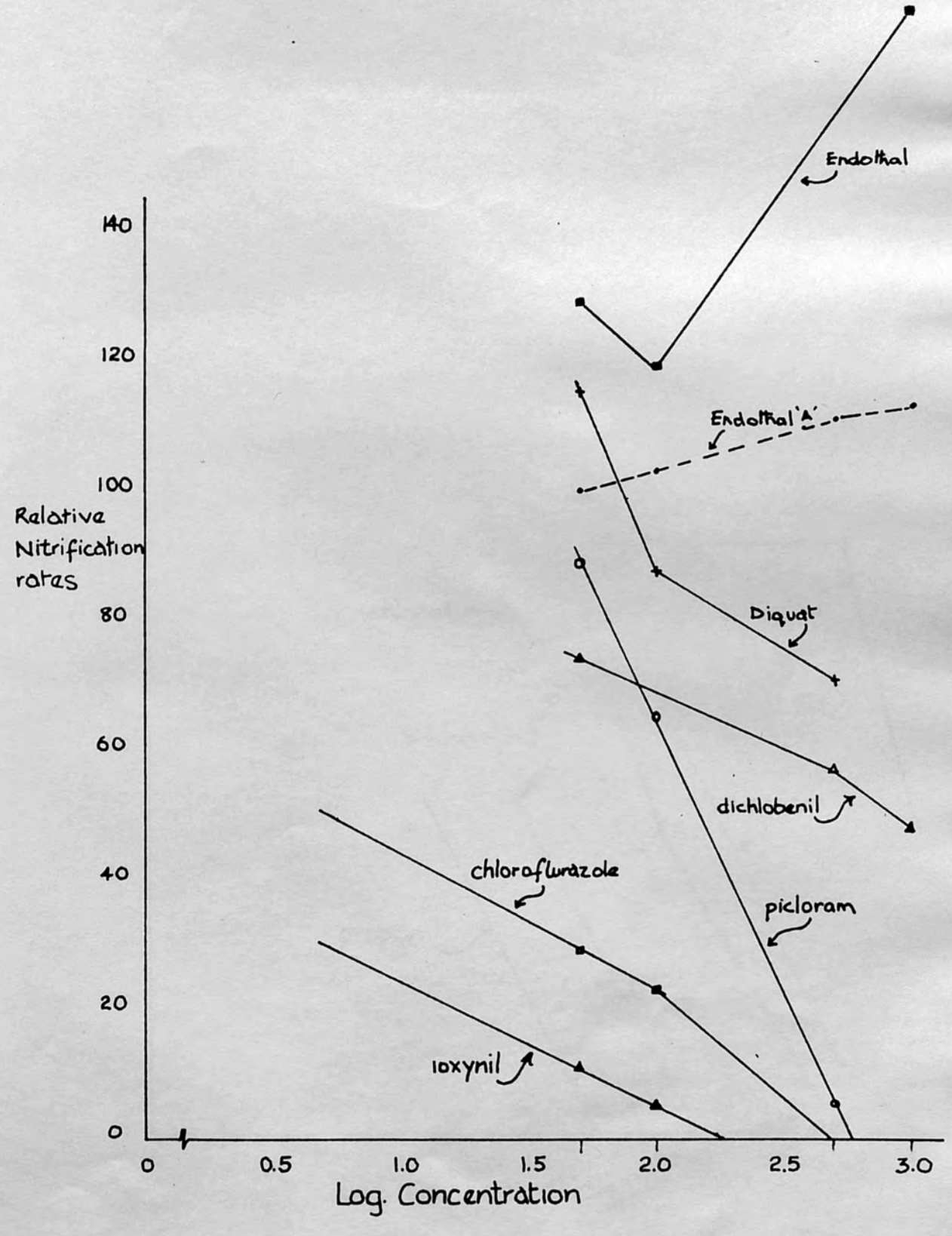
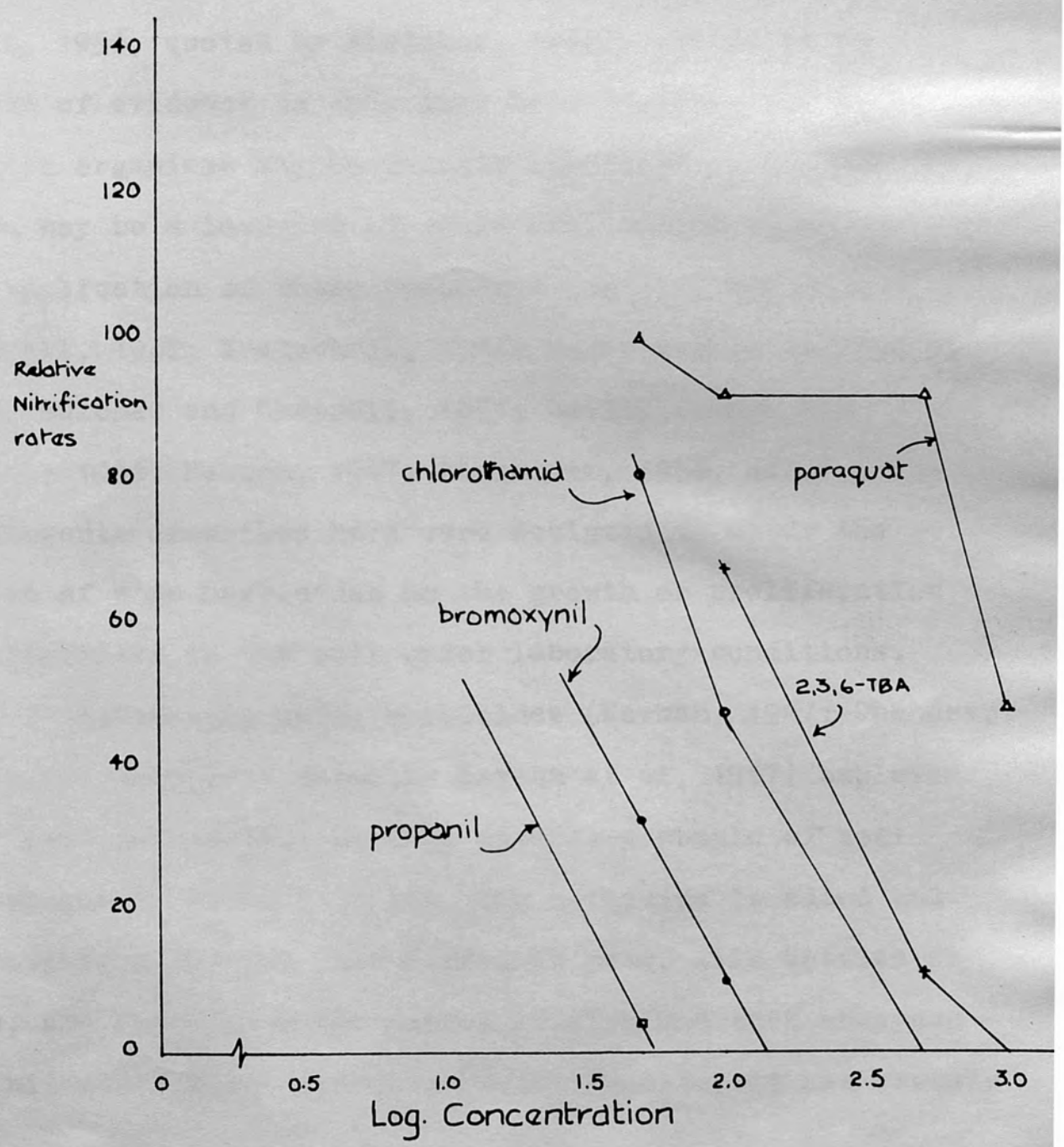


FIG 13

% OF CONTROL NITRIFICATION RATES PLOTTED AGAINST

LOG. CONCENTRATION OF HERBICIDES



THE EFFECT OF HERBICIDES ON THE GROWTH OF NITRIFIERS IN THE SOIL

INTRODUCTION

The application of chemicals for the purpose of weed control may alter deleteriously the balance of the soil eco-system; conversely, these chemicals may have beneficial bactericidal and fungicidal effects (Chappel et al, 1956, quoted by Fletcher, 1960). There is no dearth of evidence to show that heterotrophic and autotrophic organisms may be reduced numerically, or that there may be a lowering of their respiration rates on the application of these chemicals (Gamble, Mayhew and Chappell, 1962; Kratochvil, 1951; Magee and Colmer, 1956; Hale, Hulcher and Chappell, 1957; Smith, Dawson and Wenzel; 1946; Newman, 1947; Alexander, 1958, etc.). The experiments described here were designed to study the effect of some herbicides on the growth or proliferation of nitrifiers in the soil under laboratory conditions. Some investigators using herbicides (Newman, 1947; Chandra, 1964; and much more recently Bartha et al, 1967) employed the 'pot' technique. In this method, a sample of soil is brought to field capacity, the herbicide is added and thoroughly mixed and then stored in pots, milk bottles or jars, and left for a set period of time and then analysed for nitrate. While yielding useful results, it has certain

drawbacks--the soil may dry out, especially when left for rather long periods before analysis; by storing wet soil in a bottle or pot, there is the possibility that the soil at the bottom will contain more moisture than that at the top so that an even distribution of moisture is not achieved; aeration is poor, because often the practice is to stopper up the bottles, the soil is invariably disturbed at the time of sampling and there is no guarantee that one replicate sampling is the same as the next. The perfusion technique (Audus, 1946) is free of all the above drawbacks and was used in the work described here. Measurement of nitrate-nitrogen in aliquots of the perfusing fluid, taken at regular intervals are a measure of the progress of enrichment of the soil with nitrifiers.

Lees and Quastel (1946 b) have shown that when non-enriched soil is perfused with a solution of an ammonium salt, the viable nitrifying bacteria start metabolising and proliferating and further, that proliferation and nitrification take place on sites in the soil where NH_4+ is absorbed on to the soil. Nitrification proceeds at the expense of the absorbed ammonium ions in the soil and is a function of the total surface area on which ammonium is absorbed. When saturation has occurred, and, when there is an excess of NH_4+ in the system, the rate of proliferation will become constant and be

proportional to the base exchange area covered by the bacteria. The relative growth rate (R) of N viable cells is given by the expression $\frac{1}{N}\cdot\frac{dN}{dt} = R$ (relative growth rate, which is constant).

This on integration gives

$$\log N = Rt + \text{constant}$$

Now when $t = o$, $N = N_o$, and hence,

$$\log N - \log N_o = Rt$$

$$\text{or } \log \frac{N}{N_o} = Rt$$

In the experiments described in this study, it was not possible to count the number of nitrifying cells present; it was also assumed that the nitrifying capacity per cell was constant. The only means of comparing R (the relative growth rates) in the various experiments (i.e. to get the effect of the herbicides) was to use the nitrification rates. It was assumed that proliferation was exponential up to the time the rate of nitrate accumulation was linear (i.e. soil saturation). When the soil is saturated with bacteria, the total numbers of bacteria should be the same (i.e. for each soil at saturation). The exception to this would be if the herbicide had any effect on bacterial numbers at saturation (one could presumably check on this from a recovery data--using enriched soil this is not available). If these assumptions are correct, then we can take the time to reach saturation to estimate the

relative values of R. Let us take two soil samples from the same origin, N_o is the same for both. N (at time t) is also the same (soil is saturated). Therefore

$$\log \frac{N}{N_o} = R_1 t_1 \text{ and } \log \frac{N}{N_o} = R_2 t_2$$

or $R_1 t_1 = R_2 t_2$ or $\frac{R_1}{R_2} = \frac{t_2}{t_1}$

i.e. the ratio of the time lags (to saturation) is equal to the inverse ratio of the relative growth rates.

In this way, by using measurements of the lag, comparisons of relative growth rates were made.

MATERIALS AND METHODS

50 gms of fresh soil prepared as already described, together with 200 ml ammonium sulphate solution were used. Perfusion of the soil with this medium was continued for periods of about 28-30 days. 1 ml samples of the perfusate were taken daily throughout the experimental period and nitrate-nitrogen was determined as already described.

In one phase of this study, the survival of nitrifiers in soil treated with herbicide was explored. In this, the fresh soil was treated as described above (i.e. perfused with ammonium sulphate and herbicide during the first enrichment cycle of growth) but after a period of 28-30 days, the soil was washed with 3 changes of distilled water over a period of 3 days, and was then reperfused with ammonium sulphate solution only, and the nitrification activity was then followed. All determinations were made by the standard procedure adopted for this study. The results were calculated in terms of μg NO_3-N (per ml perfusate) and then compared with the activity of the nitrifiers in the fresh soil under the influence of the herbicide.

RESULTS

The results obtained in this study when ammonium sulphate solution was perfused with herbicides through non-enriched soil are given as Figs. 14-23. On the basis of the assumptions made above, i.e that the cells possessed equal nitrifying capacity, we may say that these nitrification curves (since nitrification is proportional to cell numbers), describe the pattern of the increase in cell numbers within the soil. The nitrification rate, i.e. the slope of the NO_3^- curve, is a measure of the bacterial population within the soil (i.e. the numbers); and the nitrification lag (i.e. the time taken to attain saturation of soil), is an inverse estimate of the rate of proliferation of the organisms (R = relative growth rate--see p. 36).

The results for all the herbicides tested in this study, presented as lag to saturation of soil by the nitrifying organisms are given in Table III. The length (time) of the lag phase was defined by the intercept on the time axis of a line parallel to the NO_3^- axis, drawn through the intersect of the exponential curve and the linear curve (extrapolated backwards). A diagram showing how this was drawn is given in Fig. 24, which is a reproduction of the control curve in Fig. 17. The lag values in Table III were expressed as relative to the control

FIG. 14

THE EFFECT OF CHLOROTHAMID ON THE GROWTH OF NITRIFIERS IN FRESH PERFUSED SOIL

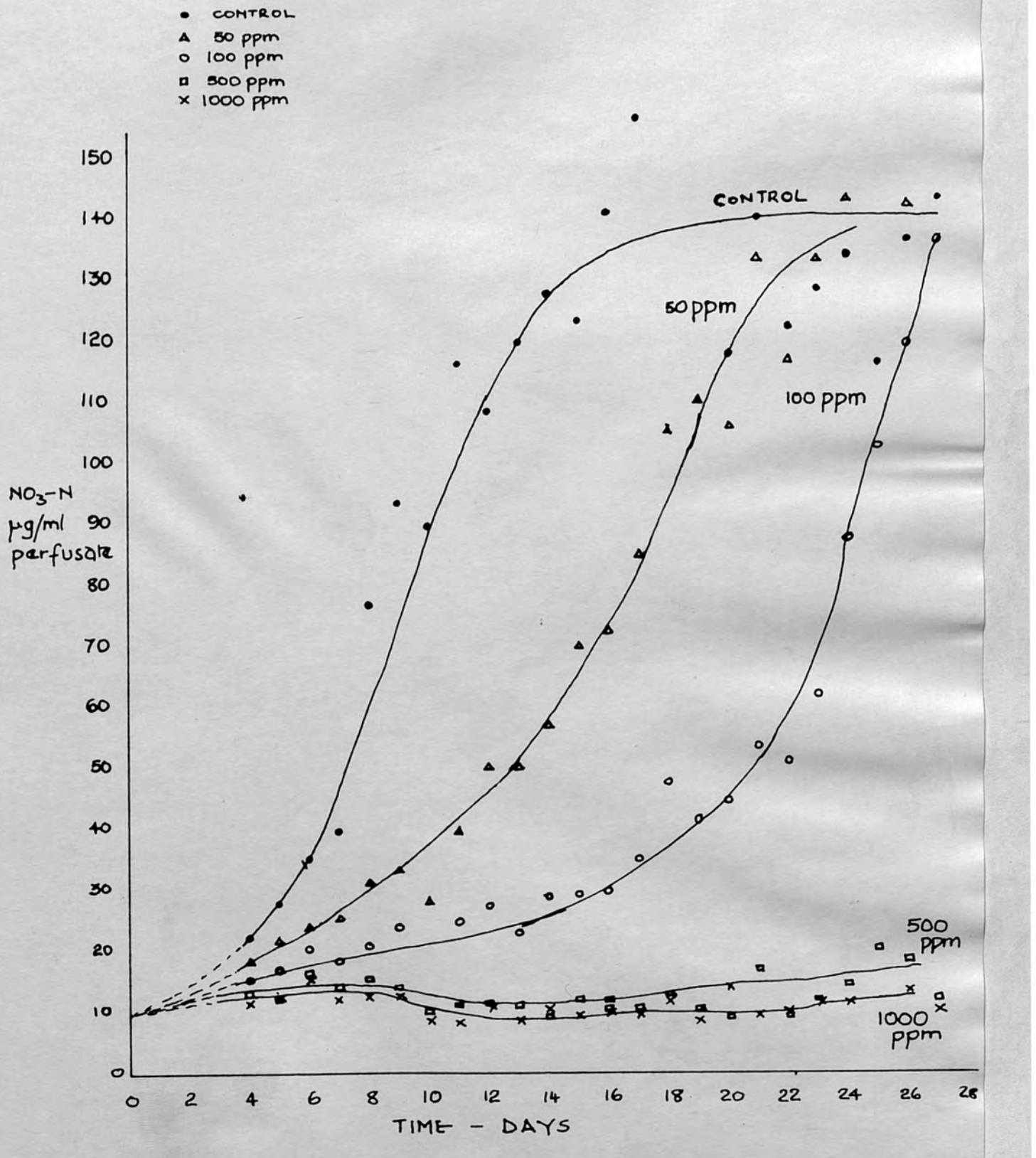


FIG 15

THE EFFECT OF PROPANIL ON THE GROWTH OF NITRIFIERS

IN FRESH PERFUSED SOIL

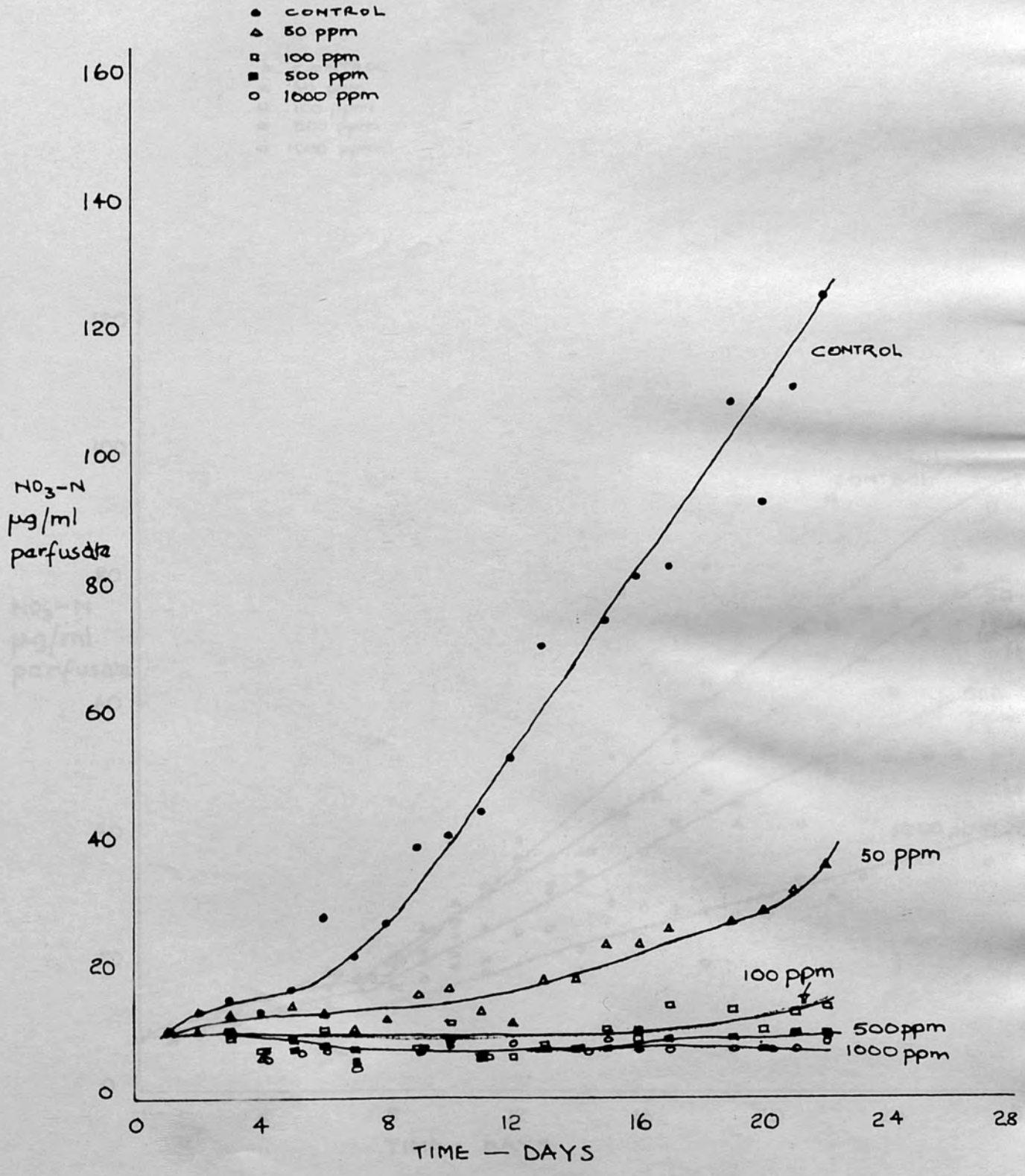


FIG. 16

THE EFFECT OF PARAQUAT ON THE GROWTH OF NITRIFIERS IN FRESH PERFUSED SOIL.

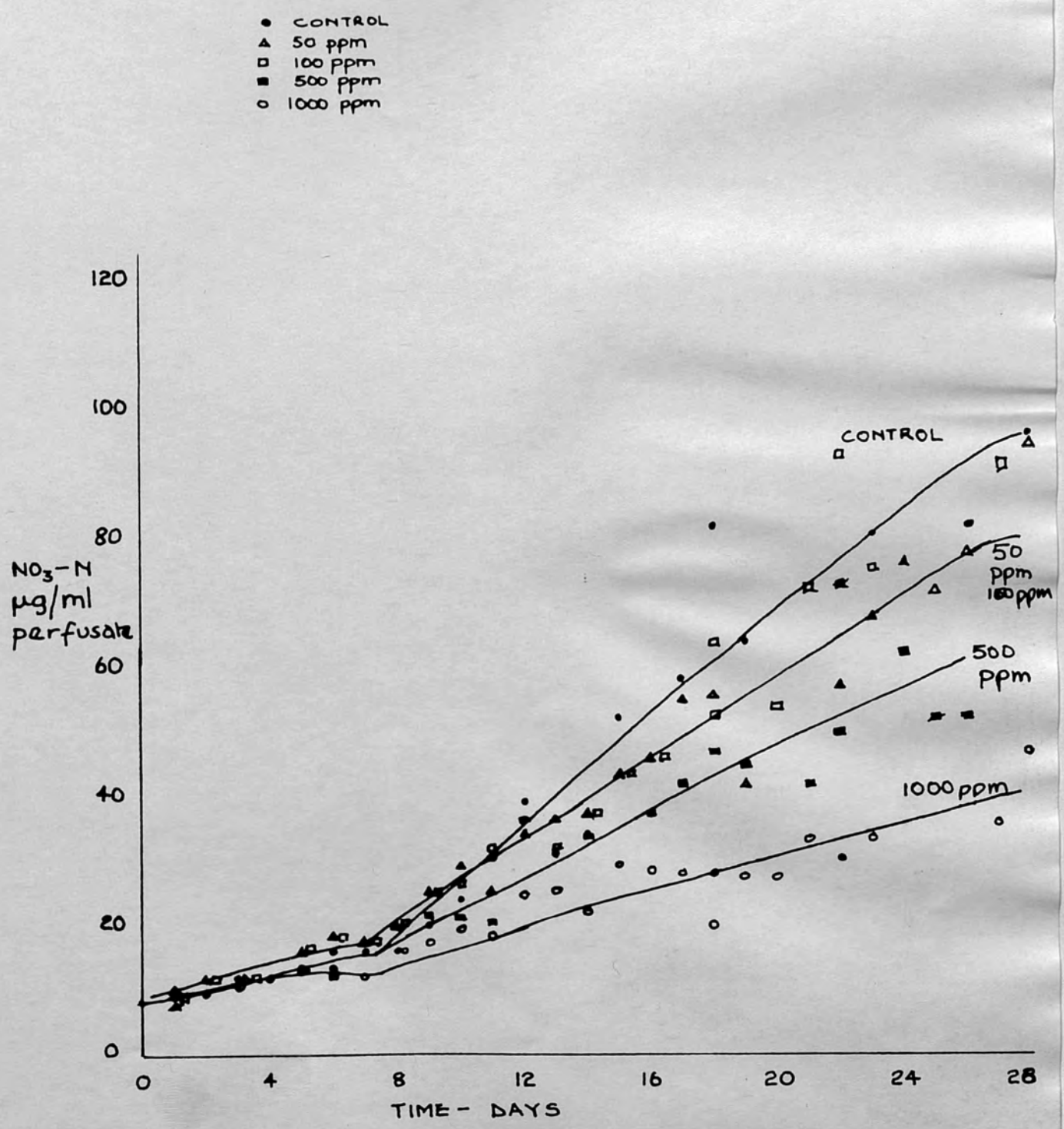


FIG 17

THE EFFECT OF PICLORAM ON THE GROWTH OF NITRIFIERS IN FRESH PERFUSED SOIL

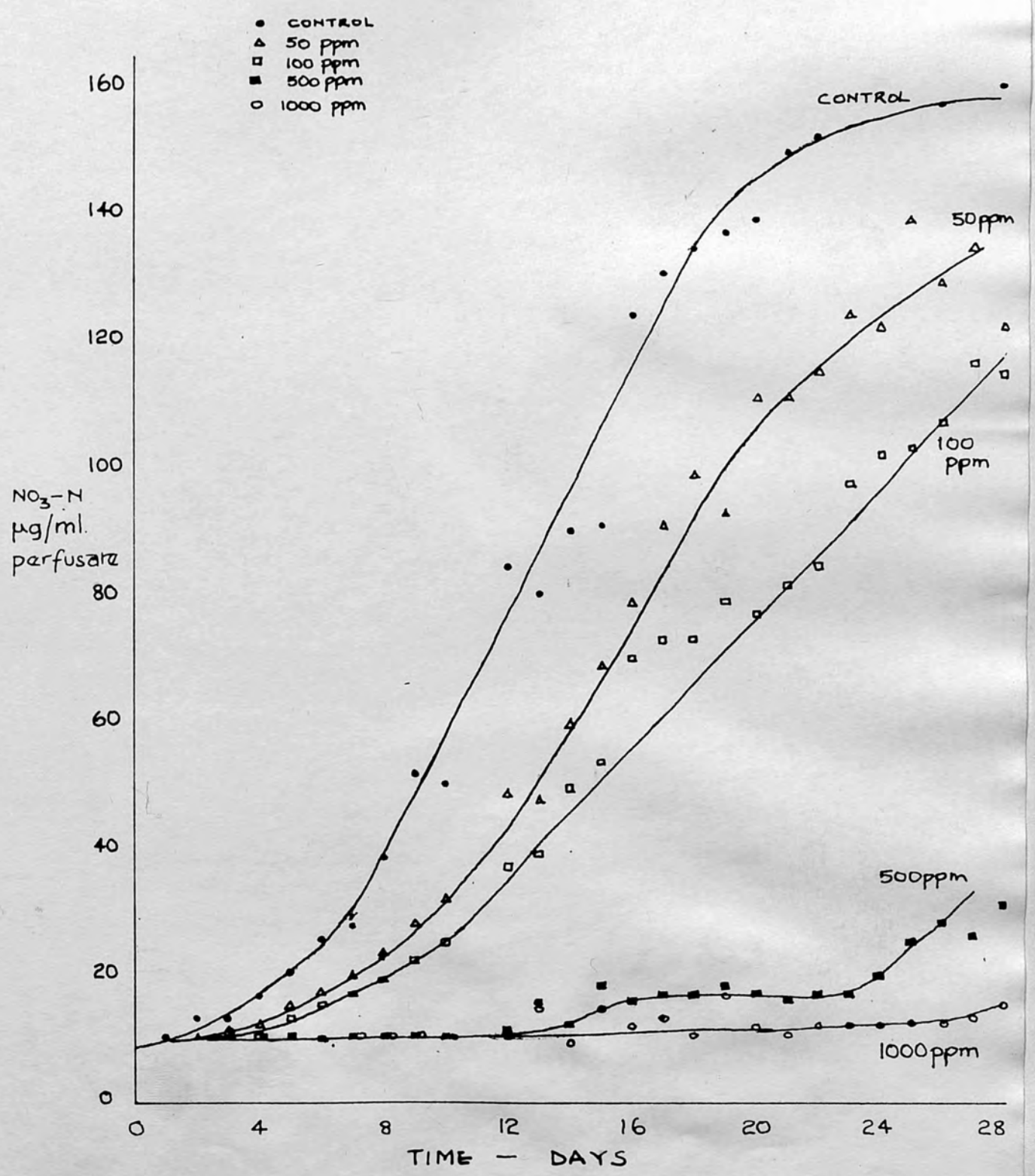


FIG.18

THE EFFECT OF BROMOXYNIL ON THE GROWTH OF NITRIFIERS IN FRESH PERFUSED SOIL.

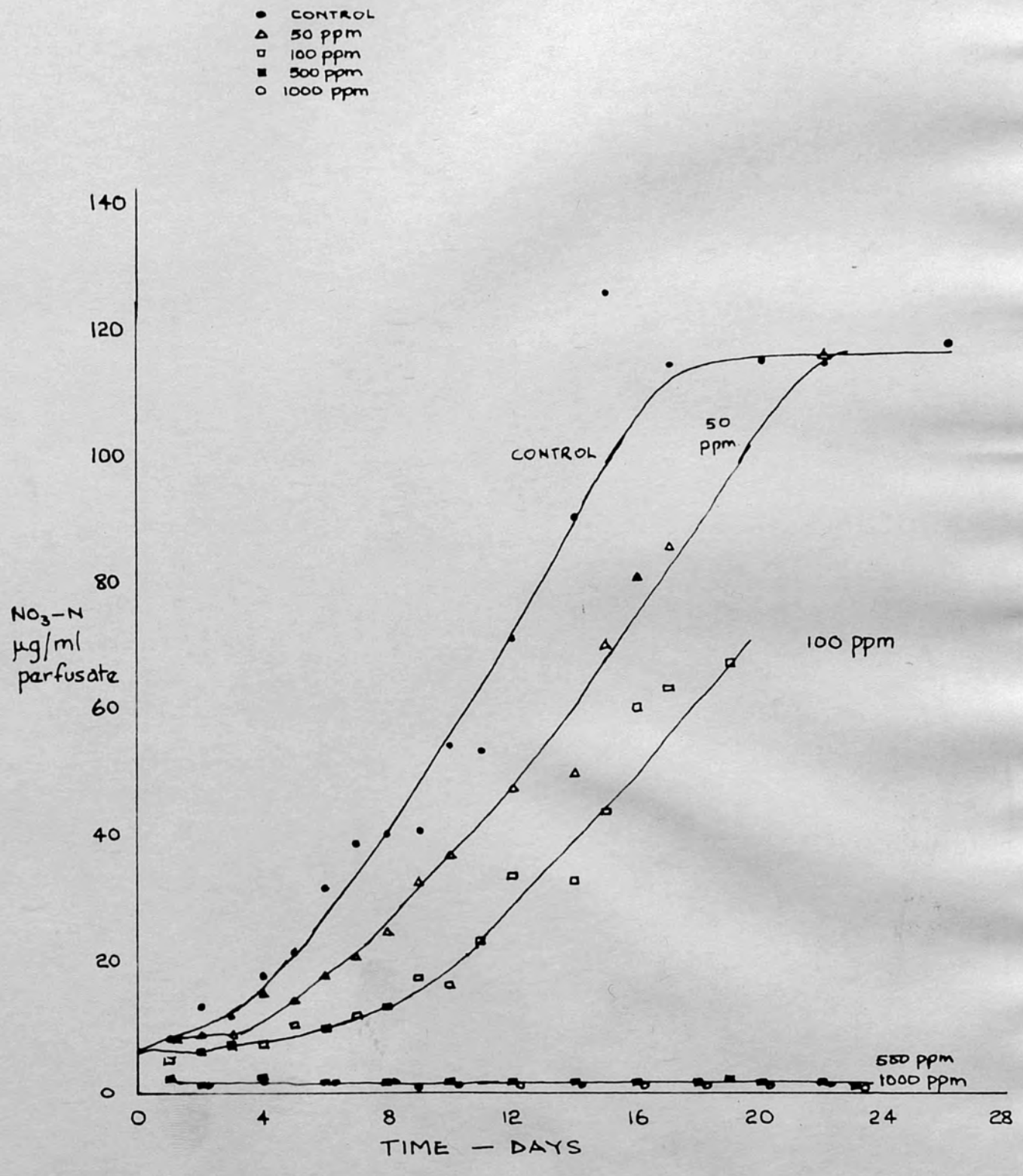


FIG. 19

THE EFFECT OF CHLOROFLURAZOLE ON THE GROWTH OF NITRIFIERS IN FRESH PERFUSED SOIL

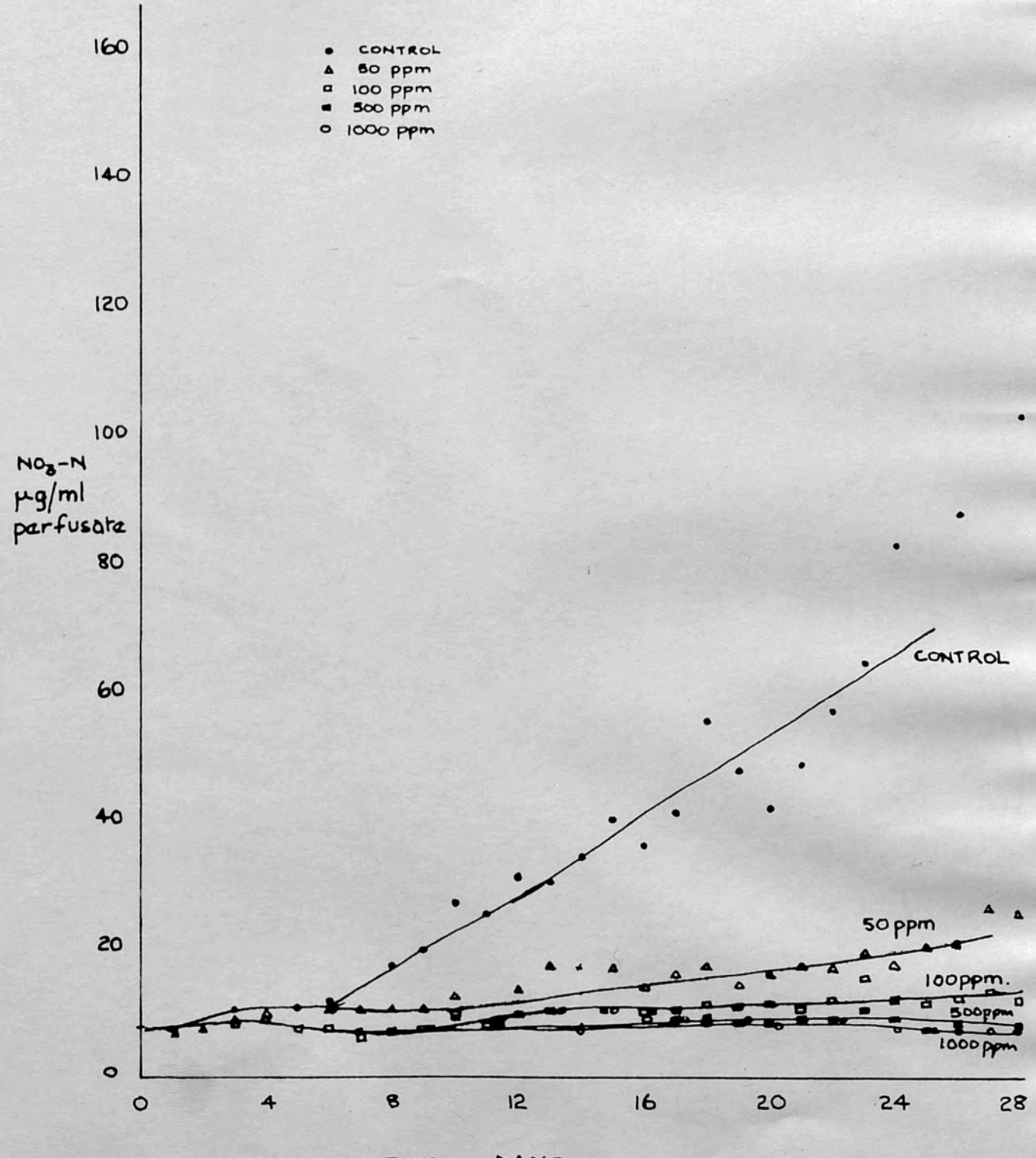


FIG 20

THE EFFECT OF DICHLOBENIL ON THE GROWTH OF NITRIFIERS IN FRESH PERFUSED SOIL

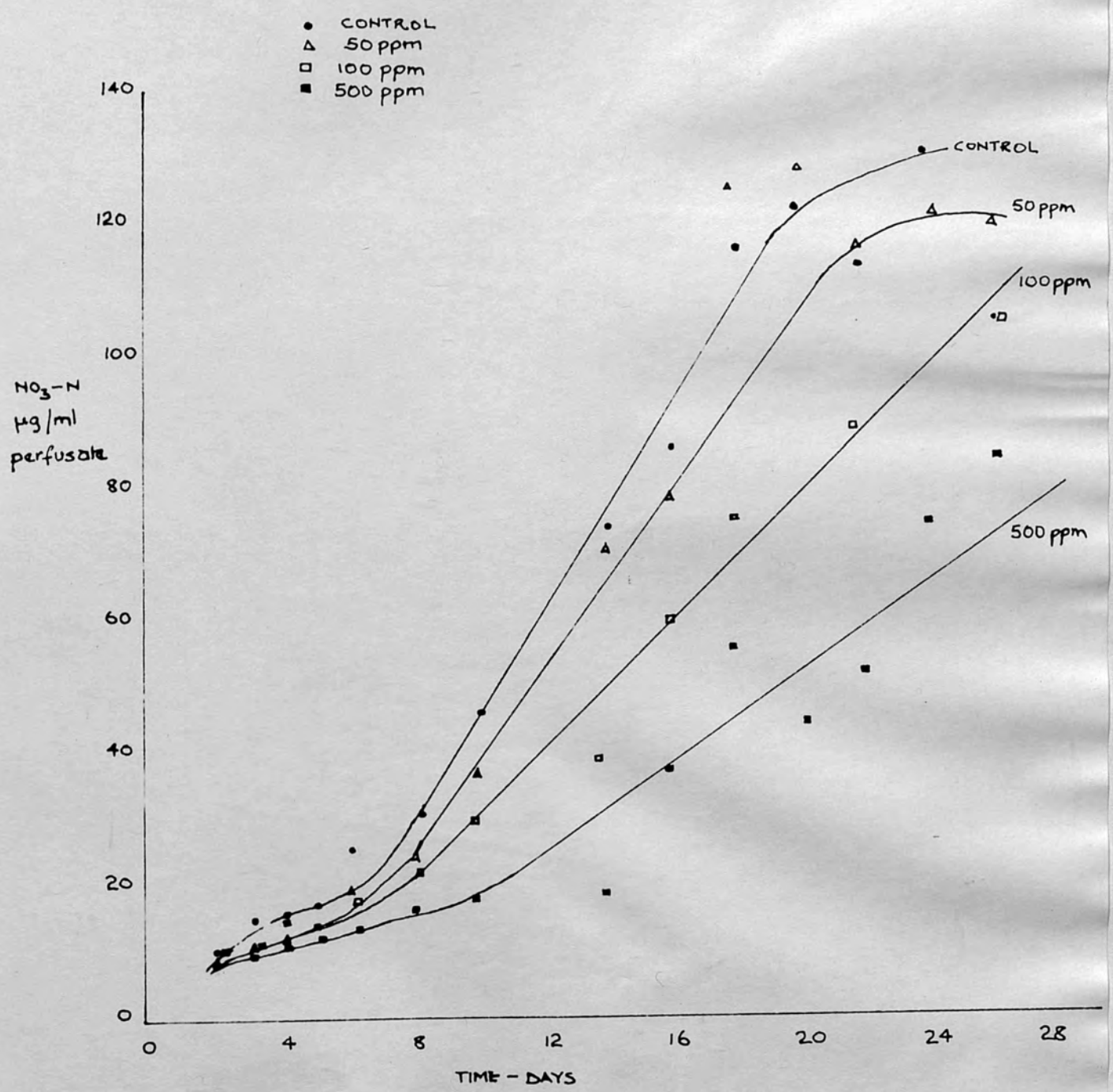


FIG. 21

THE EFFECT OF IOXYNIL ON THE GROWTH OF NITRIFIERS IN FRESH PERFUSED SOIL

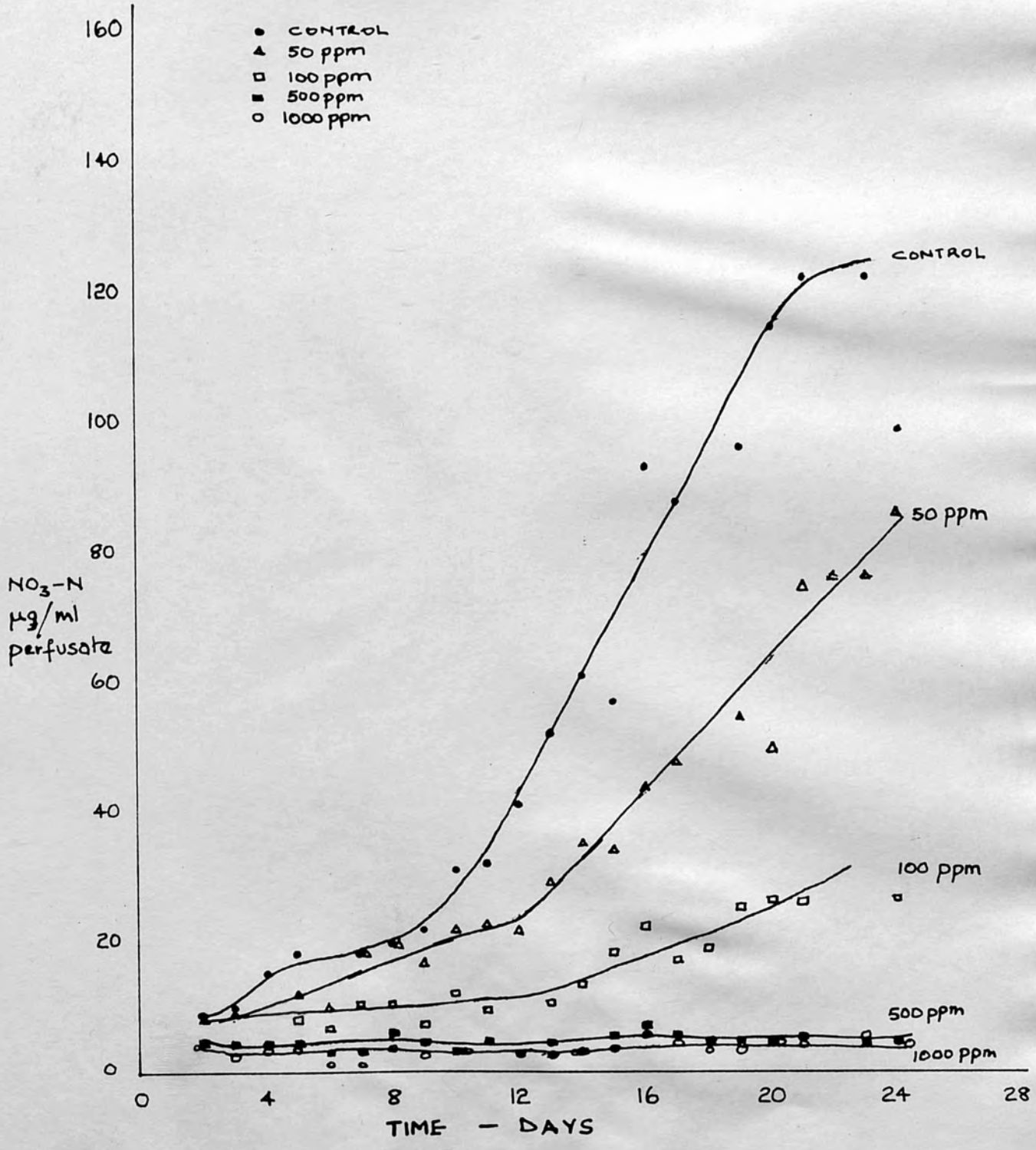


FIG 22

THE EFFECT OF ENDOTHAL ON THE GROWTH OF NITRIFIERS

IN FRESH PERFUSED SOIL

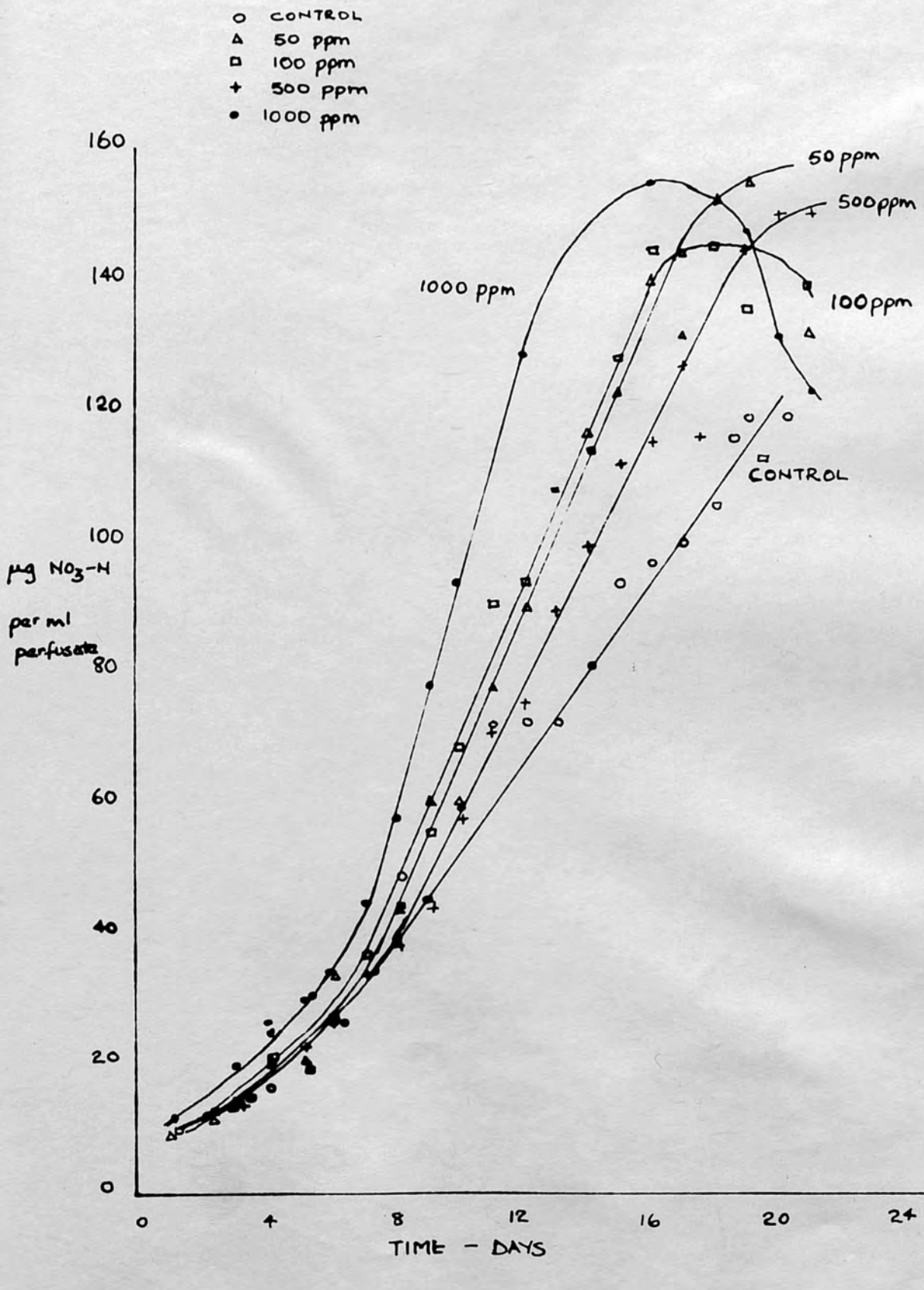


FIG 23

THE EFFECT OF 2,3,6-TBA ON THE GROWTH OF NITRIFIERS IN FRESH PERFUSED SOIL

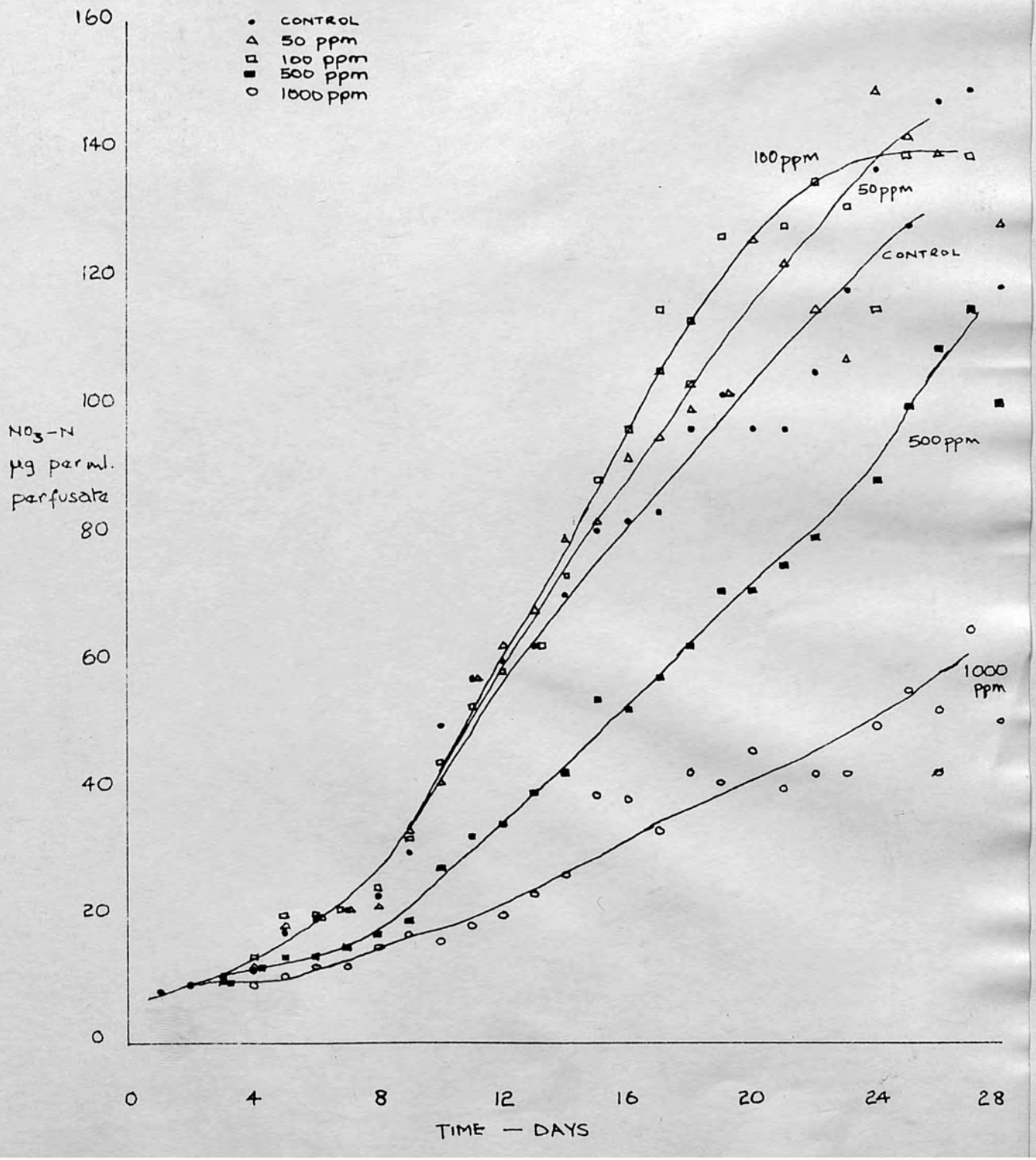


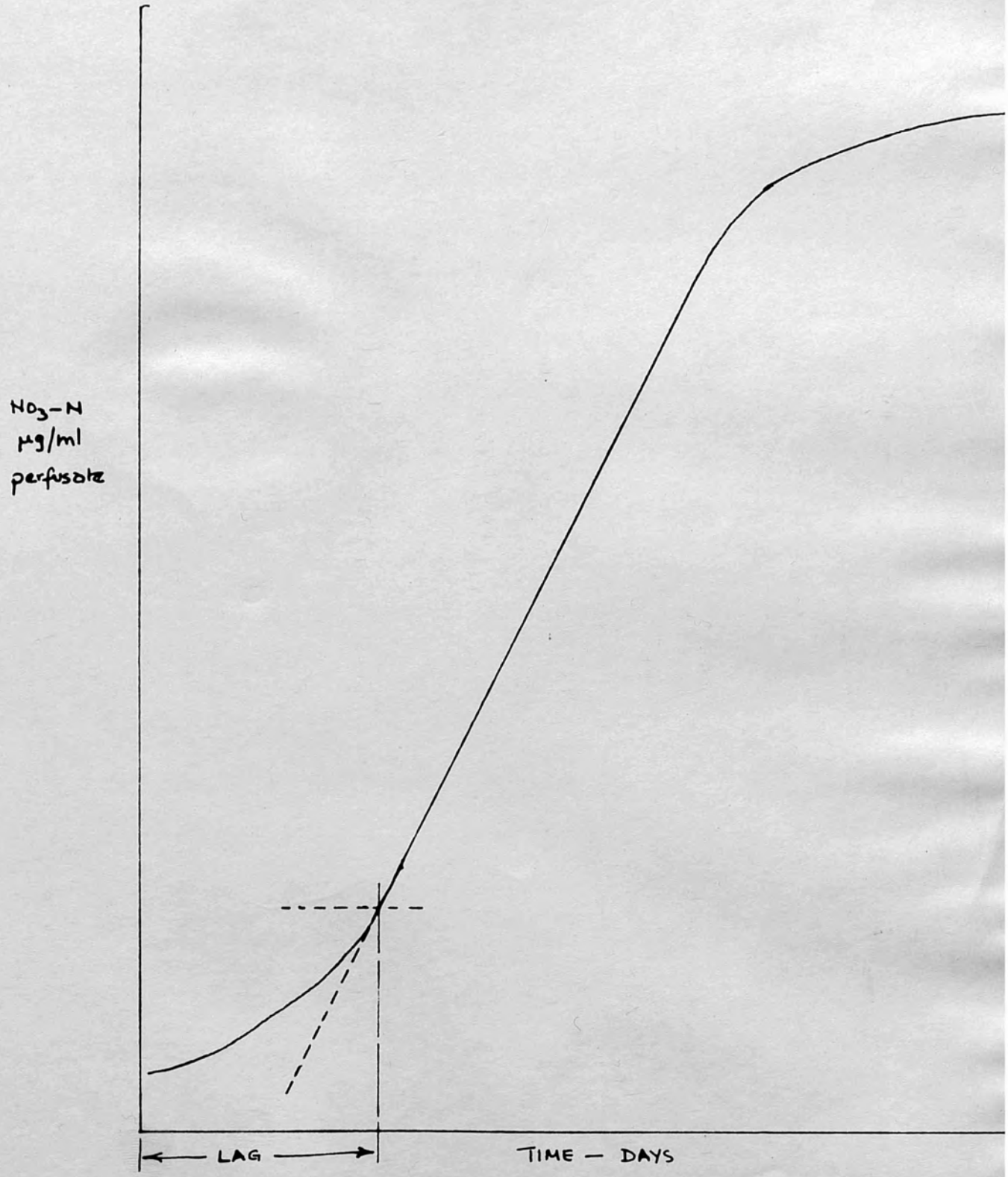
TABLE III

The lag (time to saturation) in proliferating nitrifiers in fresh soil perfused with ammonium sulphate solution in the presence of the herbicides listed at the concentrations indicated.

Herbicide	Herbicide concentration (ppm)				
	0	50	100	500	1000
			Days.		
2,3,6-TBA	9	9	9	10	11
Dichlobenil	9	10	10	12	-
Ioxynil	11	12	14	∞	∞
Paraquat	7.4	6	6	7.4	7.8
Endothal	10	8	8	10	8
Picloram	8	10	10	24	∞
Propanil	8.4	22.4	∞	∞	∞
Chlorothamid	6.2	15.4	22.4	∞	∞
Bromoxynil	6	8	11	∞	∞
Chloroflurazole	6	∞	∞	∞	∞

FIG 24

GRAPHICAL DETERMINATION OF THE LENGTH OF THE LAG PHASE



values for each herbicide, and plotted against $\log_{10}$ of the concentrations of the herbicides (Figs. 25 and 26). The toxicity of the herbicides as measured by reduction in relative growth rates were in the order Chloroflurazole > Ioxynil > Propanil > Picloram > Bromoxynil Chlorothamid > Dichlobenil > Paraquat > 2,3,6-TBA > Endothal.

In a saturated soil, provided there is no effect of herbicide on the rate of nitrification per cell, the nitrification rate will be an estimate of the populations of organisms present in the soil. It was shown in the previous section that these herbicides had a depressing effect on the nitrification process, hence, the nitrification rates (as determined by the slopes of the linear curves) are not true estimates of the population of nitrifiers in the soil on saturation. The nitrification rates (slopes of the linear curve) for the herbicides studied were determined and presented on Table IV. The values of Table IV were converted to relative nitrification rates as percentages of the control values and are presented as Figs. 27 and 28. The order of activity of the herbicides in this phase of the nitrification curves was Chloroflurazole > Ioxynil > Propanil > Picloram > Bromoxynil ≡ Chlorothamid > Dichlobenil > Paraquat > 2,3,6-TBA > Endothal.

TABLE IV

The estimates of the nitrification rate (μg NO_3-N/Day) when fresh soil is perfused with ammonium sulphate solution in the presence of the herbicides listed, at the concentrations indicated.

Herbicides	Herbicide concentration (ppm)				
	0	50	100	500	1000
2,3,6-TBA	6.6	8	10.6	4.65	2.53
Dichlobenil	7.6	7.2	5.2	4.3	-
Ioxynil	9.1	5.1	1.9	0	0
Paraquat	4.2	3.2	3.2	2.5	1.4
Endothal	7	11	11	10	17
Picloram	10.6	8.4	5.2	4	0
Propanil	7	7.1	0	0	0
Chlorothamid	14.1	11.5	14.9	0	0
Bromoxynil	8.3	6.5	5.5	0	0
Chloroflurazole	3.1	0	0	0	0

FIG 25

THE RELATIVE LENGTHS OF THE LAG PHASE PLOTTED AGAINST LOG. CONCENTRATION OF HERBICIDES

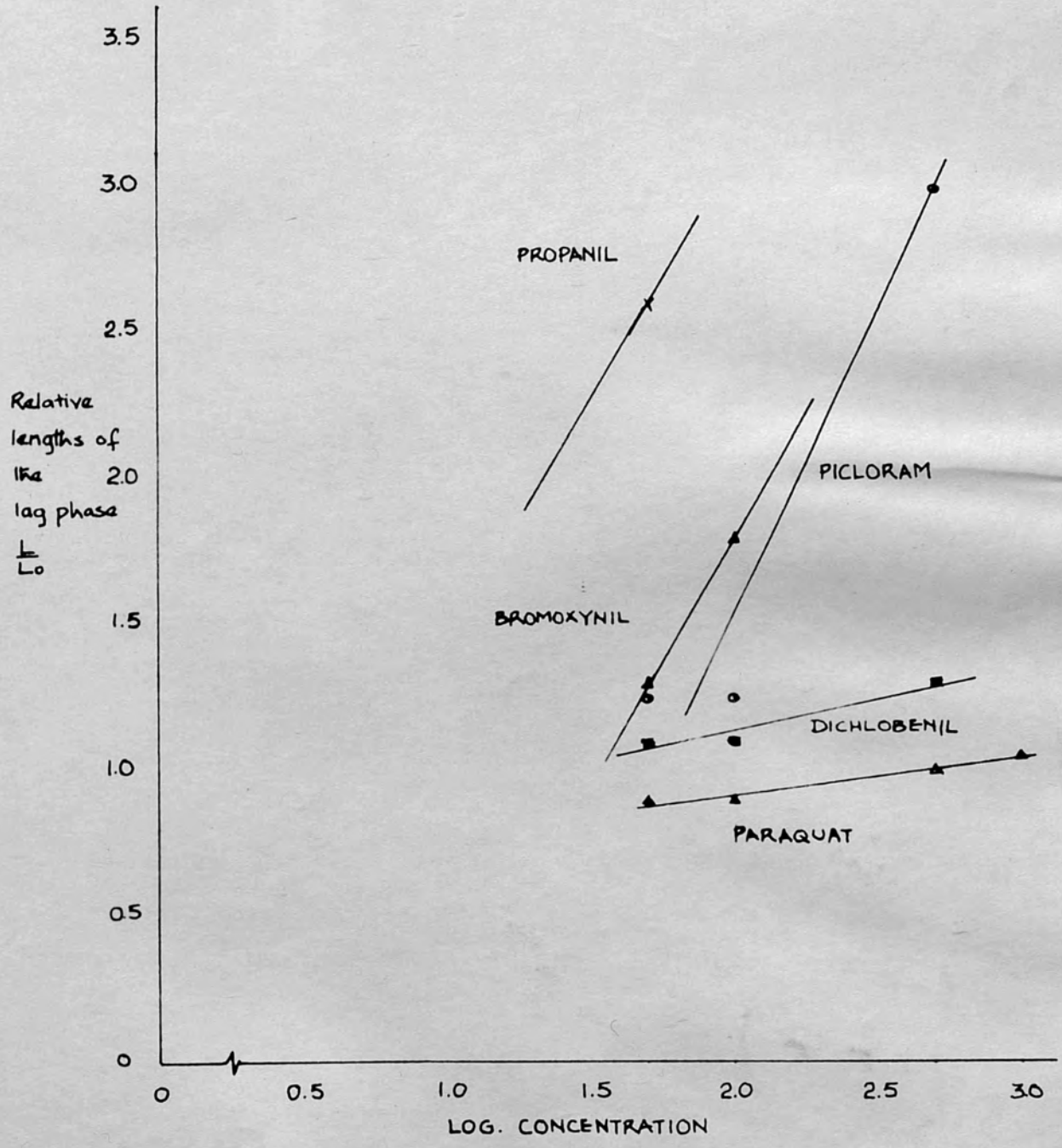


FIG 26

THE RELATIVE LENGTHS OF THE LAG PHASE PLOTTED AGAINST LOG. CONCENTRATION OF HERBICIDES

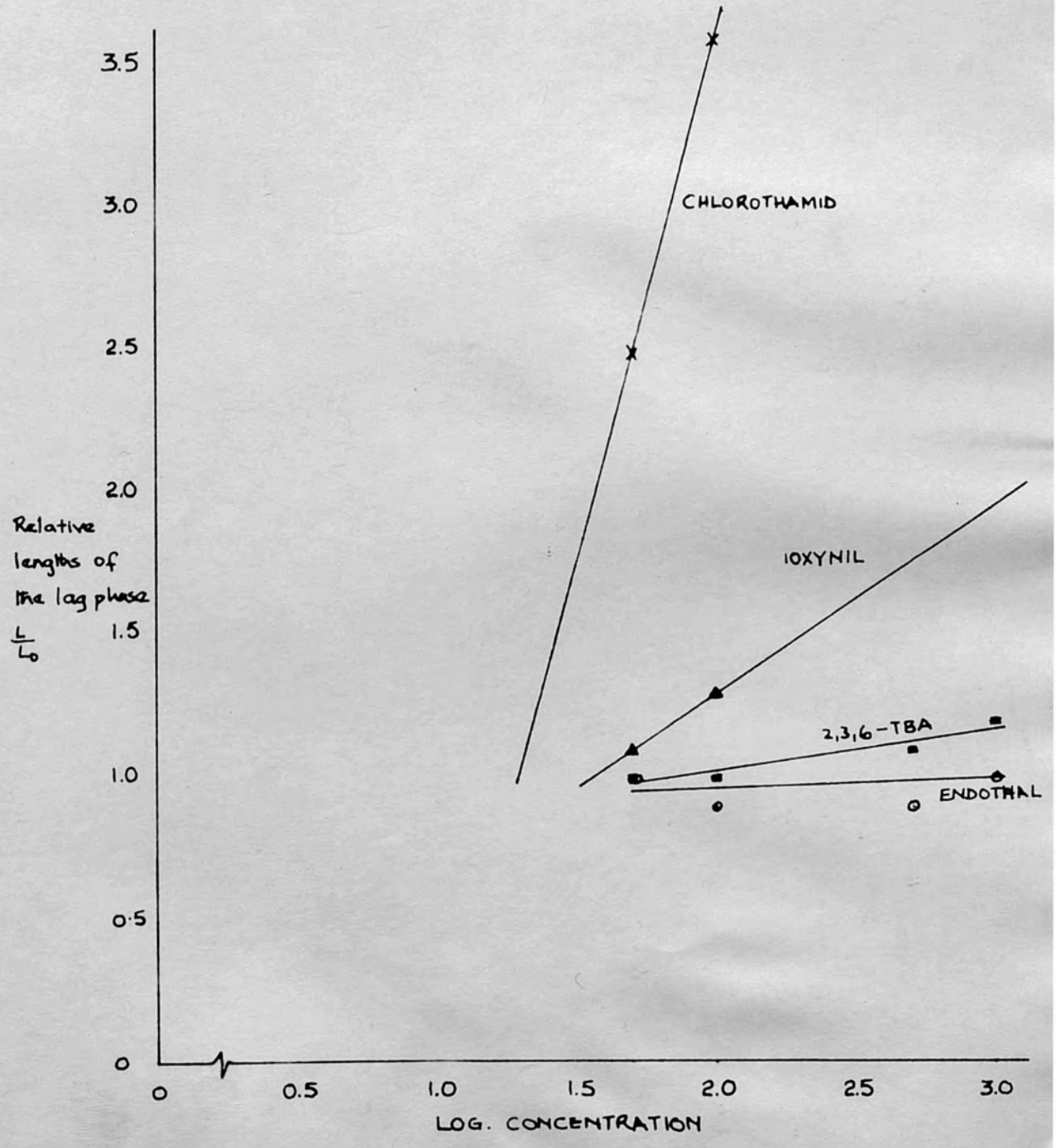


FIG 27

% OF CONTROL NITRIFICATION RATES PLOTTED

AGAINST LOG. CONCENTRATION OF HERBICIDES

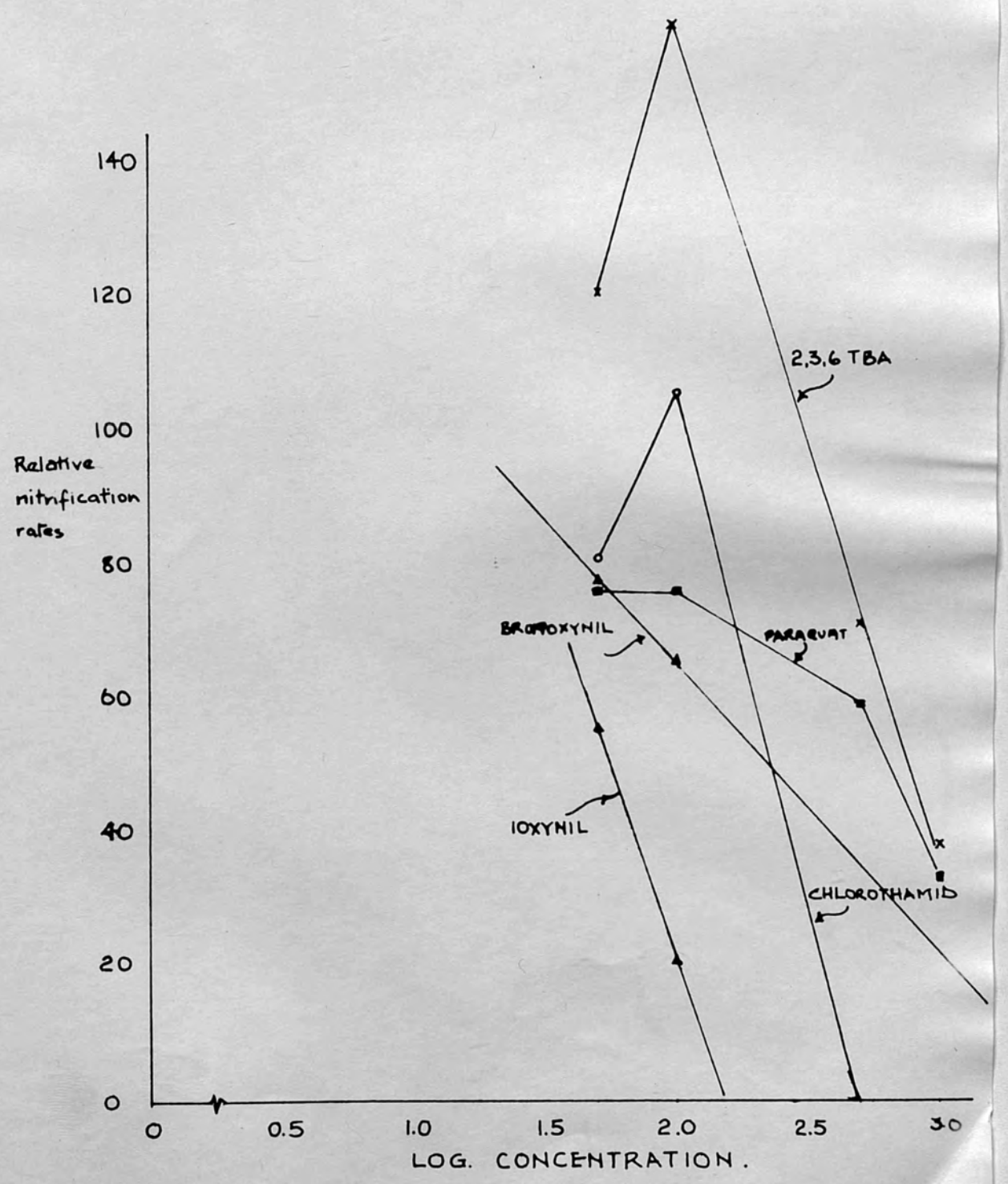
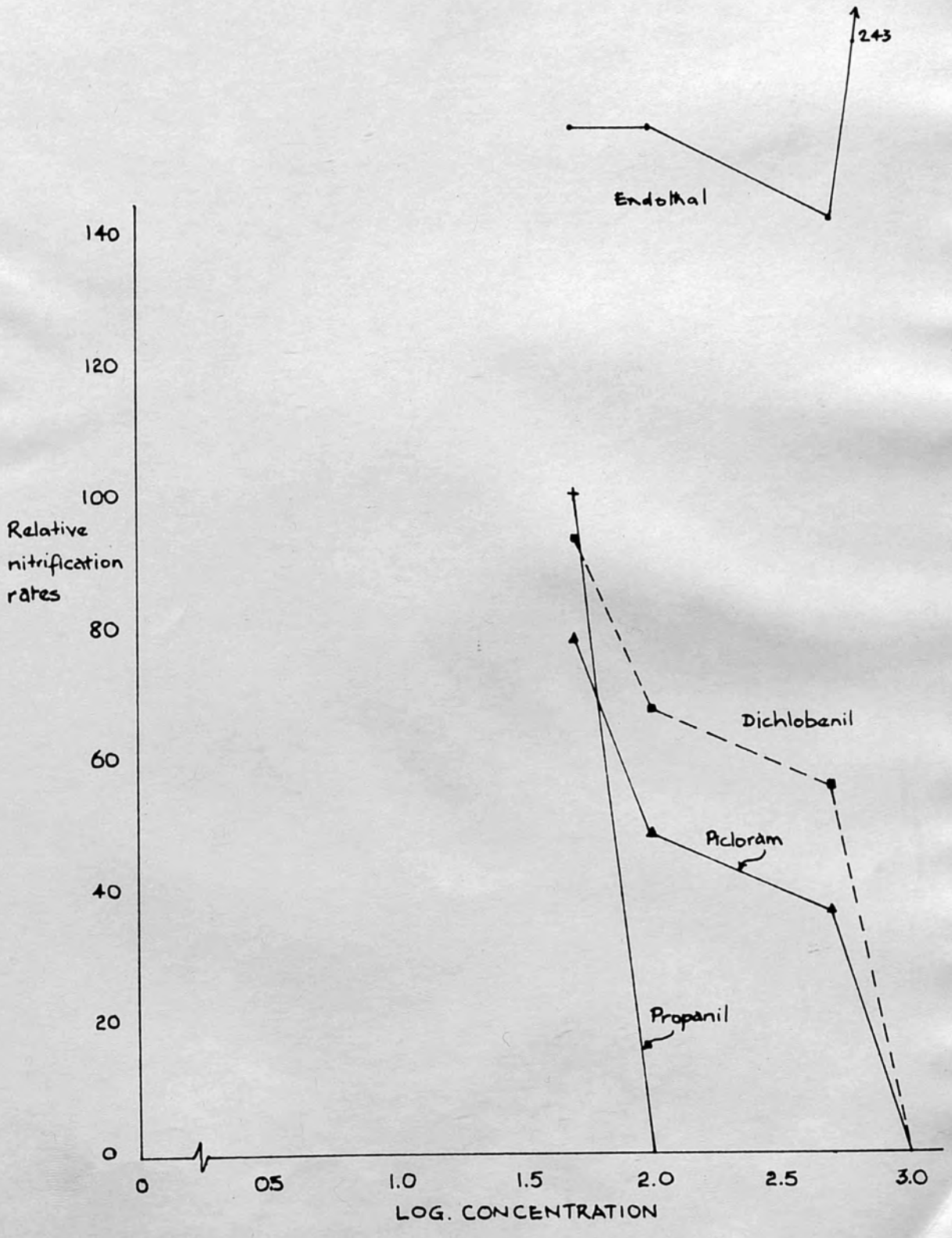


FIG 28

% OF CONTROL NITRIFICATION RATES PLOTTED

AGAINST LOG. CONCENTRATION OF HERBICIDES



The possibility of restoring nitrification activity to soil previously perfused with ammonium sulphate and herbicide for the first (and only) enrichment growth cycle was explored. The herbicides Picloram, Chlorofluorazole and Propanil were used and were perfused at the usual concentrations. The effects of 28 days perfusion on the nitrifiers in the presence of the compounds, and on the subsequent behaviour of the nitrifiers when perfused with ammonium sulphate solution in the absence of the herbicides were followed. The growth curves obtained are presented as Figs. 29, 30 and 31. For the purpose of this discussion, the term 'washed soil' will apply to the washed perfused soil while 'fresh soil' will apply to non-enriched soils as for normal treatments. The lag in growth and estimates of the nitrification rates of the exponential phase are given in Tables VI and V respectively.

The results for Picloram (Fig. 29) mean that:

(a) the treatment of soil at all concentrations up to 500 ppm had no effect on the total maximum population, since there was no lag and the nitrification rates were the same.

(b) treatment of soil at 500 ppm led to the nitrification rate being doubled, i.e. the numbers of nitrifiers were apparently doubled. The increase in nitrification may be partially explained by either (i) small amounts of residual

FIG 29

GROWTH OF NITRIFYING BACTERIA IN SOIL PREVIOUSLY PERFUSED WITH PICLORAM FOR 28 DAYS, WASHED, AND RE-PERFUSED WITH AMMONIUM SULPHATE SOLUTION

PREVIOUS TREATMENTS ARE INDICATED

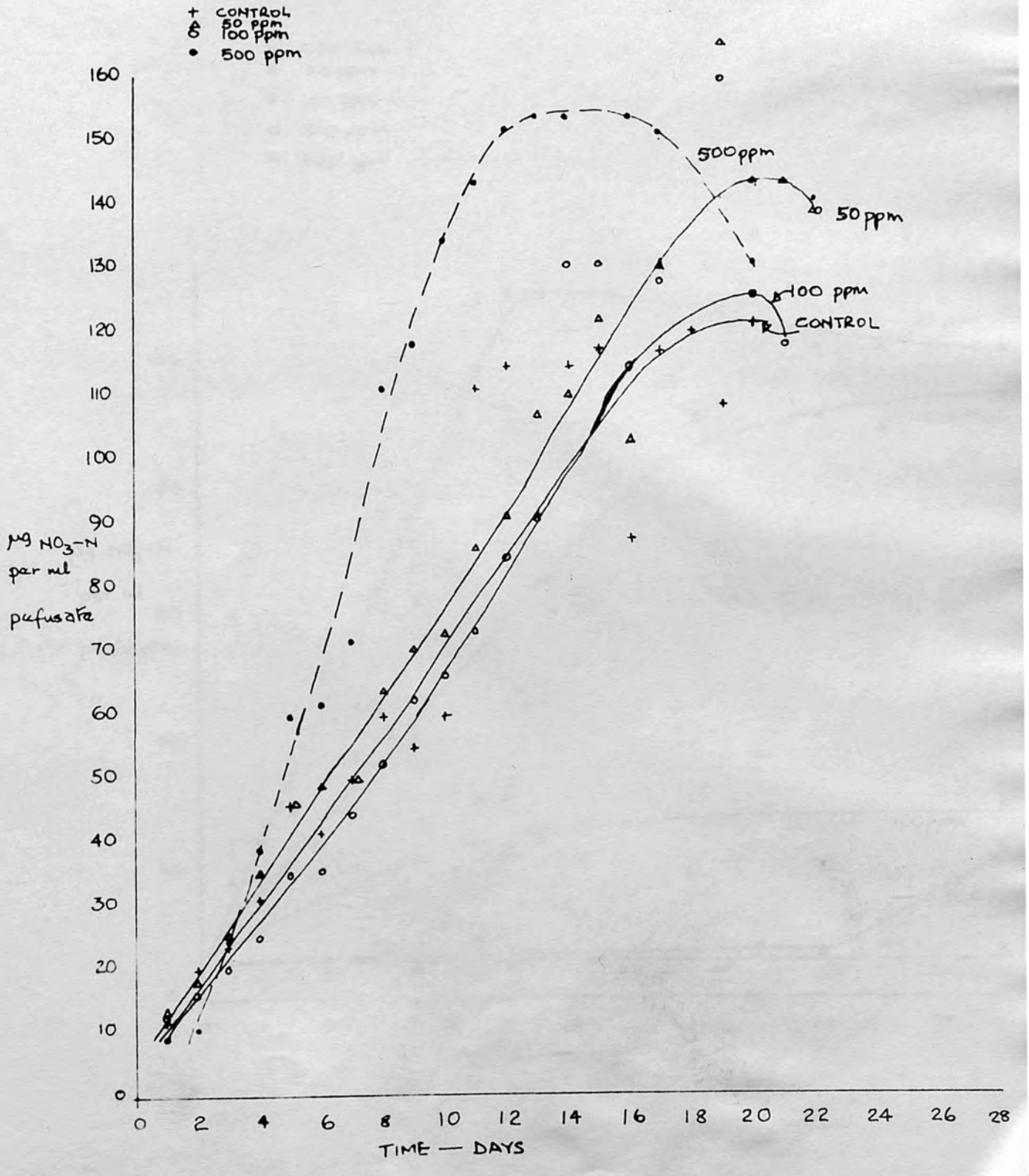


FIG 30

THE GROWTH OF NITRIFYING BACTERIA IN SOIL PREVIOUSLY PERFUSED WITH CHLOROFLURAZOLE FOR 28 DAYS, WASHED, AND REPERFUSED WITH AMMONIUM SULPHATE SOLUTION

PREVIOUS TREATMENTS ARE INDICATED

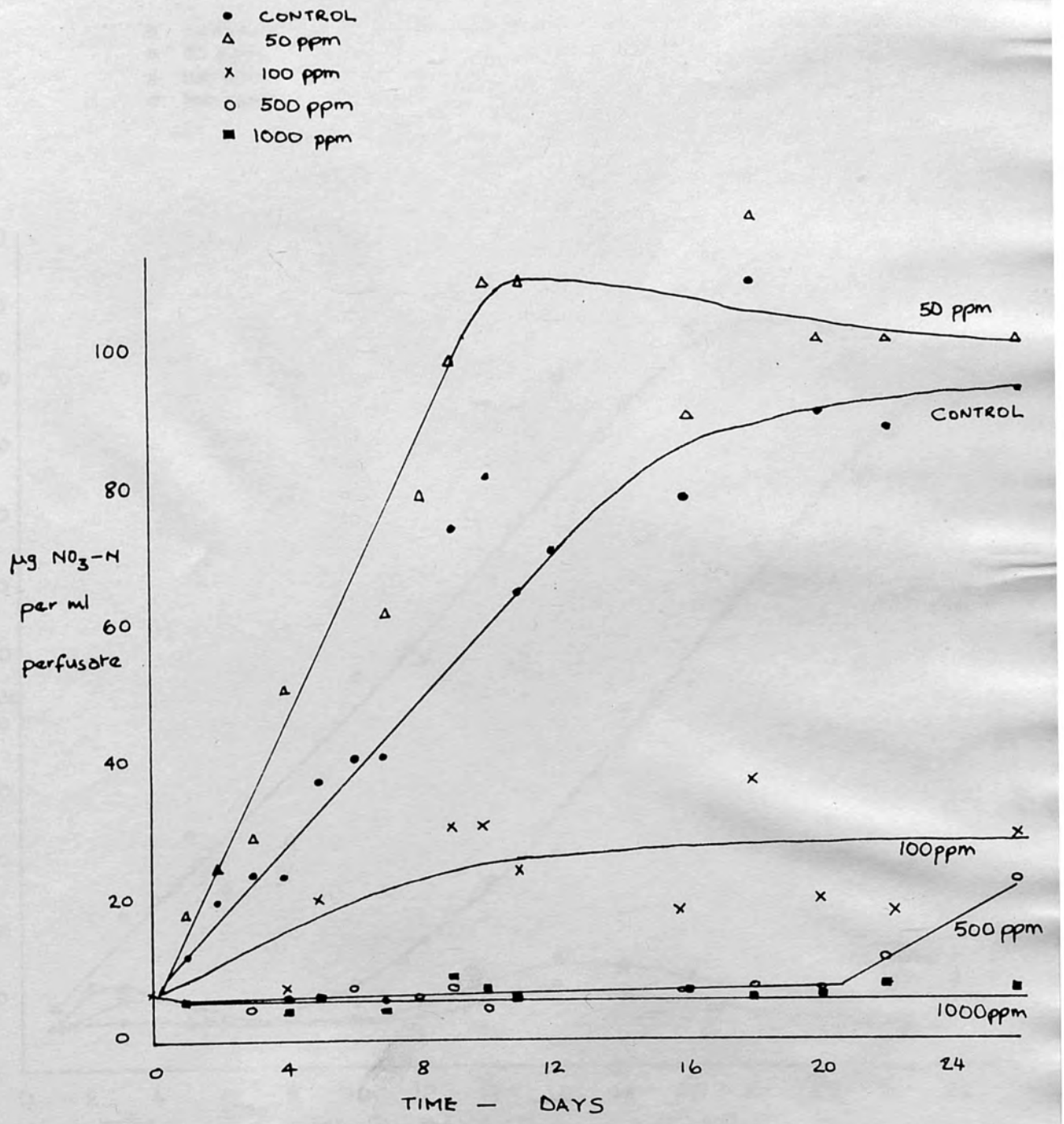


FIG 31

THE GROWTH OF NITRIFYING BACTERIA IN SOIL PREVIOUSLY PERFUSED WITH PROPANIL FOR 28 DAYS, WASHED, AND REPERFUSED WITH AMMONIUM SULPHATE SOLUTION.

PREVIOUS TREATMENTS ARE INDICATED

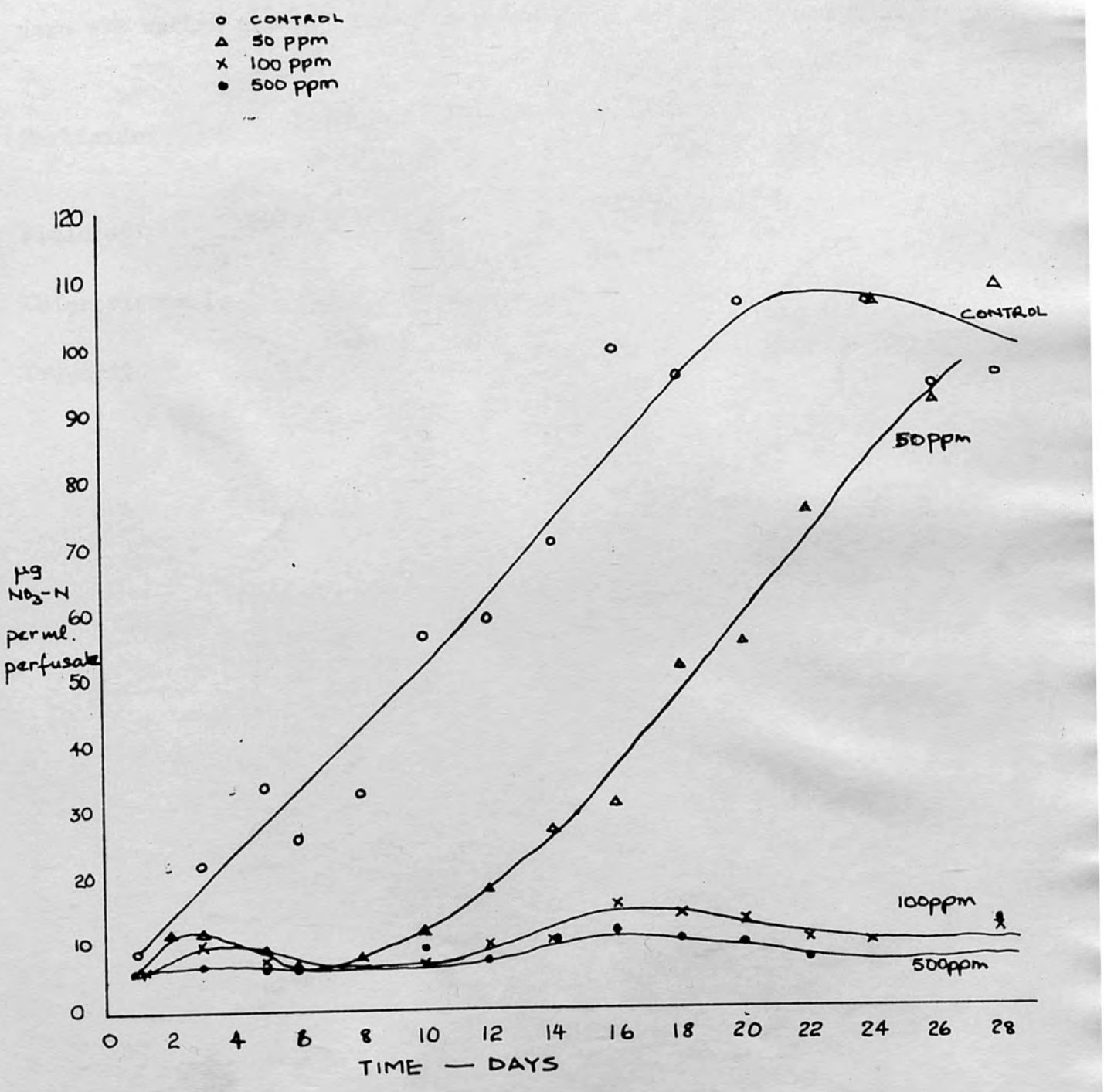


TABLE V

Estimates of the nitrification rate (μgNO_2 - N/ml/day) when fresh soil perfused with herbicide and ammonium sulphate for 28-30 days was washed and reperfused with ammonium sulphate solution only.

Herbicides	Herbicide concentration (ppm)				
	0	50	100	500	1000
Picloram	6.8	7.4	7.0	15.6	-
Chloroflurazole	5.5	10.5	3.0	3.0	0.0
Propanil	5.0	6.0	0.0	0.0	-

TABLE VI

The lag (growth rate) of nitrifiers in fresh soil previously perfused with herbicide and ammonium sulphate for 28-30 days was washed, and reperfused with ammonium sulphate solution only.

Herbicide	Herbicide concentration (ppm)				
	0	50	100	500	1000
Picloram	0	0	0	0	-
Chloroflurazole	0	0	0	20	〃
Propanil	0	10.6	〃	〃	-

herbicide actually promoted the nitrification rate or (ii) that a breakdown product of the herbicide promoted nitrification rate: both of which explanations assume that the soil had the same population as in the control.

The results for Chloroflurazole may be explained as follows:

(1) Treatment of the soil at 500-1000 ppm effectively sterilized the soil as regards nitrifiers, since there was no growth (no nitrification).

(2) Perfusion of soil with 50 ppm caused a stimulation of nitrification, most probably in the manner described for Picloram at 500 ppm (b) above.

(3) Perfusion of soil at 100 ppm--this appears to be a permanent effect on the total "saturation capacity" of the soil: the nitrification started and then stopped--these results are very queer and inexplicable.

The results for Propanil (Fig. 31) may be explained as follows:

(1) Treatment of soil at 100-500 ppm effectively sterilized the soil as regards nitrifiers.

(2) On treatment of soil with 50 ppm of this herbicide the lag greatly increased; this indicates that there is a reduction of cell numbers to well below the usual number in non-enriched soil, since the lag in the fresh soil (8.4) was more or less doubled ($\simeq$ 15 µg/day).

This is shown to be so in the following:

Let N_o be usual numbers of nitrifiers in non-enriched soil and N_o^1 = starting number of cells in 'washed'soil at 50 ppm.

Then by previous reasoning,

$$\log \frac{N}{N_o} = R_1 t \text{ and } \log \frac{N^1}{N_o^1} = R_1 t^1$$

Assuming that at saturation, $N = N^1$

then, $\log \frac{N_o}{N_o^1} = R_1(t^1-t)$, i.e. the difference in the lags is a measure of the reduction in the usual numbers of the cells. The actual value cannot be estimated since we do not know R_1.

Further possible explanations of the stimulated nitrification rates with Picloram at 500 ppm and Chloroflurazole at 50 ppm may be that the herbicides killed off other non-nitrifiers which (a) either compete for limited growth factors or (b) compete for nitrification sites and thus reduce the maximum numbers of nitrifiers in the untreated soil. From the results of Winogradski (quoted by Quastel and Scholefield, 1951), it was established that the oxidation of ammonia proceeded in two stages:

a) $NH_4^+ \longrightarrow NO_2^-$ (Nitrosomonas sp)

b) $NO_2^- \longrightarrow NO_3^-$ (Nitrobacter sp)

In studies on the effects of herbicides on soil nitrification made so far, the total nitrate production was used as an index of nitrification. This does not give any

clues as to which of the above stages are affected. If stage a) was affected, there should be little or no nitrification; on the other hand if b) was affected, there should be an accumulation of the nitrite produced in stage a). If, however, both stages were equally susceptible to or tolerant of the presence of the herbicides, there would be no nitrite accumulated, either because no nitrite was produced by Nitrosomonas or because the nitrite produced was almost immediately converted to nitrate by Nitrobacter. As the method of estimating total nitrification by presence or absence of nitrate involved partially the oxidizing of any nitrites present in the perfusate, it was not possible to tell or indicate which of the above steps was being inhibited.

Two attempts were made in this study to follow the effect of herbicides on the appearance or disappearance of nitrite in the perfusate. The herbicides used were dichlobenil and chloroflurazole. Their use was fortuitous, as they happened to be the test chemicals under study when this phase of work was thought of. Nitrite was estimated by the Greiss-Ilosvay technique.

The results with the relevant nitrate curves plotted on the same graphs are presented as Figs. 33 and 34. In Fig. 33 the amount of nitrite accumulated was

FIG 33

THE EFFECT OF DICHLOBENIL ON THE ACCUMULATIOF OF NITRATE AND NITRITE WHEN FRESH SOIL WAS PERFUSED WITH AMMONIUM SULPHATE SOLUTION

NITRATES - BOLD LINES NITRITES - BROKEN LINES

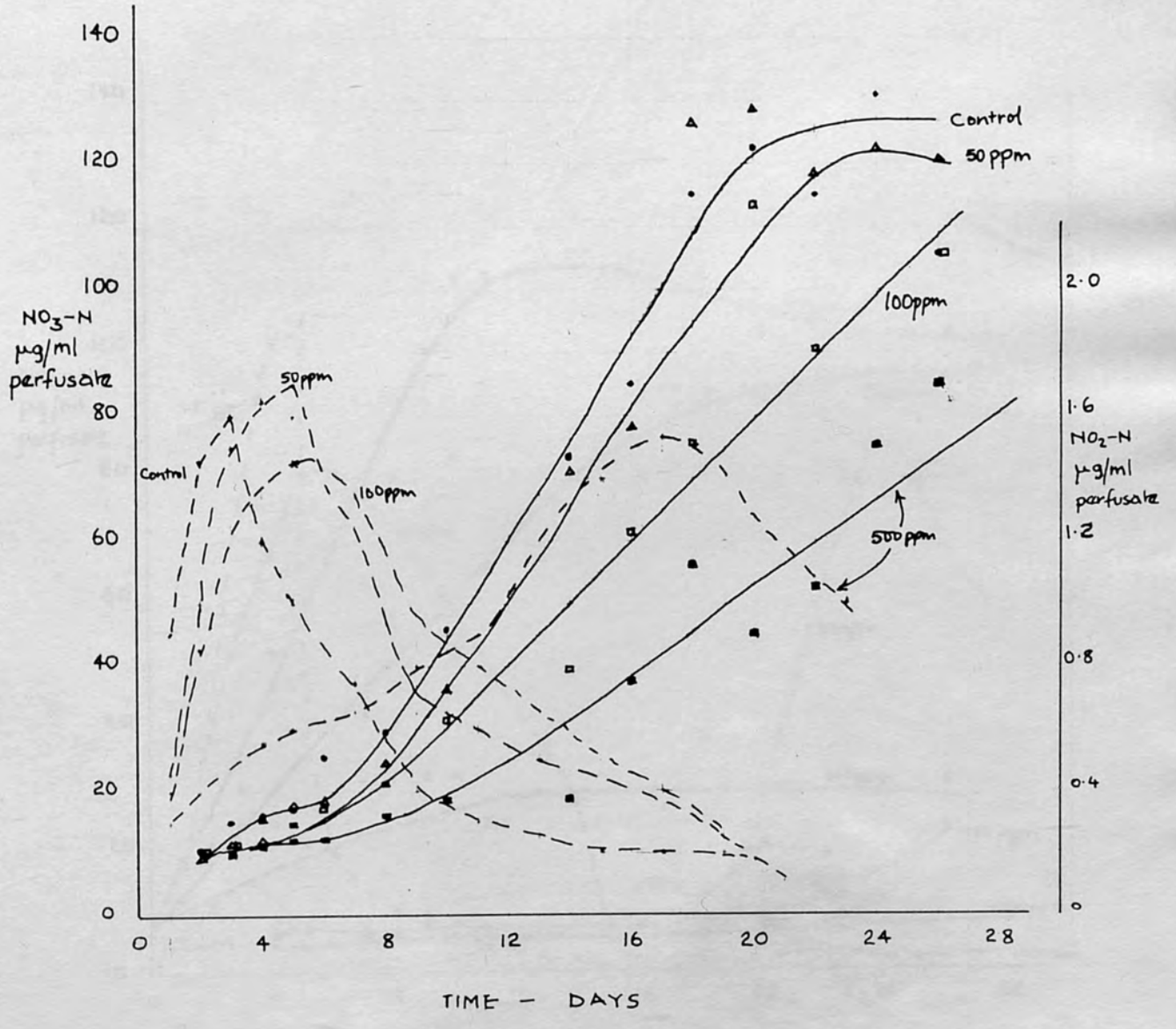
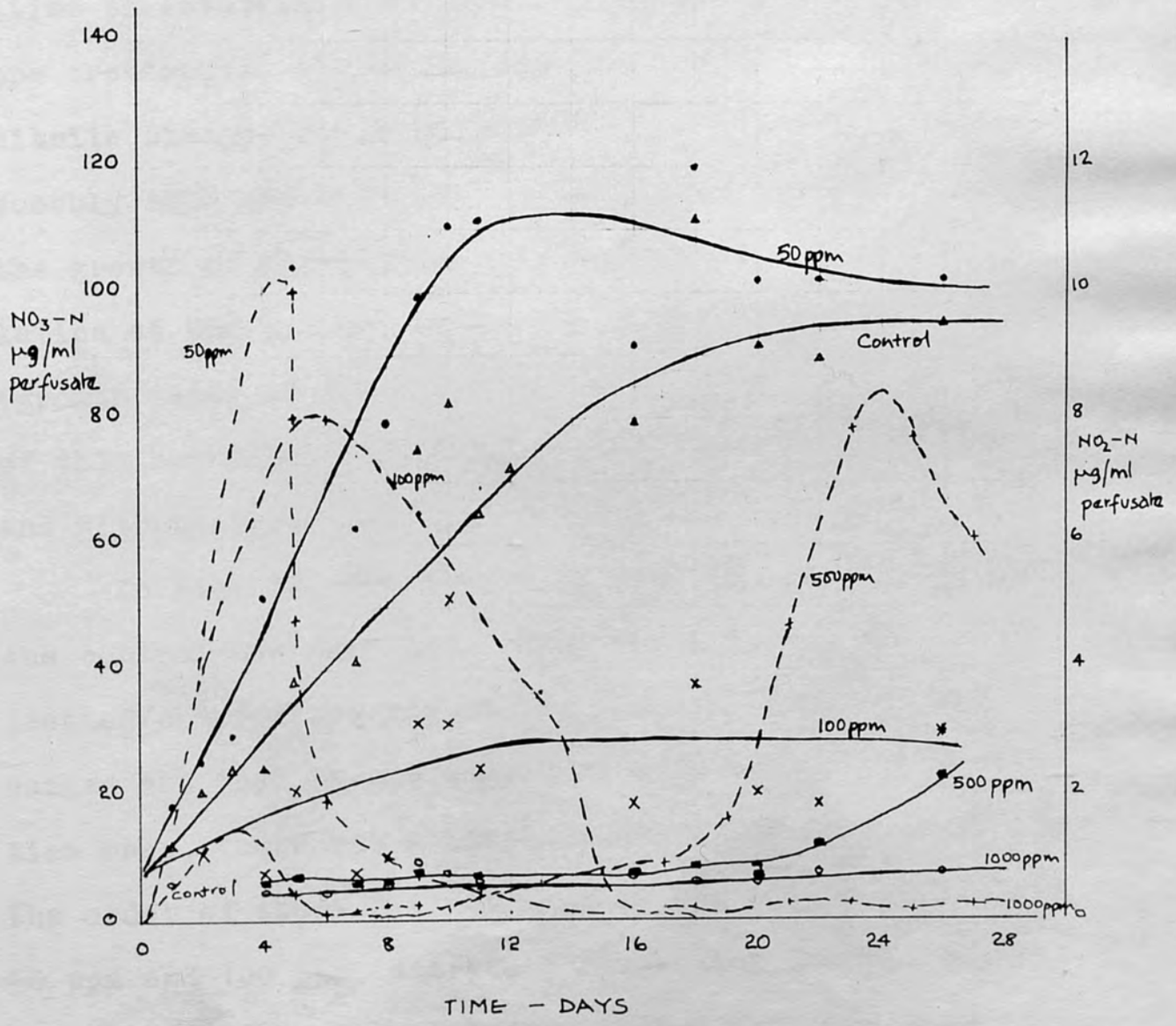


FIG 34

THE ACCUMULATION OF NITRATE AND NITRITE IN SOIL PREVIOUSLY PERFUSED WITH CHLOROFLURAZOLE FOR 28 DAYS, WASHED AND RE-PERFUSED WITH AMMONIUM SULPHATE SOLUTION. PREVIOUS TREATMENTS ARE INDICATED

NITRATES — BOLD LINES NITRITES – DOTTED LINES.



very low, only 1.6 µg/ml, but this amount was the same at all test concentrations. The maximum accumulation of nitrite was on day 3 for the control and on day 5 for both the 50 and 100 ppm--the nitrite then disappeared gradually over the next few days. The peak value of nitrite at 500 ppm was on day 17. The values for the (nitrate) lag (Table III and this figure) indicate quite clearly that there was no effect on the growth rate (time to saturation) between the control, 50 ppm and 100 ppm treatments, but at 500 ppm the lag was 12 days. The nitrite disappeared a few days after it was formed; presumably this was the time required to stimulate/activate the growth of Nitrobacter. The drift in nitrite accumulation at 500 ppm, and hence the differences in the lag (growth rate) of the nitrate curves, was due to an effect of this herbicide acting equally on both Nitrosomonas and Nitrobacter.

In Fig. 34, the amount of nitrite accumulated in the control was very low. When the soil was previously treated with chloroflurazole at 50, 100, 500 and 1000 ppm, washed and then re-perfused with ammonium sulphate solution only, there was a large accumulation of nitrite of the order of about 8 times that of the control. At both 50 ppm and 100 ppm, nitrite accumulation started almost immediately, rose to a maximum in 4-5 days and then

disappeared. At 500 ppm, nitrite accumulation started on day 18, rising to a maximum on day 24, and then fell. The large accumulation observed indicated that there was some inhibiting effect on Nitrobacter. However, the rather long period before nitrite accumulation at 500 ppm can only be due to the fact that the Nitrosomonas population was reduced to very low numbers by the previous treatment.

THE EFFECTS OF HERBICIDES ON NITRIFYING BACTERIA GROWN IN PURE CULTURES IN NUTRIENT SOLUTION

INTRODUCTION

In the general introduction to this thesis, it was pointed out that with regard to the results of the effects of herbicides on microbiological activities in the soil, there was a possibility of the toxicity (or for that matter, stimulation) being due not to the applied chemicals but to other substances into which they might have been converted in the soil, and that there was no guarantee that the applied herbicides would themselves inhibit or stimulate nitrification in pure cultures of these organisms.

There is a great lack of information in the literature, relating to the effects of herbicides on the nitrifying organisms in pure cultures. This is partly accounted for by the possibility that workers in this field may have felt that these organisms are ~~impossible~~ difficult to grow in pure cultures, especially in the absence of particulate solids, and partly to the fact that until recently pure cultures of these organisms were not easily come by and their maintenance was and is a problem.

A successful attempt was made to isolate these organisms from the soil, using a modified version of the method described by Lees (1952) using an enriched soil.

A mixed, but uncharacterized, unidentified and impure culture of the organisms was obtained. They were present at a density such that 10 gms of $(NH_4)_2SO_4$ in 4 litres of solution was completely converted to NO_3^- in less than 5 days--this was confirmed on 5 occasions. However, further work on the isolation, purification and characterisation was stopped when pure cultures of Nitrosomonas europae and Nitrobacter winogradzki were obtained from outside sources. It is unfortunate that my isolated 'mixed culture' was discarded so early, as further isolations would, it is hoped, have yielded information (a) as to what strains of these organisms were in my soil samples, and (b) on whether there were any differences or similarities between the effects of these herbicides on the organisms from my samples and those from outside sources.

From the foregoing, it was thought essential to look at the effects of some, at least, of the herbicides used in the perfuser experiments, on nitrification by Nitrosomonas and Nitrobacter. The initial aim was to confirm whether or not the conversion of ammonium to nitrite was necessarily accompanied by a corresponding increase in cell numbers in the presence of the herbicides. Estimates of cell numbers were made by use of a haemocytometer, but this was given up in view of the large numbers of counts that needed to be made. The use of a

nephelometer was also considered, but the fact that some of these chemicals were coloured ruled out its use, since the colours would have interferred with the readings. Nitrite production or utilization only depending on which of the organisms I was dealing with (nitrite production by Nitrosomonas sp and utilization by Nitrobacter sp) was the criterion used for determining growth.

Two chemicals which were not used in the perfuser series of experiments, were included in the work described here. Monuron (C:M.U 3-(p-chlorophenyl)-1,1-dimethyl urea) is a widely used herbicide, but the evidence in the literature regarding its effect on nitrification is rather conflicting--for while Gamble et al (1952), Quastel and Scholefield (1953) and Casely and Luckwill (1965) reported monuron as a strong inhibitor of nitrification, Hill et al (1955), Alexander (1958), and Hale et al (1957) found no adverse effects on the soil microflora. 4,5,6,7-tetrachloromethyl benzimidazole (supplied by Fisons) is not a herbicide as such, but has been reported as a very strong uncoupler of oxidative phosphorylation (Jones and Watson, 1965), and was included here because of its close structural relationship to chloroflurazole.

MATERIALS AND METHODS

Nitrobacter winogradzki was obtained from a source in Germany and grown in a medium made up as follows:

$NaNO_2$	2.0g
K_2HPO_4	0.04g
$MgSO_4 \cdot 7H_2O$	0.05g
$CaCO_3$	0.007
Ammonium molybdate	50µg
Distilled water	1000ml

pH to 8·2 - 8·4 with 0.1N KOH.

Nitrosomonas europae was grown from a lyophilized sample obtained from Rothamsted (7049) and grown in a medium made up as follows:

$(NH_4)_2SO_4$	0.5 - 1.0g
$CaCl_2$	0.05g
KH_2PO_4	0.2g
$MgSO_4$	0.05g
Fe-EDTA complex	0.05mg
Distilled water	1000ml

Sterile Na_2CO_3 (5%) was added aseptically to maintain pH at 8 - 8.4 using phenol red as indicator.

The cells were grown at room temperature as obtainable in the laboratory. Stock cultures were carried in conical flasks; the depth of the medium was kept under 1cm to ensure adequate aeration. Batch cultures were

grown in a 5-litre New Brunswick Co. fermentor for at least one week on every occasion, until a cloudy suspension was obtained. The bacterial cells were ~~reaped~~ harvested by centrifuging the suspension at 11,000g in an MSE-18 'High Speed' centrifuge at 10°C, when the cells are precipitated as a yellow-brown mass. The cells were washed once with water and resuspended in phosphate buffer.

50ml of medium containing 50µg NO_2-N/ml for Nitrobacter (and, in the case of Nitrosomonas, 50ml of medium containing 0.25g $(NH_4)_2SO_4$ per litre) were sterilized in 250ml conical flasks at 150 lb/sq in. for 15 mins., the appropriate herbicide concentrations were added, and then inoculated with 0.1ml of a dense suspension of Nitrosomonas or Nitrobacter. Treatments were run in duplicates. The flasks were incubated at 22°C-25°C in a horizontal reciprocal shaker to ensure good aeration, and sampled daily or every other day for the presence of nitrite--0.1ml samples were taken each time. Nitrite was estimated by the Greiss-Ilosvay technique (Lees and Quastel, 1946) using green filters, in an EEL colorimeter. The formulation of this reagent used in these experiments is as supplied by British Drug Houses Ltd, Poole, England--laboratory reagent grade. An equal volume of each of reagents I and II (sulphanilic solution and α-naphthylamine solution--in acetic acid) was mixed to give the solution used. One

criticism usually levied against this reagent is that the sensitivity of estimations is limited to 5-60µgN (Eastoe and Pollard, 1950 quoted by Follett and Ratcliffe, 1963); however, as this is the range within which the determinations were made in this study, the criticism should not apply.

RESULTS

Nitrosomonas europae: The effects of the herbicides tested at the concentrations indicated, on the conversion of $NH_4^+ \longrightarrow NO_2$ as an index of the growth of this organism, was followed by determining daily the nitrite content of the medium. As shown in Table IX, the maximum concentration of nitrite 28µg/ml was obtained within 4 days (most probably earlier), in the control cultures. Endothal at 50 and 500 ppm, and Monuron at 50 ppm had no effect on this aspect of nitrification. Monuron at 500 ppm, 2,3,6 TBA and Picloram at 50 ppm, and Ioxynil at 50 and 500 ppm produced an inhibition of 20-30%; while about 40% nitrification was obtained when 2,3,6-TBA at 500 ppm, Propanil at 5 ppm and Paraquat at 500 ppm were used. Picloram at 500 ppm and Paraquat at 50 ppm reduced nitrification by about 80-90%. I am unable to explain the results obtained with the two Paraquat concentrations. However, with Propanil at 25 ppm, 4,5,- and 4,5,6,7-Chloroflurazole both at 5 ppm and 25 ppm, the organisms were killed.

TABLE IX

Concentration of nitrite ($\mu g\ NO_2$ - N/ml) in culture solution containing Nitrosomonas europae and the herbicides listed below at the concentrations indicated. Means of 2 replicate determinations.

		Days					
		4	6	8	10	12	14
Control		28	28	28	28	29	29
Endothal	50 ppm	22	23	25	24	24	24
	500 ppm	28	32	29	28	33	32
Monuron	50 ppm	23	26	27	25	29	29
	500 ppm	12	14	17	17	17	16
2,3,6-TBA	50 ppm	15	23	16	14	17	17
	500 ppm	11	12	12	10	12	11
Picloram	50 ppm	21	20	20	22	20	20
	500 ppm	3	4	3	4	3	4
Ioxynil	50 ppm	19	20	21	19	22	19
	500 ppm	16	18	17	16	19	18
Paraquat	50 ppm	4.5	5	5	4.5	5.5	5.5
	500 ppm	8	9	9	9	9	9
Propanil	5 ppm	9	11	10	10	11	11
	25 ppm	0	0	0	0	0	0
Chloroflurazole	5 ppm	0	0	0	0	0	0
	25 ppm	0	0	0	0	0	0
4,5,6,7-tetra chloroflurazole	5 ppm	0	0	0	0	0	0
	25 ppm	0	0	0	0	0	0

TABLE X

Concentration of nitrite (ug NO_2 - N/ml) in culture solution containing Nitrobacter winogradsky and the herbicides listed below at the concentrations indicated. Means of 2 replicate determinations

Herbicides		0	1	2	3	4	5	6	7	8	10	12	14
Control		42	27	1	0	0	0	0	0	0	0	0	0
Endothal	50 ppm	45	19	1	0	0	0	0	0	0	0	0	0
	500 ppm	45	24	9	3	0	0	0	0	0	0	0	0
Paraquat	50 ppm	42	23	38	34	37	37	29	39	33	-	25	15
	500 ppm	33	24	35	28	36	36	24	44	35	-	22	19
Ioxynil	50 ppm	31	20	10	1	0	0	0	0	0	-	0	0
	500 ppm	16	13	18	14	21	21	13	22	13	-	21	13
2,3,6-TBA	50 ppm	41	24	17	1	0	0	0	0	0	-	0	0
	500 ppm	24	17	18	18	16	17	18	19	19	-	20	16
Picloram	50 ppm	35	23	1	1	0	0	0	0	0	-	0	0
	500 ppm	33	15	34	27	19	16	16	17	17	-	18	16
Monuron	50 ppm	34	17	1	0	0	0	0	0	0	-	0	0
	500 ppm	34	24	1	0	0	0	0	0	0	-	0	0
Propanil	10 ppm	57	20	27	13	4	-	2	3	4	2	2	2
	25 ppm	48	18	49	21	40	-	27	17	40	19	20	24
4,5-chloro-flurazole	10 ppm	44	32	4	1	1	-	0	0	0	0	0	0
	25 ppm	36	23	26	20	19	-	14	15	15	14	13	16
4,5,6,7-chloroflurazole	10 ppm	54	29	39	40	40	-	38	36	36	36	37	35
	25 ppm	47	19	40	38	35	-	37	32	33	25	25	28

Nitrobacter winogradzki: was grown on sodium nitrite medium and its activity in the presence of the test-herbicides was followed by measuring the nitrite content over 14 days (Table X). Paraquat at both 50 ppm and 100 ppm; Ioxynil, 2,3,6-TBA, and Picloram at 500 ppm; and both propanil and 4,5-chloroflurazole at 25 ppm, produced about 50% inhibition over 14 days, 4,5,6,7-tetra chloroflurazole virtually knocked out nitrification. Propanil at 10 ppm had residual inhibitory effects, albeit low, on day 14. The other herbicides at the concentrations used had little or no inhibitory effects after the 4th day.

Both organisms gave similar responses to the most toxic of the herbicides (the two chloroflurazole isomers and propanil). The effects of paraquat in this study were much more inhibitory than in either the work on the growth of nitrifiers in the soil or the nitrification process-- This can simply be explained as follows. In the soil studies most of the chemical was adsorbed on to the clay (Boon, 1965) but in this study, there was no adsorption, so that the chemical, even at 50 ppm, was present at an inhibitory concentration. Monuron in general had no effects on Nitrobacter but was slightly more inhibitory to Nitrosomonas. The stimulatory effects of Endothal, noted in the work with perfusers, were not confirmed with Nitrobacter; but at 500 ppm, this chemical produced a

slight stimulatory effect on nitrite production by Nitrosomonas. Previous stimulations (soil perfusion studies) may therefore be explained as follows. Nitrification is a 2-step reaction; $NH_4^+ \longrightarrow NO_2$, and $NO_2^- \longrightarrow NO_3^-$; in this, all other things being equal, the rate of nitrate production depends on the rate of nitrite production, therefore any mechanism that stimulates nitrite production will ultimately stimulate overall nitrification. On the whole Nitrosomonas $NH_4^+ \longrightarrow NO_2^-$ appeared to be more sensitive than Nitrobacter $NO_2^- \longrightarrow NO_3$ with particular regards to the effects of Chloroflurazole, Propanil, Monuron and 2,3,6-TBA.

THE EFFECTS OF HERBICIDES ON O_2 UPTAKE BY PURE CULTURES OF NITRIFYING ORGANISMS AND BY ENRICHED SOIL

INTRODUCTION

The pattern of the effects of the herbicides named, on nitrification, has so far been determined by 4 different techniques, three of which have involved the use of the perfuser with the soil studies, and the fourth the growth of the organisms in pure cultures. All these methods together do give useful information on the overall effects, but do not yield clues as to the possible effects at the respiratory level, for if this is the site of action of the herbicides, then the inhibitions would have already taken place before any effects on the NO_2^- and NO_3^- production is detected. Although precise mechanisms of respiratory inhibition by herbicides are not well understood, there seems to be a general indication that such phytotoxicants, at least in plant tissues, may be highly specific. There is, to the best of my knowledge, no report of any work involving the use of herbicides, on the respiration of the nitrifying bacteria in pure cultures. It was decided, therefore, that the effects of at least some of the herbicides so far examined, on the respiration of the nitrifying bacteria, should be looked at, and compared with what is obtainable using the same herbicides on an enriched soil. The object was to

determine the inhibitory effects (if any) of these herbicides on the utilization by the bacteria and and enriched soil, of specific substrates (NH_4^+ and NO_2^- for Nitrosomonas and enriched soil; and Nitrobacter respectively) of the nitrification process as measured by the inhibition of O_2 consumption.

MATERIALS AND METHODS

Preparation of materials.

Bacterial cells grown in nutrient solution in a 5-litre New Brunswick Fermentor and harvested, as already described, but resuspended in fresh buffer, recentrifuged and then suspended in 35ml buffer-pH 8, and stored in a refrigerator at $0^{o}C$ were used in these experiments.

The enriched soil was prepared as before, but was drained of water in the manner described by Quastel and Scholefield (1951) and then spread out to dry on a sheet of newspaper for 1 hour before use.

The Warburg apparatus (operating at 120 strokes/min.) was used to measure oxygen uptake, using standard manometric techniques (Umbreit et al, 1949). Each flask, containing 2.2ml of the reaction medium, received 0.3ml of the cell suspension, while for the enriched soil, the flasks contained 2.05ml of media and received 1.5gm of moist soil, great care being taken to see that there were no soil crumbs in the centre well.

Reaction medium. Each flask contained the following: 1.2ml phosphate buffer (2mM Na_2HPO_4—ZmM KH_2PO_4). For the work on enriched soil, this was reduced to 1.05ml on the basis of a soil volume of 0.45cc. 0.5ml 80mM $NaNO_2$ solution to give 16mM final concentration, which is the optimal for nitrite oxidation (Boon and Laudelout, 1962),

or 0.5ml 12mM $(NH_4)_2SO_4$ to give a final concentration of 2.5mM, for Nitrosomonas and enriched soil.

The NO_2^- or NH_4^+ substrate was added from the side arm. In addition, each flask contained 0.2ml, 20% KOH with a 2cm sq. filter paper in the centre well. 0.5ml of the inhibitors used in these experiments were added to the reaction medium in amounts stated with the results, and were pipetted in together with the other reactants. The flasks were placed in the Warburg apparatus and allowed to equilibrate for 15-20 minutes, before the substrates were tipped in to start the reaction. All experiments were conducted at 27°C, and run in duplicates. Random repeat experiments were carried out in the case of Nitrosomonas to confirm consistency of results.

Inhibitors and herbicides. The known inhibitors studied were sodium azide and 2,4-dinitrophenol (DNP). The herbicidal compounds were the same as for the pure culture growth experiments, and were investigated as potential inhibitors of ammonium and nitrite oxidation in the nitrification process.

RESULTS AND DISCUSSION

Oxygen uptake by enriched soil and by cell suspensions of Nitrosomonas europae and Nitrobacter winogradski in the presence of the concentrations of the herbicides stated in Tables XI, XII and XIII was measured at either 10 or 15 min. intervals over periods of 90-120 mins. Dilutions of the cell suspensions were made from time to time but the various dilutions were never weighed; therefore, the results if presented as either µl/gm dry weight or µl/ml suspension would be incomparable and not meaningful. However, since there was one set of controls for every batch of experiments (herbicides and concentrations were mixed at random into batches), the results presented as % of the control should at least be reproducible. This was found to be the case when random repeat experiments were carried out with Nitrosomonas (Table XIII).

DNP and NaF_3. DNP at 5 ppm had no effect on the respiration of Nitrosomonas (100%) but greatly stimulated the respiration of Nitrobacter (125%) while at 25 ppm, the respiration of both organisms was greatly stimulated (122% and 147% in Nitrosomonas and Nitrobacter respectively), probably due to some uncoupling of oxidative phosphorylation. Sodium azide at 25 ppm reduced oxygen uptake to less than 40% in both organisms, while at 5

TABLE XI

Effect of some inhibitors and herbicides on ammonium oxidation by enriched soil.

Herbicide or Inhibitor	Concentration		O_2 Consumed
	ppm	M	% of Control
2,4-DNP	5	$2{\cdot}72\times10^{-5}$	49
	25	$1{\cdot}36\times10^{-4}$	49
Sodium azide	5	$7{\cdot}7\times10^{-6}$	51
	25	$3{\cdot}85\times10^{-5}$	49
4,5 chloroflurazole	5	$1{\cdot}9\times10^{-5}$	102
	25	$9{\cdot}8\times10^{-5}$	46
4,5,6,7 (tetra) chloroflurazole	5	$1{\cdot}55\times10^{-5}$	93
	25	$7{\cdot}75\times10^{-5}$	80
2,3,6-TBA	50	$2{\cdot}2\times10^{-4}$	99
	500	$2{\cdot}2\times10^{-3}$	83
Ioxynil	50	$1{\cdot}3\times10^{-4}$	49
	500	$1{\cdot}3\times10^{-3}$	50
Paraquat	50	$1{\cdot}95\times10^{-4}$	146
	500	$1{\cdot}95\times10^{-2}$	106
Endothal	50	$2{\cdot}2\times10^{-4}$	104
	500	$2{\cdot}2\times10^{-3}$	98
Picloram	50	$2{\cdot}07\times10^{-4}$	93
	500	$2{\cdot}07\times10^{-3}$	93
Propanil	5	$2{\cdot}3\times10^{-5}$	106
	25	$1{\cdot}15\times10^{-4}$	91

TABLE XII

Effect of some inhibitors and herbicides on nitrite oxidation by *Nitrobacter winogradski*.

Herbicide or inhibitor	Concentration		O_2 consumed
	ppm	M	% of control
2,4-DNP	5	$2{\cdot}72\times10^{-5}$	135
	25	$1{\cdot}36\times10^{-4}$	147
Sodium azide	5	$7{\cdot}7\times10^{-6}$	71
	25	$3{\cdot}85\times10^{-5}$	34
4,5-chloroflurazole	5	$1{\cdot}95\times10^{-5}$	85
	25	$9{\cdot}8\times10^{-5}$	77
4,5,6,7-(tetra) chloroflurazole	5	$1{\cdot}55\times10^{-5}$	186
	25	$7{\cdot}75\times10^{-5}$	100
2,3,6-TBA	50	$2{\cdot}2\times10^{-4}$	90
	500	$2{\cdot}2\times10^{-3}$	65
Ioxynil	50	$1{\cdot}3\times10^{-4}$	95
	500	$1{\cdot}3\times10^{-3}$	68
Paraquat	50	$1{\cdot}95\times10^{-4}$	101
	500	$1{\cdot}95\times10^{-3}$	103
Endothal	50	$2{\cdot}2\times10^{-4}$	-
	500	$2{\cdot}2\times10^{-3}$	92
Picloram	50	$2{\cdot}07\times10^{-4}$	192
	500	$2{\cdot}07\times10^{-3}$	222
Propanil	5	$2{\cdot}3\times10^{-5}$	100
	25	$1{\cdot}15\times10^{-4}$	77

TABLE XIII

Effect of some inhibitors and herbicides on ammonium oxidation by *Nitrobacter winogradski*.

Herbicide or inhibitor	Concentration ppm	Concentration M	O_2 consumed % of control
2,4-DNP	5	$2{\cdot}72\times10^{-5}$	100
	25	$1{\cdot}36\times10^{-4}$	122
Sodium azide	5	$7{\cdot}7\times10^{-6}$	56
	25	$3{\cdot}85\times10^{-5}$	36
4,5-chloroflurazole	5	$1{\cdot}95\times10^{-5}$	75
	25	$9{\cdot}8\times10^{-5}$	13
4,5,6,7-(tetra) chloroflurazole	5	$1{\cdot}55\times10^{-5}$	65
	25	$7{\cdot}75\times10^{-5}$	17
2,3,6-TBA	50	$2{\cdot}2\times10^{-4}$	113
	500	$2{\cdot}2\times10^{-3}$	82
Ioxynil	50	$1{\cdot}3\times10^{-4}$	94 (92)
	500	$1{\cdot}3\times10^{-3}$	33
Paraquat	50	$1{\cdot}95\times10^{-4}$	65
	500	$1{:}95\times10^{-3}$	83 (81)
Endothal	50	$2{\cdot}2\times10^{-4}$	140
	500	$2{\cdot}2\times10^{-3}$	132
Picloram	50	$2{\cdot}07\times10^{-4}$	170 (172)
	500	$2{\cdot}07\times10^{-3}$	140
Propanil	25	$1{\cdot}15\times10^{-4}$	46
	100	$4{\cdot}6\times10^{-4}$	36

ppm, the values were 56% and 71% for Nitrosomonas and Nitrobacter respectively. Both DNP and sodium azide reduced the oxygen uptake to 50% in the enriched soil. The behaviour of DNP in the enriched soil may be due possibly to a concentration effect if the concentration per cell in the soil is assumed to be more than in the pure cultures; otherwise, this behaviour is difficult to explain.

4,5- and 4,5,6,7-chloroflurazole, at 5 ppm and 25 ppm, reduced respiration to 75%, 65%, 13% and 17% respectively in Nitrosomonas, but in Nitrobacter, the 4,5,6,7-derivative stimulated respiration at 5 ppm (186%) and had no effect at 25 ppm (100%); while the 4,5-derivative reduced respiration to 80% and 77% at 5 ppm and 25 ppm respectively. In the enriched soil the 4,5- had no effect on oxygen uptake at 5 ppm (102%) but reduced it to only 46% at 25 ppm; while 4,5,6,7- at both 5 ppm and 25 ppm reduced respiration to 93% and 83% respectively.

Endothal at 50 ppm stimulated respiration in Nitrosomonas (142%), but had no effect in enriched soil (104%); at 500 ppm, while stimulating Nitrosomonas (132%) it had no effect on enriched soil (98%) but reduced oxygen uptake in Nitrobacter(92%).

Paraquat, at both 50 ppm and 500 ppm, reduced oxygen uptake in Nitrosomonas 65% and 83% respectively,

had no effect on Nitrobacter (101% and 103%), but stimulated it in enriched soil (146%) at 50 ppm. Funderburk et al (1964) reported stimulation by paraquat of respiratory oxygen uptake in duckweed.

2,3,6-TBA, at 50 ppm, stimulated respiration in Nitrosomonas (113%) but had no effect on an enriched soil (99%) but reduced it in Nitrobacter (90%). This chemical at 500 ppm caused a reduction in oxygen uptake in Nitrosomonas (82%), Nitrobacter (65%) and enriched soil (83%)

Ioxynil at both 50 ppm and 500 ppm caused a reduction of respiration in Nitrosomonas (94% and 33%), Nitrobacter (95% and 68%) and in enriched soil (49% and 50%), respectively.

Picloram, at both 50 ppm and 500 ppm, strongly stimulated oxygen uptake in Nitrosomonas (170% and 140%) and Nitrobacter (192% and 222%) but reduced it in enriched soil (93% and 93%).

Propanil, at 25 ppm, reduced oxygen uptake to 46%, 77% and 91% respectively in Nitrosomonas, Nitrobacter and enriched soil. At 5 ppm there was no effect on Nitrobacter (100%) and in enriched soil (106%). 100 ppm applied to Nitrosomonas reduced respiration to 36% of the control.

Paraquat acts in the plants by the reoxidation of

the free radicals by molecular oxygen, a process which leads to the formation of peroxides (Brian et al, 1958). Because it is the salt of a strong base, it undergoes cation exchange on clay minerals, and so is virtually immediately immobilized in the soil (Boon, 1965). This to a large extent explains the more effective inhibition by paraquat of oxygen uptake by Nitrosomonas than by the enriched soil In the enriched soil, ioxynil at 50 ppm and 500 ppm together with 4,5-chloroflurazole at 25 ppm behaved in a manner identical to DNP; while ioxynil together with 4,5-chloroflurazole for Nitrobacter and with both 4,5- and 4,5,6,7-chloroflurazole for Nitrosomonas behaved in a manner different from DNP, which is a classical uncoupler of oxidative phosphorylation.

The effects of these herbicides on respiration obtained in this study is compared with results of other workers using other organisms, etc., in Table XIV.

TABLE XIV

Comparisons of the effects of herbicides on the nitrifying bacteria with those on other organisms.

Herbicide	Effect on nitrifying bacteria	Action on other organisms
4,5 chloroflurazole 4,5,6,7	O_2 uptake reduced in Nitrosomonas but stimulated in Nitrobacter	Uncoupled oxidative phosphorylation in rat liver mitochondria (Jones & Watson, 1965)
DNP	Stimulated O_2 uptake in both organisms but reduced it in enriched soil	Classical uncoupler of oxidative phosphorylation
Sodium azide	O_2 uptake inhibited, but not completely knocked out	Classical metabolic inhibitor--inhibits cytochrome oxidase
Endothal	Stimulated O_2 uptake and nitrification in Nitrosomonas	Mode of action apparently unknown
Paraquat	Stimulated respiration in enriched soil but reduced it in Nitrosomonas, and had no effect in Nitrobacter	Acts in plants by re-oxidation of free radicals by oxygen (Homer et al, 1960) Respiratory O_2 uptake stimulated in duckweed (Funderburk et al, 1964)
2,3,6-TBA	Stimulated O_2 uptake in Nitrosomonas but reduced it in Nitrobacter (50ppm)	Reduced O_2 uptake by isolated cucumber mitochondria (Foy and Penner, 1965)
Ioxynil	Reduced respiration in both organisms. Strongly inhibited nitrification rate in enriched soil	Uncoupler of oxidative phosphorylation and inhibitor of the Hill reaction in photosynthesis (Foy, 1964, quoted by Hart et al, 1964) Reduced O_2 uptake in cucumber mitochondria (Foy and

Continued, following page

TABLE XIV - continued

		Penner, 1965), inhibits Hill reaction of bean leaf chloroplasts and uncouples oxidative phosphorylation in pea shoot mitochondria (Kerr and Wain, 1964 a&b)
Picloram	Stimulated O_2 uptake in both organisms; slightly reduced it in the enriched soil	Reduced O_2 uptake in cucumber mitochondria (Foy and Penner, 1965)
Propanil	Reduced O_2 uptake in both organisms	Depressed respiration of soil and nitrification in soil (Bartha et al, 1967)

4,5,6,7-chloroflurazole (at 5 ppm) and Picloram (at 50 and 500 ppm) in Nitrobacter; Endothal and Picloram (at 50 and 500 ppm), and 2,3,6-TBA (at 50 ppm) in Nitrosomonas; together with Paraquat (at 50 ppm) in enriched soil, stimulated the uptake of oxygen in a manner similar to DNP (in both organisms) which is a classical uncoupler of oxidative phosphorylation. The stimulatory effects noted for Picloram and 2,3,6-TBA above are at variance with, while the reduction of oxygen uptake by 2,3,6-TBA (at 500 ppm in enriched soil, and Nitrosomonas, and at 50 and 500 ppm in Nitrobacter) and the reduction of O_2 uptake by Ioxynil in all instances of testing, are in agreement with the reduction of O_2 uptake in isolated cucumber mitochondria reported for these chemicals by Foy and Penner (1965). Propanil (like sodium azide) reduced oxygen uptake by the nitrifying organisms and the enriched soil; this is in agreement with the findings of Bartha et al (1965) that this chemical depressed the respiration of the soil.

DISCUSSION

The microbial population of the soil is heterogeneous, which makes any problem concerning the activities of these organisms a difficult one. One of the main difficulties is that of duplicating results, for no two soil samples will be exactly alike; this difficulty was to a large extent overcome by the use of the perfuser technique, the same sample of soil being used for each set of experiments. The study of one particular soil organism in its natural environment also presents a problem due to interaction with other microbes and possible dependence on one or more of these.

The effects of the herbicides studied on nitrification in the soil was determined both in an enriched soil and in a 'fresh' or 'non-enriched' soil.

1) The effects of the herbicides in an enriched soil are direct effects on nitrification per cell. It has been shown (Lees and Quastel, 1946 (b); and Quastel and Scholefield, 1951) that in a bacterial saturated or enriched soil, the area of proliferation is limited and cannot be extended owing to full occupancy of the available sites for proliferation; such a soil resembles very much a mixture of isolated enzymes or a suspension of resting cells and may therefore be used for studying the kinetics of substrate breakdown or metabolism.

TABLE XV

The concentrations of herbicides causing I_{50} in enriched and non-enriched soil and 2L in non-enriched soil.

Herbicides	Concentration ppm I_{50}		2L
	Enriched soil	Fresh soil	
Propanil	13	67	21
Ioxynil	<5	56	>1000
Chloroflurazole	5	<13	<13
Bromoxynil	28	224	126
Chlorothamid	95	224	36
2,3,6-TBA	160	750	>1000
Picloram	150	95	168
Dichlobenil	840	530	>1000
Paraquat	950	630	>1000
Diquat	>1000	-	-
Endothal	>>1000	>>1000	>1000

2) Herbicide effects in the fresh soil:

a) An effect on the lag must be an effect on the growth rate, whether direct or indirect.

b) An effect on <u>final</u> nitrification rate could be

i) a <u>direct</u> effect on nitrification rate per cell (final population saturated) and thus can be checked by comparison with 1) above;

ii) an effect on the final saturation numbers of cells with no effect on the nitrification per cell (also comparable with 1) above) could be due to a stimulation i.e. (more nitrification sites) or inhibition (fewer nitrification sites).

c) If b) i) is correct, then there would probably be a correlated (i.e. indirect) effect on the lag. The greater the reduction in nitrification rate per cell, the longer the lag.

d) If b) ii) is correct, then presumably, the lag would not be measurably affected since the cells would grow at the same rate to fill available nitrification sites. In fact, one might expect a small reduction in the lag.

An analysis of the effects of these herbicides on soil nitrification was made in order to determine which of the above aspects, metabolism or growth of these organisms was the primary site of action. This was done

by considering the concentrations of the herbicides necessary to cause a 50% inhibition (I_{50}) of the nitrification rate in an enriched soil or the final nitrification rate in fresh soil and the concentrations necessary to double the lag (2L). The I_{50} values for the enriched soil obtained from Figs. 12 13; those for the fresh soil obtained from Figs. 27 and 28, together with the 2L values obtained from Figs. 25 and 26 are presented as Table XV.

From Table XV, and in view of the above discussion, the following conclusions may be drawn regarding the effects of the herbicides on nitrification.

1) There was no effect on the lag except at very high herbicide concentrations, i.e. there was no effect on the growth rates. This occurred with 2,3,6-TBA, Paraquat, Dichlobenil, Ioxynil and Endothal. In Paraquat, Endothal and Dichlobenil, this is presumably because there were no inhibitory effects on nitrification. In 2,3,6-TBA and Ioxynil, there were marked effects on nitrification in enriched soil, much less than in fresh soil. This suggests that there was some adaptation of nitrifiers during the growth, i.e. the selective proliferation of a tolerant strain.

2) There were similar I_{50} values in both soils (enriched and non-enriched). This occurred with Chloroflurazole,

Picloram, Dichlobenil, Paraquat and Endothal. This means that the herbicides had no effect on maximum soil population since inhibition in fresh soil was due to a direct effect on the nitrification rate per cell. In all these herbicides the 2L values were the same as the I_{50} values, suggesting therefore, that the effect of the herbicides on the lag (growth rate) was indirect and was the result of an effect on the nitrification rate (energy flow).

3) The I_{50} (enriched soil) was much lower than the I_{50} (fresh soil); this suggests as in 2) above that the organisms were adapting during growth with the resulting selection of a more tolerant strain (again, not herbicide breakdown, as this would show in the enriched soil curves). This type of response was obtained with Propanil, Bromoxynil, Chlorothamid and also in Ioxynil and 2,3,6-TBA (already mentioned). In most of these herbicides, the growth rate (lag) reflected the nitrification rate, i.e. it was determined by it, but in Chlorothamid the lag was very much more sensitive. Thus in spite of an adaptation going on, there was still a suggestion of a direct effect of this herbicide on the growth rate (i.e. independent of energy flow from the nitrification process). The results for Chlorothamid are identical with those reported for 2,3,6-trichlorophenyl acetic acid by Mayeux and Colmer (1962), who found that the

lag phase (growth rate, cell division) was more susceptible than the enriched soil (i.e. nitrification process) to herbicide presence during the course of nitrite oxidation in the soil.

The foregoing is confirmed with Chloroflurazole and Picloram by the results of the 'recovery' of growth in washed soil. With Picloram at all test concentrations (Fig. 29) and Chloroflurazole at 0 and 50 ppm (Fig. 30) no lag was obtained; these results are in agreement that the herbicidal effects were primarily on the nitrification process.

Rough estimates of the concentration of herbicide causing half maximum rates of nitrification in pure cultures of Nitrosomonas and Nitrobacter, were obtained from Tables IX and X and are presented as Table XVI and were looked at in conjunction with Table XV. With Chloroflurazole, Picloram, Propanil and 2,3,6-TBA, the concentrations causing a 50% inhibition were the same in the soil as in the pure culture work. The reasons for the behaviour of Propanil (at least) is as follows. Bartha et al (1967) showed that this chemical, 3,4-dichloropropionanilide, was broken down in the soil into 3,4-dichloroaniline and propionic acid; they also found that the aniline moiety was as active as the Propanil molecule in inhibiting nitrification in the soil. With Paraquat, a higher

TABLE XVI

Rough estimates of the concentrations for half maximum rate of nitrification in pure cultures of Nitrosomonas and Nitrobacter.

Herbicide	$NH_4^+ \rightarrow NO_2^-$ Nitrosomonas	$NO_2^- \rightarrow NO_3^-$ Nitrobacter
Propanil	<5	$10<25$
Ioxynil	>500	$500>50$
4,5-chloroflurazole	$\ll 5$	$25>10$
2,3,6-TBA	50	$500>50$
Picloram	$500>50$	$500>50$
Paraquat	$50<500$	<50
Endothal	$\gg 500$	>500
Monuron	$500>50$	$\gg 500$
4,5,6,7-tetra-chloroflurazole	$\ll 5$	<10

concentration of this herbicide was necessary to cause equivalent inhibition in the soil than in pure cultures, this as was mentioned much earlier, was probably due to the adsorption of the chemical onto clay minerals (Boon, 1965). Ioxynil was inhibitory at a lower concentration in the soil than in the pure cultures. This is rather difficult to explain; a tentative explanation is that of an interaction of Ioxynil and the soil system, leading to the formation of a product more toxic than Ioxynil. The effects of the herbicides on both parts of the nitrification process (in pure culture work) are in general similar; however, the conversion of ammonium to nitrite (Nitrosomonas) was slightly more sensitive than the conversion of nitrite to nitrate (Nitrobacter). This would seem to be so when the results for the Benzimidazoles (4,5-di and 4,5,6,7-trichloro), Propanil, 2,3,6-TBA (and Monuron, perhaps) are considered. On the other hand, there was a stimulatory effect of Endothal on Nitrosomonas, which is in accordance with the results in Table II and Fig. 12.

The concentrations of herbicides causing a 50% inhibition of the oxygen uptake were obtained from Tables XI, XII and XIII and are presented as Table XVII, and again, were compared with Table XV. With Propanil, a higher concentration of herbicide was required to inhibit

TABLE XVII

Estimates of the concentration for 50% inhibition of oxygen uptake.

Herbicide	Enriched soil	Nitrosomonas	Nitrobacter
Propanil	$\gg 25$	25	25
Ioxynil	50	$500 > 50$	> 500
4,5-chloroflurazole	25	$25 > 5$	> 25
2,3,6-TBA	> 500	> 500	$\simeq 500$
Picloram	> 500	> 500	> 500
Paraquat	> 500	> 500	> 500
Endothal	> 500	> 500	> 500
4,5,6,7 tetra-chloroflurazole	> 25	$\simeq 5$	> 25

respiration by enriched soil, than by either pure cultures of Nitrosomonas and Nitrobacter or to reduce the rate of the nitrification process: one explanation may be that, as already pointed out (Bartha et al, 1967), the aniline moiety of Propanil is as toxic as the propanil molecule so that there is a suppression of the nitrification process (both in soil and pure cultures). However, the propionate is metabolized by soil organisms, and this could then lead to an increased respiration in the duration of the respiratory experiments. There was a greater sensitivity to herbicidal presence by the nitrification process, than by oxygen uptake, at least with Picloram, Propanil and 2,3,6-TBA. This suggests that the sites of action of these herbicides were at a more fundamental level than the site of aerobic respiration.

The I_{50} values for the enriched soil (Table XV), showed that Propanil (13 ppm), Ioxynil (<5 ppm), Chloroflurazole (5 ppm) and Bromoxynil (28 ppm) were the most toxic inhibitors; while Paraquat (950 ppm), Dichlobenil (840 ppm), Diquat (>1000 ppm) and Endothal (≫1000 ppm) were the least toxic. These values further indicate that the three dihalogenobenzonitriles differed with respect to their effects on the nitrification process, for while Dichlobenil showed no evidence of an 'adaptation,' Ioxynil and Bromoxynil had almost identical patterns.

The differences in the concentrations for Ioxynil (<5 ppm) and Bromoxynil (30 ppm) could be due to some subsidiary actions, but they do confirm the report of Wain (1964) that Ioxynil was the more active of the two; these differences may be partially related to their solubilities (Fletcher, 1946), for Carpenter et al. (1964) reported Ioxynil (14 ppmw for Na-salt) as more soluble than bromoxynil (6.1 ppmw for the K-salt).

In interpreting the results presented in this work it should be borne in mind that some of the herbicides were used as formulated chemicals while others were in their purest forms. Whichever form of chemical was used depended on what was available. Furthermore, especially with regard to the formulated chemicals, it should be pointed out that some of the observed effects of such chemicals may be attributed to materials other than the active ingredients, and also that the addition of the surfacants or emulsifiers etc. in formulated preparations may cause either a synergistic or an antagonistic effect with respect to the activity of the herbicides on nitrification, as compared with the active ingredients themselves. It would of course be interesting if the effects of the surfacants were determined separately as this would yield information as to the relative effects of the various ingredients and combinations in the formulated compound.

One advantage of working with a formulated herbicide is that this is the form in which the chemical is available to the public and since commercial formulations are applied as herbicides in the field, their effects on nitrifiers should be studied as such.

Fletcher (1956) estimated that if the actual content of active growth substance in an application on reaching the soil was 1 lb per acre, assuming no adsorption and complete solution and a 20 per cent water content of the soil, the concentration becomes 2-2.5 ppm approximately. On the basis of this, it may be concluded that the use of the herbicides tested, at normal field rates of application, is unlikely to leave any permanent detrimental effects on the nitrifying organisms. However, there is the possibility that a lack of weeds in an environment, especially when pre-emergence control is adopted, might alter the organic matter status of the soil and that the nitrification or any other soil process, for that matter, might be adversely impinged upon.

BIBLIOGRAPHY

ALEXANDER, M. Herbicides and soil micro-organisms
FARM RESEARCH 24:15 (1958)

AUDUS, L.J. A new perfusion apparatus
NATURE (LONDON) 158:419 (1946)

AUDUS, L.J. Microbial breakdown of herbicides in soil
HERBICIDES AND THE SOIL: E.K. Woodford and G.R. Sagar (eds.) pp 1-19 (Blackwell Scientific Publications, Oxford) (1960)

BARTHA, R., LANZILOTTA, R.P. and PRAMER, D.
Stability of some pesticides in soil
APPL. MICROBIOL. 15:67-75 (1967)

BLACKMAN, G.E. A comparison of certain plant-growth substances with other selective herbicides
NATURE (LONDON) 155:498 (1945)

BOON, B., and LAUDELOUT, H.L.
Kinetics of nitrite oxidation by Nitrobacter winogradski
BIOCHEM. J. 85:440-447 (1962)

BOON, W.R. The chemistry and mode of action of the bipyridylium herbicides diquat and paraquat
OUTLOOK IN AGRICULTURE IV: 163-170 (1965)

BRIAN, R.C., HOMER, R.F., STUBBS, I. and JONES, R.L.
A new herbicide, 1,1'-ethylene-2,2'-dipyridilium dichloride
NATURE (LONDON) 181:446-447 (1958)

CARPENTER, K., COTTRELL, H.J., DE SILVA, W.H., HEYWOOD, B.J., LEEDS, W.G., RIVETT, K.F. and SOUNDY, M.L.
Chemical and biological properties of two new herbicides - ioxynil and bromoxynil
WEED RES. 4:175-195 (1964)

CASELEY, J.C. and LUCKWILL, L.C.
The effect of some residual herbicides on soil nitrifying bacteria
ANN. REP. Long Ashton Res. Sta. 1964:78-86

CHANDRA, P. Herbicidal effects on certain microbial activities in some brown soils of Saskatchewan
WEED RES. 4:54-63 (1964)

EYLAR, O.R. and SCHMIDT, E.L.
A survey of heterotrophic micro-organisms from the soil for ability to form nitrite and nitrate
J. GEN. MICROBIOL. 20:473 (1959)

FLETCHER, W.W. Effect of hormone herbicides on the growth of Rhizobium trifolii
NATURE (LONDON) 177:1244 (1956)

FLETCHER, W.W. The effect of herbicides on soil micro-organisms
HERBICIDES AND THE SOIL: E.K. Woodford and G.R. Sagar (eds.) pp 20-62 (Blackwell Scientific Publications, Oxford) (1960)

FOLLET, M.J. and RATCLIFFE, P.W.
Determination of nitrite and nitrate in meat products
J. SCI. FD. AGRIC. 14:128-144 (1963)

FOY, C.L. and PENNER, D.
Effect of inhibitors and herbicides on tricarboxylic acid cycle substrate oxidation by isolated cucumber mitochondria
WEEDS 13:226-231 (1965)

FUNDERBURK, H.H. and LAWRENCE, J.M.
Mode of action and metabolism of diquat and paraquat
WEEDS 12:259-264 (1964)

GAMBLE, S.J.R., MAYHEW, C.J. and CHAPPELL, W.E.
Respiration and plate counts for determining the effects of herbicides on soil micro-organisms.
SOIL SCI. 74:347-350 (1952)

HALE, M.G., HULCHER, F.H. and CHAPPELL, W.E.
The effects of several herbicides on nitrification in a field soil under laboratory conditions
WEEDS 6:331-341 (1958)

HAMNER, C.L. and TUCKEY, H.B.
The herbicidal action of 2,4-dichlorophenoxyacetic and 2,4,5-trichlorophenoxyacetic acid on bind weed
SCIENCE 100:154 (1944)

HART, R.D., BISHOP, J.R. and COOKE, A.R.
Discovery of ioxynil and its ability in the United States
PROC. 7th Brit. Weed Contr. Conf. I:3-7 (1964)

HILL, G.D., MCGAHEN, N.W., BAKER, N.W., FINNERTY, D.W., and BINGEMAN, C.W.
The fate of substituted urea herbicides in agricultural soils.
AGRON. J. 47:93-103 (1955)

HOMER, R.F., MEES, G.C. and TOMLINSON, T.E.
Mode of action of dipyridyl quaternary salts as herbicides
J. SCI. FD. AGRIC. 11:309 (1960)

KERR, M.W. and WAIN, R.L.
The uncoupling of oxidative phosphorylation in pea shoot mitochondria by 3,5-diiodo-4-hydroxy benzonitrile (ioxynil) and related compounds
ANN. APPL. BIOL. 54:441-446 (1964 a)

KERR, M.W. and WAIN, R.L.
Inhibition of the ferricyanide-Hill reaction of isolated bean leaf chloroplasts by 3,5-diiodo-4-hydroxy benzonitrile (ioxynil) and related compounds
ANN. APPL. BIOL. 54:447-- (1964 b)

KRATOCHVIL, D.E. Determinations of the effect of herbicides on soil micro-organisms
WEEDS 1:25-31 (1951)

LEES, H. The biochemistry of nitrifying organisms I. The ammonia oxidising systems of Nitrosomonas
BIOCHEM. J. 52:134-139 (1952)

LEES, H. and QUASTEL
Biochemistry of soil nitrification I. Kinetics of, and effects of poisons on, nitrification, as studied by a soil perfusion technique

BIOCHEM. J. 40 803-815 (1946 a)

LEES, H. and QUASTEL, J.H.
Biochemistry of soil nitrification
2. The site of nitrification
BIOCHEM. J. 40:815-823 (1946 b)

MAGEE, L.A. and COLMER, A.R.
Some effects of 2,4-D upon Azobacter as measured by respiration inhibition
WEEDS 4:124-130 (1956)

MAYEUX, J.V. and COLMER, A.R.
The effects of 2,3,6-trichlorophenylacetic and 2,2-dichloropropionic acids on nitrite oxidation
APPL. MICROBIOL. 10:206-210 (1962)

NEWMAN, A.S. The effect of certain plant growth regulators on soil micro-organisms and microbial processes
PROC. SOIL SCI. SOC. AMER. 12: 217-221 (1947)

NUTMAN, P.S., THORNTON, H.G. and QUASTEL, H.J.
Inhibition of plant growth by 2,4-dichlorophenoxyacetic acid and other plant growth regulators
NATURE (LONDON) 155:498-500 (1945)

QUASTEL, J.H. Soil metabolism
PROC. ROY. SOC. B 143:159-178 (1955)

QUASTEL, J.H. and SCHOLEFIELD, P.G.
Biochemistry of nitrification in the soil
BACT. REVS. 15:1-53

QUASTEL, J.H. and SCHOLEFIELD, P.G.
Urethanes and soil nitrification
APPL. MICROBIOL. 1:282-287 (1953)

SCHMIDT, E.L. Nitrate formation by a soil fungus.
SCIENCE 119:197 (1954)

SLADE, R.E., TEMPLEMAN, W.G. and SEXTON, W.A.
Plant growth substances as selective weed killers
NATURE (LONDON) 155:497-8 (1945)

SMITH, N.R., DAWSON, V.T. and WENZEL, M.E.

The effect of certain herbicides on soil micro-organisms
PROC. SOIL SCI. SOC. AMER.
10:197-201 (1946)

SNELL, F.D. and SNELL, C.T.
Colorimetric methods of analysis
623 pp (Chapman and Hall, Publ., London
(1936)

UMBREIT, W.W., BURRIS, G.R. and STAUFFER, J.E.
Manometric techniques and tissue metabolism
(Burgess Publishing Co., Minneapolis)
(1949)

WAIN, R.L. Ioxynil, a new selective herbicide
PROC. 7th Brit. Weed Contr. Conf.
I:1-2 (1964)

ZAKI, M.A. Studies on certain herbicides and their behaviour in soil and plant
Ph.D. Thesis, University of London
(1965)

ZIMMERMAN, P.W. and HITCHCOCK, A.E.
Substituted phenoxy benzoic acid growth substances and the relation of structure to physiological activity
Contrib. Boyce Thompson Institute
12:321-344 (1942)