

Effects Of Some Herbicides On Soil Nitrifiers and
Nitrification

By

Malinie Ratnaweera

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Bedford College,
Regent's Park,
London, NW1 4NS.

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ERRATA

1. Nitrosomonas europaea needs to be corrected as Nitrosomonas europaea.
2. Nitrobacter winogradski needs to be corrected as Nitrobacter winogradskyi.

throughout.

ABSTRACT

The herbicides Ioxynil, Bromoxynil, NPH 1320, Totril, Dicamba, Tricamba, Trifluralin, Oxadiazon, Legurame, M & B 9057 and M & B 9555 were tested for their effects on the process of nitrification in soil as well as in pure cultures.

An improved version of the perfusion apparatus was developed and the perfusion technique was used as the principal experimental method in the soil studies in which the effects of different herbicides on the rates of nitrification in soils previously enriched with nitrifiers and in fresh soils continuously perfused with the herbicides were estimated. These estimates were made use of in the assessment of the degree of toxicities of the herbicides under consideration, on the metabolic rates per cell of nitrifiers and on their degree of proliferation in soil. An attempt was also made to study the possible differential effects of these herbicides on the two main groups of chemoautotrophic soil nitrifiers and the results obtained revealed that the metabolic activities of Nitrosomonas populations in soil were much less sensitive to the lower concentrations of many of the herbicides tested when compared with the sensitivities shown by the Nitrobacter populations, of the same soils, to the toxic effects of the same herbicides. But the rates of metabolic activities of Nitrosomonas populations were found to be the factor limiting the overall rates of the nitrification process in soils treated with the higher concentrations of most of these herbicides.

The nitrification experiments carried out with cell suspensions of pure cultures of Nitrosomonas Europa and Nitrobacter Winogradski indicated that the herbicides exerted differential effects on the metabolic activities of these two organisms even in artificial media.

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The only other method used in studying the effects of herbicides on soil nitrification involved the measurement of the rates of oxygen uptake by samples of enriched soil treated with known concentrations of herbicides, making use of the conventional Warburg respirometric technique.

The qualitative effects of most of the herbicides on the activities of nitrifiers, grown in artificial media and in the natural medium of soil, were found to be essentially similar although the concentrations effective in causing these toxicities in pure cultures were much less than the concentrations needed to cause similar levels of toxicities in soil media.

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CHAPTER 1

General Introduction

The use of chemicals for weed control is quite evidently a practice which existed even in the ancient times, though much was not known about the scientific aspects of this field. During these times people were in the habit of applying salt, ashes and various other industrial by-products to road sides and pathways to eradicate the unwanted vegetation. Later on as far back as the 19th century many inorganic salts have been utilised for the same purpose.

Although it was known by then that certain chemicals were harmful to plants, the selective effects of these chemicals and the practical implication of this selectivity in the control of weeds in agricultural crops was not fully realised until the late 19th century. In fact the first observation of selective weed killing was accidental and dates back to 1896, when a French vineyard was sprayed with copper sulphate as a preventive measure against fungal infection of the vines. This spray when drifted into the adjacent grain field was found to kill, selectively, the broadleaf weeds within the cereal crop.

Many years later major developments in weed control began with the discovery of auxin type of chemicals such as substituted phenoxy and benzoic acids with selective toxicities. In view of the very low concentrations of these chemicals, e.g. 2 methyl, 4-chlorophenoxy acetic acid (MCPA) by Slade, Templeman & Sexton (1945), 2, 4-dichlorophenoxy acetic acid (2,4-D) by Nutman, Thornton & Quastel (1945) and 2, 4-5 Trichlorophenoxy acetic acid (2,4,5-T) by Hamner & Turkey (1944), needed to bring about marked toxicities, it was realised that there was a possibility of such substances added to or produced in soils would have significant influences on the nature and composition of plant associations growing on them. Suggestions by Beal (1944) and Mitchell

& Hamner (1944) about this possibility of using synthetic growth substances as selective weed killers also came around the same time independently of each other.

a) 'Herbicides', their mode of action and use in agriculture

In the present context the term 'herbicide' is used to denote a large and a diverse group of chemicals, in addition to the above mentioned basic ones, with the common property of eradicating all vegetation in waste land or of selectively killing weeds in agricultural crops without seriously injuring the surrounding crop. It has been currently estimated that there are about 125 or more herbicides commercially available. A major proportion of these are not of the original auxin type, and could be roughly categorised under 10-12 main groups on their basic chemical structure.

As a result of the tremendous amount of research done in the recent past, on selective and non-selective herbicides, its use for weed control has become a routine practice in maintaining clean agricultural crops. Considering the mode of activity of these chemicals they fall into two major categories, namely

- 1) Contact and translocated herbicides;
- 2) Residual herbicides.

Those of the first category are the 'post-emergence' herbicides and are primarily directed as a spray at the young foliage and stems whereas those belonging to the second category are the 'pre-emergence' herbicides that are applied directly to the soil. In whatever manner they are used, sooner or later these herbicides find their way into the soil.

Although these chemicals are used for pest control purposes, they may influence the general soil microbiological and chemical properties once they reach the soil. It has generally been assumed that herbicides applied at normal field rates do not affect soil biological processes.

But there are conflicting reports in literature which indicate that under certain conditions normal application of these chemicals could be detrimental to at least some of these biological processes. These observed effects on soil microbes, whether favourable or unfavourable, are dependent on

- i) how long they can persist in the soil, which in turn is determined by a large number of parameters such as the environmental factors, water content, aeration and pH of soil etc.,
- and ii) whether the breakdown products (if there are any) will have the same type of effects on the soil microbial populations, as does the parent substance itself.

In view of the probable hazards which could be brought about by the widespread use of weed killers in vast quantities it has become essential, in the interest of both herbicide industry and farmers, to explore the influences of herbicides on soil micro-organisms that govern the cyclic processes, in nature, responsible for the availability of plant nutrients such as minerals, C, N and S etc.

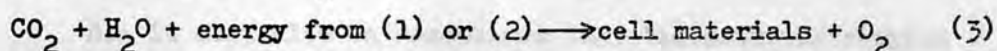
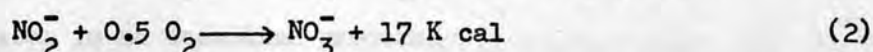
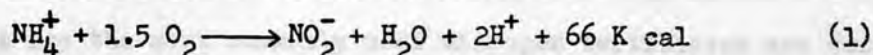
With this objective in mind the effects of several herbicides on the 'nitrification' of ammonium-N applied to soil under controlled laboratory conditions were studied.

b) Soil Nitrification and its role in soil fertility

'Nitrification' is a process that takes place in all fertile soils whereby ammonium-N, normally added as fertilizers, is converted to nitrate -N. The biological nature of the nitrification process in general was demonstrated quite conclusively as far back as 1877 by Schloesing & Muntz (quoted by Stephenson 1930). In their simple experiments they filled a tube, 1 metre long, with sand and chalk previously sterilized by incineration and sewage water was poured daily into the upper end of the tube. As a result they found that the ammonia content of the effluent suddenly started to disappear and fell to nothing after having remained

constant for twenty days. They then showed that antiseptics like Chloroform inhibited this ability of nitrification which normally continued for four months once started. Later they found that the nitrification in these tubes could be restored by pouring in some washings from garden soil. The same authors found that nitrification could also be stopped by heating the tubes to 100°C and yet restarted by addition of soil washings. However it was not until 10-15 years later ^{that} ~~were~~ three independent workers Frankland and Frankland (1890), Warrington (1891) and Winogradski (1890-1891 - quoted by Stephenson 1930) ^{were} able to isolate the nitrite oxidizing organisms.

It is now known that at least in soils of more or less neutral reaction this process is mediated by two main types of micro-organisms working sequentially, namely Nitrosomonas species capable of oxidizing ammonium-ion to nitrite and Nitrobacter species capable of oxidizing nitrite so formed to nitrate. Both these types of microbes are obligate chemoautotrophic bacteria which derive their energy from the respective primary inorganic oxidations that are coupled to cellular biosynthetic reactions involving the reduction of carbon-dioxide by mechanisms very similar to those of the carbon reduction cycle in photosynthetic organisms. Therefore the overall process can be outlined as reactions 1, 2 and 3 where step (1) refers to Nitrosomonas activity, step (2) refers to Nitrobacter activity and the third step refers to the reductive assimilation of carbon-dioxide common to both organisms.



Even though nitrification is considered as the most important of all the natural systems, producing nitrogen in forms available to plants, the importance of other processes like fixation by root nodules of leguminous plants ~~+~~ (i.e. Rhizobium species) and Clostridium spp. and

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fixation by aerobic bacteria - Azotobacter spp. - must always be remembered as these are the only processes by which the atmospheric nitrogen is assimilated^{by} the plants. Schmidt (1954) and Eylar & Schmidt (1959) have reported that certain amount of nitrification could be carried out by some other soil organisms too. But the part played by these so-called 'non-classic' nitrifiers is generally overlooked in view that their contribution to total nitrification in soil is quantitatively small.

Various workers in the field of agricultural sciences seem to have different views regarding the importance of the process of nitrification in relation to soil fertility. It has been reported by Spratt and Gasser (1970a) that Wheat grew faster with ammonium than with nitrate nitrogen. But later Spratt and Gasser (1970b) found that in the presence of adequate water wheat grew and yielded best with fertilizer-nitrogen supplied as nitrate even though ammonium nitrogen increased yields as much as or more than by nitrate when wheat was grown with a restricted supply of water during the growing season. Good and Carter (1965) showed that agricultural crops like tomato, beans, squash were stunted when grown in fumigated soil and he attributed this effect to the lack of nitrate, based on his finding that the yields of these crops have been largest when half the fertilizer-N was applied as nitrate.

From the above mentioned works and also from general practical experiences reported by many workers it is clear that as far as the soil fertility is concerned the nitrification process whereby ammonium salts which are the most commonly used nitrogen fertilizers are converted to nitrates is an essential process.

But it must also be remembered that excessive nitrifying capacity of a cropped soil may be disadvantageous because both nitrite and nitrate ions are susceptible to loss by leaching and denitrification whereas ammonium nitrogen is held by the base exchange complexes of the soil and it is neither removed due to leaching nor react to form gaseous

products that are lost from the soil except that ammonia could be lost when soil becomes excessively alkaline.

Therefore the usefulness of a study dealing with an investigation of the possible effects of some herbicides on nitrification could mainly be twofold, namely:

- a) To know which herbicides are extremely toxic to nitrifiers at normal field rates so that use of lethal concentrations of such chemicals could be avoided; and
- b) To get an idea about which of these herbicides have very mild toxic effects on nitrifying organisms since such chemicals can almost certainly be used to enhance the efficiency of utilization of nitrogenous fertilizers in tropical regions where rainfall and microbial activity are high.

The second possible use of inhibitors of nitrification for increasing fertilizer efficiency is a very well appreciated fact even though its practical applications have not so far been well mentioned in the literature. There are few reports on this field by Prasad (1968), Prasad et al. (1966) and Patric et al. (1968). Cleve & Goring (1962) and Gasser (1970) have reported some work on the possibility of using chemicals like 2-Chloro-6 (Trichloro methyl) pyridine (N-Serve) and a variety of other chemicals for this purpose.

c) Methods used in studying the effects of herbicides on nitrification

Various pesticides including many herbicides have been tested by several workers for their effects on soil nitrification. Most of these studies used field trials or determinations using the soil perfusion apparatus developed by Lees & Quastel (1946). Some other workers like Quastel & Scholefield (1951), Hale, Hulcher & Chappel (1957), Farmer, Benoit & Chappel (1965), Debona (1967), Thompson & Corke (1969) and Corke & Thompson (1970) used perfusion technique only or the perfusion technique along with the conventional Warburg manometric technique to measure the

effects of herbicides on soil nitrification. Still other workers, namely Newman (1947), Chandra (1964) and much more recently Goring et al. (1967) and Bartha et al. (1967) have used the 'pot technique' for this same type of assessment of the effects of herbicides on soil nitrifying capacity.

The 'pot technique' in one form or the other has been the most widely used method for the study of nitrification in soil under laboratory conditions. Here the soil samples are brought to field capacity by the addition of nutrient solution, which is either free of the inhibitor or containing the inhibitor at desired concentrations, and then stored in stoppered jars. Soil samples taken from these jars, after being left for set periods of time following the treatments, are normally analysed for NO_3^- nitrogen. Although this method is of great value for some agronomic purposes, it is less suitable for detailed biochemical study of nitrification process in soil. This is due to many drawbacks inherent in the technique as discussed in great detail by Lees & Quastel (1946a). Few of the important points in which the 'pot' technique proves to be unsatisfactory for biochemical studies on soil nitrification are as follows: soil samples may tend to dry during long experimental periods; the soil in the bottom of the jars may get water logged due to draining of water to the bottom thereby causing an uneven distribution of moisture; soil invariably gets disturbed during sampling and also aeration is normally poor due to the containers being kept closed as a measure to prevent excessive evaporation from surface soils.

It is now well accepted that the technique proved to be biochemically the most useful and adaptable in studying the effects of various inhibitors on soil nitrification process is the 'soil perfusion' or the 'soil percolation' technique by Lees & Quastel (1946) and Audus (1946). In these soil perfuser systems, which are operated by negative or positive pressures, a solution of an ammonium salt is perfused and reperfused through a column of soil that is kept aerated by the air dragged through the column along with the perfusing solution. Later

Wright & Clark (1969) modified this conventional perfusion apparatus so that the necessity to make use of positive or negative pressures for its operation could be avoided. This new set-up was operated by a multichannel peristaltic pump which could keep a maximum of six 'per-fuser units' going at the same time under identical experimental conditions. An account of this original as well as the modified perfusion apparatus will be given in the next chapter on Materials and Methods.

In their original work on soil nitrification Lees & Quastel (1946b) made it clear that the presence of soil itself is necessary for appreciable nitrification to take place and that the soil surface acts specifically by supplying NH_4^+ held in base exchange complexes. They then concluded that nitrification probably takes place at those sites on the soil surface where base exchangeable ammonium ions are located. Considering the above findings to be correct it was concluded that the rate of soil nitrification is in part a function of the base exchange capacity of the soil and the process is restricted to specific sites on the soil surface.

The same authors using the perfusion apparatus then followed the kinetics of nitrification process as it takes place in soil. In these experiments samples of soil were packed in soil columns (details of the experimental methods will be discussed in Chapter 2) and ammonium sulphate solutions containing a known concentration of ammonium ions were perfused through each of these columns. The perfusing liquid or the perfusate was sampled at regular intervals and analysed for NH_4^+ , NO_2^- and NO_3^- ions. The effects of different poisons such as chloretone, ethyl urethane, quinones, cadmium and chlorates on nitrification were then assessed by comparing the rate of nitrification in a soil sample perfused with a solution of an ammonium salt containing the inhibitor, at known concentration, with the rate in an identical sample of soil perfused with a similar solution of ammonium salt that has no inhibitor. As a result

of these kinetic studies the following important details regarding soil nitrification were revealed.

During the initial perfusion of a sample of an air dried soil there is likely to be a lag period before any ammonium is nitrified, the length of the lag period being dependent on whether the soil has recently carried out any appreciable amount of nitrification or not. Initially small amounts of NO_2^- start to appear with the build up of a population of Nitrosomonas. This formation of NO_2^- by Nitrosomonas stimulates the growth of Nitrobacter which then oxidises the NO_2^- to NO_3^- . Thereafter the oxidation of ammonium yields nitrate alone because the Nitrobacter population of a soil, under normal conditions, is capable of oxidising all NO_2^- nitrogen as fast as it is formed. This initial phase of nitrification is of long duration since ~~the exponential~~ a lag phase is involved, and eventually a condition arises, on reperfusion with fresh ammonium solution, where the sites of proliferation on the soil particles are saturated with the nitrifying organisms. Such a soil is termed 'enriched' or 'saturated' soil where nitrification takes place with no initial lag and at relatively rapid rates that remain constant during subsequent perfusions with fresh ammonium salt solutions. In this condition the soil could be assumed to behave like an enzyme system, with no proliferation of the relevant organisms taking place except presumably to replace the dead cells.

Some workers such as Quastel & Scholefield (1953), Otten, Dawson & Schreiber (1957), Eno (1962), Caseley & Luckwill (1965), Corke & Thompson (1970) etc. studying the effects of herbicides on soil nitrification made use of 'fresh' soil to assess the herbicidal effects whereas others like Debona (1967) used enriched soil in their experiments. The results obtained in the first type of experiments would show the effects of herbicides on proliferation of nitrifying bacteria while the results obtained with enriched soil would only show any effects on nitrification process per se.

Still other workers e.g. Quastel & Scholefield (1951) and Hale, Hulcher & Chappel (1958) removed the enriched soil from the perfusers and measured the oxygen consumption by samples of it transferred into Warburg vessels. The inhibitors were tested, making use of this method, by the measurements of rates of respiration being made with and without the addition of any specific inhibitor into the Warburg flasks containing the soil samples and the substrates.

The methods discussed above are the main ways in which kinetics of soil nitrification and the effects of various inhibitors, including a number of herbicides, on the process of nitrification itself have been studied so far. In addition to these, the effects of a few herbicides on the metabolic activities of nitrifying organisms grown in pure cultures have been looked at by Debona (1967), San Clemente (1968 & 1970) and Garretson & San Clemente (1968).

d) The present problem and methods of approach

In spite of the extensive reviews written by Audus (1960) and Fletcher (1960) on the general microbial effects of herbicides, some of the field and laboratory studies carried out on this same subject by different workers have given rise to conflicting results.

A survey of literature shows that these controversial views regarding the herbicidal effects on soil micro-organisms still exist even when a small group of organisms such as those responsible for soil nitrification only are considered leaving aside the vast number of other microbes included under the general term 'micro-organisms'. For example Carini (1963) and Casely & Luckwill (1965) have reported that Diuron and Neburon are ineffective as inhibitors of nitrification while Dubey (1969) working on the same herbicide diuron has shown that it inhibits nitrification at concentrations as low as 20 p.p.m. when applied to soils of low to intermediate nitrifying capacity even though concentrations up to

100 p.p.m. had no effects on soils of high nitrifying capacity. As pointed out in the above example, the effects of the same chemical could vary depending on the efficiency of the soil in relation to its nitrifying capacity. Similarly the effects also vary greatly with the type of chemical and on the dosage.

On the other hand if one considers the speed at which new herbicides are produced and put on the market, it becomes evident that there is a great deal of work which has to be done to assess any deleterious effects these new chemicals might have on soil micro-organisms and their activities that are of significance in soil fertility and plant nutrition. This seems essential because almost all commercial herbicidal chemicals are tested more carefully only for their economic benefits and few other possible major hazards with very little or no regard at all to nevertheless important processes like soil nitrification mediated essentially by micro-organisms.

The objective of the present investigation is to study the effects of a range of newer herbicides on the nitrification process, with the view that such a study would certainly go a long way to augment the existing knowledge of the actions of herbicides so far studied, as regards their effects on soil nitrification. From much of the work reported up to date it is not very clear whether the effects observed have been directly on the growth and viability of the organisms themselves or on the enzymes controlling the biochemical processes involved in nitrification. Although it is simple and logical to conclude that an inhibition of the latter would automatically retard growth, few other factors like the possibility for the organisms to develop some degree of adaptation to the herbicide and also the possible changes in the herbicide itself during long term perfusion would obstruct such a simple conclusion. Therefore the experiments described in this thesis were aimed at distinguishing between these two possible actions and at the same time to find out

whether the herbicides used in these experiments have any differential effects on the activities of the two major groups of organisms, namely Nitrosomonas spp. and Nitrobacter spp., concerned in the nitrification process. So far very little or no attempt has been made to evaluate any such differential effects the herbicides might have on the two individual steps involved in nitrification, the first of which constitutes the conversion of NH_4^+ to NO_2^- and the second that converts the NO_2^- to NO_3^- as was discussed earlier in this chapter.

Enriched soil samples saturated with equilibrium populations of Nitrosomonas species and Nitrobacter species were used in the experiments carried out here to investigate the effects of herbicides on the overall nitrification process and the effects of the added herbicides on the steady rates of nitrification of these saturated soils were regarded as giving a fair indication of their actions on the process. On the other hand 'fresh' or unenriched soil samples were used in the experiments carried out to investigate the effects of herbicides on the proliferation of nitrifying organisms and the kinetics of accumulation of nitrite and nitrate were used as an approximation to the growth kinetics of Nitrosomonas species and Nitrobacter species populations.

In the above mentioned methods, the herbicides are usually incorporated into the perfusates and thus the observed effects may or may not be due to the added substance itself depending on whether the herbicide under consideration undergoes any changes after coming in contact with soil. For example Beynon & Wright (1968) have shown that Chlorothiamid is not very resistant to microbial attack and it can get rapidly converted into Dichlobenil within four weeks after soil application. They have also shown that the subsequent disappearance of Dichlobenil is much slower and according to Sheets, Harris & Smith (1968) Dichlobenil residues could be detected in treated soils even after a period of one year. However Dichlobenil is only about one-tenth as inhibitory to nitrification as Chlorothiamid according to Debona & Audus (1970). The fate of the herbicide

Propanil (3,4-dichloropropionanilide) in soil is also known to some extent and according to Bartha et al. (1967) 3,4-Dichloropropionanilide gets broken down to 3,4-dichloroaniline and propionic acid. They have also found that the aniline moiety was as active as Propanil molecule in inhibiting the nitrification process in soil. But unfortunately the nature and the effectiveness of the breakdown products as soil nitrification inhibitors are not completely known for most of the other herbicides and therefore the inhibitory effects exerted by these herbicides on the rates of the soil nitrification process could not be considered, with certainty, as being due to the added herbicide alone. However these soil experiments give an idea about what may happen in practical agriculture.

Studies on nitrification process as it takes place in pure cultures of Nitrosomonas and Nitrobacter species are not complicated by such interfering factors (as discussed above) that might occur in soil. Therefore in spite of the difficulties in maintaining ~~continuous~~ cultures of these organisms, under strictly autotrophic conditions, the effects of some herbicides (that has not been studied so far) on the respiratory rates and short term nitrification rates (where no growth is involved) of these organisms were investigated. An attempt was also made to study the effects of some of the herbicides on the growth of these organisms in pure culture. The experimental details pertaining to the pure culture experiments will be discussed later in this thesis.

These pure culture studies yield desirable information regarding the real toxicities of the added herbicides on the nitrification process even though the results obtained with the nitrifiers growing on artificial media may not always be extrapolated with certainty to the field where, with soil as the medium, the environment and the surrounding population as a whole are exceedingly complex.

All the different types of experiments mentioned above ~~are~~ are

designed to evaluate the effects of a range of herbicides on

- a) Rates of nitrification in enriched soil;
- b) Growth rates or proliferation of the nitrifying bacteria, Nitrosomonas and Nitrobacter in fresh soils perfused with ammonium sulphate solutions;
- c) The rates of oxygen uptake by enriched soils;
- d) Rates of oxidation of ammonium and nitrite ions by pure cultures of Nitrosomonas and Nitrobacter respectively during short term nitrification experiments which does not involve any growth of these organisms;
- e) Rates of oxygen uptake by Nitrosomonas and Nitrobacter cell suspensions;
- f) The growth rates of Nitrosomonas and Nitrobacter cultures as measured by rates of formation and oxidation of nitrite respectively, were carried out during the present investigation.

CHAPTER 2

Materials and Methods

Section A. Nitrification in Soil

1. Preparation of Soil

The soil used throughout the nitrification experiments was obtained from a fallow plot in the Botany garden of Bedford College, Regent's Park, London N.W.1. The very top layer (about 1 cm.) of soil was scraped off and the soil just below this layer up to a depth of 7.5 cms. was collected. This soil was passed through 13 mm. mesh riddle to remove large stones and plant debris and then air dried under laboratory conditions. When dry the soil was sieved to separate the crumbs of size ranging from 2-4 mm. This fraction was stored in air tight polythene containers and used whenever needed.

2. Perfusion technique

a) Introduction

The basic idea of the perfusion technique which was not unlike the modern method has been used since the very early times by Schloesing & Muntz (1877) in their simple but original experiments that demonstrated quite conclusively the biological origin of nitrification process. Here they used a tube one metre long filled with sand and chalk, previously sterilized by incineration, and sewage water was poured daily into the upper end of the tube. The effluent collected at the bottom of the tube was analysed for ammonium and nitrate ions.

After this original work on soil nitrification there has been an apparent lack of interest in this field which may have been partly due to the assumption that the organic components of soil would exercise a variety of indeterminable effects on nitrification and partly due to the lack of a suitable experimental technique for the study of the biochemical changes in soil. Many years later Lees & Quastel (1946a) developed the

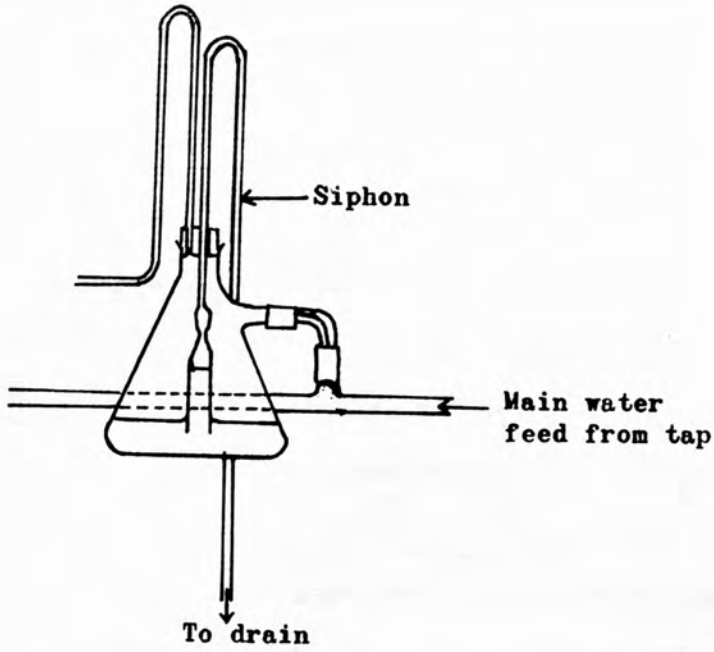
conventional perfusion apparatus that made possible the study, within certain defined limits, of nitrification process as it actually occurs in soil.

The perfusion apparatus described by Lees & Quastel (1946) consisted of two parts, namely

- a) The reciprocator, and
- b) The perfusion unit.

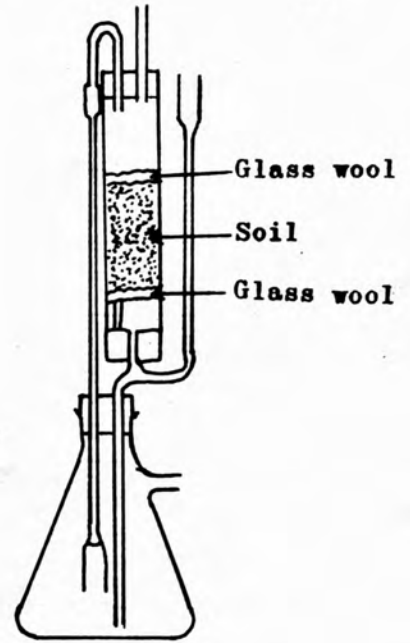
The reciprocator (Fig. 1(a)) consisted of a 300 ml. Buchner flask fitted with a rubber bung and two tubes as shown. Tap water is run steadily and slowly through the side arm via a capillary tube so that the flask alternately fills up and then rapidly empties by means of a siphon tube into a drain as in the Soxhlet apparatus. At each of these cycles air is first slowly forced out and then rapidly sucked in. This alternating air-flow was used to drive the perfusion unit proper shown in Fig. 1(b).

The perfusion unit consisted of a glass tube 10 x 1 in. having a glass wool plug at the bottom to hold 20-50 gm. of sieved soil. A further glass wool plug at the top of the soil column was used to minimize puddling of the soil surface. The bottom of this tube was fitted with a one holed rubber bung into which was inserted the side arm of a T piece, the main tube of which continued vertically upwards for about 10 in. to terminate in a wide mouth, and vertically downwards through the bung almost to the bottom of a 300 ml. Buchner reservoir. Through the other hole of the reservoir flask bung was a water delivery or 'lift' tube which was a straight piece of glass tube 16 in. long with a widened lower end. The upper end of this tube was connected by a rubber tubing to a bent 4 in. capillary (1 mm. bore) which passed through one hole in the bung at the top of the soil column. The other hole in this bung was occupied by a 6 in. piece of fine capillary communicating with the atmosphere.



The reciprocator

Fig. 1 (a)



The perfusion unit

Fig. 1 (b)

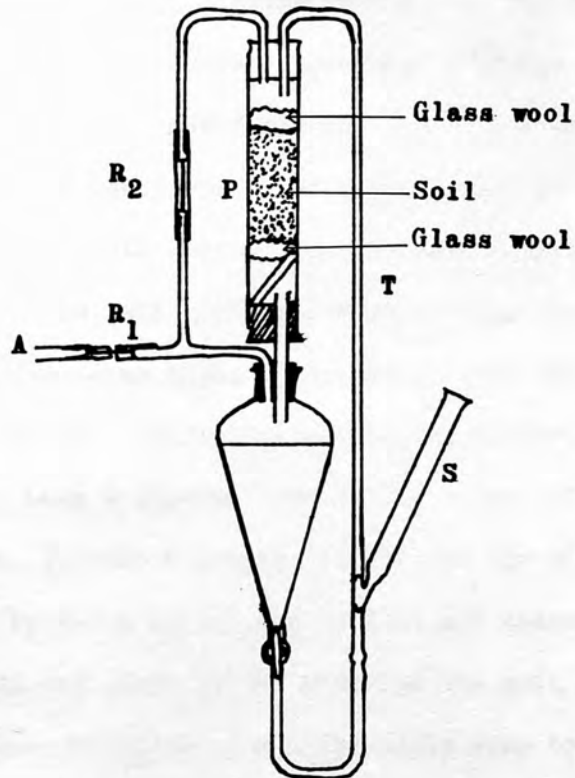


Fig. 2

To start percolation the soil was saturated with part of a measured volume of the solution to be studied and the remainder of the solution was put into the reservoir flask. The lift tube of the flask was then adjusted so that its wide end just touches the reservoir fluid and the side arm of the reservoir flask was connected with the air line of the reciprocator. Briefly, the above description deals with the main points about the original perfusion apparatus and a detailed account of its operation procedure and modifications are discussed by Lees & Quastel (1946a).

Later in the same year Audus (1946) simplified this technique while still retaining the essential features of the original apparatus. The design of this is shown in Fig. 2. In his version of the perfusion apparatus Audus (1946) made use of a small constant suction applied at A (Fig. 2) by means of a suction pump. This suction regulated and transmitted back through the lengths of the thermometer tubing R_1 and R_2 and the soil column P to the perfusion solution in T causes air to be drawn in at the base of the side tube S thereby detaching a column of solution that could be drawn up the tube T and discharged on to the top of the soil column. In essence the reciprocator of the original perfusion apparatus was replaced by a small suction pump in this modification.

The above mentioned soil perfusion systems that are operated by negative or positive pressures have, up to date, found wide application in the study of soil microbial activities such as the studies on, the nitrification process by Lees & Quastel (1946), the effect of herbicides on nitrification by Hale, Hulcher & Chappel (1957) and the microbial breakdown of herbicides by Audus (1960) and Kaufman and Kearney (1965).

Recently Wright and Clark (1969) modified the soil perfusion system, once again, by incorporation of a peristaltic pump to circulate the liquid thus eliminating the dependence on positive or negative pressures. The perfusion system used in the present investigation is

basically similar to that of Wright & Clark (1969), in that it is operated by means of peristaltic pumps, but with few more added modifications and was developed during the course of my work presented in this thesis.

b) The modified perfusion apparatus and its operation procedure

The soil column consists of 50 gr. of 2-4 mm. portion of sieved air dried soil supported with a pad of glass wool on a perforated perspex disc kept in position half way inside a 60 cm. long glass tube. This glass tube is 3 cm. in diameter along the top 45 cm. length and the lower portion is blown into a narrower tube of uniform diameter as shown in Fig. 3.

This soil column is connected to a Wolf bottle by means of a rubber bung, fitting its 29/32 ground glass neck and through which the narrow end of the tube could pass so as to reach well beyond the liquid level C. This Wolf bottle with a 500 ml. capacity serves as the reservoir for the perfusate. One of the two small necks of the reservoir is fitted with a rubber bung through which passes a narrow piece of glass tubing long enough to reach well into the perfusate. This glass tubing is then connected to the 'pump unit' with (Portex) translucent, non toxic, vinyl tubing of 4 mm. bore (supplied by the Portex Ltd., Kent, England). The perfusate is carried through this tube from the reservoir to the pump unit which is fitted on to the frame above each soil column, Fig. 3. Another piece of similar tubing takes the perfusate from the pump to the top of the soil column. These two pieces of tubing (one bringing perfusate into the pump unit and the other taking it back to the top of the soil column) are connected with each other by means of a piece of silicone rubber tubing (9.5 cm. long, 4 mm. internal diameter and supplied by Esco (Rubber) Ltd.) placed in position inside the tube track and pressing against the frictionless pump rollers made of PTFE, Fig. 4.

A series of these small pump units (12) are connected to a single,

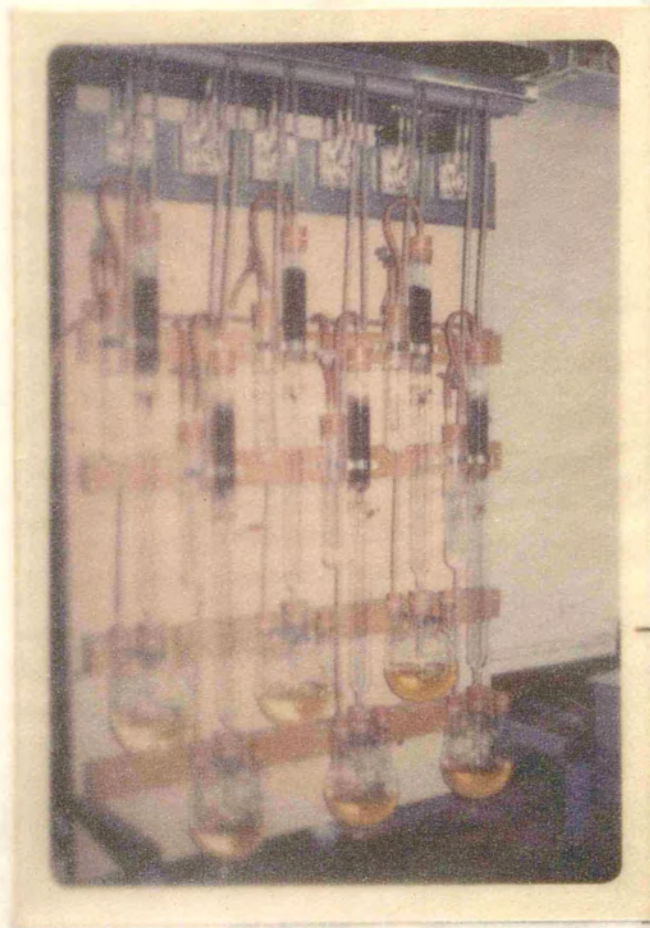


Fig. 3

Perfusion Apparatus

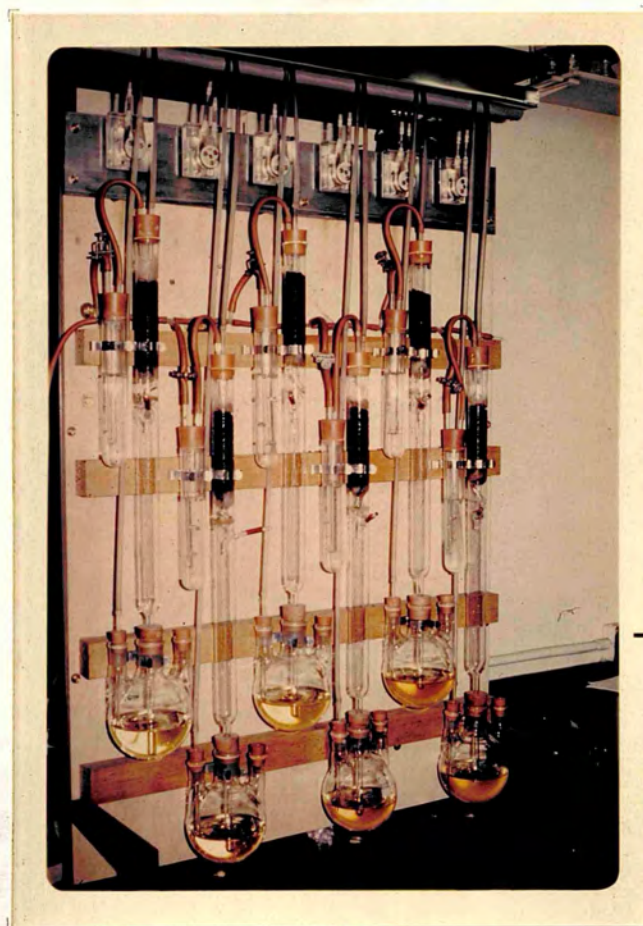


Fig. 3

Perfusion Apparatus

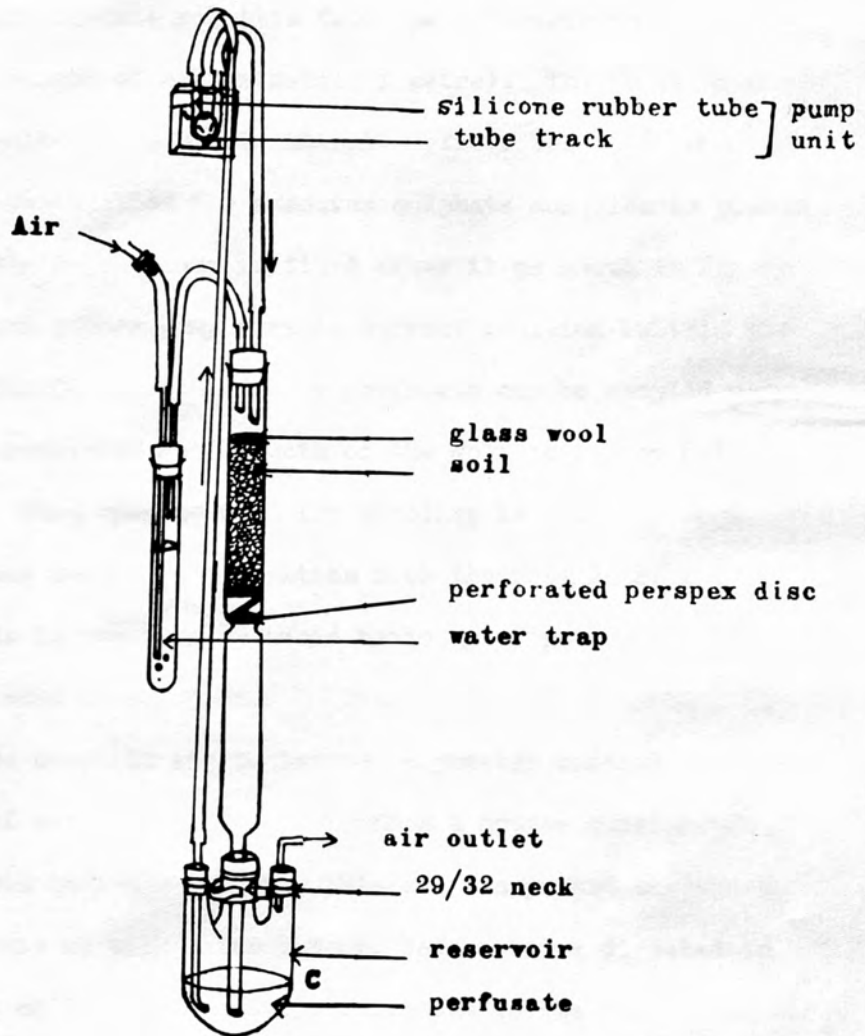


Fig. 4
Perfuser Unit

geared motor^(Parvalux) working at a normal output speed of 15 r.p.m. Since it was desired to have an approximate flow rate of 175 ml./hr. the motor was geared up $1:1\frac{1}{2}$ times to give a resulting output speed of $22\frac{1}{2}$ r.p.m. When this motor is turned on, the pump rollers start to rotate, rolling in succession against the silicone rubber tubing in the tube track and the pumping action thus created quite efficiently maintained a continuous flow of the ammonium sulphate solution from the reservoir to the top of the soil column (a height of approximately 1 metre). The front disc of the pump unit prevented any possible slipping off of the pump tube.

To start circulation the ammonium sulphate solution is placed in the reservoir, the soil column is fixed above it as shown in Fig. 3 and with the silicone rubber pump tube in correct position (within the tube track) the motor is turned on. The perfusate can be sampled very easily through the remaining small mouth of the Wolf bottle by using an automatic pipette. This opening used for sampling is normally kept closed to prevent any possible evaporation from the reservoir, but leaving a small hole in the bung to avoid build up of pressure due to the passage of air used in aeration.

To improve aeration and to introduce greater control over the rate of aeration, of each soil column, air from a source outside the laboratory was passed into the system. This air was passed through a water trap to saturate it with water vapour, before being directed to the soil column, so as to minimise evaporation due to the passage of air.

Some of the advantages of this modified perfusion system are as follows.

- 1) With the use of one motor several soil columns can be perfused at the same time with the same or different solutions. Also identical rates of perfusion can be maintained in every perfuser unit since all the pump units are operated with the same motor.
- 2) Use of air saturated with water vapour for aeration minimises evaporation. This device also permits the use of any other gas

if desired in place of air.

- 3) Rate of aeration can be controlled easily, and independently of the circulation of the perfusate.
- 4) Sampling of the perfusate is very easy.
- 5) It needs very little or no adjustment or attention during long term experiments although it was found necessary to renew the silicone rubber pump tubes about once in 3-4 weeks.
- 6) The pump units being fixed above the reservoirs restricted the possibility of fine sediment (escaping through the glass wool plug) entering the pump tubes and blocking them.

Also 7) The tubes can be disconnected from the pump without disconnecting from the remainder of the apparatus thus allowing sterilization of the complete circulating unit to be possible if necessary.

3. Chemical Analysis of NO_2^- and NO_3^- - N

a) NO_2^- - N

The sample to be tested (e.g. 1 ml. perfusate) was diluted to 11 ml. with deionised water and 2 ml. of mixed Griess-Ilosvay's reagent were added. (Equal volumes of Griess-Ilosvay's reagent I and II were mixed just before use.) After at least half an hour the colour intensity of the reaction mixture was read with a Unicam 1400 absorptiometer at 520 nanometres wave-length. The NO_2^- - N contents of the samples ($\mu\text{g NO}_2^- / \text{ml. perfusate}$) were determined from the colorimeter readings by comparison with a standard sodium nitrite calibration curve, Fig. 5. The test samples obtained from the perfuser units were diluted accordingly since the direct proportionality between the concentration of nitrite - N and the optical density measured by the colorimeter tend to depart from linearity beyond nitrite - N concentrations of $5 \mu\text{g/ml.}$

b) NO_3^- - N

Estimation of nitrate - N was carried out according to the method used by Lees & Quastel (1946) with the H_2O_2 step omitted. The

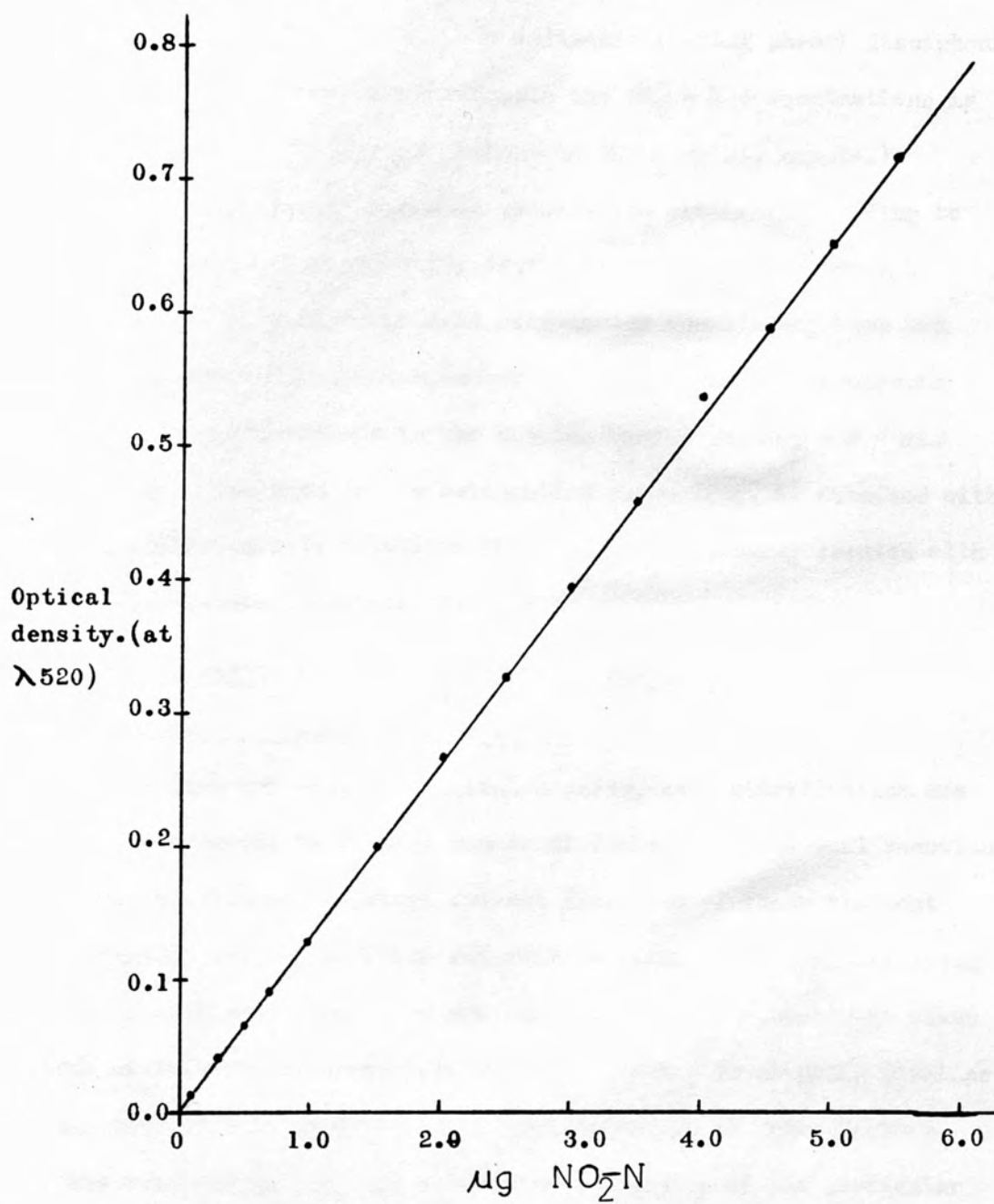


Fig. 5 Calibration curve for the quantitative estimation of Nitrite-nitrogen.

H_2O_2 oxidation step was conveniently omitted since other workers have reported that concentrations of nitrite - N up to at least 700 $\mu\text{g/ml}$. sample does not interfere with NO_3^- - N estimations using phenol disulphonic acid reagent. (In the present experiments the NO_2^- - N concentrations in the test samples never exceeded a maximum of 30-40 $\mu\text{g/ml}$. sample.)

The phenol disulphonic acid reagent was prepared according to Snell & Snell (1936). The intensity of the yellow colour produced by reaction with phenol disulphonic acid reagent and ammonia solution was measured using the Unicam absorptiometer at 410 millimicron wavelength and the NO_3^- - N concentrations in the samples tested ($\mu\text{g NO}_3^-$ - N / ml. perfusate) were calculated from a calibration curve (Fig. 6) obtained with standard potassium nitrate solutions that had been similarly treated with the phenol disulphonic acid reagent.

4. Practical Considerations

a) Factors affecting nitrification in soil

Just like any other biological process, soil nitrification ~~is~~ is known to be affected by a large number of factors such as soil reaction, temperature, aeration and moisture content etc. Out of these the most important factors are the aeration and soil reaction since the nitrifying organisms, as they are known up to now, are aerobic chemoautotrophs whose biological activities are optimal in soils of neutral to slightly alkaline reaction. However soil aeration is in turn dependent on other factors such as the moisture content and waterholding capacity of the particular soil and also to a large extent on the size of the soil particles which determine the volume of soil pore spaces.

It has already been discussed by Broadbent & Stojanovic (1952) that low partial pressures of O_2 in soil reduces or even completely stops any nitrification of added ammonium - N at the same time increasing nitrogen losses due to denitrification. As the soil particle size has a direct influence of gaseous exchange it is not very surprising to note

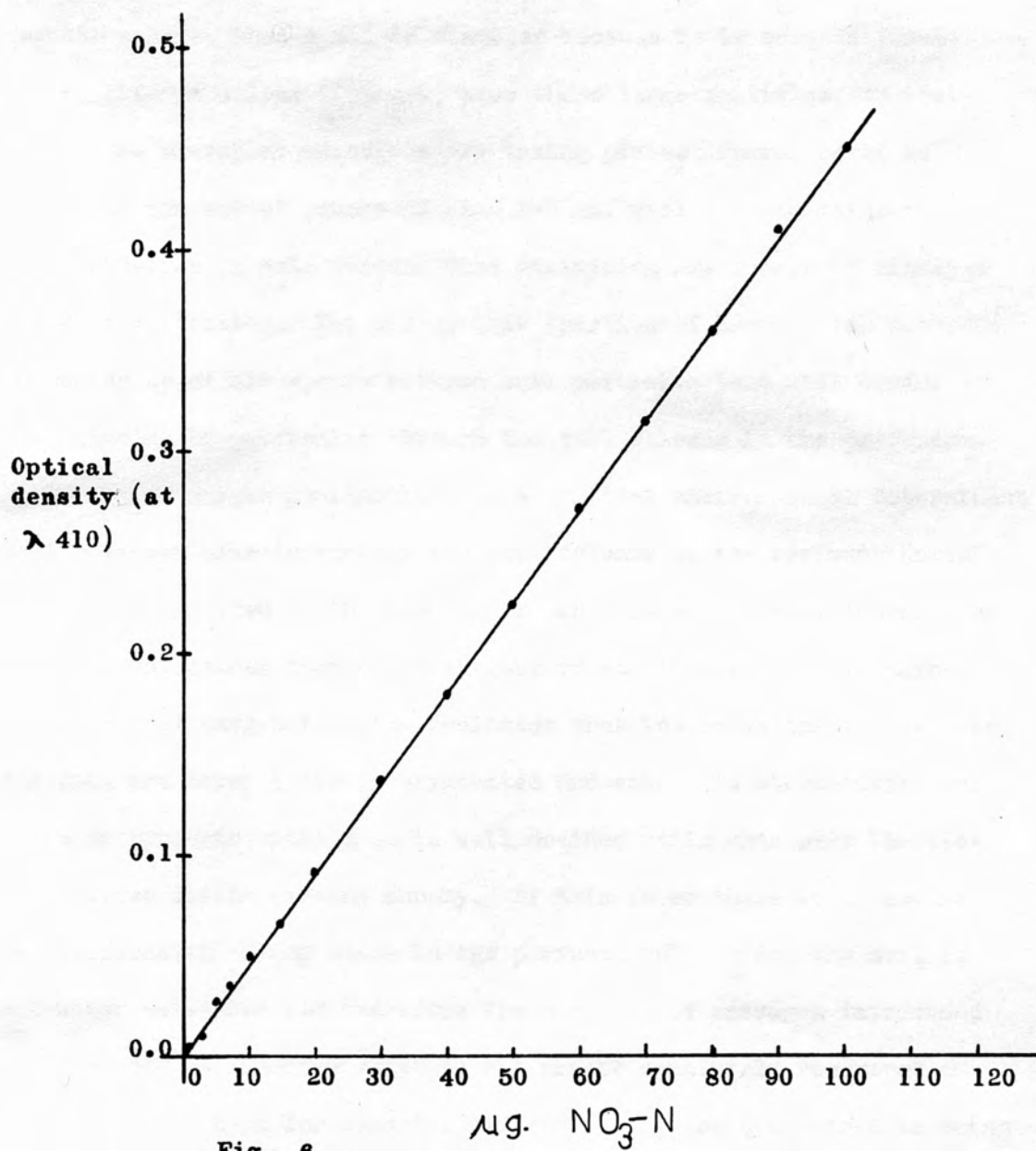


Fig. 6

Calibration curve for the quantitative estimation
of Nitrate - nitrogen.

that nitrogen losses from soil tend to decrease with increasing particle diameter which results in improved aeration. Yet it has been pointed out by Nommik (1956) that denitrification is favoured by mechanical aggregates greater than 4 mm. in diameter because it is more difficult for O_2 to diffuse a long distance, pass these large particles, to the centres where microbial reactions are taking place. Therefore it is evident that the use of crumbs of size 2-4 mm. will help to maintain improved aeration in soil columns thus minimising any losses of nitrogen due to denitrification. The use of this fraction of crumbs also prevents any blocking up of air spaces between soil particles that will hinder the percolation of perfusates through the soil columns in the perfusers.

Since oxygen availability is a critical environmental determinant of soil nitrogen transformations the soil columns of the perfuser units were kept well aerated by the passage of air from an external source. But the demonstrations that nitrogen gets volatilized from soils maintained at normal oxygen tensions indicates that the pores and interstices in the soil are never entirely oxygenated and anaerobic micro-environments exist at microscopic sites even in well drained soils when ever the biological oxygen demand exceeds supply. If this is so there would always be denitrification taking place in the perfuser units where the soil is almost water saturated and therefore the quantity of nitrogen introduced into the system as ammonium sulphate may not be completely recovered as NO_3^- . This unaccounted for fraction, if any, has to be considered as being lost by biological and chemical volatilization.

Warrington (1878, 1879, 1884 & 1891) in a very early series of experiments has shown that light inhibited the second stage of nitrification when crude mixed cultures of nitrifiers, supplied with ammonia, were exposed to light thus resulting in an accumulation of only $NO_2^- - N$. The conditions of the above observation applies quite well to the soil experiments since a sample of soil while being perfused with ammonium sulphate will be exactly equivalent to a crude mixed culture of nitrifiers.

Therefore all the soil perfusion experiments were carried out in the dark so as to avoid any possibility of such undesirable inhibitions of Nitrobacter activity. Perfusions being carried out in the dark also helped in preventing the growth of algae in the tubes and the dark conditions necessary for these experiments were achieved by screening the manifold of perfusers all round with black polythene.

b) Limitations of the perfusion technique

Although perfusion technique has proved to be biochemically the most suitable for studying the inhibitors of nitrification and the kinetics of nitrification itself, as it takes place in soil, it is not without its limitations and shortcomings. But when compared to the other methods, available for this type of study, perfusion technique has only a very few or no serious drawbacks. In fact the only two limitations are:

- a) Removal of solution for analysis inevitably decreases the total volume of perfusate and there is no corresponding decrease in the amount of soil to compensate for this. This error could be reduced to a very low level by taking care to remove as small quantities as possible, and by having a large initial volume of perfusate.
- b) The apparatus is suitable only for studies conducted at a soil moisture content a little short of water logging. Although many microbial activities are rapid and optimal at this water content (aeration also being optimal) the impossibility of working at any other moisture level is certainly a drawback.

5. Analysis of Data

a) Statistical analysis

Effects on nitrification in enriched soil

The nitrification rates in enriched soils remain almost steady over long periods of time provided the nitrate-nitrogen concentrations in the perfusates are not allowed to build up above toxic limits and also sufficiently high concentrations of ammonium ions are maintained

in the systems to satisfy the requirements of the bacterial enzymatic activities. This statement is well substantiated by the findings of Lees & Quastel (1946a) where they concluded that bacteria saturated soils behave like an enzyme system with no proliferation of the relevant organisms taking place except presumably to replace dead cells. The time course of accumulation of nitrate nitrogen in the perfusates of enriched soil samples should therefore be linear over the first few days. In the experiments reported in this thesis, where enriched soil samples were used to follow the time course of nitrification, it was found that this linear phase of nitrifying activity ended in 4-6 days in the control soils, by which time the available ammonium ion concentration begins to limit the process. This same type of linearity was shown by all the samples of enriched soil even when they were under the influence of a range of concentrations of the different herbicides investigated. It was therefore possible to obtain an accurate estimate of the nitrification rates in these soils by a simple linear regression analysis of the data (i.e. the time course of accumulation of $\text{NO}_3^- - \text{N}$ and $\text{NO}_3^- + \text{NO}_2^- - \text{N}$ in the perfusates) where the gradients of the fitted regression lines or the regression coefficients were taken as the estimates of the nitrification rates. Fig. 7 shows a family of such fitted regression lines representing the nitrate nitrogen accumulation, with time, in a series of enriched soil samples perfused with ammonium sulphate solutions containing a range of known concentrations of Ioxynil.

It has already been discussed in Chapter 1 that the nitrification process in soil supplied with ammonium ions is known to be completed through two main steps namely the oxidation of ammonium ions by Nitrosomonas spp. resulting in the formation of nitrite ions and the oxidation of these nitrite ions by Nitrobacter spp. to form nitrate - N. Although the formation of nitrate-nitrogen in soils, supplied with ammonium ions, are mainly dependent on the metabolic activities of

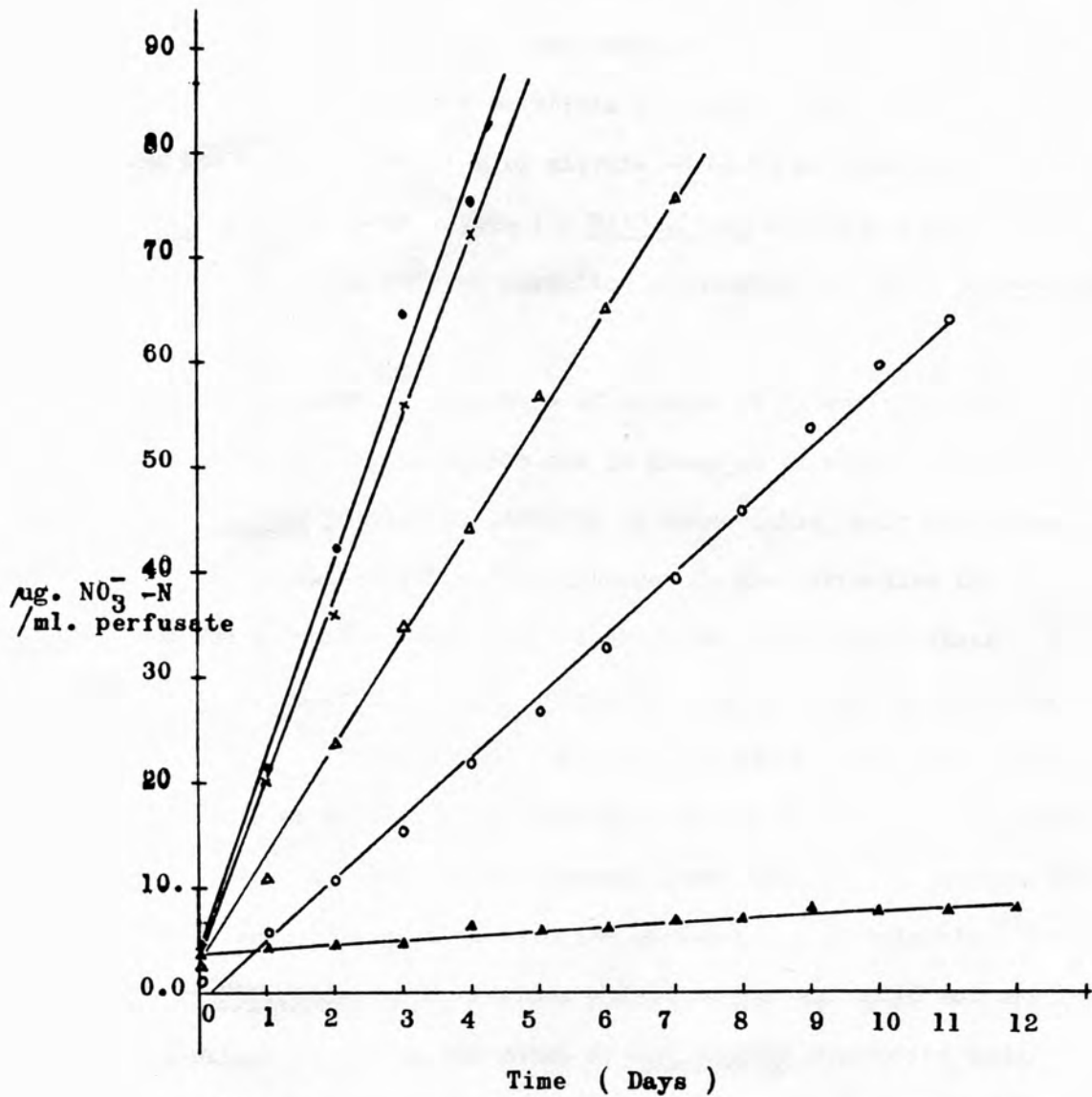


Fig. 7

Time course of accumulation of $\text{NO}_3\text{-N}$ in the perfusates when enriched soil samples were perfused with Ammonium sulphate solutions containing a range of concentrations of Ioxynil.

• - control, x - 10 ppm, Δ - 50 ppm, ○ - 100 ppm, ▲ - 500 ppm.

Nitrobacter spp., as mentioned above, the rate of accumulation of nitrate-nitrogen could not always be taken as a measure of the rate of metabolic activities of the Nitrobacter populations present in these soils. This is because the rate of formation of nitrate -N would be dependent on the amount of nitrite -N available for Nitrobacter activity which in turn is dependent on the rate of formation of nitrite -N due to Nitrosomonas activity.

Thus the rates of formation of nitrate -N by enriched soil samples supplied with ammonium ions can be taken as an estimate of the rates of Nitrobacter population activity in these soils, only when there is accumulation of excessive nitrite-nitrogen in the perfusates in addition to the accumulation of nitrate-nitrogen, since under these conditions the rates of metabolic activities of Nitrobacter populations would not be limited by the availability of nitrite -N. But when there is very little or no nitrite -N accumulation in the perfusates of enriched soils percolated with solutions of ammonium ions, the rates of accumulation of nitrate -N would only give an estimate of the rates of metabolic activities of Nitrosomonas populations and therefore one could not arrive at any conclusions regarding the rates of Nitrobacter activities under these conditions. On the other hand the rates of accumulation of $\text{NO}_3^- + \text{NO}_2^- - \text{N}$ in the perfusates (under conditions of excessive nitrite accumulation) would always give an estimate of the rates of metabolic activities of Nitrosomonas populations in these soils. Therefore the metabolic rates of Nitrosomonas populations in enriched soil samples under control conditions and under the influence of different herbicide concentrations were determined by linear regression analysis of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ accumulation data just as in the previous case. The series of fitted lines derived by regression analysis of the $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ accumulation data observed in the perfusates of the same enriched soil samples mentioned above (i.e. in the same enriched soil samples treated with the known

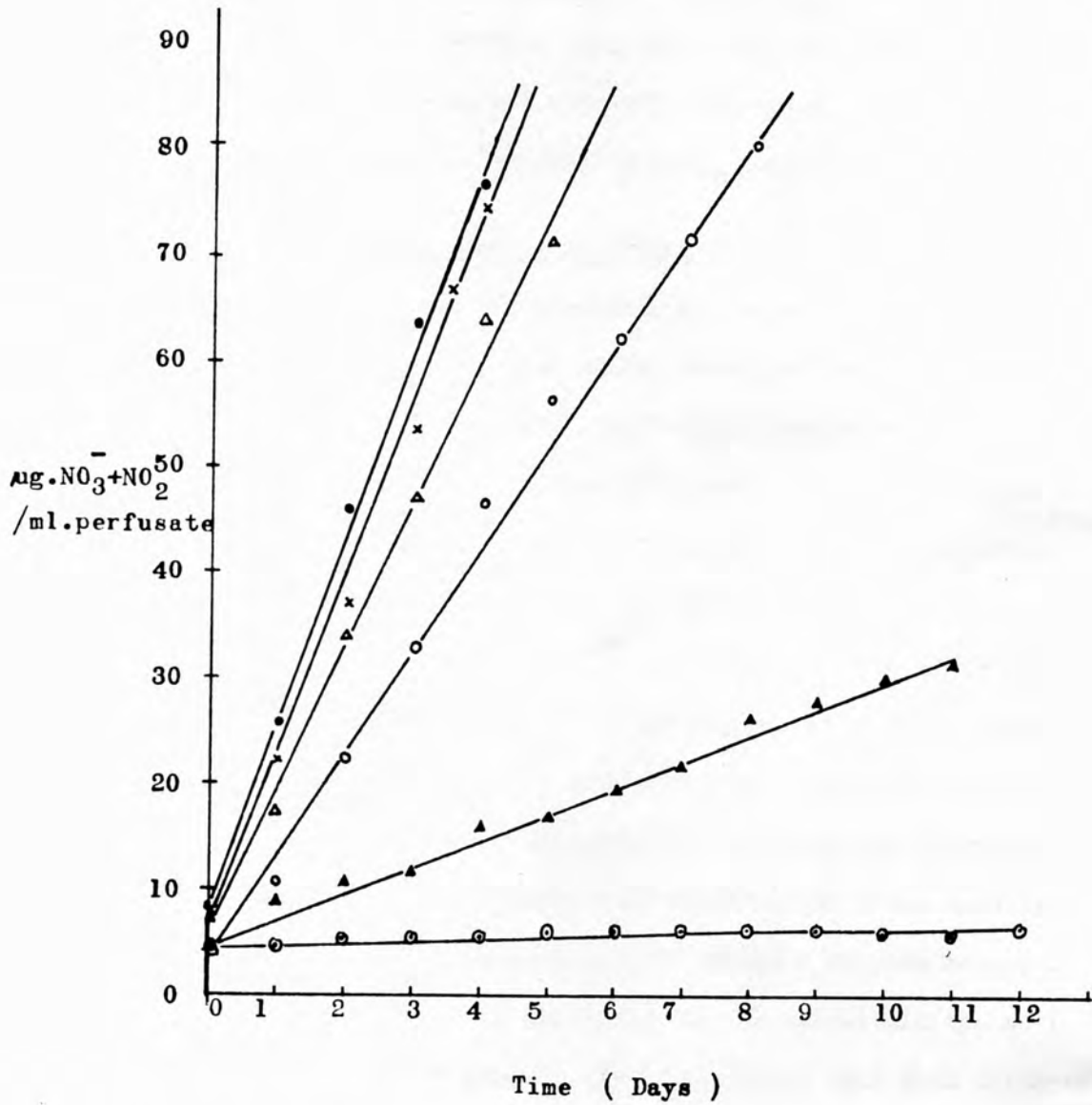


Fig. 8

Time course of accumulation of $\text{NO}_2^- + \text{NO}_3^- - \text{N}$

in the perfusates when enriched soil samples were perfused with Ammonium sulphate solutions containing a range of concentrations of Ioxynil.

● - control, x - 10 ppm, △ - 50 ppm, ○ - 100 ppm,
▲ - 500 ppm, ● - 1000 ppm.

concentrations of Ioxynil) are presented in Fig. 8.

The effects of the individual concentrations of the different herbicides, on the rates of Nitrosomonas and Nitrobacter population activities in enriched soil samples, supplied with ammonium ions, were then calculated as the ratio of the relevant regression coefficients to the regression coefficients of the corresponding controls.

Effects on population growth

Regarding the growth of nitrifying bacteria Seifert (1966) came to the conclusion that they show linear growth in soil. Siström (1962) showed that a direct proportionality exists between the quantity of energy gained during substrate oxidation and the production of biomass of bacteria. It is also known that the nitrifying bacteria obtain all the necessary energy by oxidation of ammonia and nitrite nitrogen. Thus considering that the growth of bacterial biomass depends on the multiplication of the bacterial cells, the production of nitrates could be a good index for the growth of the population of nitrifying bacteria in soil. However the nitrites and nitrates thus produced may serve as a source of nitrogen for numerous other soil microbes and there usually exists a difference between the actual amounts produced and the observed increase in the nitrate content of the soil. But in almost all the soil perfusion experiments reported here it has been assumed that this difference is negligible and the nitrite and nitrate accumulation during soil perfusion is mainly due to the metabolism of proliferating nitrifying organisms granting no interfering factors.

When 'fresh' or non enriched soil is perfused with an ammonium salt solution the course of nitrate accumulation takes a curvilinear path, starting from low values at the commencement of perfusion and then rising in a roughly exponential fashion. In control soils this exponential phase of nitrification ends in about 8-12 days after which it reaches a linear phase. At this point the soil is presumably saturated with

nitrifying organisms and the rate of nitrification becomes maximal and almost constant. Later on as the ammonium ion - substrate concentration becomes limiting the nitrification rates tend to decline.

Considering the kinetics of nitrification in controlled soils it can be reasonably assumed that the rate of nitrification per cell under saturating substrate conditions normally remain unchanged during the whole of the enrichment process. Based on this assumption and on the work of Seifert (1968), the rate of nitrification at any time up to the point when substrate becomes limiting, could be taken as a reasonable measure of the size of the nitrifying population at that time. In addition to these, many of the workers in this field so far have always assumed that P , which is the mean metabolic activity per viable cell, is a constant during proliferation. Quastel & Scholefield (1951) have shown an approximate agreement between the experimental results and the mathematical consideration of the kinetics of nitrite oxidation in soil assuming the value of P to be constant and this finding would strongly support the above assumption. On the other hand the same authors have shown that the mean metabolic activity per cell is increased in response to the exposure of nitrifying soils to an increased initial concentration of nitrite. To explain this discrepancy they concluded that such increases in metabolic activity per cell is normally acquired by the cells before proliferation commences and is most probably during the lag period. Thus in the present analysis too the mean metabolic activity per cell (P) is assumed to be a constant during the course of population growth or enrichment. Considering this assumption to hold good, the rate of nitrification at any time in the presence of a herbicide could also be taken as a measure of the size of the nitrifying population. But if the herbicide does affect the nitrification rate per cell of these organisms, then the rates of nitrification in the fresh soil samples would have to be modified by a factor equivalent to the degree to which the value of P itself is

affected by the herbicide, before these overall nitrification rates are taken as a measure of the size of the nitrifying populations.

These above mentioned assumptions have been used as the basis in the analysis of nitrification curves for fresh soil. As the first step in assessing the influence of herbicides on the growth of nitrifiers in soil, a fourth degree polynomial regression analysis of the nitrate accumulation data was carried out using the library subroutine BMD05.R from the scientific subroutine package (360A - CM - 03X) of the CDC 6600 computer programme library. By means of this analysis smooth curves were fitted passing through theoretically predicted points for the progress of nitrate accumulation in each soil sample.

A family of progress curves, analysed in this way and presenting the time course of accumulation of nitrate nitrogen in a series of samples of fresh soil treated with a range of concentrations of NPH 1320 (25% W/V Bromoxynil octanoate) is shown in Fig. 9. (In these curves the lines are drawn through the theoretically predicted y values where as the actual points in the figure represent the observed y values at the corresponding intervals of time x.)

The values of the four regression coefficients thus calculated namely C_1 , C_2 , C_3 and C_4 in the reverse order along with the value for the intercept of y axis on x axis for each line were then fed into another computer programme designed to calculate the slopes of the smooth curves over each successive time interval. Here the values corresponding to any three consecutive points on x and y axes were considered at a time and the slope of the curve between these three points was determined assuming a linear approximation provided the three points chosen are close together.

$$\frac{\Delta y}{\Delta x} = \frac{\Delta (\text{theoretical NO}_3^- \text{ - N concentration})}{\Delta \text{ time}} = \frac{y_2 - y_1}{x_2 - x_1}$$

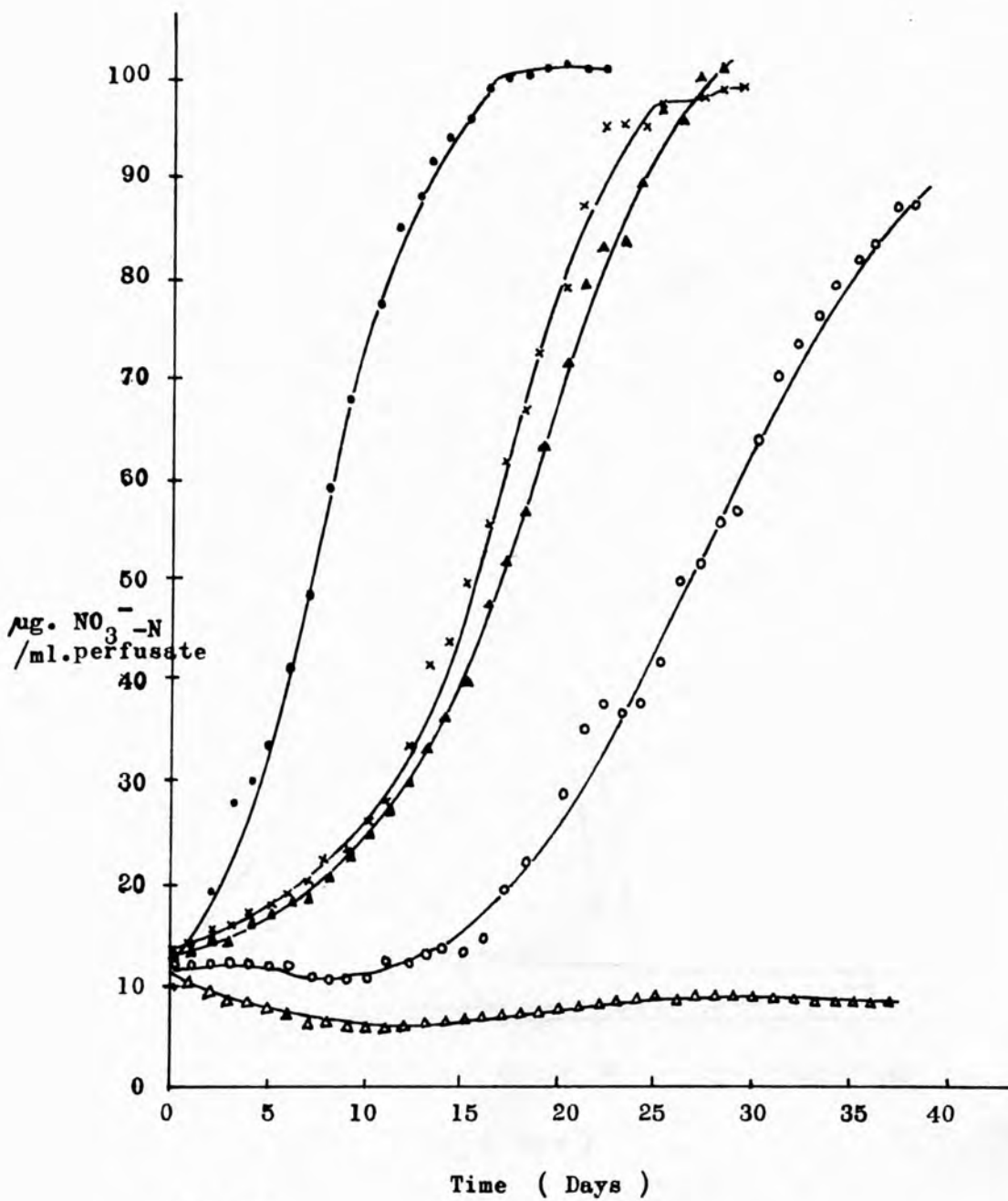


Fig. 9.

Time course of accumulation of $\text{NO}_3^- \text{N}$ when fresh soil samples were perfused with Ammonium sulphate solutions containing a range of concentrations of NPH 1320.

● - control, x - 10 ppm, ▲ - 50 ppm, ○ - 100 ppm,
 △ - 500 ppm.

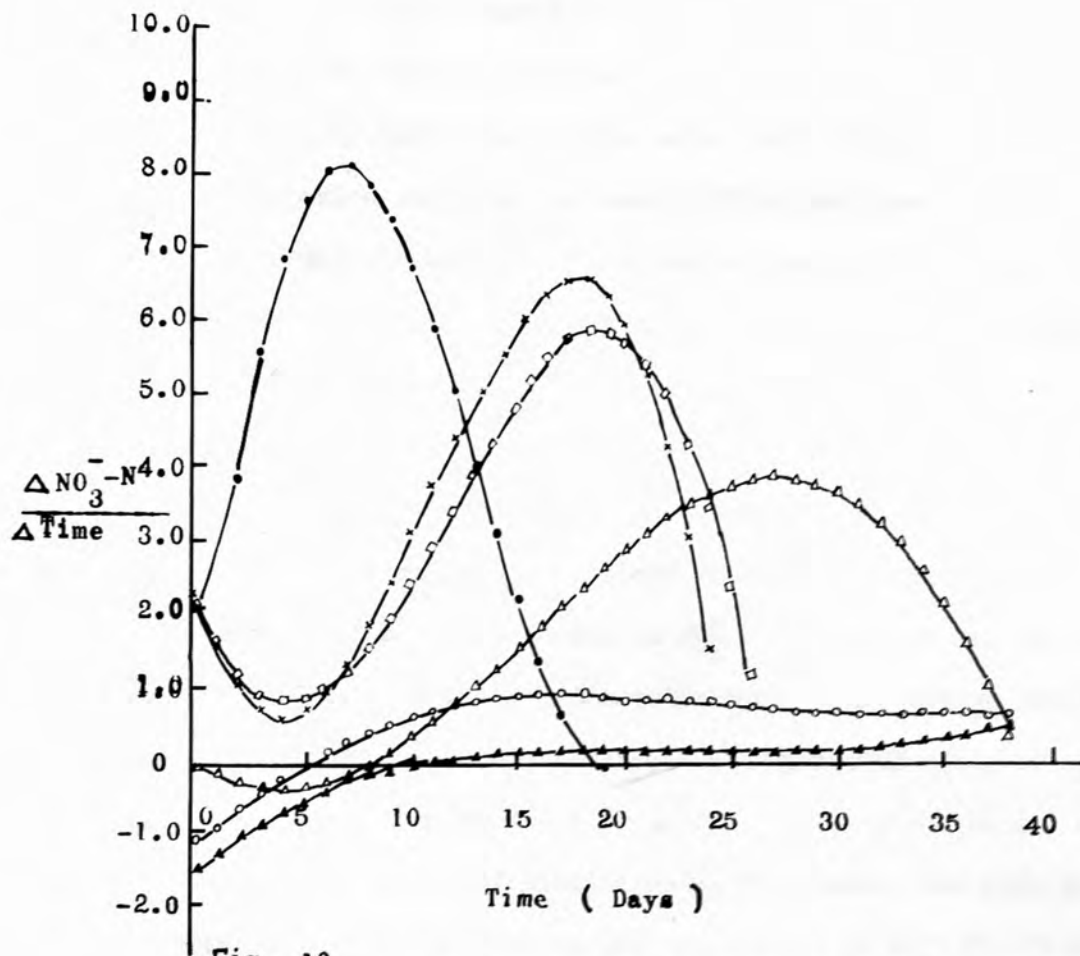


Fig. 10

Rates of formation of NO_3^- -N in fresh soils perfused with Ammonium sulphate solutions containing NPH 1320.

• - control, × - 10ppm, □ - 50 ppm, ▲ - 100 ppm,
○ - 500 ppm, ▲ - 1000 ppm.

These slopes calculated between every three consecutive points on the progress curves were then plotted against time (x) to give the required differential graphs or the 'rate' curves. The series of 'rate' curves corresponding with the smoothed progress curves for nitrate-nitrogen accumulation in NPH 1320 treated soils are shown in Fig. 10. From these differential graphs the maximum rates of formation of NO_3^- - N in a particular sample of soil as well as the time to reach these maximum rates could be obtained.

It must be mentioned at this point that although this type of polynomial regression analysis for curve fitting and then plotting the differential curves, making use of a linear approximation between the consecutive points on the smoothed progress curves, is the best possible way to bring out a clear picture of the kinetics of nitrification in control soils and in soils treated with inhibitors, this may not give an exact picture of what is going on in the soils treated with heavy doses of inhibitors. For example if we go back to Fig. 10 it must be remembered that the negative values for the rate of NO_3^- - N formation are in fact not real and they are a result of the polynomial curve fitting procedure.

Almost all the workers so far, involved in the study of soil nitrification process and its inhibitors, have tried to assess the effects of herbicides on the growth of nitrifiers by determining the rate of accumulation of nitrate -N which is the end product of nitrification process. As was discussed earlier in this chapter, this type of assessment is justified by the findings of Seifert (1968) who concluded that the level of nitrification of a soil can be used as an index of the size of the nitrifying population present in that particular soil. But the results from much of this work do not give any information regarding which stage of nitrification is affected more by the particular herbicides under investigation.

As already discussed the nitrification process in normal soils

of alkaline to neutral reaction is completed in two consecutive stages, the first stage being mediated by Nitrosomonas spp. whereas the second stage is mediated by Nitrobacter spp. In other words, when a fresh soil sample is perfused with a solution of an ammonium salt under normal conditions, the ammonium ions first get oxidised to nitrite-nitrogen due to the metabolic activities of the proliferating Nitrosomonas populations and the nitrite that is formed is known to stimulate the growth of Nitrobacter populations responsible for the oxidation of nitrite -N to nitrate -N. It is also known that once stimulated, the Nitrobacter population in an ordinary soil sample is capable of utilising immediately all the nitrite produced by Nitrosomonas, leaving no room for excessive nitrite accumulation. Thus only extremely low concentrations of nitrite lasting for periods of very short duration would be observed in fresh soil samples where the proliferation of Nitrosomonas and Nitrobacter populations proceed at normal rates. From the above mentioned facts it is clear that the overall rate of the nitrification process in a control soil under normal conditions is limited by Nitrosomonas activity.

On the other hand if a sample of fresh soil is perfused with an ammonium salt solution containing an inhibitor (individual herbicides in this case) and if this substance is effective in inhibiting Nitrosomonas activity in soil then there would be no nitrite or nitrate formed regardless of whether the Nitrobacter population too is affected or not. But if the added inhibitor is more effective in suppressing Nitrobacter activity then there would be an accumulation of nitrite nitrogen, the concentration of the nitrite accumulated being dependent on the differential effects the particular herbicide, under consideration, exert on the metabolic activities of the growing Nitrosomonas and Nitrobacter populations. However if both the stages are equally susceptible to or tolerant of the presence of the herbicides there again no nitrite would be accumulated, either because no nitrite is being produced by Nitrosomonas

or because the nitrite produced gets immediately converted to nitrate by Nitrobacter. Thus it is clear that the first stage of nitrification is the rate determining step of the enrichment cycle (enrichment with nitrifiers) whenever there is no nitrite accumulation in the perfusates of fresh soil samples perfused with ammonium sulphate solutions; a condition similar to what was discussed in dealing with nitrification rates of enriched soils.

Considering the maximum rates of nitrification attained by a freshly perfused soil sample as representing the size of the saturating nitrifier populations (Seifert 1968 - discussed before) it is logical to assume that the maximum rate of formation of nitrite -N as an estimate of the size of the saturating population of Nitrosomonas. But under the experimental conditions used in the present investigation, all the nitrite-nitrogen produced due to Nitrosomonas activity or a fraction of it gets oxidised to nitrate -N due to Nitrobacter population activities, going on following the Nitrosomonas population activities and therefore the maximum rate of accumulation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ (in cases where excessive nitrite accumulation was observed in the perfusates) or the maximum rates of formation of $\text{NO}_3^- - \text{N}$ (in cases where there were no accumulations of nitrite -N in the perfusates) by freshly perfused soil samples were taken as the measure of the size of the saturating populations of Nitrosomonas established in them. In the same way the maximum rates of formation of nitrate -N in the perfusates where excessive nitrite-nitrogen accumulations could be observed, were taken as the estimates of the sizes of the saturating populations of Nitrobacter spp. in these soil samples. As in the case of enriched soil samples discussed before, the size of the saturating populations of Nitrobacter spp. could not be estimated in soil samples where there were no accumulations of nitrite -N observed in the perfusates.

The above reasoning was made use of in the attempt to assess

the differential effects of several herbicides on the growth of Nitrosomonas and Nitrobacter species in fresh soil perfused with ammonium sulphate solutions containing known concentrations of the herbicides. Here the amount of NO_2^- - N / ml. perfusate was also determined daily corresponding to the determinations of the concentrations of NO_3^- - N / ml. perfusate. The variation of the concentration of NO_2^- - N with time was followed by its appearance and disappearance in the perfusates and the observed time course of formation of NO_2^- - N in the perfusates of control soil along with the other five samples of soil perfused with ammonium sulphate solutions containing the known concentrations of NPH 1320, under identical experimental conditions are presented in Fig. 11. (These are the NO_2^- - N accumulation curves obtained with the same soil samples in which the time course of accumulation of NO_3^- - N was observed to follow the pattern shown in Fig. 9.) Fig. 12 presents the family of smoothed curves showing the time course of accumulation of $(\text{NO}_3^- + \text{NO}_2^-)$ - N in these fresh soil samples (treated with NPH 1320) and here too the data showing the variation of the $(\text{NO}_3^- + \text{NO}_2^-)$ - N concentrations were analysed by the polynomial curve fitting procedure discussed previously. Fig. 13 presents the differential or the 'rate' curves corresponding to the progress curves shown in Fig. 12.

Finally the 'rate' curves obtained by the analysis of both the sets of data (i.e. the data for the time course of accumulation of NO_3^- - N and the data showing the time course of accumulation of $(\text{NO}_3^- + \text{NO}_2^-)$ - N) were used to assess the inhibitory effects of the herbicides on population growth of Nitrosomonas and Nitrobacter and the two important parameter of these 'rate' curves which were made use of in these comparisons are:-

1. The maximum values for the nitrification rates which would give the maximum sizes attained by the populations of nitrifying bacteria in control soils and in the soils that are under the influence of

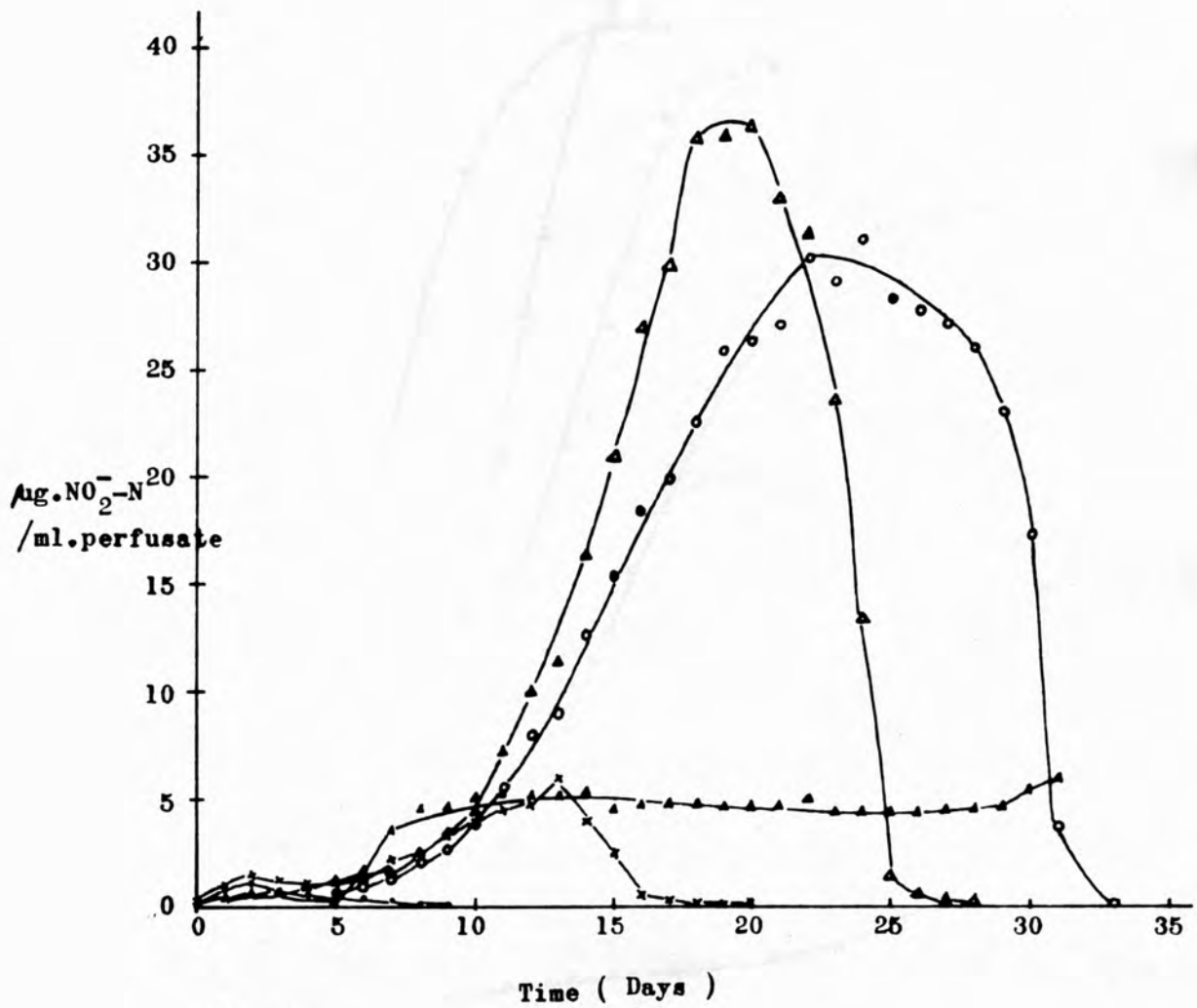


Fig. 11

Time course of accumulation of $\text{NO}_2^- \text{N}$ when fresh soil samples were perfused with Ammonium sulphate solutions containing a range of concentrations of NPH 1320.

● - control, x - 10 ppm, Δ - 50 ppm, ○ - 100 ppm
▲ - 500 ppm.

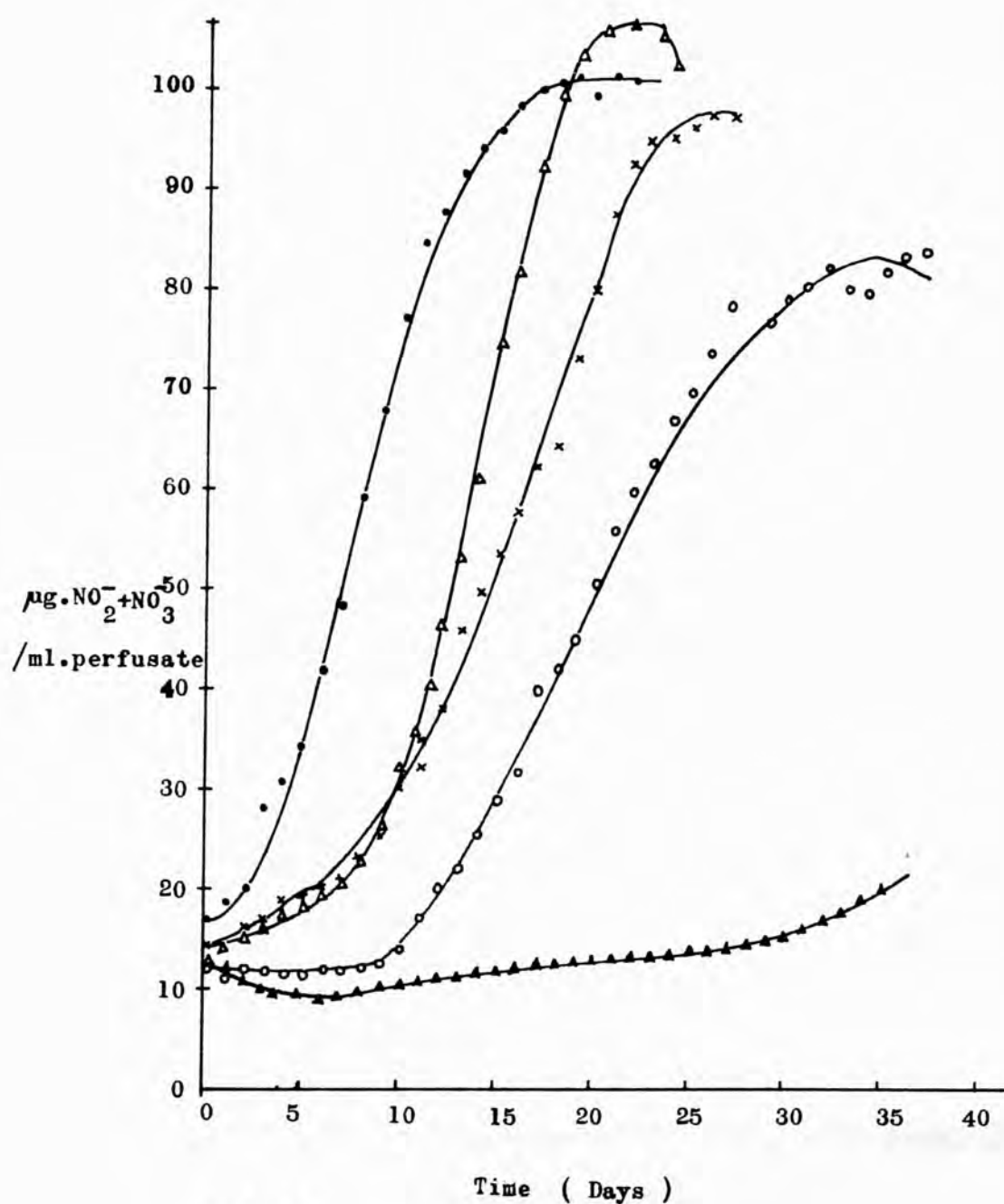


Fig. 12

Time course of accumulation of $\text{NO}_3^- + \text{NO}_2^- - \text{N}$ when fresh soil samples were perfused with Ammonium sulphate solutions containing a range of concentrations of NPH 1320.

● - control, × - 10 ppm, Δ - 50 ppm, ○ - 100 ppm,
▲ - 500 ppm.

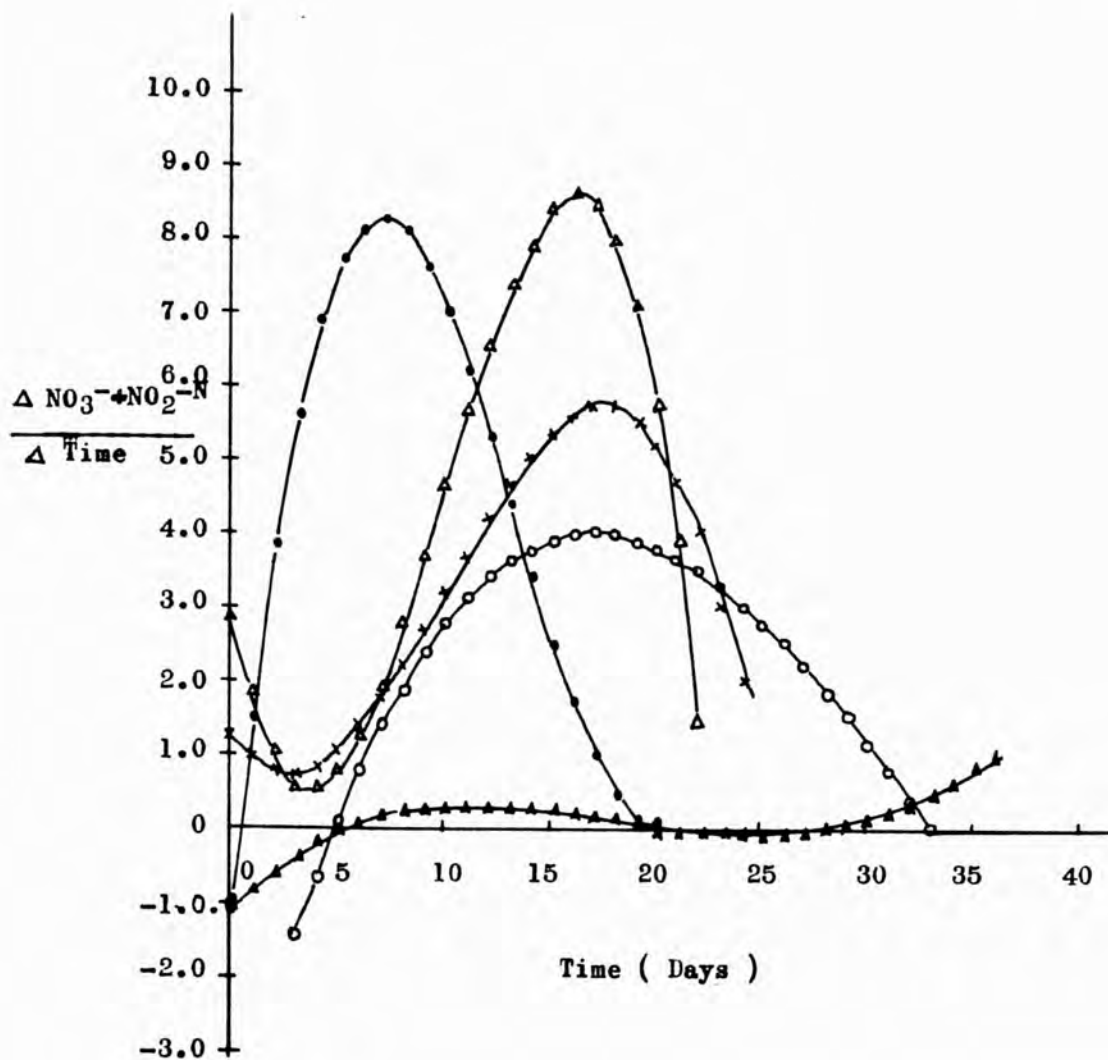


Fig. 13

Rates of formation of $\text{NO}_3^- + \text{NO}_2^- \text{ N}$ in fresh soil perfused with Ammonium sulphate solutions containing NPH 1320.

- - control, × - 10 ppm, Δ - 50ppm, ○ - 100 ppm,
- ▲ - 500 ppm.

known concentrations of the herbicides, bearing in mind the conditions under which these rates could be taken as measures of the saturating population sizes of Nitrosomonas and Nitrobacter species (discussed earlier in this Chapter).

And 2. The time (T) taken for the 'rate' curves to reach maximum values. This can be taken as a measure of the time to saturation, on the assumption that when the rate curves reach their maximum heights the soil samples are saturated with the nitrifying organisms under the particular experimental conditions. Thus the value for $\frac{1}{T}$ would be an estimate of the rate of proliferation of the population concerned under the corresponding experimental conditions.

Oxygen uptake by enriched soils

The rates of oxygen uptake by enriched soils (perfused with $(\text{NH}_4)_2\text{SO}_4$ solution supplied during enrichment) with ammonium ions as respiratory substrate in the presence and in the absence of the added inhibitors were measured using the conventional Warburg technique. The experimental details relevant to these measurements would be discussed in Chapter 5. In these experiments the amounts of oxygen consumed by the soil samples suspended in buffer and respiring in the presence of the substrate with or without the added inhibitor in the reaction media were measured as a function of time in minutes, all the measurements being made in terms of the pressure changes in the closed arm of the manometers as described by Umbreit et al. (1949).

Quastel & Scholefield (1949) showed that the rate of oxygen uptake by an enriched soil sample in the presence of the substrate used in enriching the soil usually remain constant over long periods of time. Therefore it was possible in these respiration experiments too (just as in the case of the previous experiments where nitrification rates in enriched soils were studied) to perform a linear regression analysis

on the data showing the consumption of oxygen with time. The gradients of these fitted regression lines were taken as the values for the rates of oxygen uptake by the particular soil samples and these rates were in terms of μ litres of O_2 consumed / sample / min..

b) Theoretical implications of the perfusion technique

As was discussed before Lees & Quastel (1946a) have shown that a soil sample which has been enriched with nitrifying organisms by previous perfusion with ammonium salt solution behave essentially like an enzyme system with steady rates of metabolic activity. Thus if such a soil is treated with a known herbicide concentration the degree of inhibition of the rate of nitrification would represent the effect of that particular herbicide concentration on the metabolic activity per cell (P) of the nitrifying organisms. The only possible drawback in expressing these estimates in relation to the added herbicide concentrations is that the herbicide solution is bound to get diluted by the additional amount of moisture already present in enriched soil. Unfortunately there is no simple way of correctly accounting for the amounts of moisture present in these enriched soils or overcoming this discrepancy by other means.

On the other hand when a 'fresh' soil sample is perfused with an ammonium salt solution containing a known concentration of a herbicide, the situation becomes more complex in trying to evaluate the effect of this added herbicide on the growth rates of the nitrifying organisms. As mentioned before, the maximum rates of nitrification obtained in such experiments are generally taken as the estimates of the size of saturating population modified by the degree to which the rate of nitrification per cell is affected by the particular herbicide concentration. Thus granting no interfering factors, other than the effect of the herbicide on metabolic rate per cell, the maximum rate of nitrification (x) observed in fresh soil sample perfused with ammonium salt solution containing a particular herbi-

cide concentration should be equal to the value obtained by multiplying the maximum rate of nitrification (a) in the control fresh soil sample perfused with ammonium sulphate solution only, under identical experimental conditions, by the rate of nitrification (b) (expressed as percentage of the corresponding control value) in an enriched soil sample of similar size and treated with a similar concentration of the same herbicide. Therefore if $x = a \text{ times } b$, it can be assumed that the reduction in maximum rate of nitrification observed in the treated fresh soil is due to the effect of the herbicide on metabolic rate per cell and not due to any effects on the growth rates of these organisms. But if the value of x is less than the product of (a) and (b) then it would be clear that the herbicide concentration, to which the soil sample is subjected during the enrichment phase, undoubtedly affects the growth or proliferation of the nitrifying organisms concerned, thereby reducing the observed size of the saturating population of nitrifiers in a particular soil sample, carrying out nitrification under a specified set of experimental conditions; well below the theoretically expected value.

Still another possibility exists where the observed value of x could be greater than the product of (a) and (b). In such a situation it is difficult to say whether the added herbicide is inhibiting or stimulating the growth rates of nitrifiers in the treated soil sample because these observed high values could well be due to many factors such as

- a) possible break down of the herbicide forming less toxic products,
- b) elimination of other organisms competing for the base exchange sites in soil (nitrifiers could not grow in the absence of available base exchange sites)
- and c) a possible development of resistance in the nitrifying organisms concerned, as a result of being in constant contact with the herbicides during growth phase etc.

c) Presentation of results

Soil being an extremely complex system it is hard to imagine the possibility of getting two soil samples identical in all respects even if these samples are collected from exactly the same locality at the same time. This variability seems to be an inherent difficulty, in dealing with soils and faced by all the workers in this field. The soil used in the experiments described in this thesis were normally collected in bulk so as to be sufficient for a number of experiments and these large soil samples were stored in polythene bags after being air dried. At the time of starting an experiment all the soil samples (50 gm. each) needed for the series of perfusers were taken from the same stock. But the nitrifying capacity of these stock samples may or may not change on storage and therefore the results obtained from one set of experiments could not be confidently compared with the results from another set of experiments, for example using a different herbicide at equivalent concentrations but performed a week or two later. This difficulty could be overcome by having a control soil sample along with each of the series of treated samples and expressing the results in every case as the percentage of the control value. Such a presentation would also to some extent account for any changes in the rates of nitrification due to inherent variations that would be present in the different batches of soil samples which might have direct bearing on the degree of actual effects due to the added herbicide. Therefore all the results from the different parameters used namely:-

- 1) The maximum rates of nitrification attained in fresh soils;
- 2) The reciprocal value of the time to reach these maximum rates of nitrification in fresh soil (rates of saturation of the soil samples with nitrifying organisms while under the influence of different herbicide concentrations);
- 3) The rates of nitrification in enriched soils;

and

- 4) The rates of oxygen uptake in enriched soils,

were all expressed as percentage values of the corresponding controls in dealing with comparisons of the effects of different herbicides applied to soil at known concentrations.

As these experiments are carried out under ordinary laboratory conditions which usually show slight fluctuations in temperature there might be small variations in nitrification rates due to temperature effects and this type of variation would also be accounted for by presenting the results as percentage values of the corresponding controls.

Section B. Nitrification in Pure Cultures

1. Cultivation of Organisms

a) Nitrobacter Winogradski

A sample broth culture of Nitrobacter Winogradski was obtained from Rothamsted Experimental Station, Harpenden, Herts., and stock cultures of it were grown in liquid medium of the following composition:

NaNO ₂	2.0 g.
NaCl	0.5 g.
MgSO ₄	0.05 g.
K ₂ HPO ₄	0.05 - 0.1 g.
Ammonium molybdate	50 µg.
CaCO ₃	8 mg.
Fe(chelate) - a trace	1 mg.
Distilled water	1000 ml.

The pH of this medium was carefully adjusted to 8.6 with dilute potassium hydroxide before autoclaving so that the pH would have been fallen to about 7.8 after sterilization.

b) Nitrosomonas Europaea

A broth culture of this organism too was obtained from Rothamsted Experimental Station and was grown in a liquid medium made

up as follows (Skinner & Walker 1961):

$(\text{NH}_4)_2\text{SO}_4$	0.5 g.
KH_2PO_4	0.07 g.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.05 g.
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.05 g.
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.001 g.
Distilled water	1000 ml.

The pH of the medium was maintained at 8-8.4 using a sterile solution of 5% sodium carbonate. Phenol red was included in the medium to act as a pH indicator.

Both the cultures were incubated in the dark at 25°-30°C. Stock cultures as well as batch cultures were grown in conical flasks; the depth of the medium being maintained around 1 cm. so as to ensure adequate aeration.

2. Preparation of Cell Suspensions

Nitrobacter as well as Nitrosomonas cells used in all the pure culture experiments were obtained from respective cultures which have been incubated for approximately 2 weeks. During this period of growth the substrate ion concentrations in the cultures, namely the nitrite ion concentration in Nitrobacter cultures and the ammonium ion concentration in Nitrosomonas cultures, were maintained at sufficiently high levels by the addition of sterile solutions of sodium nitrite and ammonium sulphate respectively. Special care was also taken to maintain the pH of the Nitrosomonas cultures by the addition of sterile sodium carbonate solution.

Nitrobacter and Nitrosomonas cells were harvested separately from these rapidly growing cultures by spinning them down at 11,000 g. for half an hour using a high speed M.S.E. centrifuge the temperature of which was maintained at 10°C. The bacterial pellets were then washed once with sterile water at the centrifuge so as to free the cells from any

ammonium or nitrite ions and then resuspended in sterile phosphate buffer of pH 7.8. These cell suspensions were prepared fresh for every experiment since it was found that these nitrifying organisms could not survive even overnight under low temperature storage conditions in a refrigerator at 4°C.

3. Experimental Techniques

The different criteria used in the assessment of the effects of herbicides on the nitrifying organisms grown in pure cultures are as follows:

- a) The rates of oxygen consumption by Nitrosomonas and Nitrobacter measured by:
 - 1) using conventional Warburg respirometry, and
 - 2) polarographic measurement of oxygen tensions using oxygen electrode.
- b) The rates of nitrification by Nitrosomonas and Nitrobacter in separate short term experiments lasting only for a period less than two hours.
- c) The growth rates of the nitrifiers in pure cultures.

The experimental details pertaining to each of the above techniques will be discussed later in the relevant chapters.

4. Analysis of Data

a) Statistical analysis

(1) The rates of O₂ consumption

In the experiments carried out using the Warburg respirometers, the amount of oxygen taken up by the bacterial suspensions in each Warburg vessel, in the presence and in the absence of the added herbicide, were measured in terms of the pressure changes in the closed manometer arm (Umbreit et al. 1949). These measurements were taken at regular intervals of 10-15 minutes over a period of 1½ to 2 hours. Since the relationship

between the amount of oxygen consumed and time was found to be linear during the experimental period, it was possible to perform a linear regression analysis on the observed data showing the amount of oxygen consumed as a function of time. This same type of linearity was also shown by the oxygen consumption data of the Nitrosomonas as well as the Nitrobacter cell suspensions treated with all concentrations of different herbicides and therefore it was easy to obtain an accurate estimate of the rates of oxygen uptake, in the controls and in the treated cell suspensions, by calculating the gradients of the fitted regression lines.

Fig. 14 shows a family of regression lines for a series of samples of a cell suspension of Nitrosomonas Europaea respiring in the presence of a range of concentrations of Legurame and 10 mM. ammonium sulphate while Fig. 15 represents the corresponding family of regression lines for a series of samples of a cell suspension of Nitrobacter Winogradski respiring in the presence of Legurame and 5 mM. sodium nitrite solution.

(2) Short term Nitrification rates

2.a) Oxidation of NH_4^+ by Nitrosomonas Europaea

Here the amounts of nitrite formed in terms of $\mu\text{g NO}_2^- \text{-N}$ formed / reaction vessel / 90 minutes were measured in every series of reaction mixtures set up with Nitrosomonas cell suspensions under the influence of known concentrations of different herbicides. (The experimental details regarding the setting up of these reaction mixtures are as discussed in Chapter 6.) The nitrite -N concentrations thus measured can be directly plotted against logarithms of the herbicide concentrations so as to be presented in the form of a graph. Fig. 16 shows such a presentation of a set of data obtained from an experiment where samples of a Nitrosomonas cell suspension were treated with a range of concentrations of Legurame.

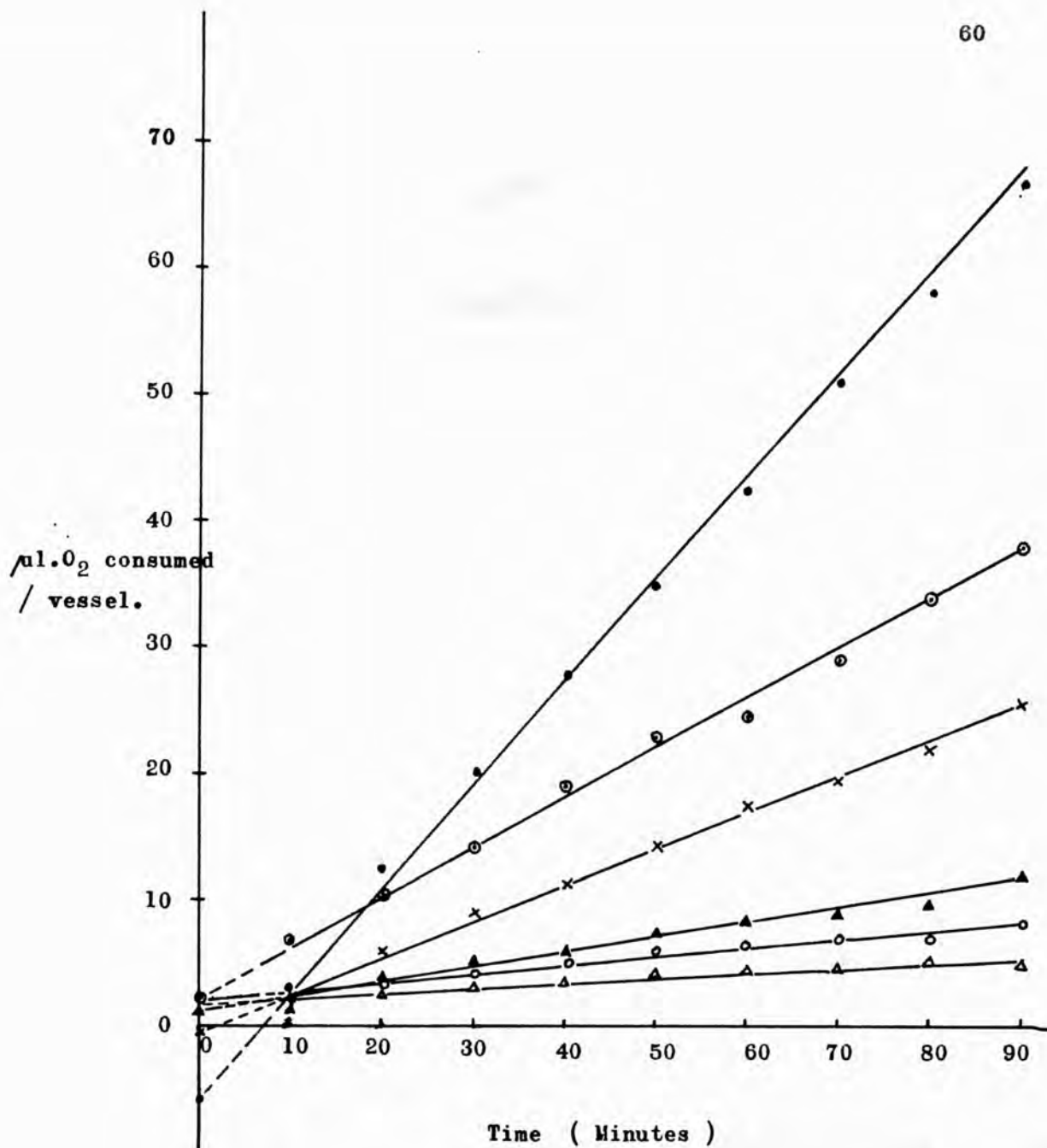


Fig. 14

Rates of Oxygen uptake ($\mu\text{l. O}_2/\text{vessel}/\text{min.}$) in washed cell suspensions of Nitrosomonas Europaeae supplied with Ammonium sulphate solutions containing a range of concentrations of Legurame.

● - control, ○ - 5 ppm, × - 10 ppm, ▲ - 50 ppm
 ○ - 100 ppm, △ - endogenous rate.

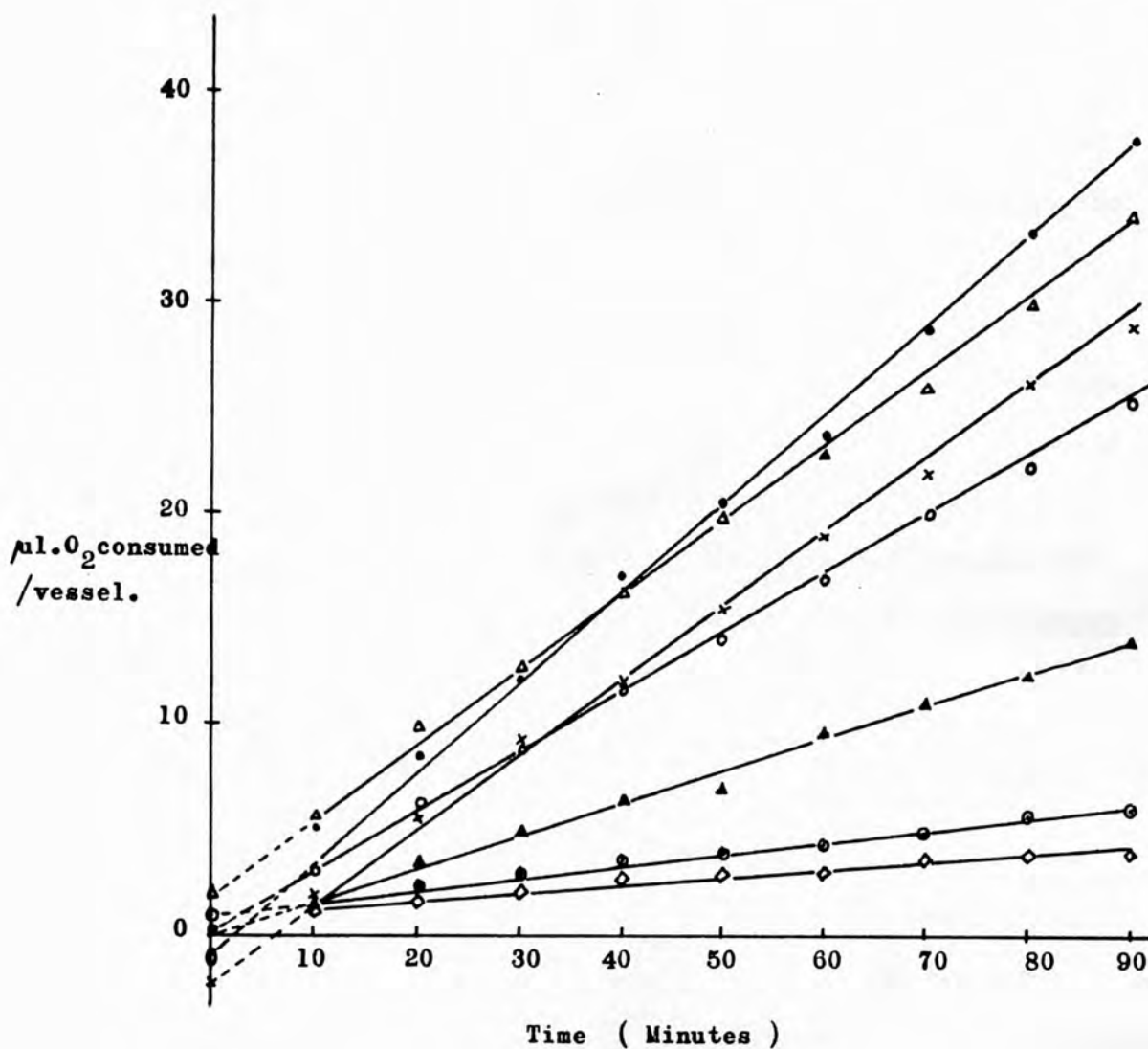


Fig. 15

Rates of Oxygen uptake ($\mu\text{l. O}_2/\text{vessel}/\text{min.}$) in washed cell suspensions of Nitrobacter Winogradski supplied with Sodium nitrite solutions containing a range of concentrations of Legurame.

● - control, Δ - 10 ppm, x - 50 ppm, ○ - 100 ppm,
 ▲ - 250 ppm, ⊙ - 500 ppm, ⊠ - endogenous rate.

2.b) Nitrite oxidation by Nitrobacter Winogradski

In the case of reaction mixtures set up with Nitrobacter Winogradski, the amounts of nitrite remaining in the media after the incubation period of 60 min. were estimated by the use of Griess Ilosvays technique. The amounts of nitrite thus estimated were subtracted from the original concentration of nitrite added into the reaction media, so as to get at the amount of nitrite that has been oxidised by the Nitrobacter cell suspensions in the presence and in the absence of the added herbicide. These results too could be presented in the form of a graph just as in the above case and Fig. 17 shows such a diagram presenting the amounts of nitrite oxidised by samples of a Nitrobacter cell suspension treated with the same range of concentrations of Legurame as used with the Nitrosomonas cell suspensions.

(3) Growth rates of Nitrifiers in liquid cultures

The time course of growth of Nitrosomonas and Nitrobacter cell suspensions were followed by making use of the amounts of NO_2^- - N formed or oxidised / ml. culture / day since these measures were taken as the biochemical indices of cell growth and metabolism of the two organisms respectively. (The reasons for using this parameter as an index of growth would be discussed later in Chapter 8, while the statistical analysis of the data would only be dealt with here.)

As early as 1918 Buchanan, summarising much of the previous work, illustrated the different phases of growth of a bacterial suspension by a sigmoid curve in which the logarithms of the numbers of living (viable) organisms are plotted against time. All the work up to now, too, has confirmed this sigmoid pattern followed by the bacteria during the growth cycle.

In the present experiments where the accumulation of nitrite -N with time was followed in growing Nitrosomonas cultures it was found that the course of nitrite accumulation took a sigmoid path starting from low

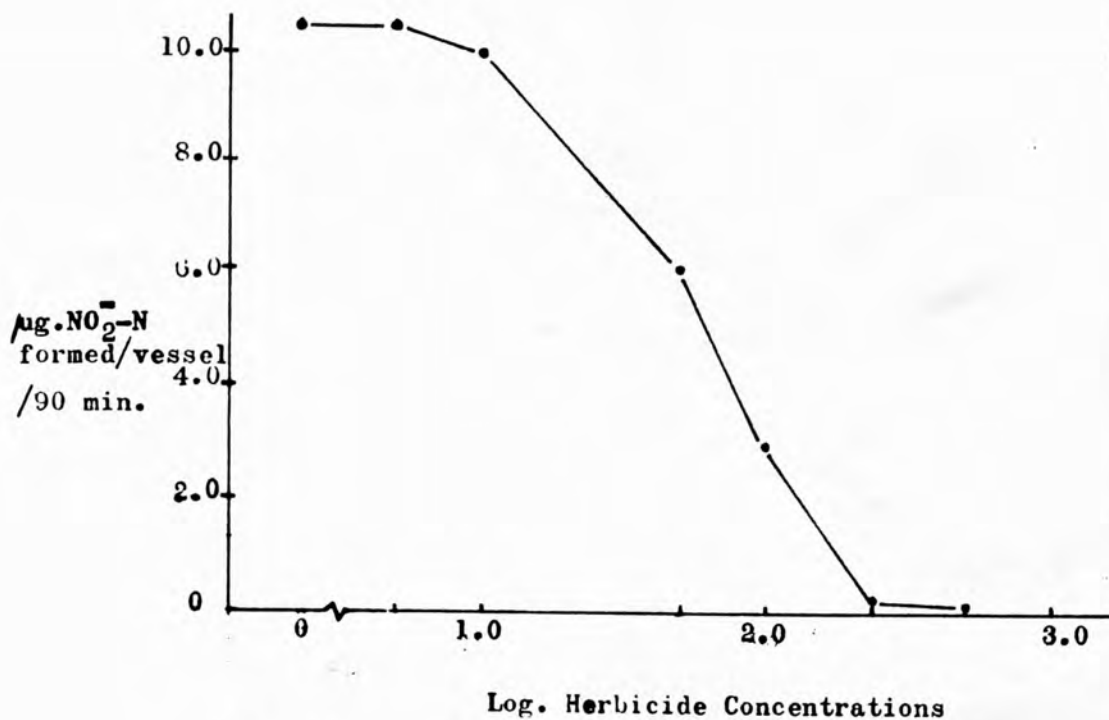


Fig. 16 Nitrite formations in washed cell suspensions of Nitrosomonas Europaea incubated for 90 min. at 25^oc in presence of a range of concentrations of Legurame.

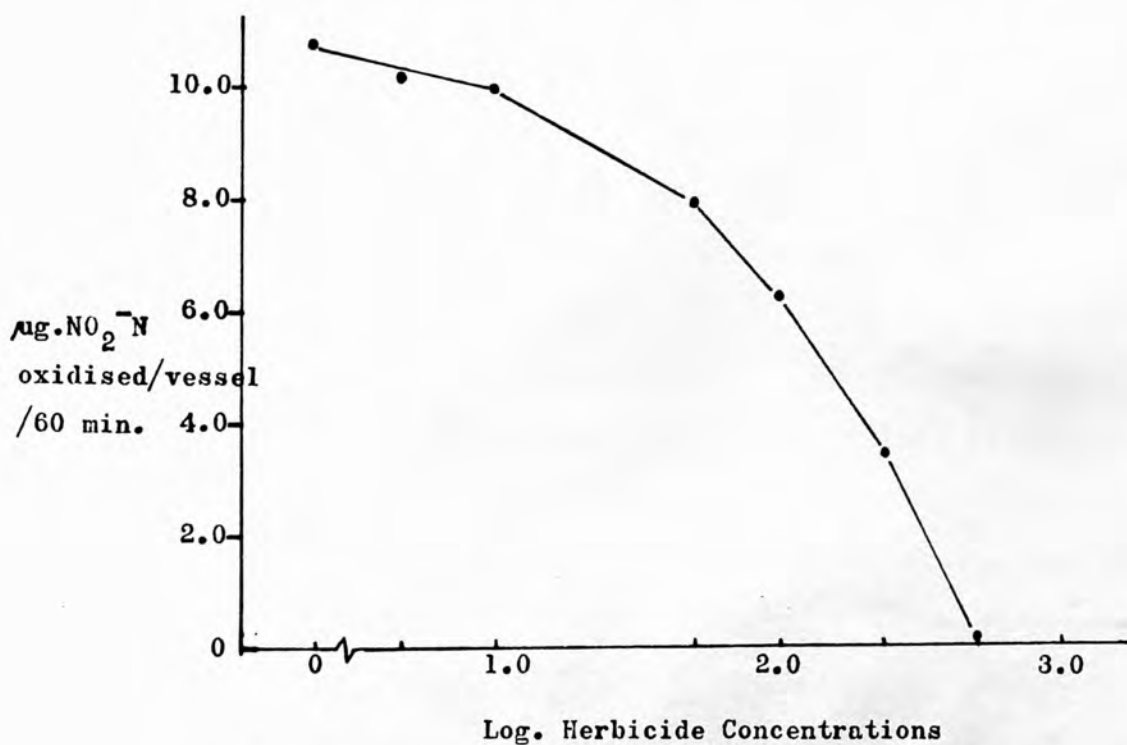


Fig. 17 Nitrite oxidations in washed cell suspensions of Nitrobacter Winogradski incubated for 60 min. at 25^oc in the presence of a range of concentrations of Legurame

values at the start of the experiments and then rising in a roughly exponential fashion. Figure 18 presents a family of such curves obtained with Nitrosomonas cell suspensions growing in culture media containing different known concentrations of Totril. This condition was found to be exactly similar to that occurred during nitrate accumulation in fresh soil samples perfused with ammonium sulphate solutions. Thus the nitrite accumulation data obtained from these pure culture growth experiments were also analysed by means of a polynomial curve fitting procedure followed by differential analysis and plotting $dNO_2^- - N$ formed / ml. culture medium / day against time in days. The principle behind this analysis is the same as described earlier in this chapter in dealing with the effects of herbicides on the growth of nitrifying populations in fresh soils perfused with ammonium sulphate solutions. Figure 19 presents the differential graphs corresponding to the growth curves shown in Figure 18.

In the case of the experiments carried out to assess the effects of herbicides on the growth of Nitrobacter Winogradski, the progress of growth was followed by estimating the residual $NO_2^- - N$ concentrations in the culture media, at one day intervals. A typical set of such data showing the time course of disappearance of $NO_2^- - N$ in a series of cultures of Nitrobacter Winogradski growing under controlled conditions and under the influence of known different concentrations of NPH 1320 are presented in Figure 20. Due to the sharp discontinuity of data setting in with substrate depletion (as seen from Fig. 20) a polynomial curve fitting procedure followed by differential analysis which was described in connection with the analysis of data of the previous experiments was judged to be inappropriate in this case, for the calculation of the rates of oxidation of $NO_2^- - N$ by growing cultures of Nitrobacter Winogradski. Therefore the rates of oxidation of $NO_2^- - N$ by these cultures had to be calculated from the direct experimental data as the difference between the observed residual concentrations of $NO_2^- - N$ corresponding to times t_1 and t_2 divided by the time interval, i.e. $t_2 - t_1$ (t_1 and t_2 being two consecutive times at which

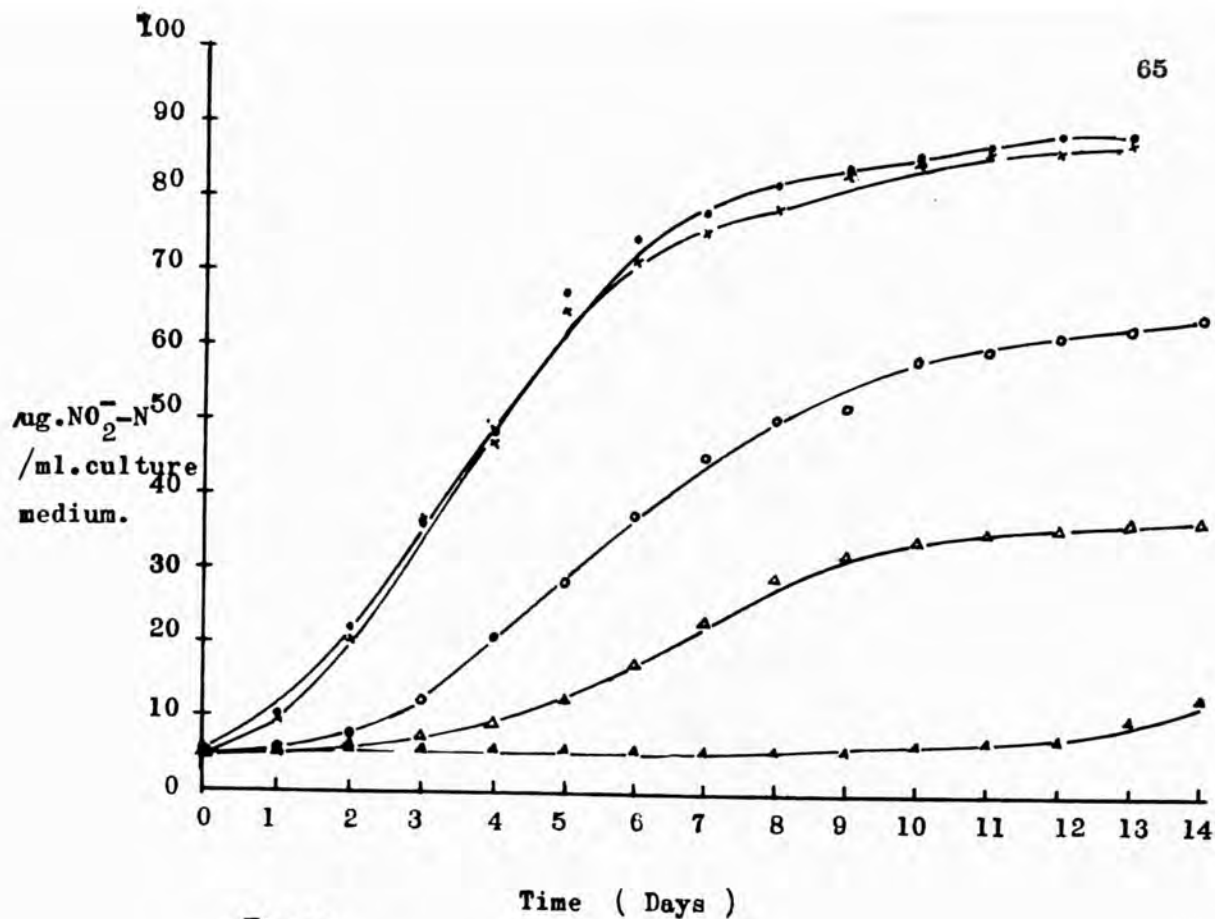


Fig.18

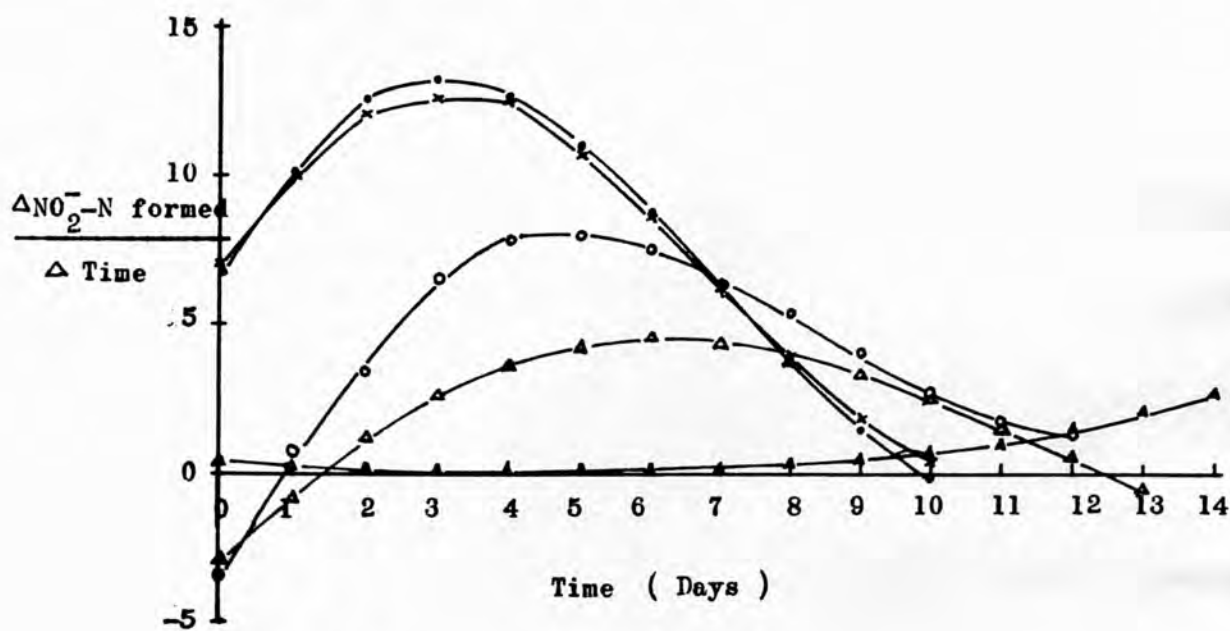


Fig. 19

Fig.18 Time course of accumulation of NO_2^- -N in growing cultures of Nitrosomonas Europaea incubated in the presence of different concentrations of Totril.

Fig.19 Differential curves showing the effect of Totril on the rates of formation of NO_2^- -N by Nitrosomonas.

• - control, x- 1 ppm, o- 10 ppm, Δ- 50 ppm, ▲- 100 ppm.

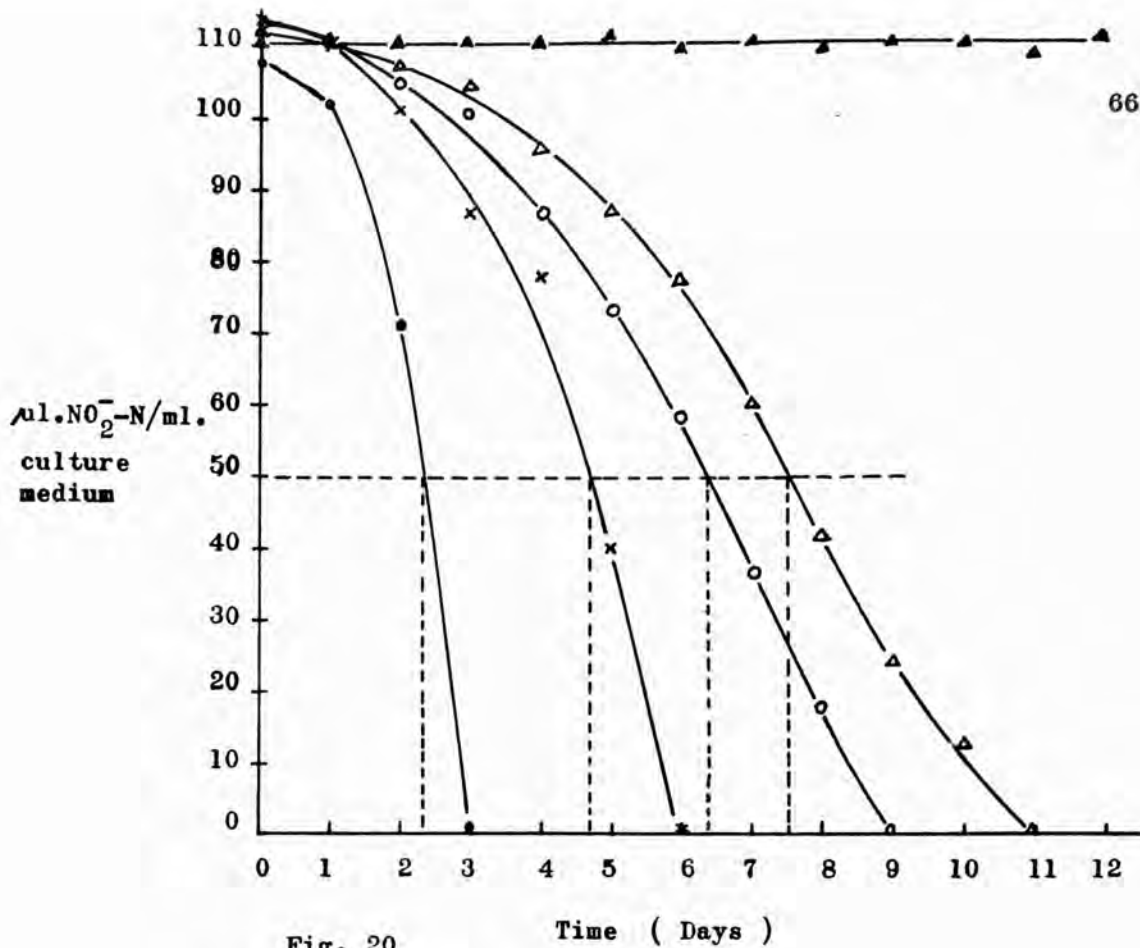


Fig. 20

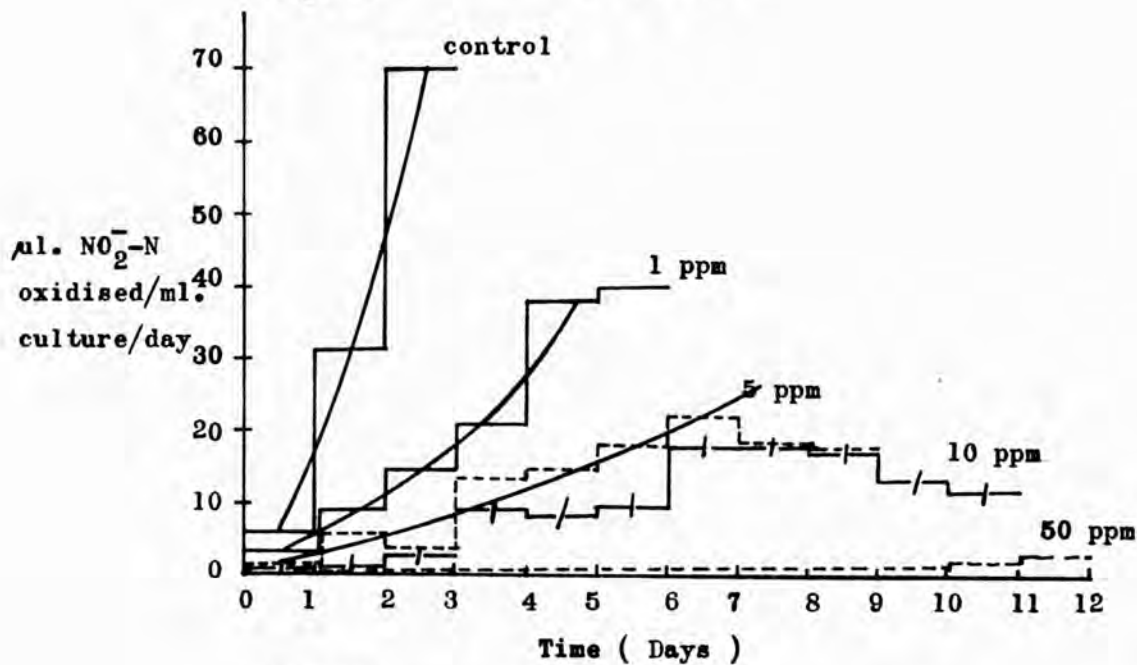


Fig. 21

Fig. 20. Time course of oxidation of NO_2^- -N in pure cultures of Nitrobacter Winogradski incubated in the presence of different concentrations of NPH1320

Fig. 21. Rate curves showing the effect of NPH 1320 on the maximum rates of oxidation of NO_2^- -N by Nitrobacter.

• - control, x - 1 ppm, o - 5 ppm, Δ - 10 ppm, ▲ - 50 ppm.

the NO_2^- - N estimations were made and $t_2 - t_1$ being always equal to unity in these experiments). A typical set of stepped rate curves presenting the variation of the rates of NO_2^- - N oxidation (calculated as above) by the cultures of Nitrobacter growing in media containing different concentrations of NPH 1320 are shown in Figure 21.

But it is evident from Figures 20 and 21 that, at least in the control culture and in the cultures treated with lower concentrations of NPH 1320 (e.g. 1 p.p.m.), the maximum rates of oxidation of NO_2^- - N were not reached because of the exhaustion of the substrate. Therefore the maximum rates of NO_2^- - N oxidation by cultures of Nitrobacter Winogradskii growing in liquid media containing different concentrations of NPH 1320, when expressed as percentage values of the observed maximum rate of oxidation of NO_2^- - N in the control culture would result in values much higher than what they ought to be. To overcome this discrepancy it was decided to consider the rates of oxidation of NO_2^- - N in each of the culture media while they still had an appreciable amount of nitrite remaining in them. This was done by first drawing a smooth curve passing through the mid points (or as close as possible to the mid points as shown in Fig. ~~21~~²¹) of the stepped rate curves and then reading off from these smooth curves, the rates of NO_2^- - N oxidation by the corresponding cultures at times when the media had a standard concentration of nitrite remaining in them. (In the case of the Nitrobacter cultures growing in media containing different concentrations of NPH 1320 - Fig. 20, the rates of NO_2^- - N oxidation were calculated at that time when the different media had 50 μg . of NO_2^- - N remaining per ml. culture.)

In these pure culture growth experiments too the rates of nitrification (i.e. the rates of formation of NO_2^- - N in Nitrosomonas cultures and the rates of oxidation of NO_2^- - N in Nitrobacter cultures) were taken as the measure of the sizes of the appropriate bacterial cell populations in liquid cultures, making the assumptions:

- a) that the rate of nitrification per cell remains constant during growth,
- and b) the nitrification rate per cell is unity and is not affected by the herbicides.

Thus the rates of nitrification (expressed as percentage values of the corresponding controls) in cultures growing under control conditions and under the influence of different herbicide concentrations were taken as the parameter indicating the effects of the range of herbicides tested, on the proliferation of Nitrosomonas and Nitrobacter cells in pure cultures. Bearing the above assumption (b) in mind it must be emphasized that these maximum rates of nitrification only represent values modified by the degree to which the nitrification rate per cell is affected by the particular herbicide just as in the case of the population growth measurements in fresh soil samples perfused with ammonium sulphate solutions.

b) Presentation of results

Batch cultures of Nitrosomonas Europaea and Nitrobacter Winogradski grown for the same length of time under exactly similar conditions were used in making the cell suspensions for all the above mentioned pure culture experiments. Also the bacterial cell pellets obtained by spinning down the contents of each of the culture flasks were always suspended in the same volume of the buffer solution even though an attempt was not made to make the dilutions so as to result in cell suspensions having equal optical densities or having similar values for mg. dry weight or fresh weight of cell material /ml. of the suspension. Therefore the results if presented as the amount of nitrite -N formed or oxidised / ml. cell suspension (i.e. $\mu\text{g NO}_2^- - \text{N} / \text{ml. cell suspension}$) was not thought to be meaningful because the cell densities in the different suspensions were not strictly comparable. However since there was a set of controls for every set of experiments it was decided to express the results (i.e. the rate of nitrite formation or rate of nitrite oxidation / reaction vessel ,

specific period of time) as percentage values of the corresponding control. By carrying out random repeat experiments it was found that the results expressed as percentage values of their controls were in fact reproducible within small limits of experimental errors.

Blackman (1952) and Sampford (1952) have both used the method of probit analysis for assessing relative toxicities of a variety of toxicants on the same organism or several organisms. They have reported that if the inhibitory effects of a particular toxicant on one or more biological systems are determined over a wide range of dosages and if the relation between the appropriate response and function of the dose or concentration of the toxicant generally follow a sigmoid pattern then the methods of probit analysis are suitable for precise estimations of the values of LD_{50} , that is the dosage that kills 50% of the population.

In most of the different types of experiments carried out with soil and pure cultures of nitrifying bacteria - Nitrosomonas and Nitrobacter, during the present investigation too it was found that the values representing the degrees of inhibition of different parameters expressed as percentage values of the corresponding controls followed a sigmoid pattern when plotted against the logarithms of the inhibitor concentrations used. In such cases a simple probit analysis of the inhibition data (expressed as % of control), calculated by the previously discussed statistical methods, was carried out to obtain the I_{50} values (concentrations of the herbicides causing 50% inhibition of the activities of nitrifiers) for different herbicides with respect to different parameters used. In few of the experiments (as would be seen from later diagrams) where the rate curves (in which different rates expressed as percentage values of corresponding controls are plotted against logarithms of the appropriate herbicide concentrations) followed almost straight lines and not sigmoid curves, the I_{50} concentrations were estimated by eye without carrying out any probit analysis.

The probit analysis was done by making use of the CDC 6600

computer library subroutine PROBT and the values for I_{50} concentrations thus calculated were used as the final estimates in assessing relative toxicities of a range of herbicides on soil nitrifiers grown in pure cultures and also on nitrification process in soil itself.

CHAPTER 3

Effects of Herbicides on Nitrification in Enriched Soil

Introduction

Lees & Quastel (1945) reporting the remarkable inhibitory effects of potassium chlorate on the biological conversion of ammonium to nitrate have shown that if a fresh soil with a relatively low population of nitrifying organisms was treated with ammonium sulphate and potassium chlorate, the oxidation of ammonium ions proceeded unhindered while the oxidation of the resulting nitrite was considerably delayed thereby causing appreciable accumulation of NO_2^- in the soil. The same authors then showed that soils previously enriched by perfusion with ammonium salts or nitrite were capable of oxidising nitrite almost as well in the presence of chlorate as in its absence.

Later Lees & Quastel (1946a) and Quastel & Scholefield (1951) confirming the inhibitory actions of urethanes on nitrification have in addition found that ammonium oxidation by Nitrosomonas growing in fresh soil is more sensitive to ethyl urethane poisoning than is ammonia oxidation by a Nitrosomonas population already established in an enriched soil.

Both these reports point to the fact that the growth of nitrifiers (Nitrosomonas spp. and Nitrobacter spp.) in a freshly perfused soil were more sensitive, to the toxic effects of the added inhibitor, than the oxidations of ammonium and nitrite ions by already established populations of Nitrobacter and Nitrosomonas in an enriched soil. Thus it was thought that an investigation of the effects of the herbicides, listed in table I, on the process of nitrification in enriched soil was rather appropriate, to establish whether these herbicides too show any differential effects on the oxidation of ammonium and nitrite ions as opposed to their effects on the growth of nitrifier populations in soil - which would be dealt with in chapter 4.

Table 1

Herbicides used in the Investigation

(P = pure compound; F = formulated products)

Common name		Chemical name	Source
Ioxynil	P	4-hydroxy 3,5-diiodobenzonitrile (sodium salt)	May & Baker
Totril (25% w/v. S _N 403)	F	Ioxynil octanoate	May & Baker
Bromoxynil (PAFD 574)	P	4-hydroxy 3,5-dibromobenzonitrile (potassium salt)	May & Baker
NPH 1320 (25% w/v. PAFD 1150)	F	Bromoxynil octanoate	May & Baker
Dicamba (Banavel D)	P	3,6-dichloro-2-methoxy benzoic acid	Vesicol Chemi- cal Corporation
Tricamba	P	3,5,6-Trichloro-2 methoxy benzoic acid	Vesicol Chemi- cal Corporation
Terbacil	P	5, chloro-6-methyl-3 butyl uracil	Du Pont
Trifluralin (44.5% w/v)	F	2,6, dinitro N, N dipropyl 4-trifluoromethyl aniline	Lilly Research Centre
Phenmedipham	P	3-(methoxy carbonyl amino) phenyl N- (3' methyl phenyl) carbamate	Schering AG Berlin
Chlorbufam	P	1 methyl prop-2 ynyl-N-(3-chloro- phenyl) carbamate	-
M&B 9057	P	Methyl-4-aminobenzene sulphonyl carbamate	May & Baker
M&B 9555	P	Methyl-4-methoxy carbonylamino benzene-sulphonyl carbamate	May & Baker
Oxadiazon (17,623 HP)	F	2-tertiary butyl-4-2'-4' dichloro- 5' isopropyloxy phenyl 1,3,4 oxadiazoline-5one	May & Baker
Legurame (Carbetamide)	F	N-phenyl-1(ethyl carbamoyl-1)- ethyl carbamate-D isomer	May & Baker

Since the time Lees & Quastel (1946) found that enriched soils behave essentially similar to an enzyme system, with no proliferation of the relevant organisms taking place except presumably to replace dead cells, there has been a vast amount of literature accumulated on the effects of different potential inhibitors, including many herbicides, on the nitrification process in enriched soil. But as previously mentioned in chapter 2, it has not been made clear from much of this work, whether the overall effects observed were due to any inhibition of the oxidation of ammonium ions mediated by Nitrosomonas spp. or else due to an inhibition of the oxidation of nitrite ions mediated by Nitrobacter spp.

The work reported here was thus carried out with the view to disclosing any differential effects the herbicides under investigation might have on the metabolic activities of Nitrosomonas and Nitrobacter populations in enriched soils.

Experimental procedure

Soil columns enriched with nitrifying organisms were prepared by continuous perfusion with ammonium sulphate solution ($4 \times 10^{-3}M$) using the soil perfusion apparatus described in chapter 2. The nitrate content of the perfusates were determined at appropriate intervals until the nitrate -N concentrations became steady, usually in about 12-18 days. The soil columns were then drained, washed with about 2 changes of distilled water to remove any nitrates and nitrites that might be held in the soil and then reperfused with fresh ammonium sulphate solutions. This process was repeated until constant maximum rates of nitrification were achieved after which the soil samples were considered to be saturated with nitrifying organisms and ready for use. In the preliminary experiments it was found that the soil samples used in the present investigation attained constant maximum rates of nitrification by the end of the second round of perfusion with fresh ammonium sulphate solutions. The soil

samples when saturated with nitrifying organisms were washed quickly by perfusion with several successive changes of distilled water until no nitrate-nitrogen could be detected in the washings.

A series of such soil columns each containing 50 gm. of enriched soil were prepared at the same time and perfused with 250 ml. of ammonium sulphate solution ($4 \times 10^{-3}M$) containing known different concentrations (0, 10, 50, 100, 500 and 1000 p.p.m.) of a particular herbicide. The progress of nitrification in these soil samples were then followed, over a period of at least two weeks, by daily analysis of 1 ml. aliquots of each of the perfusates separately, for their nitrite and nitrate-nitrogen contents, by using the standard techniques discussed earlier in chapter 2.

Results

In control soil samples, which were not under the influence of any herbicide, the linear phase of nitrification usually ended in 4-5 days by which time the nitrification process was almost complete and checked due to substrate depletion. As would be expected there was very little or no nitrite accumulation in the control soil samples while different degrees of nitrite accumulation over varying periods of time were detected in the perfusates of treated soil samples depending on the herbicide and its dosage.

The data showing the concentrations of nitrate-nitrogen and the concentrations of $(NO_3^- + NO_2^-) - N$ in the perfusates at regular intervals of time until the end of their linear phases of accumulation were then analysed separately by means of a simple regression analysis and the regression coefficients thus obtained were taken as the mean rates of nitrification due to the activities of the population of Nitrobacter spp. and Nitrosomonas spp. respectively in these enriched soil samples treated with different herbicide concentrations.

Tables 2 and 3 present the rates of formation of nitrate -N and $(NO_3^- + NO_2^-) - N$, respectively, by previously enriched soil samples on

reperfusion with ammonium sulphate solutions containing different herbicides at known concentrations. (In discussing the results of this chapter the term enriched soil will be used to denote soils that have been perfused with ammonium sulphate solutions during the enrichment phase.)

Figures 22 and 23 represent the variations of the rates of formation of nitrate -N, by the enriched soil samples treated with different herbicides (expressed as percentage values of the corresponding controls), with the logarithms of the herbicide concentrations used while figures 24 and 25 represent the variation of the rates of formation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ by the same soil samples (expressed as percentage values of the corresponding controls), with the logarithms of the appropriate herbicide concentrations.

Considering the trends of the rate curves presented in figures 22 and 23 it was evident that the rates of formation of nitrate -N (expressed as percentage values of the corresponding controls) in enriched soil samples treated with different herbicides tend to decrease with increase in the concentrations of the herbicides. Also the rate curves obtained with herbicides showing higher toxicities towards the activities of nitrifying organisms in enriched soil samples were seen to decline steeply within a narrow range of concentrations. For example when an enriched soil sample was perfused with an ammonium sulphate solution containing * Ioxynil at a concentration of 10 p.p.m., the rate of accumulation of nitrate -N (as % of control) was as high as 93% whereas the rates of formation of nitrate -N in similarly enriched soil samples perfused with ammonium sulphate solutions containing Ioxynil concentrations equivalent to 50 p.p.m., 100 p.p.m. and 500 p.p.m. were found to be only around 57%, 32% and 2% respectively (Fig. 22). From the curves presented

* Pesticides mentioned in the text are chemically identified in Table 1.

Table 2

Rates of NO_3^- - N formation in soils previously enriched with $(\text{NH}_4)_2\text{SO}_4$ and later reperfused with ammonium sulphate containing different herbicides listed at the concentrations indicated below.

(All rates calculated in terms of $\mu\text{g NO}_3^-$ - N formed/ml. perfusate/day.)

Herbicides		Herbicide concentrations in p.p.m. —→						
		Control	10	50	100	250	500	1000
1)	Ioxynil (P)	18.55	17.25	10.60	5.99	-	0.31	0.00
2)	Bromoxynil (P)	15.15	14.40	7.65	4.99	-	0.12	0.00
3)	NPH 1320 (F)	26.00	25.80	23.00	16.80	3.27	0.01	-
4)	Totril (F)	17.95	17.10	16.35	12.53	4.61	1.31	-
5)	Tricamba (P)	16.00	14.47	12.84	12.05	-	10.46	9.03
6)	Dicamba (P)	17.10	18.25	17.75	12.14	-	11.84	9.71
7)	Legurame (F)	17.35	16.25	13.79	10.39	-	5.47	0.67
8)	Oxadiazon (F)	22.85	22.55	18.30	16.95	-	9.64	3.43
9)	Trifluralin (F)	17.10	16.65	13.70	11.93	-	6.67	4.41
10)	9555 M & B (P)	14.29	15.91	13.79	13.58	-	13.51	13.86
11)	9057 M & B (P)	23.15	20.30	18.10	17.40	-	16.10	16.05
12)	Terbacil (P)	16.51	11.24	10.94	9.63	-	5.76	2.52

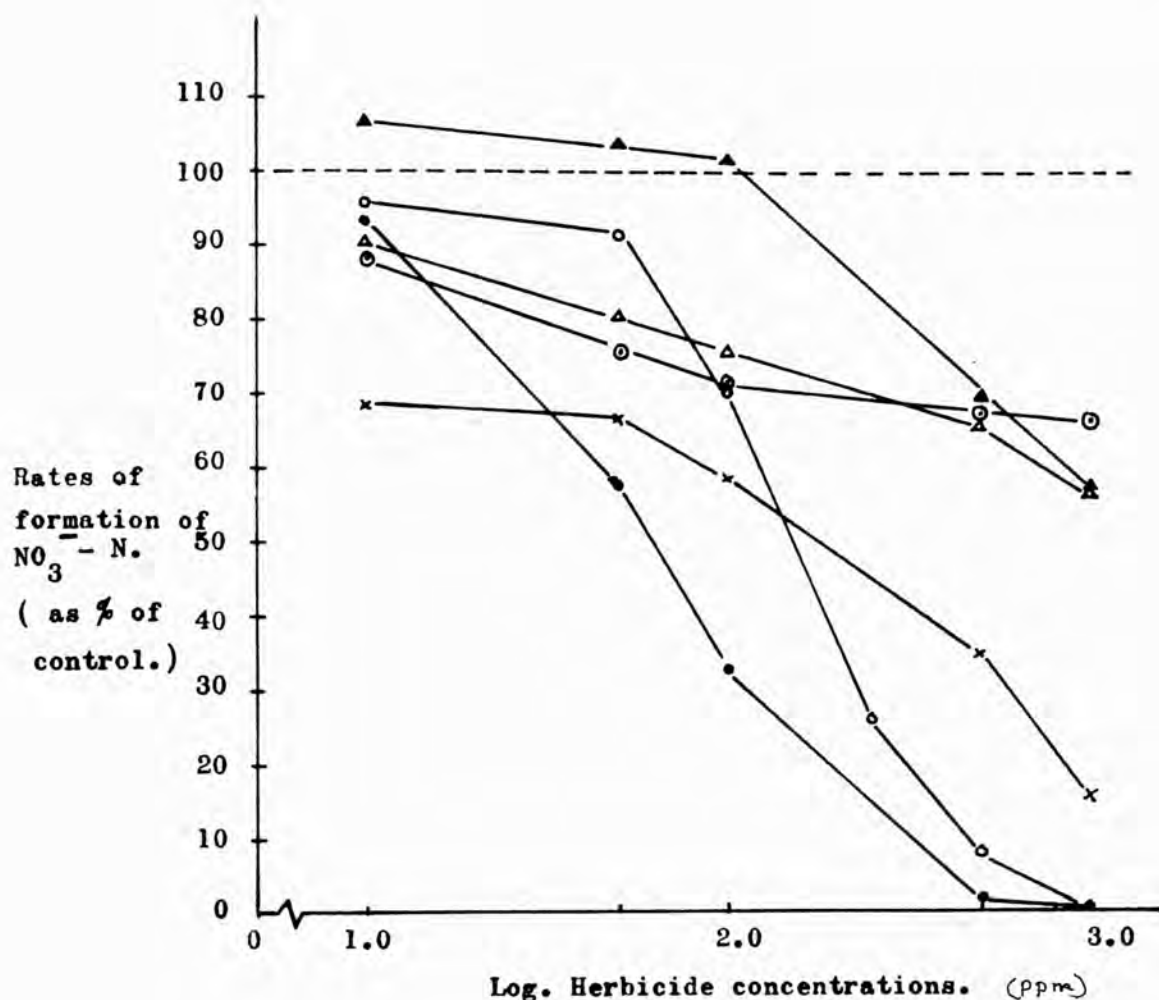


Fig. 22

Rates of accumulation of $\text{NO}_3^- \text{-N}$ ($\mu\text{g. NO}_3^- \text{-N}$ formed/ml. perfusate/day) when enriched soil samples were perfused with fresh Ammonium sulphate solutions containing different herbicides at known concentrations. (All rates expressed as percentage values of the corresponding controls.)

• - Ioxynil, ○ - Totril, x - Terbacil, Δ - Tricamba, ▲ - Dicamba, ⊙ - M & B 9057.

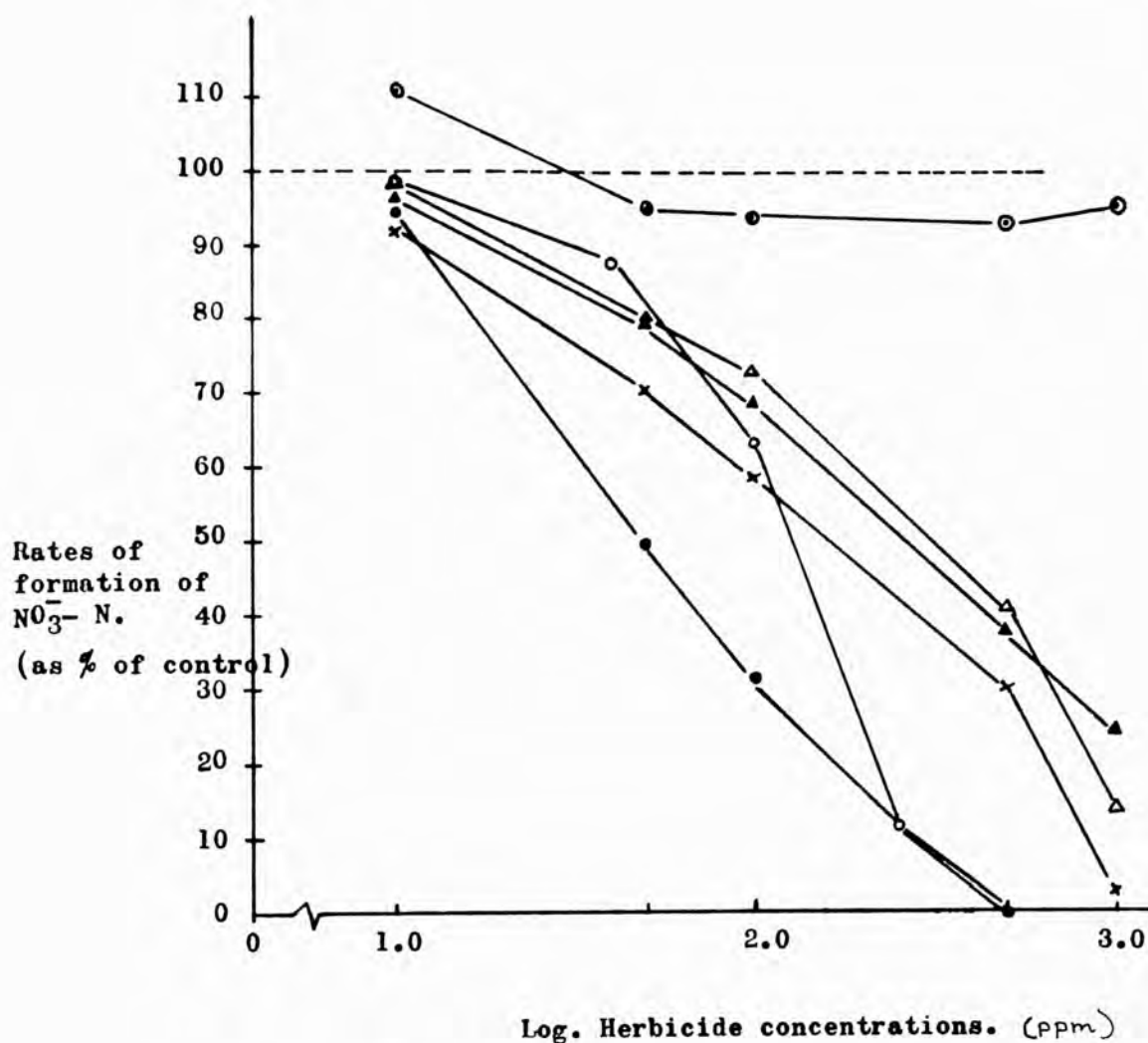


Fig. 23

The rates of accumulation of $\text{NO}_3^- \text{N}$ ($\mu\text{g. NO}_3^- \text{N}$ formed/ml.perfusate/day.) when enriched soil samples were perfused with fresh Ammoniumsulphate solutions containing different herbicides at known concentrations. (All rates expressed as percentage values of the corresponding controls.)

- - Bromoxynil, ○ - NPH 1320, x - Legurame,
- ▲ - Trifluralin, △ - Oxadiazon, ⊙ - M&B 9555.

in figures 22 and 23 and also from the herbicide concentrations effective in causing 50% inhibition (I_{50}) of the rates of formation of nitrate nitrogen presented in Table 4(a) it is clear that the three herbicides Totril, Bromoxynil and NPH 1320 too are highly effective as regards their effects on nitrate accumulation in enriched soils perfused with ammonium sulphate solutions, the I_{50} concentrations being as low as $2.96 \times 10^{-4}M$ (147 p.p.m.), $2.1 \times 10^{-4}M$ (60 p.p.m.) and $2.9 \times 10^{-4}M$ (90 p.p.m.) respectively. (The I_{50} concentrations for Ioxynil and Bromoxynil were estimated by eye whereas those for Totril and NPH 1320 were estimated by probit analysis.)

On the other hand when enriched soil samples were perfused with different concentrations of the herbicides Oxadiazon, Legurame, Trifluralin and Terbacil, the variation of the rates of formation of nitrate-nitrogen (expressed as % of controls) with the logarithms of the herbicide concentrations were found to decline rather slowly over a wide concentration range (10-1000 p.p.m.) as seen from the appropriate rate curves presented in Figures 22 and 23. These herbicides also showed higher I_{50} concentrations (estimated by probit analysis) as compared with the previously mentioned herbicides and were in the respective order of $7.68 \times 10^{-4}M$ (265 p.p.m.), $6.14 \times 10^{-4}M$ (145 p.p.m.), $8.6 \times 10^{-4}M$ (290 p.p.m.) and $5.3 \times 10^{-4}M$ (115 p.p.m.).

In the case of the remaining herbicides Dicamba, Tricamba, M & B 9057 (Asulam) and M & B 9555 the concentrations effective in causing 50% inhibition of the rate of formation of nitrate -N were found to exceed the maximum concentrations (1000 p.p.m.) tested in these experiments. It is also evident from the rate curves shown in Fig. 22 that there was a slight stimulation of nitrate accumulation in enriched soil samples treated with low concentrations of Dicamba ranging up to 100 p.p.m. M & B 9057 too seems to stimulate slightly or exert no effect at all on the rate of formation of nitrate -N in enriched soils even at the highest concentrations tested.

Table 3

Rates of $\text{NO}_3^- + \text{NO}_2^- - \text{N}$ formation in soils previously enriched with $(\text{NH}_4)_2\text{SO}_4$ and later reperfused with ammonium sulphate containing different herbicides listed at concentrations indicated below.

(All rates calculated in terms of $\mu\text{g NO}_3^- + \text{NO}_2^- - \text{N} / \text{ml. perfusate/day.}$)

Herbicides		Herbicide concentrations in p.p.m. —————>						
		Control	10	50	100	250	500	1000
1) Ioxynil	(P)	18.08	17.53	14.23	9.62	-	2.47	0.09
2) Bromoxynil	(P)	14.78	12.81	13.73	9.62	-	2.41	-
3) NPH 1320	(F)	25.78	25.41	23.18	18.31	10.44	5.62	-
4) Totril	(F)	17.49	16.96	16.02	15.16	13.09	6.98	-
5) Tricamba	(P)	12.87	14.17	12.63	11.61	-	10.27	9.44
6) Dicamba	(P)	16.59	17.98	17.55	16.81	-	14.28	9.68
7) Legurame	(F)	18.23	16.19	13.59	11.58	-	6.39	0.76
8) Oxadiazon	(F)	22.76	22.37	18.00	16.83	-	9.27	3.29
9) Trifluralin	(F)	16.82	17.20	13.51	11.71	-	11.41	4.26
10) 9555 M & B	(P)	18.21	17.40	17.18	17.65	-	16.46	15.39
11) 9057 M & B	(P)	23.60	20.83	17.79	16.94	-	15.95	15.78
12) Terbacil	(P)	16.51	11.24	10.94	9.63	-	5.74	2.52

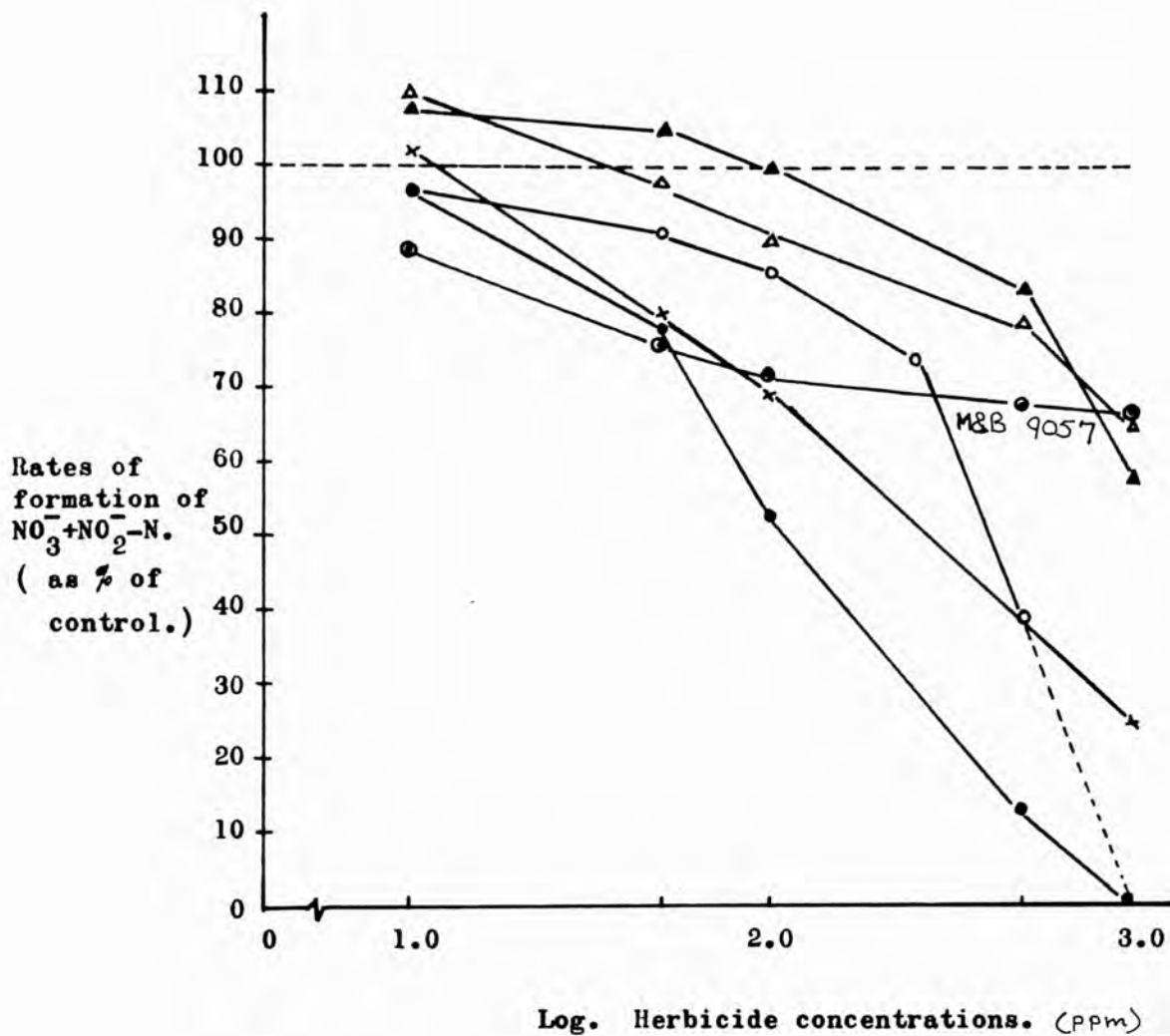


Fig. 24

The rates of accumulation of $\text{NO}_3^- + \text{NO}_2^- - \text{N}$ ($\mu\text{g. NO}_3^- + \text{NO}_2^- - \text{N}$ formed/ml.perfusate/day.) when enriched soil samples were perfused with fresh Ammonium sulphate solutions containing different herbicides at known concentrations. (All rates expressed as percentage values of the corresponding controls.)

● - Ioxynil, ○ - Totril, × - Trifluralin,
 ▲ - Tricamba, △ - Dicamba, ⊙ - M & B 9057.

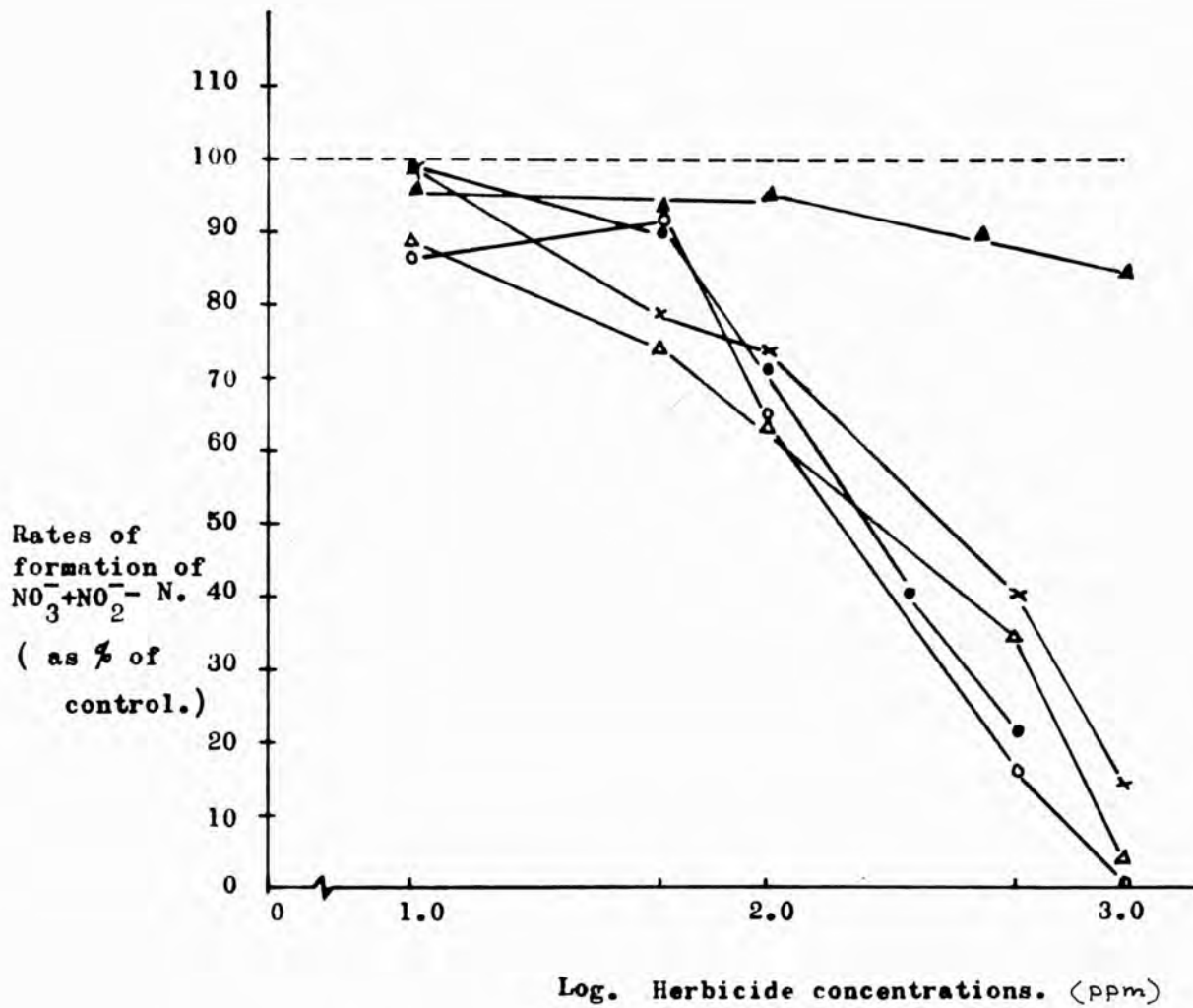


Fig. 25

The rates of accumulation of $\text{NO}_3^- + \text{NO}_2^- \text{ N}$ ($\mu\text{g. NO}_3^- + \text{NO}_2^- \text{ N formed/ml.perfusate/day.}$) when enriched soil samples were perfused with fresh Ammonium sulphate solutions containing different herbicides at known concentrations. (All rates expressed as percentage values of the corresponding controls.)

o - Bromoxynil, • - NPH 1320, x - Oxadiazon,
Δ - Legurame, ▲ - M & B 9555.

The normal trends of the curves shown in Figures 24 and 25 representing the variations in the rates of accumulation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ (expressed as percentage values of the corresponding controls) with logarithms of the herbicide concentrations too were essentially similar to those discussed above, the only difference being in the relatively high values for the rates of accumulation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ observed in the soil samples treated with some of the herbicide concentrations as compared with the rates of formation of $\text{NO}_3^- - \text{N}$ in these same soil samples. But in both the cases, i.e. in the case of the curves showing the variation of the rates of formation of nitrate $-\text{N}$ and the rates of formation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ with the logarithms of the different concentrations of M & B 9057 (Asulam) it was found that the curves levelled off beyond 100 p.p.m. concentration. However I am unable to give any satisfactory explanation for this rather peculiar observation.

Although the rates of accumulation of $\text{NO}_3^- - \text{N}$ in the perfusates of enriched soil samples percolated with fresh ammonium sulphate solutions containing different herbicide concentrations were found to be affected to varying degrees by these herbicides (results from figures 22 and 23) it is not possible to conclude, from these results only, the degree of inhibition of the metabolic activities of Nitrobacter populations due to the herbicidal effects. As discussed in Chapter 2, the amount of nitrite $-\text{N}$ accumulation observed in a particular perfusate too must be considered before one could deduce the rate of metabolic activities of Nitrobacter population in that soil. For example the Fig. 26 presents a family of curves showing the time course of accumulation of $\text{NO}_3^- - \text{N}$ and the corresponding changes of nitrite $-\text{N}$ concentrations in the perfusates of a series of enriched soil samples percolated with ammonium sulphate solutions containing a range of known concentrations of Ioxynil. The build up of nitrite $-\text{N}$ detected here indicates clearly that the Nitrobacter activity in these soil samples were inhibited to a greater extent, by Ioxynil, in

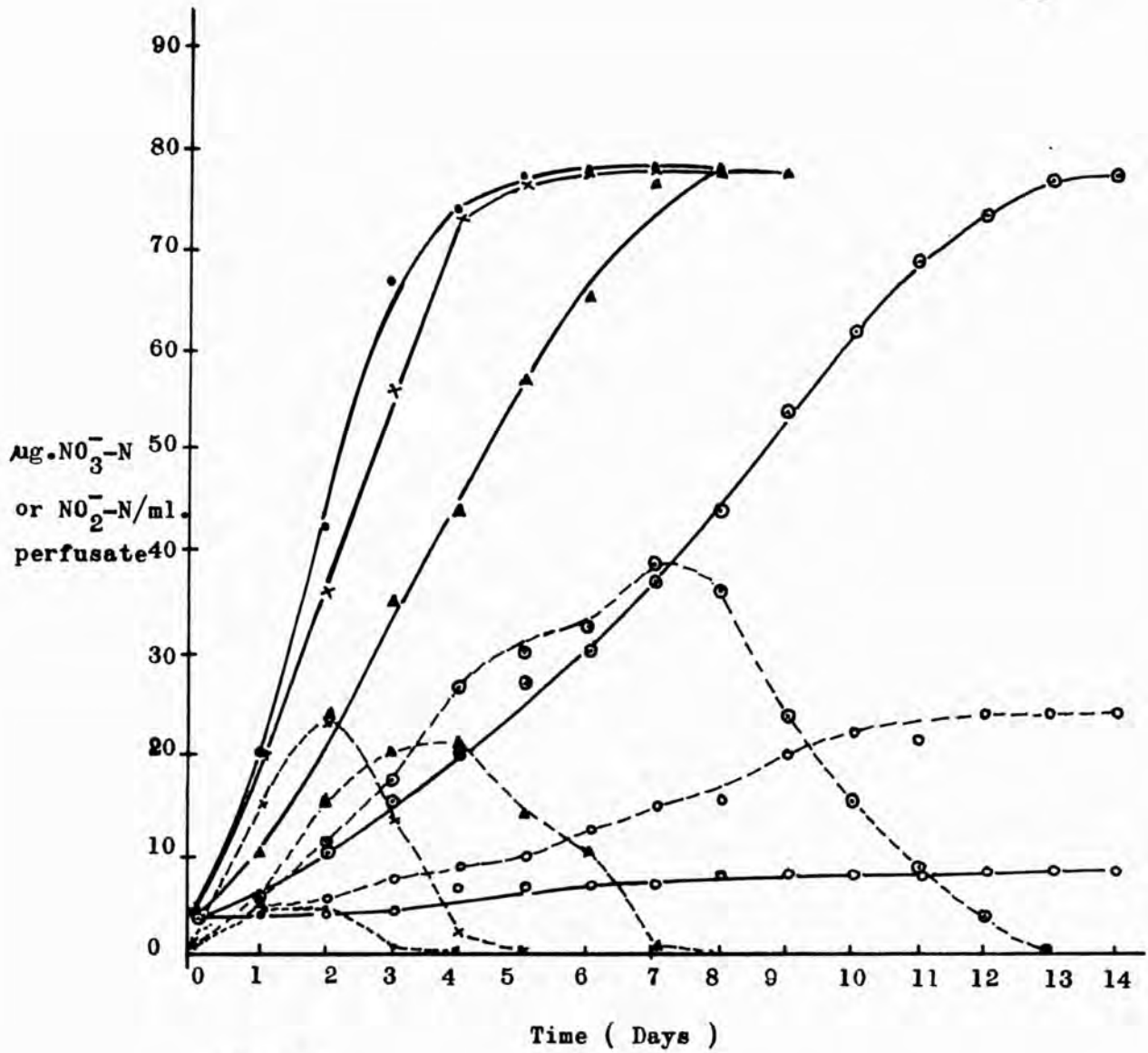


Fig. 26

Time course of accumulation of Nitrate- N (continuous lines) and Nitrite - N (broken lines) in a series of enriched soil samples perfused with Ammonium sulphate solutions containing a range of concentrations of Ioxynil

● - control, x - 10 ppm, ▲ - 50 ppm, ⊙ - 100 ppm,
○ - 500 ppm.

comparison to the extent to which the Nitrosomonas activity in the same soil samples were affected. In fact a fairly high concentration of nitrite -N (24 $\mu\text{g/ml}$ perfusate) was found to be accumulated (by the fourteenth day) in the perfusate of the enriched soil sample treated with 500 p.p.m. solution of Ioxynil at which concentration the accumulation of nitrate -N was almost completely stopped. From the higher value obtained for the concentration of Ioxynil effective in causing 50% inhibition of the rate of accumulation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ (table 4(b)) too it is clear that the oxidation of ammonium ions by Nitrosomonas populations in enriched soil treated with Ioxynil was less sensitive than the oxidation of nitrite ions by the Nitrobacter population of the same soil samples, the respective concentrations causing the 50% inhibition of the two stages of nitrification being $3.2 \times 10^{-4} \text{M}$ (117 p.p.m.) and $1.6 \times 10^{-4} \text{M}$ (60 p.p.m.).

But, enriched soil sample treated with some other herbicides for example Legurame, applied at concentrations ranging from 10 p.p.m. to 1000 p.p.m., did not show excessive nitrite -N accumulations in the perfusates as could be seen from the nitrite and nitrate accumulation curves presented in Fig. 27. The low nitrite accumulations are probably due to the activities of Nitrosomonas populations in these soil samples being more sensitive or equally sensitive to the toxic effects of Legurame, in comparison with the sensitivity of the Nitrobacter population activities in the same soils. As would be expected under these circumstances, the herbicide concentration effective in causing 50% inhibition of the rates of accumulation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ worked out to be almost the same as the concentration effective in causing 50% inhibition of the rates of formation of $\text{NO}_3^- - \text{N}$ (table 4). Thus it could be concluded that the rate of metabolic activities of Nitrosomonas populations is the factor limiting the nitrification rates in enriched soil samples perfused with ammonium sulphate solutions containing different concentrations of Legurame. In this case the variation in the rates of formation of $\text{NO}_3^- - \text{N}$ as well as

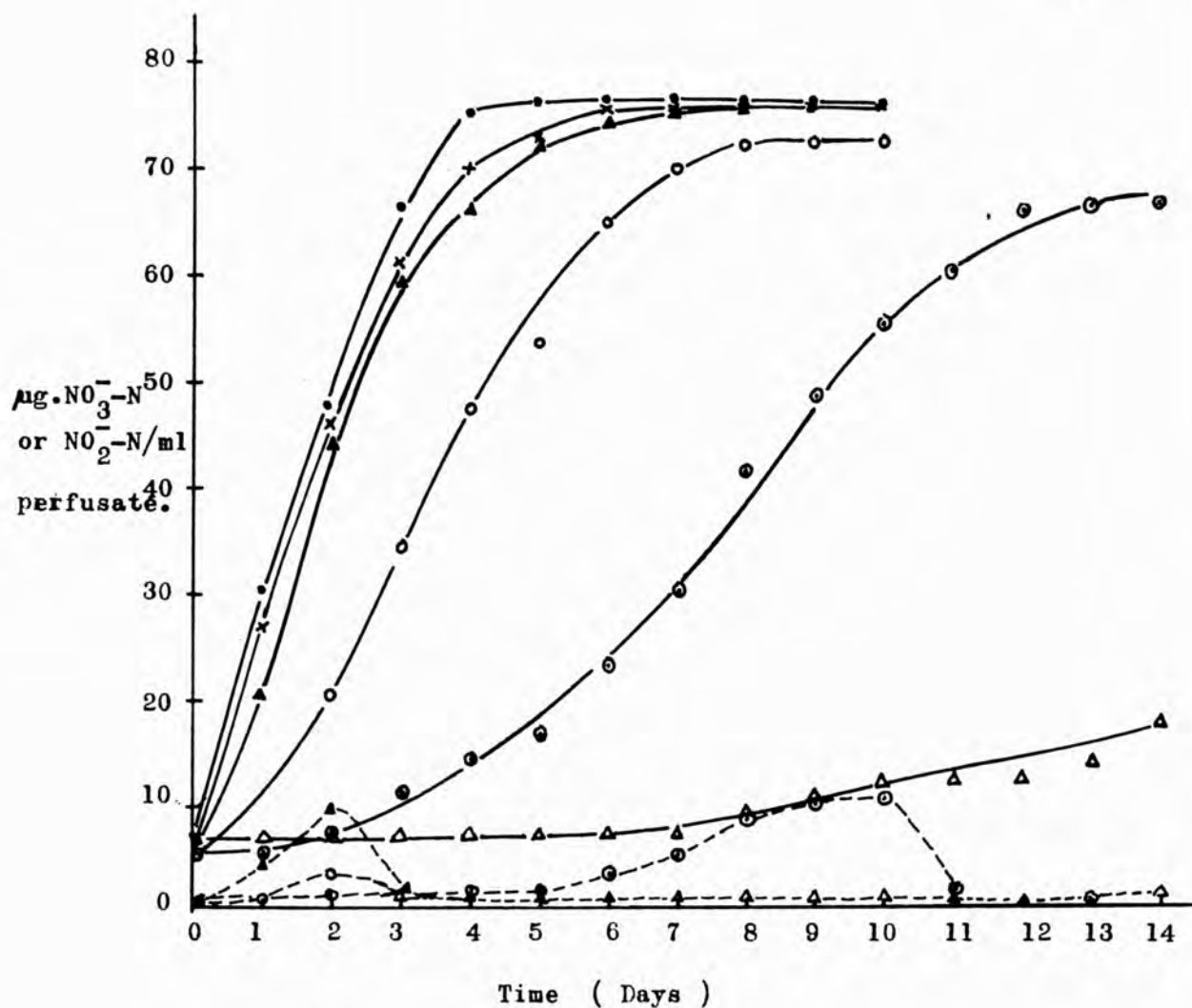


Fig. 27

Time course of accumulation of Nitrate- N (continuous lines) and Nitrite - N (broken lines) in series of enriched soil samples perfused with Ammonium sulphate solutions containing a range of concentrations of Legurame.

● - control, × - 10 ppm, ▲ - 50 ppm, ○ - 100 ppm,
⊙ - 500 ppm, △ - 1000 ppm.

the variation in the rates of accumulation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ with the different concentrations of Legurame (from Figs. 23 and 25) would only indicate the variation of the rates of metabolic activities of Nitrosomonas populations or the effects of this herbicide on the Nitrosomonas population activity in treated soil samples whereas the variations in the rates of formation of $\text{NO}_3^- - \text{N}$ and $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ in the soil samples treated with Ioxynil (previous case) would indicate the effects of known concentrations of Ioxynil on the respective activities of Nitrobacter and Nitrosomonas populations in these soils.

The above mentioned type of differential effects exerted by herbicides, on the activities of Nitrosomonas and Nitrobacter populations in enriched soil samples were also seen in the case of some of the other herbicides tested. For instance, it could be seen from figures 28 and 29 (i.e. the curves showing the maximum concentrations of $\text{NO}_2^- - \text{N}$ accumulated / mL perfusate when enriched soil samples were perfused with ammonium sulphate solutions containing different herbicides at various concentrations) that there were fairly high concentrations of $\text{NO}_2^- - \text{N}$ accumulated in the perfusates of the enriched soil samples supplied with ammonium sulphate solutions containing NPH 1320, Totril or Bromoxynil, whereas the concentrations of $\text{NO}_2^- - \text{N}$ did not build up to such high levels in the perfusates of similarly enriched soil samples percolated with the remaining herbicides under consideration namely Oxadiazon, Trifluralin, Terbacil, Tricamba, Dicamba, M & B 9057 (Asulam) and M & B 9555.

The higher concentrations of Bromoxynil, NPH 1320 and Totril (in addition to Ioxynil discussed before) effective in causing 50% inhibition (I_{50}) of the rates of accumulation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$, as compared with their concentrations effective in causing 50% inhibition of the rates of formation of $\text{NO}_3^- - \text{N}$ (table 4) in treated enriched soil samples were in accordance with the observed accumulations of high $\text{NO}_2^- - \text{N}$ concentrations and thus it could be concluded that the oxidation of ammonium ions by the

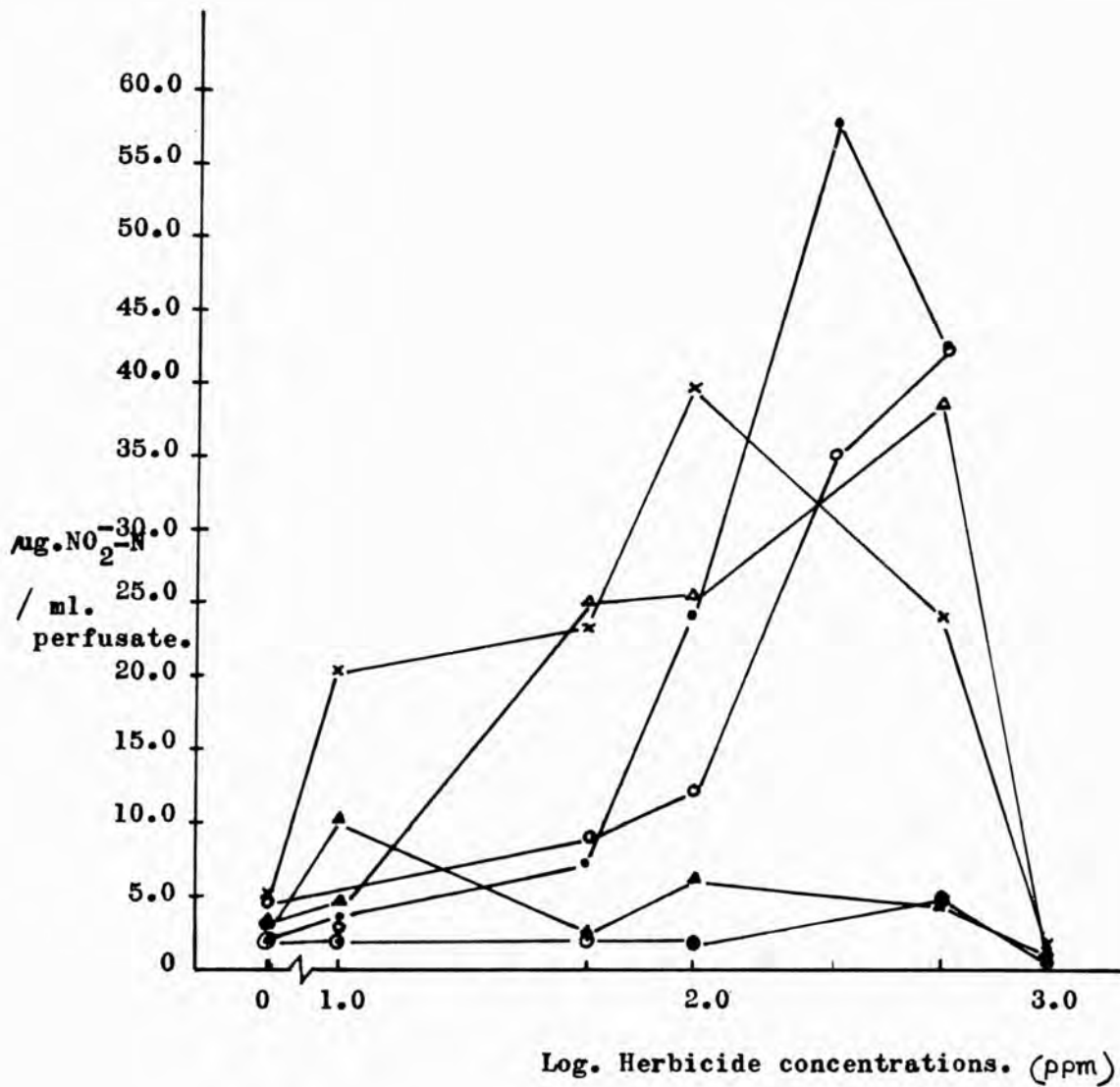


Fig. 28

The maximum concentrations of Nitrite- N accumulated in the perfusates when enriched soil samples were perfused with fresh Ammonium sulphate solutions containing different herbicides at known concentrations -S.

• - NPH 1320, ○ - Totril, x - Ioxynil,
 Δ - Bromoxynil, ▲ - Trifluralin, ⊙ - Dicamba.

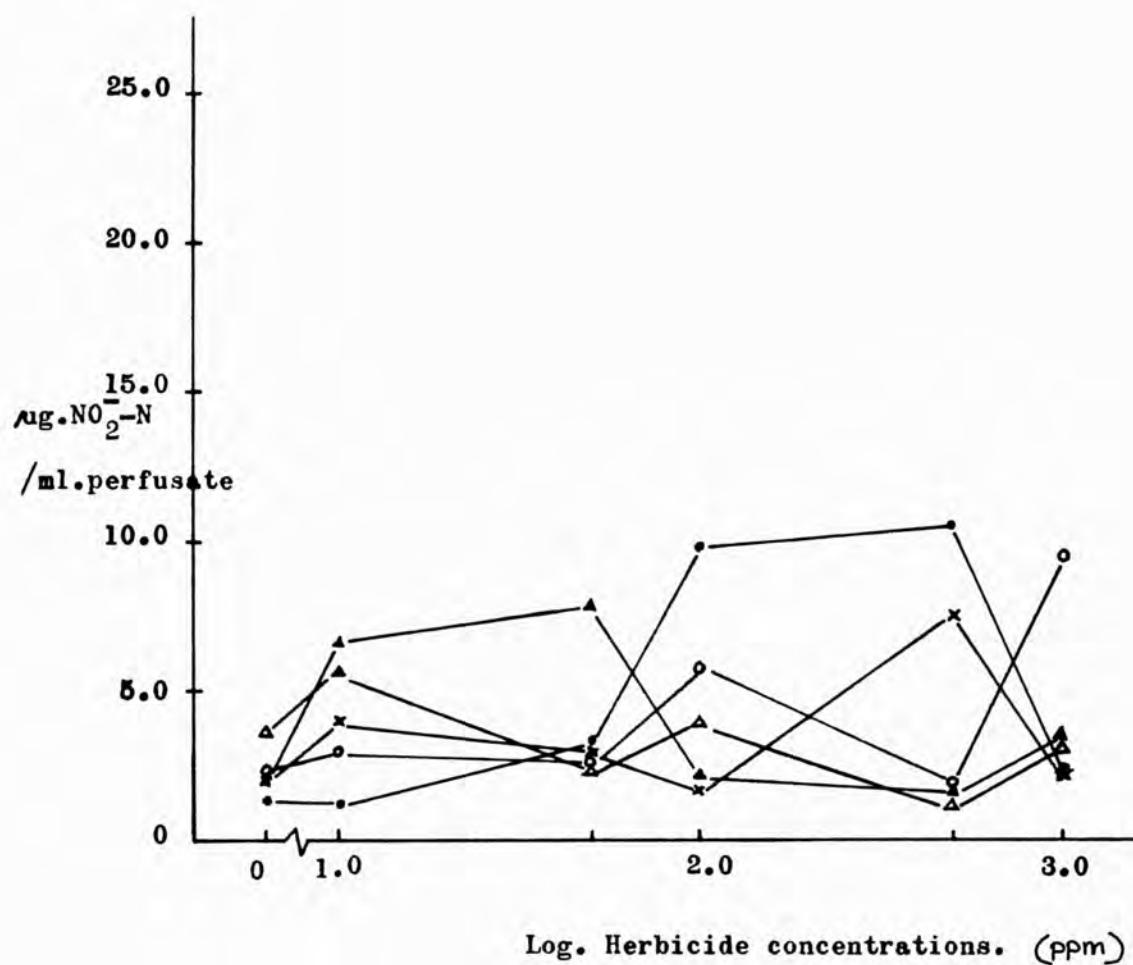


Fig. 29

The maximum concentrations of Nitrite - N accumulated in the perfusates when enriched soil samples were perfused with fresh Ammonium sulphate solutions containing different herbicides at known concentration

• - Legurame, x - Oxadiazon, ▲ - M & B 9057,
 △ - M & B 9555, o - Tricamba.

Table 4

The molar concentrations of different herbicides effective in causing 50% inhibition of the rates of formation of $\text{NO}_3^- - \text{N}$ and the rates of formation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ in enriched soil.

Herbicides	(a)	(b)
	Concentrations effective in causing 50% inhibition of rates of $\text{NO}_3^- - \text{N}$ formation	Concentrations effective in causing 50% inhibition of rates of formation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$
Ioxynil	1.6×10^{-4} M	3.2×10^{-4} M
Totril	2.96×10^{-4} M	10.0×10^{-4} M
Bromoxynil	2.1×10^{-4} M	5.0×10^{-4} M
NPH 1320	2.96×10^{-4} M	4.9×10^{-4} M
Legurame	6.1×10^{-4} M	6.6×10^{-4} M
Oxadiazon	7.7×10^{-4} M	7.1×10^{-4} M
Trifluralin	8.6×10^{-4} M	9.8×10^{-4} M
Terbacil	5.3×10^{-4} M	5.3×10^{-4} M
Tricamba	39.4×10^{-4} M *	39.4×10^{-4} M *
Dicamba	45.2×10^{-4} M *	45.2×10^{-4} M *
M & B 9555	43.9×10^{-4} M *	43.9×10^{-4} M *
M & B 9057	53.8×10^{-4} M *	53.8×10^{-4} M *

* These were the highest concentrations tested and equivalent to 1000 p.p.m.

saturation populations of Nitrosomonas (the first stage of the nitrification process) in enriched soils were much less sensitive to the toxic effects of Ioxynil, Bromoxynil, NPH 1320 and Totril when compared with the sensitivity of the Nitrobacter populations responsible for nitrite ion oxidations in the same soil samples.

From the above discussion it follows that the rates of formation of nitrate -N observed in enriched soils treated with Ioxynil, Bromoxynil, NPH 1320 and Totril (from table 2) would indicate the rates of metabolic activities of the Nitrobacter populations in these soils and therefore the degree of inhibition of each of these rates with respect to the rates of formation of nitrate -N in the corresponding controls would give an estimate of the toxic effects exerted by these herbicides on the Nitrobacter population activity. Similarly the rates of formation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ observed in the same soil samples (from table 3, and expressed as % of control values) would give an estimate of the toxic effects of Ioxynil, Bromoxynil, NPH 1320 and Totril on the metabolic activities of Nitrosomonas populations in enriched soil.

On the other hand the concentrations of the herbicides Legurame, Trifluralin, Oxadiazon and Terbacil effective in causing 50% inhibition of the rates of accumulation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ were found to be almost the same as the concentrations of these herbicides effective in causing 50% inhibition of the rates of formation of $\text{NO}_3^- - \text{N}$ (table 4) and this finding along with the observed low concentrations of $\text{NO}_2^- - \text{N}$ (Figures 28 and 29) accumulated in the perfusates of the soil samples treated with these herbicides would indicate either, that the specific metabolic activities of both nitrifying populations - Nitrosomonas spp. and Nitrobacter spp., are more or less equally sensitive or the metabolic activities of Nitrosomonas species are slightly more sensitive (i.e. the first stage of nitrification involving the oxidation of ammonium ions becoming the rate determining step) to Legurame, Oxadiazon and Terbacil.

Also the rate of formation of $\text{NO}_3^- - \text{N}$ and the rates of formation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ in enriched soils treated with these herbicides (from tables 2 and 3) when expressed as percentage values of the corresponding controls would only indicate the extent of the toxic effects on the activities of Nitrosomonas populations without giving any estimate regarding their effects on the Nitrobacter population activities.

The I_{50} concentrations of the remaining four herbicides Tricamba, Dicamba, M & B 9057 (Asulam) and M & B 9555 with respect to the activities of both the nitrifying populations in enriched soils were found to be beyond the maximum concentrations tested (1000 p.p.m.) and the concentrations of $\text{NO}_2^- - \text{N}$ observed in the perfusates too were extremely low (Figs. 28 and 29). Thus it could be concluded that the rates of nitrification in enriched soils treated with these herbicides, of very low toxicities, too are limited by the activities of Nitrosomonas populations.

CHAPTER 4The Effect of Several Herbicides on the Growth
of Nitrifying Bacteria in SoilIntroduction

The fact that nitrification in soil is essential to maintain the fertility of agricultural soils has drawn the attention of many scientists to explore any detrimental effects on the growth of nitrifying organisms, that might result from the widespread use of weed killers in maintaining weed free crops. This type of investigation has become essential since the action of pesticidal chemicals is not always limited to the immediate objective of killing a particular pest. Harmless or beneficial soil organisms too may be killed or temporarily reduced in numbers and also the qualitative nature of the soil population may be altered for varying periods of time as a result of herbicide actions.

Lees & Quastel (1946), Quastel & Scholefield (1949) and Lees (1954) have reported that very low concentrations of potassium chlorate prevented proliferation of Nitrobacter while Nitrosomonas population growth was unchecked. Smith, Dawson & Wenzel (1946) found that strains of Nitrosomonas were more sensitive to very high concentrations of 2,4-D than were the Nitrobacter spp. However they found that both groups recovered in 10-40 days following applications of 200 lb/acre of 2,4-D. Later several studies on phenoxy herbicides reviewed by Audus (1964) have shown that at normal rates of application the maximum effects are very small and transitory. Recently Debona & Audus (1970) have shown that a range of herbicides including Propanil, Ioxynil and Chloroflurazole etc. exert marked toxic effects on the nitrifying capacity of fresh soil samples supplied with an ammonium salt solution under laboratory conditions.

As was discussed earlier the aim of the present investigation

is to test a number of newer herbicides (which has not been tested so far) for their effects on the population growth of nitrifiers and at the same time to establish whether any of these herbicides have differential effects as regards the population growth of the two main types of nitrifying organisms namely Nitrosomonas spp. and Nitrobacter spp.

Herbicides used

All the herbicides used in the present investigation, their purity and sources are given in table 1.

Experimental procedure

The basic technique made use of in investigating the effects of herbicides, under consideration, on the population growth of nitrifiers in fresh soil involved the perfusion apparatus which consisted of a series of identical perfuser units as described in Chapter 2. 50 gm. samples of air dried garden soil (2-4 mm. fraction) were placed in each of these perfuser units with 250 ml. of ammonium sulphate solution ($4 \times 10^{-3}M$) in the reservoir. The ammonium Sulphate solution was allowed to percolate through the soil, the continuous stream of the perfusate dripping on to the soil column being maintained by the pump. The soil columns were kept aerated by passage of water saturated air, from an external source, at a steady rate. All the experiments were carried out at room temperature and in the dark.

The herbicides under investigation were added directly into the percolating fluid at desired concentrations of the active ingredients (0, 10, 50, 100, 500 and 1000 p.p.m.) and 2 ml. aliquots of the perfusates were removed daily from the reservoirs for analysis. One millilitre portion of each of the samples were analysed for their nitrite-nitrogen contents while the remaining 1 ml. aliquots were analysed for their nitrate-nitrogen contents. The methods of analysis for NO_2^- and NO_3^- nitrogen in the perfusates were as described in Chapter 2.

Results

Polynomial regression analysis of the nitrate-nitrogen and $(\text{NO}_2^- + \text{NO}_3^-)$ -nitrogen accumulation data were carried out separately, as was discussed in Chapter 2, for each series of experiments performed with every one of the herbicides under investigation. The maximum rates of formation of nitrate-nitrogen and the maximum rates of accumulation of $(\text{NO}_3^- + \text{NO}_2^-)$ -nitrogen were then obtained from the corresponding differential curves, and these values for the respective maximum rates are presented in tables 5 and 6. Figures 30 and 31 represent graphically the variation of the maximum rates of formation of nitrate nitrogen (expressed as percentage values of the corresponding controls) with the logarithms of the respective herbicide concentrations while figures 32 and 33 represent the variation of the maximum rates of accumulation of $(\text{NO}_3^- + \text{NO}_2^-)$ -nitrogen in the same soil samples (also expressed as percentage values of the corresponding controls) when plotted similarly against the logarithms of the different herbicide concentrations used.

From the inhibition curves presented in Figures 30 and 31 it is clear that most of the herbicides at the lowest concentrations tested (i.e. 10 p.p.m.) caused only slight inhibitions of rates of formation of $\text{NO}_3^- - \text{N}$, in fresh soil, as compared with the inhibitions caused by the higher concentrations (500 and 1000 p.p.m.), where the rates of formation of nitrate nitrogen were found to be almost insignificant. However with more toxic herbicides such as Ioxynil, Bromoxynil, Totril and NPH 1320 the rates of formation of nitrate -N in these freshly perfused soil samples were significantly inhibited even at the lowest concentration of 10 p.p.m., the observed maximum rates of formation of $\text{NO}_3^- - \text{N}$ being around 82%, 83%, 82% and 88% respectively.

Considering the nitrification rate curves for Tricamba and Dicamba (from Figs. 31 and 30) it can be seen that these two herbicides were effective in causing only relatively low degrees of inhibition in

Table 5

Maximum rates of NO_3^- - N formation attained in fresh soils perfused with 0.003 M $(\text{NH}_4)_2\text{SO}_4$ in the presence of the herbicides listed.

(All rates are in terms of $\mu\text{g NO}_3^-$ - N formed/ml. perfusate/day.)

		Herbicide concentrations in p.p.m. —→					
Herbicides		Control	10	50	100	500	1000
1) Ioxynil	(P)	14.63	11.96	9.14	7.30	0.85	0.02
2) Bromoxynil	(P)	10.96	9.15	8.52	7.43	4.38	0.12
3) NPH 1320	(F)	8.10	6.62	5.90	3.91	0.89	0.61
4) Totril	(F)	7.92	6.99	6.19	5.77	1.36	-
5) Legurame	(F)	18.12	17.08	13.70	12.88		
		14.62				6.05	3.25
6) Oxadiazon	(F)	12.51	10.90	10.56	9.81	8.51	6.75
7) Trifluralin	(F)	11.59	15.06	17.22	18.55	9.51	4.64
		13.17	13.77	15.16	19.77	8.56	5.31
8) Tricamba	(P)	10.64	11.12	10.62	7.86	-	5.36
9) Dicamba	(P)	10.62	10.64	8.32	8.02	-	6.23
10) Terbacil	(P)	8.60	7.29	7.32	6.46	5.03	0.24

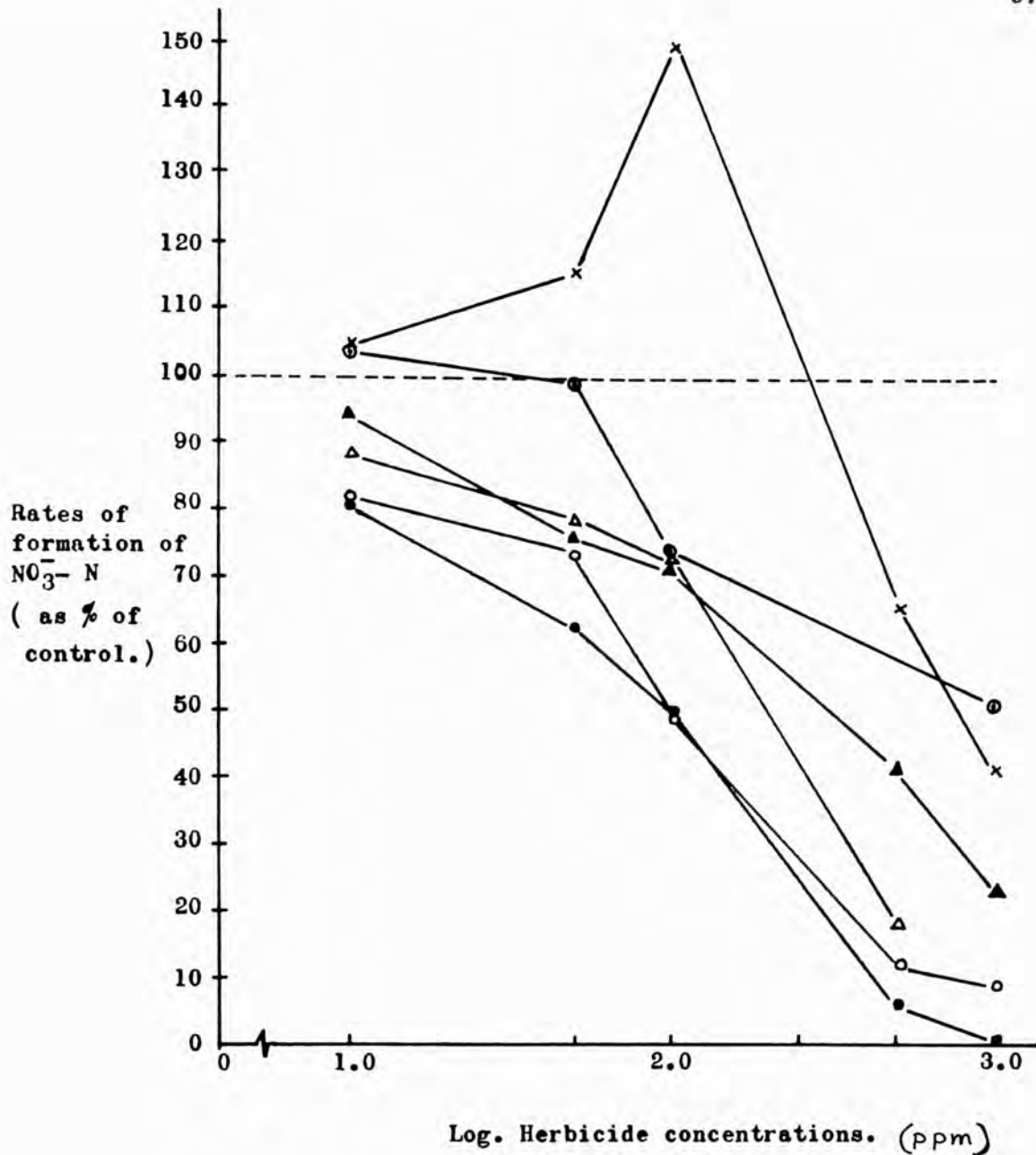


Fig. 30

Maximum rates of formation of NO_3^- -N ($\mu\text{g. NO}_3^-$ -N formed /ml.perfusate/day) in fresh soil perfused with Ammonium sulphate solutions containing different herbicides at a known range of concentrations.

(All rates expressed as percentage values of the corresponding controls.)

●- Ioxynil, ○- NPH 1320, △- Totril, ▲- Legurame,
⊙- Tricamba, × - Trifluralin.

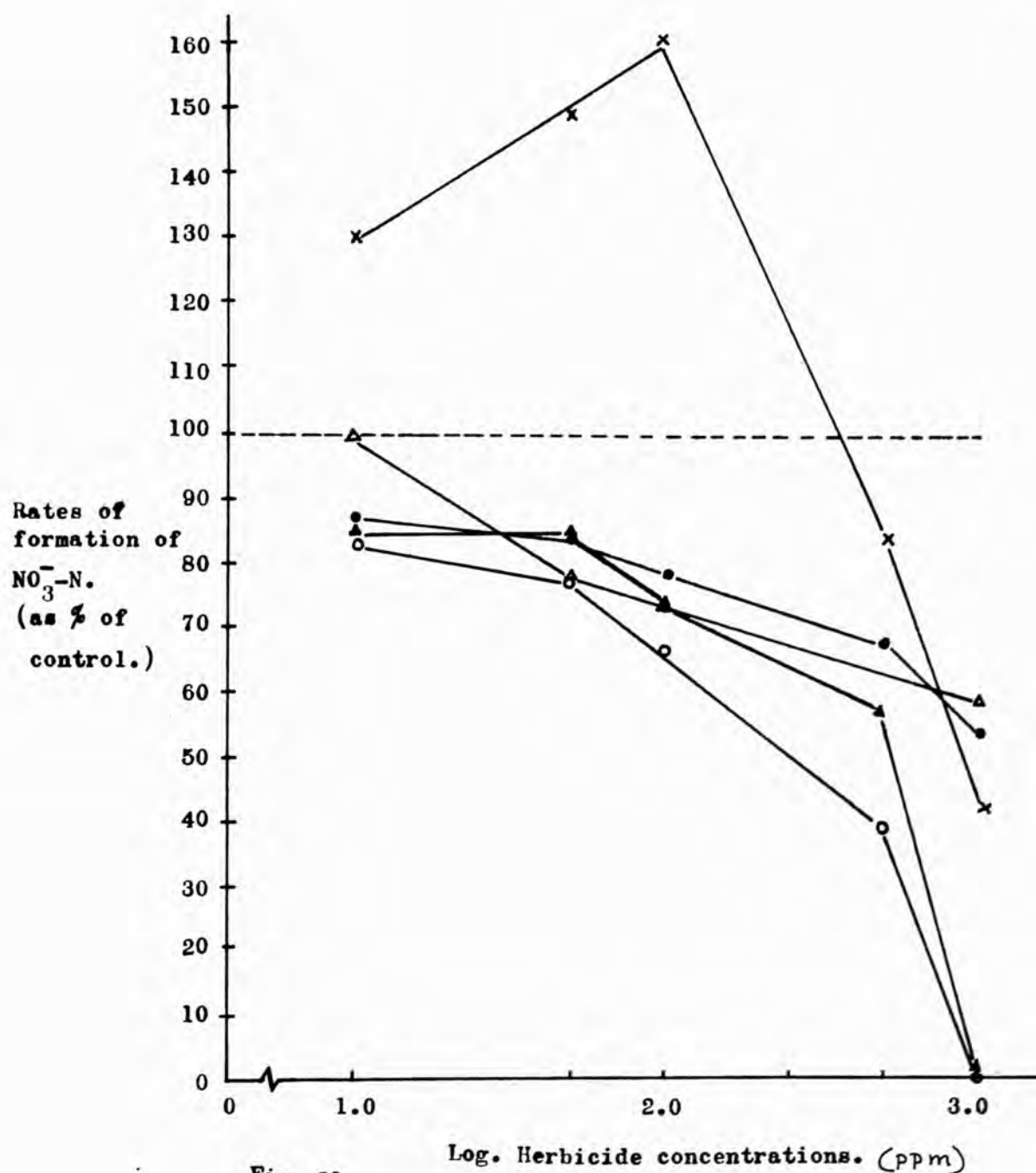


Fig. 31

Maximum rates of formation of NO_3^- -N ($\mu\text{g. NO}_3^-$ -N formed/ml.perfusate/day) in fresh soil perfused with Ammonium sulphate solutions containing different herbicides at a range of known concentrations. (All rates expressed as percentage values of the corresponding controls.)

o - Bromoxynil, Δ - Dicamba, \blacktriangle -Terbacil,
 • - Oxadiazon, x - Trifluralin.

the maximum rates (expressed as percentage of corresponding controls) of formation nitrate nitrogen even when applied to freshly perfused soils at concentrations as high as 1000 p.p.m., the percentage inhibitions of rates of NO_3^- - N accumulation in soils treated with 1000 p.p.m. solutions being only around 48% and 41% respectively. On the other hand Ioxynil, Bromoxynil, NPH 1320, Totril and Terbacil applied at similar concentrations (1000 p.p.m.) were found to be effective in causing almost complete inhibition of accumulation of nitrate nitrogen in fresh soils perfused with ammonium sulphate solutions.

Out of the three remaining herbicides, tested in this part of the present investigation, Legurame was found to have only very mild inhibitory effects at lower concentrations, the degree of inhibition in the rate of formation of nitrate nitrogen in fresh soil treated with 10 p.p.m. solution of this herbicide being around 6%. But when applied at higher concentrations, in the range of 500 and 1000 p.p.m., Legurame greatly reduced the maximum rates of formation of nitrate nitrogen in fresh soils resulting in 59% and 78% inhibitions respectively.

Oxadiazon differed from the other herbicides, discussed so far, in that it caused the rate of formation of nitrate nitrogen to be reduced to around 87% (13% inhibition) when applied at a low concentration of 10 p.p.m. whereas when incorporated into the perfusate, at higher concentrations equivalent to 500 and 1000 p.p.m. of active ingredient, it was only effective in reducing the maximum rates of formation of nitrate nitrogen to 68% and 54% (32% and 46% inhibitions) respectively. This narrow variation in the degree of inhibition of the maximum rates of formation of nitrate nitrogen, in freshly perfused soil samples, over a wide concentration gradient ranging from 10 p.p.m. to 1000 p.p.m., could only be explained satisfactorily by assuming that a large fraction of this herbicide probably gets adsorbed on to the upper layers of the dry soil samples packed in the soil columns so that even when incorporated

Table 6

Maximum rates of formation of $(\text{NO}_3^- + \text{NO}_2^-)$ - N attained in fresh soils
 perfused with $0.003 (\text{NH}_4)_2\text{SO}_4$ in the presence of the herbicides listed.

(All rates expressed in terms of $\mu\text{g NO}_3^- + \text{NO}_2^-$ - N formed/ml. perfusate/day.)

Herbicides	Herbicide concentrations in p.p.m. \longrightarrow					
	Control	10	50	100	500	1000
1) Ioxynil	14.38	11.19	9.56	9.08	1.57	0.41
2) Bromoxynil	10.85	9.12	11.27	8.28	4.93	0.96
3) NPH 1320	8.83	5.72	8.73	4.02	1.01	-
4) Totril	7.78	6.64	8.38	3.58	1.60	-
5) Legurame	18.02	15.99	18.16	12.57		
	14.38				6.01	4.25
6) Oxadiazon	12.64	10.11	10.42	9.42	8.38	6.64
7) Trifluralin	14.54	14.86	19.24	25.91	9.15	5.87
	12.94	14.35	15.91	20.59	7.52	4.77
8) Tricamba	10.30	10.79	10.41	8.54	-	6.41
9) Dicamba	10.41	10.30	8.21	7.52	-	6.14
10) Terbacil	8.34	6.60	6.90	6.20	5.70	0.61

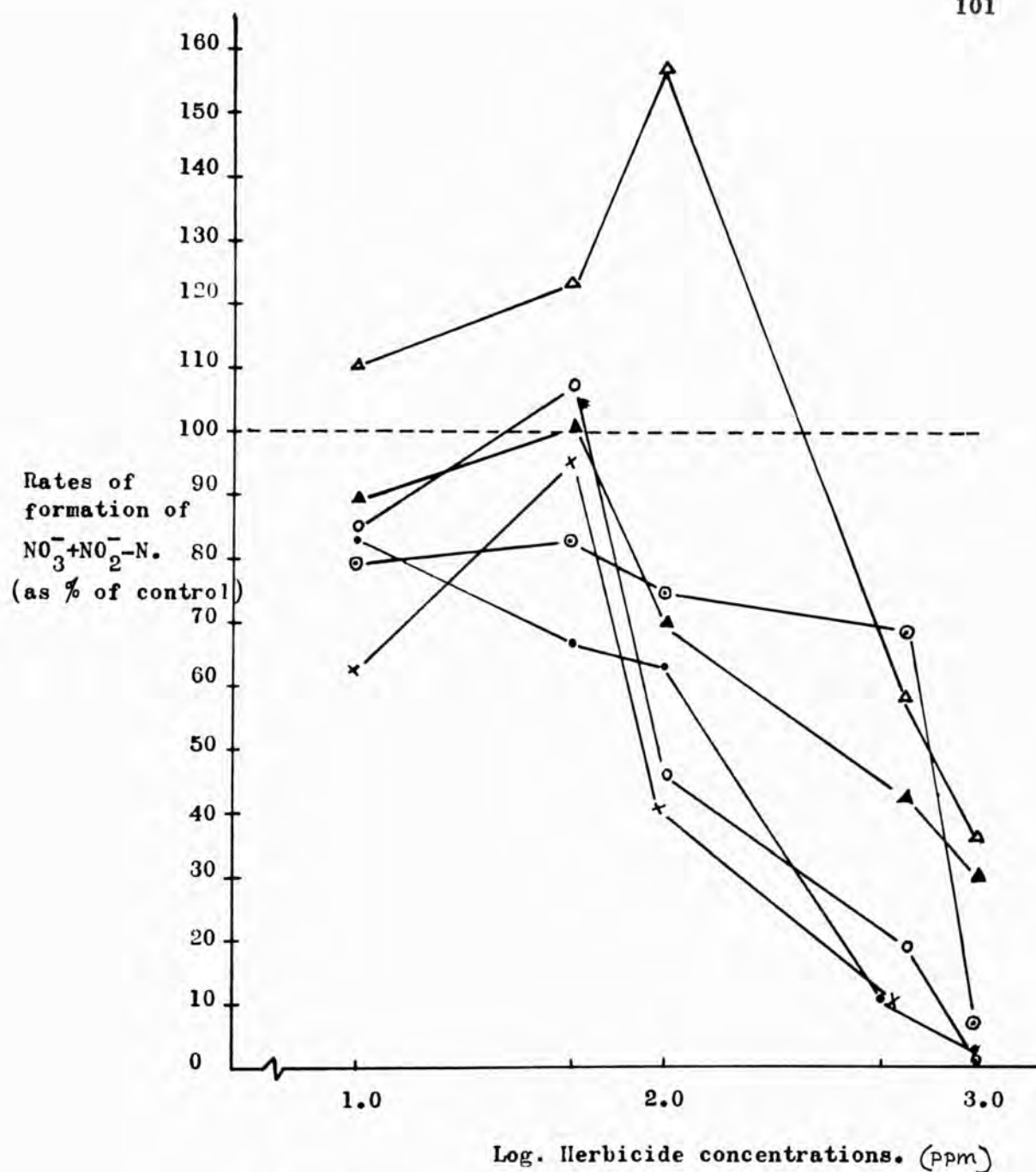


Fig. 32

Maximum rates of formation of $\text{NO}_3^- + \text{NO}_2^- - \text{N}$ ($\mu\text{g. NO}_3^- + \text{NO}_2^- - \text{N}$ formed/ml.perfusate/day.) in fresh soil perfused with Ammonium sulphate solutions containing different herbicides at a range of known concentrations. (All rates expressed as percentage values of the corresponding controls.)

● - Ioxynil, ○ - Totril, x - NPH 1320,
 ⊙ - Terbacil, ▲ - Legurame, Δ - Trifluralin.

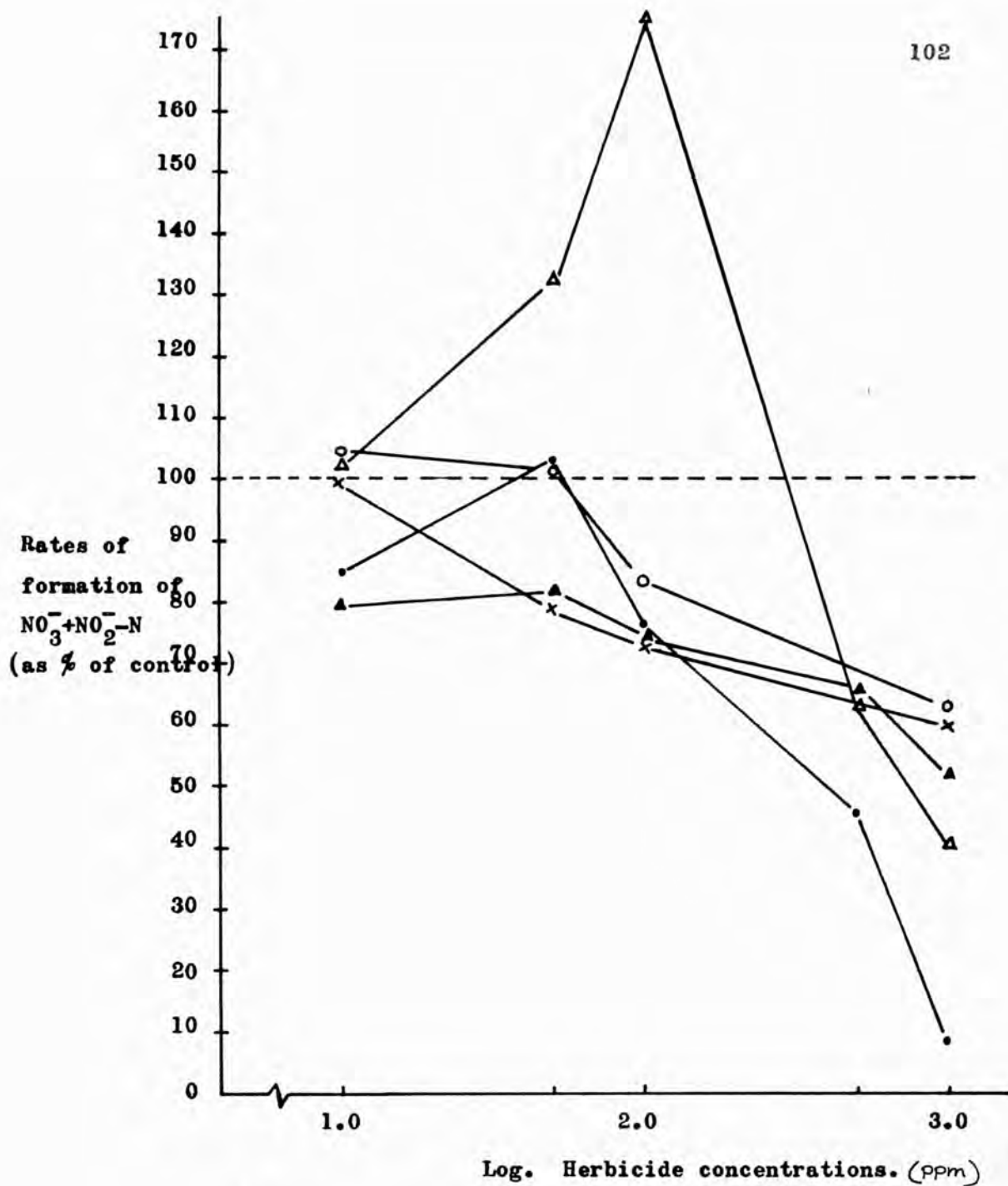


Fig. 33

Maximum rates of formation of $\text{NO}_3^- + \text{NO}_2^- - \text{N}$ ($\mu\text{g. NO}_3^- + \text{NO}_2^- - \text{N}$ formed/ml.perfusate/day.) in fresh soil perfused with Ammonium sulphate solutions containing different herbicides at a range of known concentrations. (All rates expressed as percentage values of the corresponding controls.)

- - Bromoxynil, ▲ - Oxadiazon, x - Dicamba,
- - Tricamba, △ - Trifluralin.

into the perfusate at very high concentrations, only a small fraction of it would reach the lower layers of soil in the columns, during percolation. Thus the lower layers of soil, in these soil columns, would only be exposed to extremely low concentrations of Oxadiazon and the establishment of an effective nitrifying population in these layers during perfusion would proceed at fairly normal rates resulting in high overall rates of formation of nitrate nitrogen observed. This explanation would be supported by the findings of Desmoras et al. (1970) who have reported that a large fraction of about 90% of Oxadiazon remained adsorbed in the superficial layer (0.5 cm.) of soils treated with the formulation 17,623 R.P. which happened to be the same formulation of Oxadiazon used in the present perfusion experiments.

Trifluralin incorporated into the perfusates, of freshly perfused soil samples, at concentrations up to 100 p.p.m. (10, 50 and 100 p.p.m.) was found to stimulate the maximum rates of formation of nitrate nitrogen and these increased rates of nitrate nitrogen formations (rates expressed as percentage values of the corresponding controls) can be seen clearly from figures 30 and 31. It is also evident from both the Trifluralin inhibition curves (Figures 30 and 31) that this herbicide stimulated NO_3^- - N formation only slightly at 10 p.p.m. concentration while the stimulation was significantly high (the maximum rates of NO_3^- - N formation expressed as percentage of the corresponding controls being around 150-160%) when Trifluralin was applied to fresh soil at a concentration of 100 p.p.m. active ingredient. But at still higher concentrations, equivalent to 500 and 1000 p.p.m. active ingredient, the rates of nitrate -N formation were found to fall sharply to values around 70% and 40% of the control, respectively. It is hard to give a satisfactory explanation for the above observations since no such stimulations in the rates of nitrate formation were observed when enriched soil samples were perfused with ammonium sulphate solutions containing equivalent concentrations of

Trifluralin. Owing to this same discrepancy a possibility of a breakdown product acting as a preferential substrate for nitrifiers at lower concentrations, too has to be ruled out. However Curl et al. (1969) have shown that when cultures of Fusarium oxysporum spp. vasinfectum growing on sterile sandy loam and clay soils were treated with Trifluralin concentrations ranging from 0-40 $\mu\text{g/g}$. soil, the production of Chlamydo spores and also the percentage germination of these spores were generally higher for all Trifluralin treatments than for the controls. They also found that there was a concomitant increase in the general microbial population in natural soils (not sterilised) treated with low Trifluralin concentrations where as population growth declined at higher concentrations. Thus, the stimulation in the rates of nitrate nitrogen accumulation in fresh soils observed during the present investigation could be considered as due to low concentrations of Trifluralin being effective in stimulating the establishment of nitrifying populations in these soil samples.

It has already been discussed in Chapter 2 that the maximum rates of nitrification attained by samples of fresh soil perfused with ammonium sulphate solutions (containing known concentrations of herbicides ranging from 0-1000 p.p.m.) give an estimate of the size of saturating populations of nitrifying organisms, modified by a factor dependent on the effects of different herbicide concentrations on the rates of metabolic activity per cell of these organisms. But as reasoned out in the same chapter, the maximum rates of accumulation of nitrate -N could not be taken as giving an estimate of the size of saturating populations of Nitrobacter established in these soils without considering the nitrite -N accumulations detected in their perfusates. Thus it follows that the varying degrees of nitrite -N accumulations observed in the perfusates of fresh soil samples treated with different herbicide concentrations have to be looked into before arriving at any conclusions regarding the effects of the herbicides, under consideration, on growth of Nitrobacter populations in these soils.

The maximum concentrations of nitrite -N observed in the perfusates of treated fresh soil are presented graphically in Figures 34 and 35 where these maximum nitrite -N concentrations expressed as $\mu\text{g NO}_2^- \text{ - N / ml. perfusate}$ are plotted against the logarithms of the corresponding herbicide concentrations.

It is known that in fresh soils, supplied with ammonium ions under ordinary conditions, the nitrite-nitrogen that is formed first stimulates the activity of Nitrobacter populations responsible for the formation of nitrate-nitrogen and once stimulated the Nitrobacter population in an ordinary garden soil sample is capable of utilising immediately all the nitrite -N produced due to the activity of Nitrosomonas populations. Thus only extremely low concentrations of nitrite -N lasting for periods of very short duration would be observed in fresh soil samples where the activities of Nitrosomonas and Nitrobacter populations are proceeding at normal rates and this explains the low concentrations of nitrite -N in control soils as presented in Figures 34 and 35. In other words the rates of nitrification in a normal soil is limited by the activity of Nitrosomonas population. (Same conclusion as arrived at in Chapter 3.) It follows from this conclusion that the first stage of nitrification would be the rate determining step of the enrichment cycle in treated soils too whenever there is no nitrite nitrogen accumulation observed in the perfusates of these treated soil samples. But when there is some degree of accumulation of nitrite -N observed in the perfusates of treated soils, it would indicate that the particular herbicide under consideration does affect the population growth of Nitrobacter spp. to a greater extent than the population growth of Nitrosomonas spp.

Bearing the above conclusions in mind as the background for reasoning it can be seen clearly from Fig. 34 that Ioxynil, Totril, NPH 1320, Legurame and Oxadiazon did start to show up significant differential effects on the growth of Nitrosomonas and Nitrobacter populations in fresh

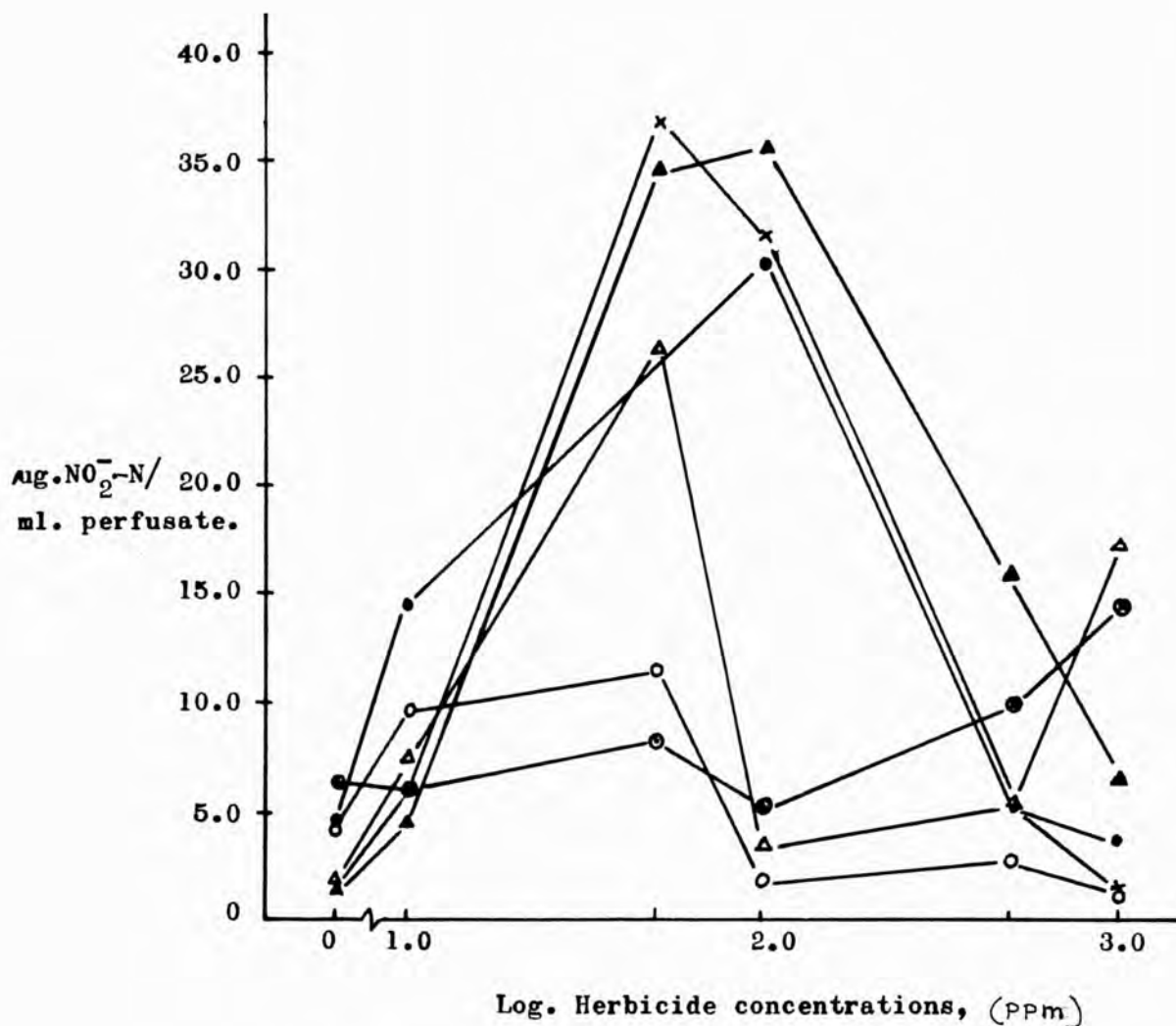


Fig. 34

The maximum concentrations of Nitrite- N accumulated in the perfusates when fresh soil samples were perfused with Ammonium sulphate solutions containing different herbicides at known concentrations.

- - Ioxynil, × - NPH 1320, ▲ - Totril,
- △ - Legurame, ○ - Oxadiazon. ⊙ - Trifluralin.

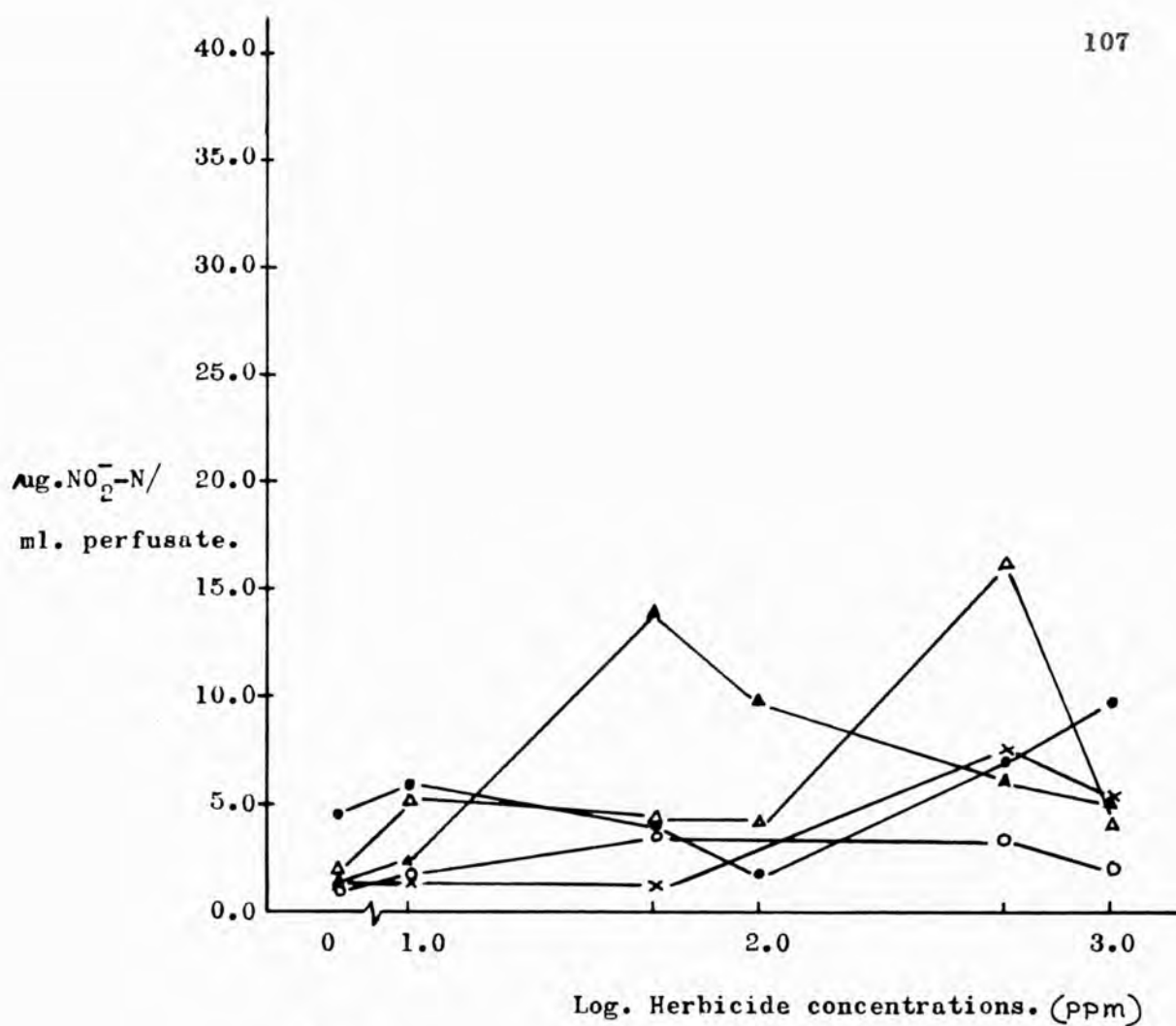


Fig. 35

The maximum concentrations of Nitrite--N accumulated in the perfusates when fresh soil samples were perfused with Ammonium sulphate solutions containing different herbicides at known concentrations.

● - Trifluralin, × - Tricamba, ○ - Dicamba,
 Δ - Terbacil, ▲ - Bromoxynil.

soil samples where these herbicides were incorporated into the percolating ammonium salt solution at a concentration equivalent to 50 p.p.m. In the case of soil samples treated with Ioxynil, NPH 1320 and Totril, these differential effects could still be seen even at a higher concentration equivalent to 100 p.p.m. whereas the differential effects did fade away rapidly at still higher concentrations in the range of 500 to 1000 p.p.m. Thus it can be concluded that the growth of Nitrobacter populations in fresh soil samples perfused with ammonium sulphate solutions were significantly affected by Ioxynil, NPH 1320 and Totril at concentrations around 50 p.p.m. (Fig. 30) whereas the proliferation of Nitrosomonas populations continued even when the soil samples were treated with a higher concentration, equivalent to 100 p.p.m., of these herbicides showing that the growth of Nitrobacter populations, in samples of fresh soil treated with Ioxynil, NPH 1320 and Totril at 50 p.p.m. and 100 p.p.m. concentrations were inhibited more than the growth of Nitrosomonas populations in the same soils. It also follows from the above conclusions that the maximum rates of formation of $\text{NO}_3^- - \text{N}$ (from Table 5) and the maximum rates of formation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ (from Table 6) in fresh soil samples treated with Ioxynil, NPH 1320 and Totril at the lower concentrations ranging from 10 p.p.m. to 100 p.p.m. could be taken as measures of the sizes of saturating populations of Nitrobacter spp. and Nitrosomonas spp. respectively established in these soils whereas the same nitrification rates expressed as percentage values of the corresponding controls (Figs. 30 and 32) would give an indication of the extent to which the proliferation of Nitrosomonas and Nitrobacter populations are affected by these herbicide concentrations.

Significantly high concentrations of $\text{NO}_2^- - \text{N}$ could be detected in the perfusates of soil samples treated with Legurame, Oxadiazon and Bromoxynil only when these herbicides were used at a concentration equivalent to 50 p.p.m. active ingredient, whereas the maximum concentrations

of NO_2^- - N in the perfusates of soil samples treated with the remaining concentrations (i.e. 10, 100, 500 and 1000 p.p.m.) of the same herbicides were not much different from the concentration of NO_2^- - N accumulated in the corresponding control soils (Figs. 34 and 35). These observations would indicate that the nitrification rates in samples of fresh soil treated with Legurame, Bromoxynil and Oxadiazon applied at lower concentrations around 10 p.p.m. and at higher concentrations around 100, 500 and 1000 p.p.m. are limited by the population growth and metabolism of Nitrosomonas spp. Thus in the case of these herbicides the maximum rates of formation of NO_3^- - N (Table 5) as well as the maximum rates of formation of $(\text{NO}_3^- + \text{NO}_2^-)$ - N (Table 6) would only give an estimate of the size of the saturating populations of Nitrosomonas spp. without giving any indication regarding the effect of these herbicides on the size of the saturating populations of Nitrobacter spp. established in the same soil samples.

It is also evident from Figures 34 and 35 that the maximum concentrations of NO_2^- - N observed in fresh soil perfused with ammonium sulphate solutions containing Trifluralin at concentrations equivalent to 10, 50 and 100 p.p.m., of active ingredient, were less than the NO_2^- - N accumulations observed in the corresponding controls; the lowest concentration of NO_2^- - N observed being in the perfusates of soil samples treated with 100 p.p.m. Trifluralin. But slightly higher concentrations of NO_2^- - N could be observed in the perfusates of soil samples treated with Trifluralin concentrations equivalent to 500 and 1000 p.p.m. These low nitrite concentrations observed in fresh soils treated with Trifluralin concentrations, up to 100 p.p.m., could be considered as a clear reflexion of the enhancement of Nitrobacter population growth in these soils. This conclusion is also in accordance with the stimulations of the maximum rates of formation of NO_3^- - N observed in these soils, which was discussed previously with reference to the results presented in Figures 30 and 31.

The curves showing the variations of the maximum rates of formation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ with logarithms of the different Trifluralin concentrations (Figs. 32 and 33) point to the fact that the rates of accumulation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ were also stimulated in the fresh soil samples treated with Trifluralin concentrations ranging from 10 p.p.m. to 100 p.p.m., the degree of stimulations being of a similar order as those observed with the rates of formation of $\text{NO}_3^- - \text{N}$ dealt with previously. This observation along with the low concentrations of $\text{NO}_2^- - \text{N}$ detected in the perfusates of the same soil samples (Figs. 34 and 35) shows that the overall nitrification rates in soils treated with Trifluralin too were limited by the rates of activities of their Nitrosomonas populations.

The low $\text{NO}_2^- - \text{N}$ accumulations observed in the fresh soil samples treated with varying concentrations of Tricamba and Dicamba (Fig. 35) indicate clearly that these two herbicides also exerted very little differential effect on the proliferation of Nitrosomonas and Nitrobacter populations. On the other hand the small variations in rates of accumulation of $\text{NO}_3^- - \text{N}$ and $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ (expressed as percentage values of the corresponding controls) observed with increasing concentrations of Tricamba and Dicamba (Figs. 30, 31 and 33 respectively) show that these two herbicides do not inhibit the population growth of nitrifying bacteria in freshly perfused garden soil.

Considering the differential toxic effects of Terbacil on the growth of nitrifying bacteria in soil, it was evident that there was significant amount of $\text{NO}_2^- - \text{N}$ accumulation only in the case of the soil sample treated with 500 p.p.m. concentration of this herbicide and thus it could be concluded that Terbacil when applied at a concentration equivalent to 500 p.p.m. was effective in inhibiting the growth of Nitrobacter populations to a greater extent than the growth of Nitrosomonas populations whereas at all the other concentrations tested, namely 10, 50,

100 and 1000 p.p.m., Terbacil did not cause excessive accumulations of NO_2^- - N in the perfusates showing that the nitrification rates in fresh soil treated with these concentrations of Terbacil were limited due to the inhibition of the proliferation of Nitrosomonas populations.

Finally it could be concluded that the overall nitrification rates in fresh soil samples perfused with ammonium sulphate solutions, containing varying concentrations of the herbicides tested, were limited mainly due to a greater inhibition of the growth and metabolic activities of Nitrosomonas populations except in the case of the soil samples treated with Ioxynil, NPH 1320, Totril and Bromoxynil applied at several concentrations ranging from 10 to 500 p.p.m. where the growth and metabolic activities of Nitrobacter populations were found to be inhibited to a greater extent than the growth of Nitrosomonas populations.

As discussed previously in Chapter 2, in dealing with the analysis of data, the only other general parameter derived from the observations made during the experiments described in this chapter was the time taken for the rates of accumulation of NO_3^- - N and $(\text{NO}_3^- + \text{NO}_2^-)$ - N to reach their maximum values. The lengths of these time intervals for the rates of formation of NO_3^- - N and $(\text{NO}_3^- + \text{NO}_2^-)$ - N, in control soils as well as in treated soils to reach their maxima, under conditions of excessive NO_2^- - N accumulation in the perfusates (same reasoning as discussed before), were then considered to be the time taken for the freshly perfused soil samples to become saturated with Nitrosomonas and Nitrobacter populations, limited by the available substrate concentrations and the specific experimental conditions used.

The derived data representing the time (days) for the accumulation of NO_3^- - N and $(\text{NO}_3^- + \text{NO}_2^-)$ - N to reach the maximum rates are given in Tables 7 and 8 respectively. From the values presented in Table 7 it could be seen that all the herbicides tested except Trifluralin, up to concentrations equivalent to 100 p.p.m., were effective in increasing the time

intervals (T) taken by the treated soil samples to attain maximum rates of formation of $\text{NO}_3^- - \text{N}$. Similarly the results presented in Table 8 show that all the herbicides under consideration excepting Trifluralin and Oxadiazon, applied at concentrations ranging up to 100 p.p.m. and 50 p.p.m. respectively, were effective in delaying the time lag to attain maximum rates of formation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$. However the magnitude of the inhibitory effects, caused by different herbicides, on the rates of proliferation ($\frac{1}{T}$) of the nitrifying populations differed greatly depending on the concentrations as well as the nature of the particular herbicide under consideration and this variation in the toxic effects of different herbicides could be seen clearly from Figures 36 and 37 where the reciprocal of the time (i.e. $\frac{1}{T}$ expressed as % of the corresponding control values) to reach maximum rates of formation of $\text{NO}_3^- - \text{N}$ and $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ in each of the soil samples are plotted against the appropriate herbicide concentrations.

From the discussion dealt with so far, it is clear that the time taken for the rates of accumulation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ to reach maximum values would represent the time taken for these soil samples to establish saturating populations of Nitrosomonas spp., under a specific set of experimental conditions. Thus it follows that the variation of the reciprocal values of these time lags with herbicide concentrations (Fig. 37) would indicate the variation of the rates of saturation of freshly perfused soils with respect to Nitrosomonas populations, or in other words the variation in the rates of proliferation of Nitrosomonas populations with increase in herbicide concentrations.

Considering the results presented in Figure 37 more closely it can be seen that the highest toxicities on the proliferation rates of Nitrosomonas in freshly perfused soils were exerted by NPH 1320 and Totril, with the observed rates of Nitrosomonas population growth in soil samples treated with these two herbicides at the lowest concentration tested (10 p.p.m.) being as low as 40% and 39% respectively. However the herbi-

Table 7

Time (days) to reach maximum rates of NO_3^- - N formation in fresh soils treated with different herbicides listed below at the concentrations indicated.

Herbicide concentrations in p.p.m. —→

Herbicides	Control	10	50	100	500	1000
1) Ioxynil	3.0	5.4	4.9	6.5	21.0	∞
2) Totril	5.6	17.3	18.4	27.6	32.0	∞
3) NPH 1320	6.7	17.5	19.5	26.5	36.0	∞
4) Oxadiazon	3.5	4.7	6.4	7.8	7.4	9.2
5) Legurame	3.0	3.2	4.4	5.0	11.5	21.0
6) Trifluralin	3.5	2.6	1.4	0.6	8.4	12.5
	2.9	2.4	2.3	1.1	6.8	9.2
7) Tricamba	4.0	4.5	4.2	5.6	-	6.4
8) Dicamba	4.2	4.0	5.6	5.5	-	8.5
9) Bromoxynil	2.8	3.5	4.2	10.0	21.0	∞
10) Terbacil	5.1	5.9	6.1	9.5	16.6	∞

Table 8

Time (days) to reach maximum rates of formation of $(NO_3^- + NO_2^- - N)$ in fresh soils treated with different herbicides listed below at the concentrations indicated.

Herbicide concentrations in p.p.m. →

Herbicides	Control	10	50	100	500	1000
1) Ioxynil	2.0	2.5	2.4	2.4	21.0	∞
2) Totril	6.6	17.1	16.2	22.4	36.0	∞
3) NPH 1320	7.0	17.5	16.2	17.2	37.0	∞
4) Oxadiazon	3.0	0.9	0.9	7.7	7.2	9.2
5) Legurame	2.0	2.6	2.8	4.6	10.5	18.0
6) Trifluralin	2.1	1.8	0.8	-	2.4	9.4
	3.1	1.7	0.7	0.6	7.6	12.5
7) Tricamba	4.1	4.5	4.3	4.8	-	4.8
8) Dicamba	4.2	3.9	5.6	5.5	-	8.3
9) Bromoxynil	2.6	2.8	5.3	7.5	21.0	∞
10) Terbacil	4.9	5.8	5.7	9.7	16.5	∞

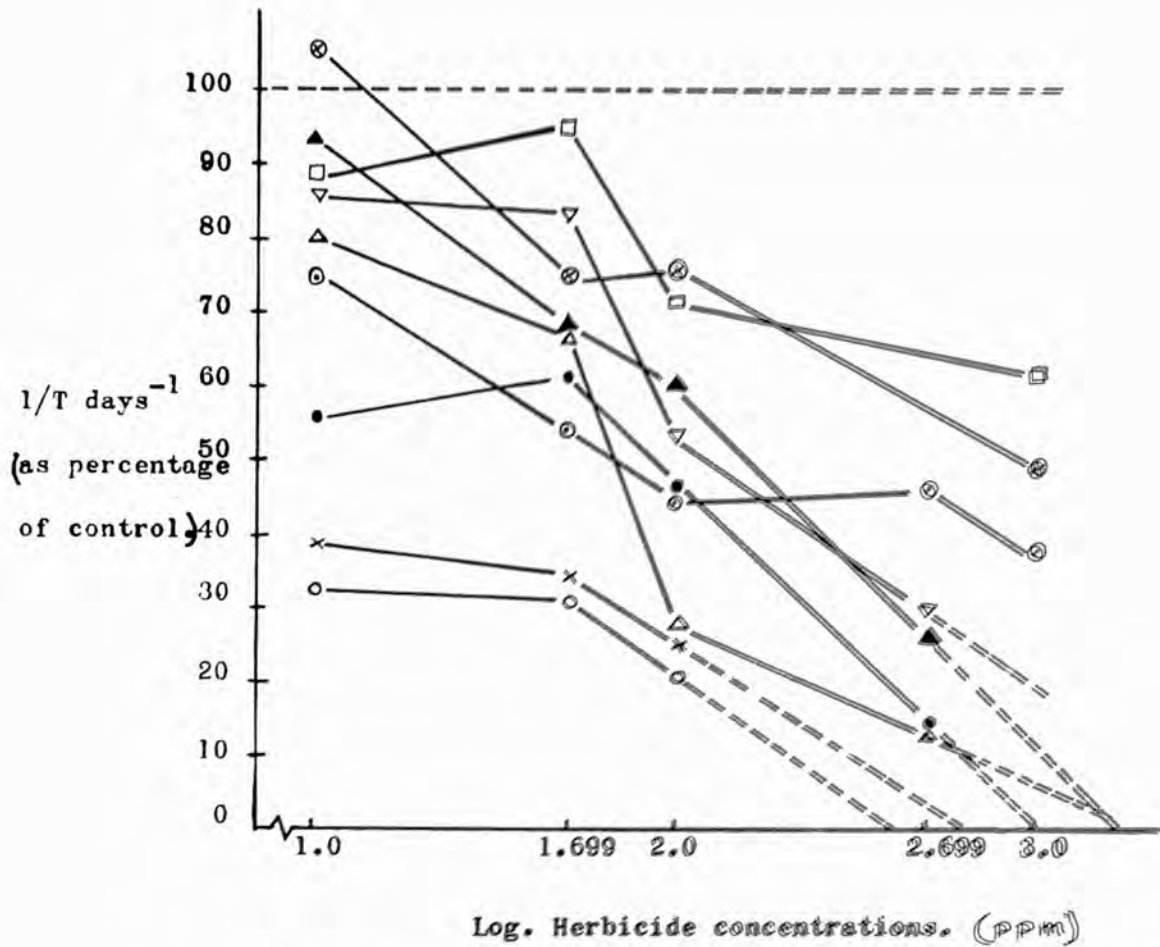


Fig. 36

Reciprocal values of the time lags ($1/T \text{ days}^{-1}$) to attain maximum rates of formation of $\text{NO}_3^- - \text{N}$ in samples of fresh soil perfused with Ammonium sulphate solutions containing different herbicides at known concentrations. (All values expressed as percentages of the corresponding controls)

○ - Totril, x - NPH 1320, ● - Ioxynil,

⊙ - Oxadiazon, △ - Bromoxynil, ▲ - Legurame,

▽ - Terbacil, □ - Tricamba, ⊗ - Dicamba.

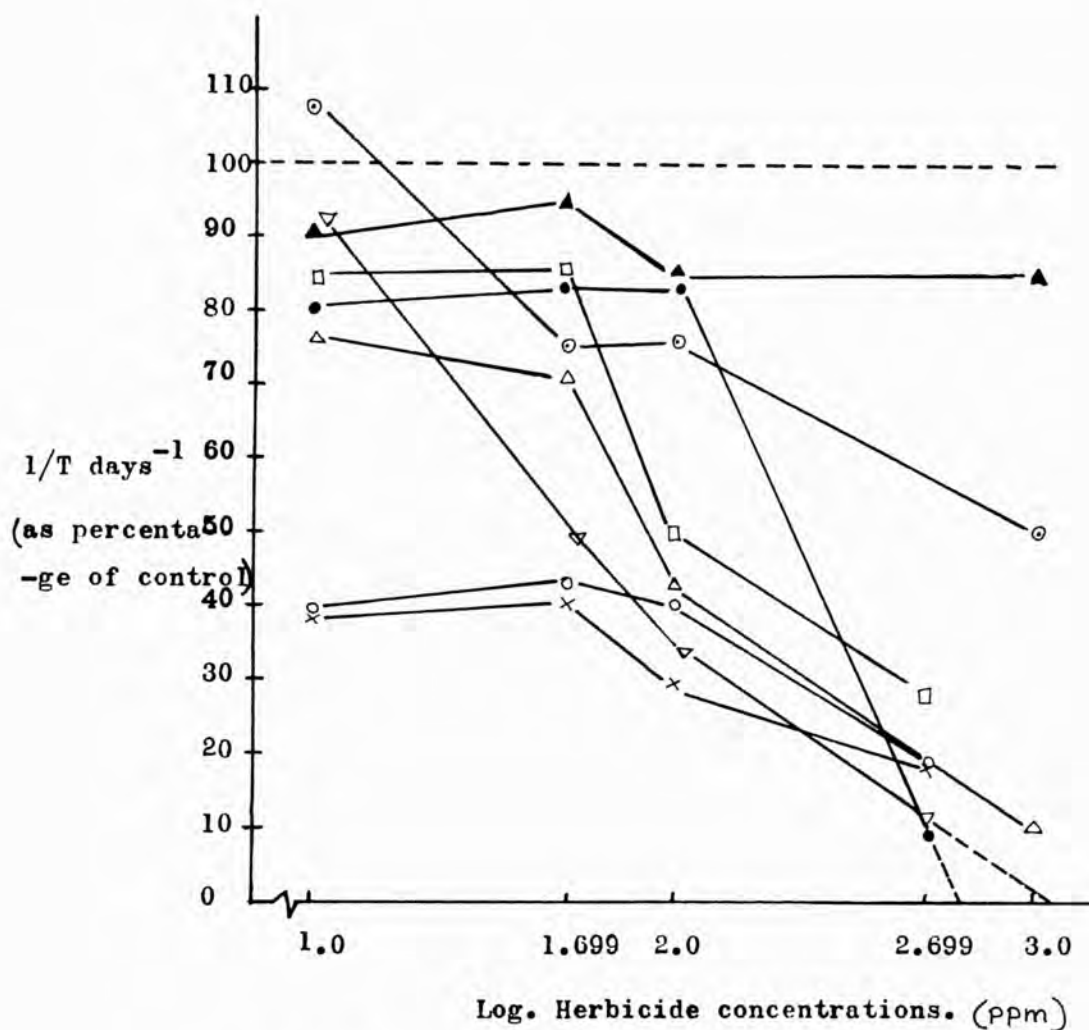


Fig. 37.

Reciprocal values of the time lags ($1/T \text{ days}^{-1}$) to attain maximum rates of formation of $\text{NO}_3^- + \text{NO}_2^- - \text{N}$ in samples of fresh soil perfused with Ammonium sulphate solutions containing different herbicides at known concentrations. (All values expressed as percentages of the corresponding controls.)

x - Totril, o - NPH 1320, • - Ioxynil,
 Δ - Legurame, ▽ - Bromoxynil, □ - Terbacil,
 ▲ - Tricamba, ⊙ - Dicamba.

cides such as Tricamba, Dicamba and Oxadiazon which were previously found to exert only low toxicities in determining the maximum rates of accumulation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ (i.e. the maximum size of the Nitrosomonas populations) attained in the treated fresh soil samples, had fairly low toxicities even upon the proliferation rates of the Nitrosomonas populations, the rates of saturation being considerably high around 85%, 51% and 33% respectively at the highest concentration (1000 p.p.m.) tested - Figure 37.

The rates of proliferation of Nitrosomonas populations were found to be greatly enhanced in the fresh soil samples that were perfused with ammonium sulphate solutions containing Trifluralin up to concentrations of 100 p.p.m. active ingredient, with the highest rates around 500% being achieved in the soil samples under the influence of 100 p.p.m. This observation is in accordance with the previously discussed stimulations in the maximum size of the saturating populations of Nitrosomonas attained in these same soils.

The results presented in Figure 36 show that the rates of attaining maximum rates of formation of $\text{NO}_3^- - \text{N}$ too were affected most by the two herbicides Totril and NPH 1320. Since there were excessive $\text{NO}_2^- - \text{N}$ accumulations observed in the perfusates of fresh soil samples percolated with ammonium sulphate solutions containing Totril, NPH 1320 and Ioxynil, applied at concentrations ranging from 10 - 500 p.p.m., Bromoxynil and Legurame applied at concentrations equivalent to 50 p.p.m., and Terbacil applied at a concentration of 500 p.p.m. (discussed previously), the reciprocal values of the time lags to attain maximum rates of formation of $\text{NO}_3^- - \text{N}$ in each of these soils would give a measure of the rate of proliferation of their Nitrobacter populations. Thus comparing the results presented in Figure 36 with those presented in Figure 37 it can be concluded that the rates of proliferation of Nitrobacter populations were inhibited slightly more than the rates of proliferation of Nitrosomonas populations, by Totril,

NPH 1320 and Ioxynil etc. mentioned above.

No conclusions could be made regarding the degree of inhibition caused by the remaining herbicides (Tricamba, Dicamba, Trifluralin etc.) on the rates of proliferation of Nitrobacter populations in the treated fresh soils, as in the case of the previously discussed results where no conclusion could be made regarding the effects of these herbicides on the size of the saturating populations of Nitrobacter established in fresh soils perfused with ammonium sulphate solutions.

When a series of fresh soil samples were perfused with sodium nitrite solution (0.006 M sodium nitrite solutions being used in place of the ammonium sulphate solutions used in the case of previous experiments) containing a range of concentrations of NPH 1320, it was observed that the progress of nitrification in the control soils as well as the treated soil samples (the progress of nitrification being measured by the amounts of NO_2^- - N oxidised / ml. perfusate) were much faster than those observed when similar soil samples were perfused with ammonium sulphate solutions containing equivalent concentrations of the same herbicide, under identical experimental conditions. A family of such progress curves obtained with a series of fresh soil samples percolated with sodium nitrite solutions containing 0, 10, 50, 100, 500 and 1000 p.p.m. NPH 1320 are presented in Figure 38 whereas the differential curves corresponding to these progress curves are presented in Figure 39. From these differential curves it is evident that NPH 1320 was effective in causing a marked degree of inhibition in the maximum sizes of the saturating populations of Nitrobacter attained in the treated soil samples (I_{50} concentration estimated by probit analysis being around 45 p.p.m.) although the progress of Nitrobacter population growth (progress of NO_2^- - N oxidation) showed a fair amount of recovery (Fig. 38) even when the soil samples were under the influence of NPH 1320 concentrations as high as 500 and 1000 p.p.m.

Comparison of the above mentioned progress curves (i.e. the curves

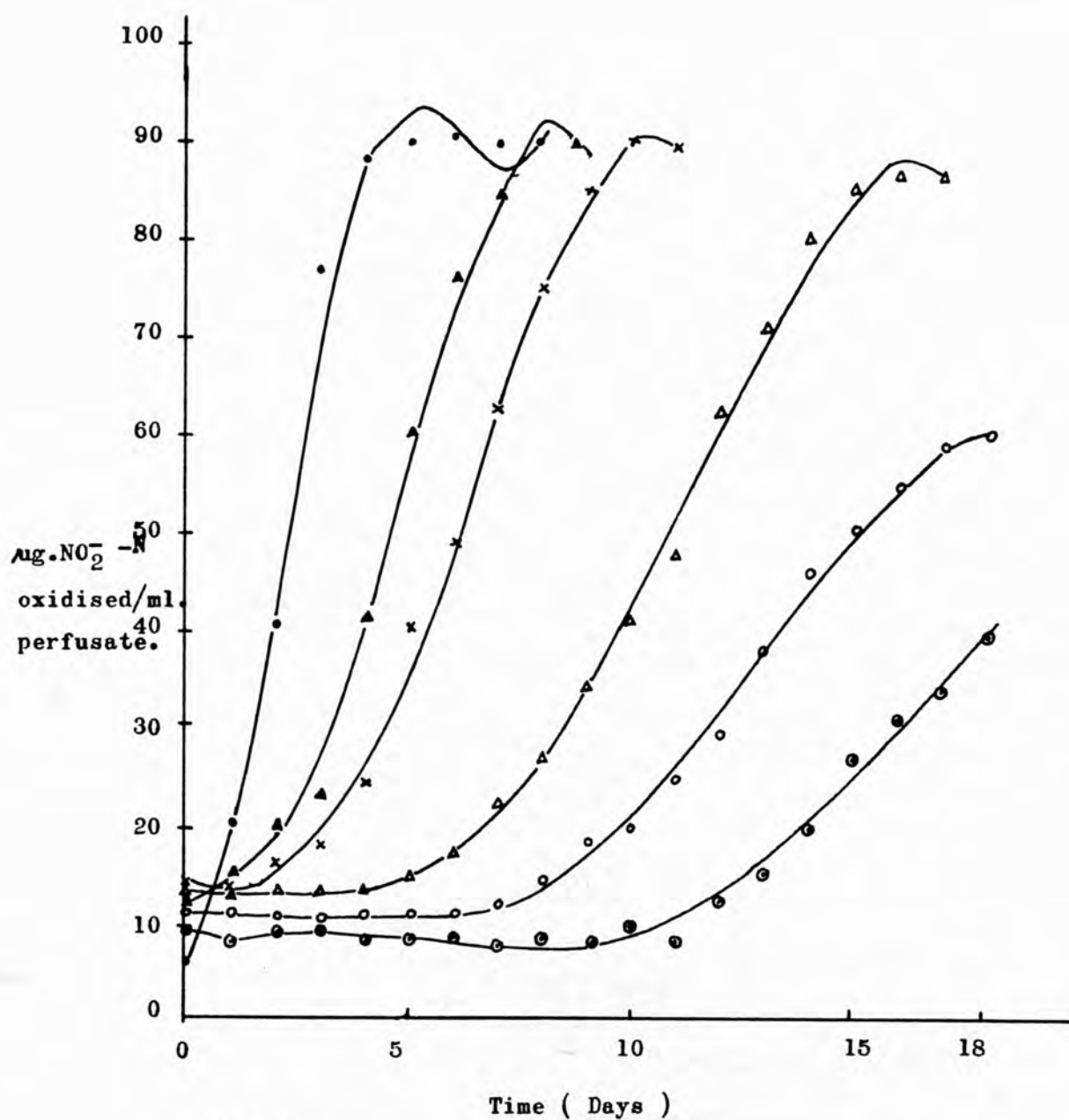


Fig. 38

Time course of oxidation of Nitrite-N in a series of soil samples perfused with Sodium nitrite solutions containing a range of concentrations of NPH 1320.

- - control, ▲ - 10 ppm, × - 50 ppm, △ - 100 ppm,
- - 500 ppm, ⊙ - 1000 ppm.

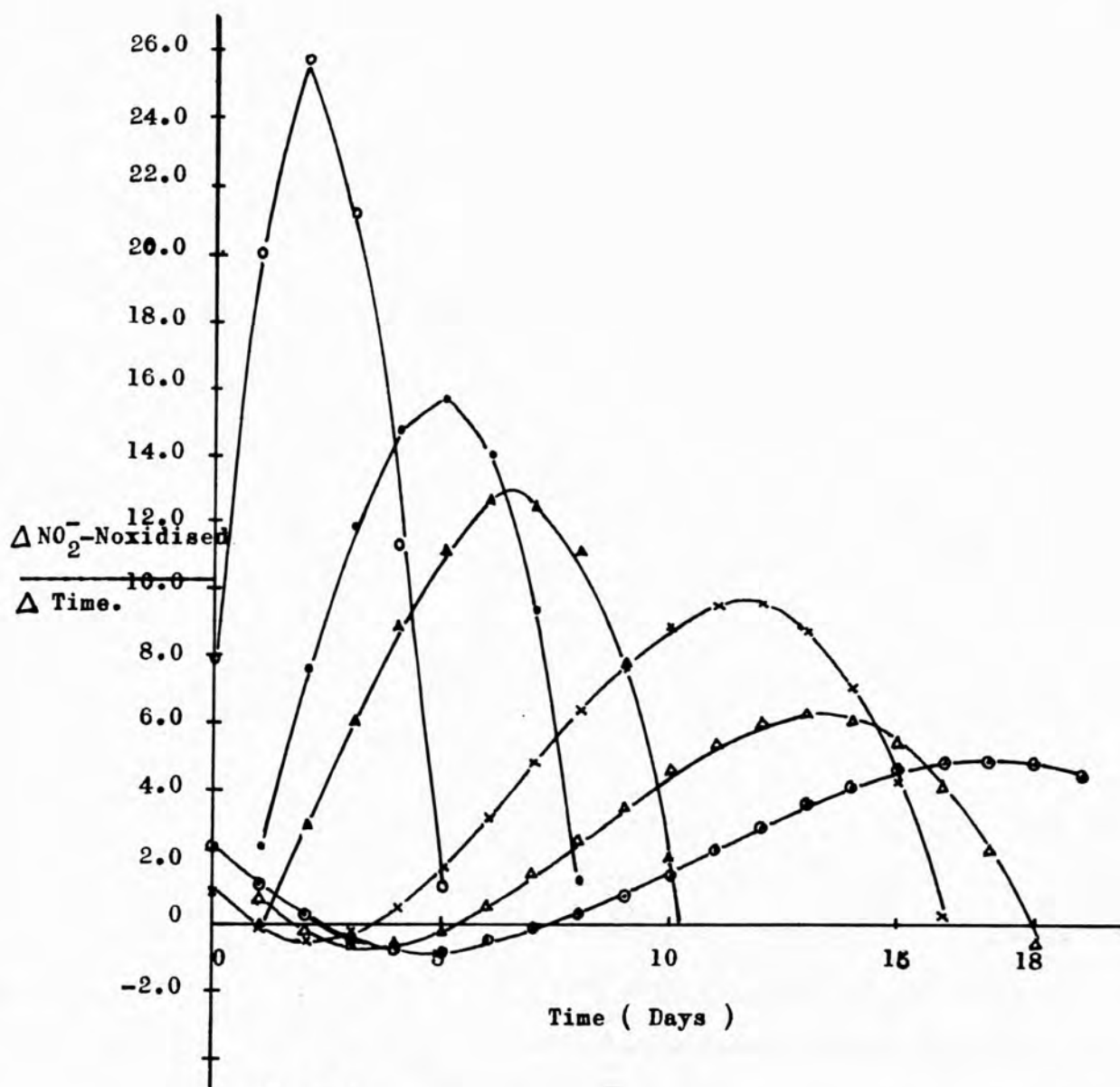


Fig. 39

Differential curves showing the rates of Nitrite oxidation ($\mu\text{g. NO}_2^- \text{-N oxidised/ml. perfusate/ day.}$) in the fresh soil samples perfused with Sodium nitrite solutions containing NPH 1320 at a range of concentrations.

○ - control, ● - 10 ppm, ▲ - 50 ppm, × - 100 ppm,
 △ - 500 ppm, ⊙ - 1000 ppm.

from Fig. 38) with those presented in Figure 9 does indicate clearly that the progress of oxidation of $\text{NO}_2^- - \text{N}$ in freshly perfused soils supplied with nitrite ions (i.e. the progress of Nitrobacter population growth) showed a tendency to proceed fairly rapidly withstanding the toxic effects of the applied NPH 1320 concentrations (a considerable degree of recovery in the progress of nitrification being achieved by the fifteenth day) whereas the progress of $\text{NO}_3^- - \text{N}$ accumulation in the freshly perfused soils supplied with ammonium sulphate solutions containing a range of concentrations of NPH 1320 (concentrations similar to those used in the case of soils perfused with sodium nitrite solutions), showed only a slight degree of recovery even after 35 days, especially in the soil samples treated with higher concentrations of the order of 500 p.p.m. (Fig. 9). Thus it follows that the proliferation of Nitrobacter populations in fresh soil samples treated with NPH 1320 recovered faster when the necessity for the first stage of nitrification mediated by Nitrosomonas spp. was eliminated by the direct supply of nitrite ions which is needed by Nitrobacter spp. as the specific substrate for their metabolic activities. It must also be mentioned here that this conclusion is in agreement with the previously discussed fact that the inhibition of the growth of Nitrosomonas populations limits the rates of nitrification in fresh soils perfused with ammonium sulphate solutions containing higher concentrations (500 and 1000 p.p.m.) of NPH 1320, or in other words that the proliferation of Nitrosomonas spp. in fresh garden soils were more sensitive than the proliferation of Nitrobacter spp., to the toxic effects of this herbicide.

In practical agriculture, the herbicides are normally applied to unenriched soils and these herbicides may remain in the treated soils for long periods of time or they may disappear, the rate of disappearance being assisted by many factors such as volatilization, photodecomposition, drainage and biological decomposition etc. Having found that Ioxynil was the most toxic herbicide (out of the range of herbicides tested in the

present investigation) with respect to its effects, on the proliferation of nitrifying populations in freshly perfused soils and on the rates of metabolic activities of the saturating populations of nitrifying organisms in enriched soil, it was thought appropriate to follow the time course of detoxication of Ioxynil in the perfusate of a fresh soil sample percolated with a solution of Ioxynil containing an initial concentration of 100 $\mu\text{g}/\text{ml}$. In this experiment too, the perfusion technique and all the other experimental conditions were maintained exactly the same as those described for the previous experiments.

The estimations of the concentrations of Ioxynil in the perfusates at regular intervals of time were done by means of a biological assay technique based on the inhibition of the longitudinal growth of the roots in garden cress seedlings (Lepidium Sativum). The basic principles and all the experimental details made use of in this biological assay technique were identical to those described by Audus (1951).

A standard root growth inhibition curve was constructed by estimating the degree of longitudinal growth of cress roots grown in Ioxynil solutions of known concentrations ranging from 0.01 p.p.m. to 10 p.p.m., as a percentage of the average lengths of the roots in control seedlings (i.e. Root growth index) and plotting these root growth indices against the logarithms of the corresponding Ioxynil concentrations (Fig. 40).

$$\text{Root growth index} = \frac{\text{Root growth in Ioxynil solution}}{\text{Root growth in control}} \times 100$$

Samples of perfusate (1 to 2 ml.) were removed for analysis at two day intervals and a series of 3-4 different dilutions of these samples were made in a standard inorganic culture medium (Arnon & Hoagland, 1940) of pH 6.0. Five culture tubes, each containing 20 seeds (refer Audus, 1951) were set up for every dilution along with a set of five additional tubes containing Hoagland culture medium, with no Ioxynil, as the corresponding controls. After incubation in the dark at 25°C for a period of four days,

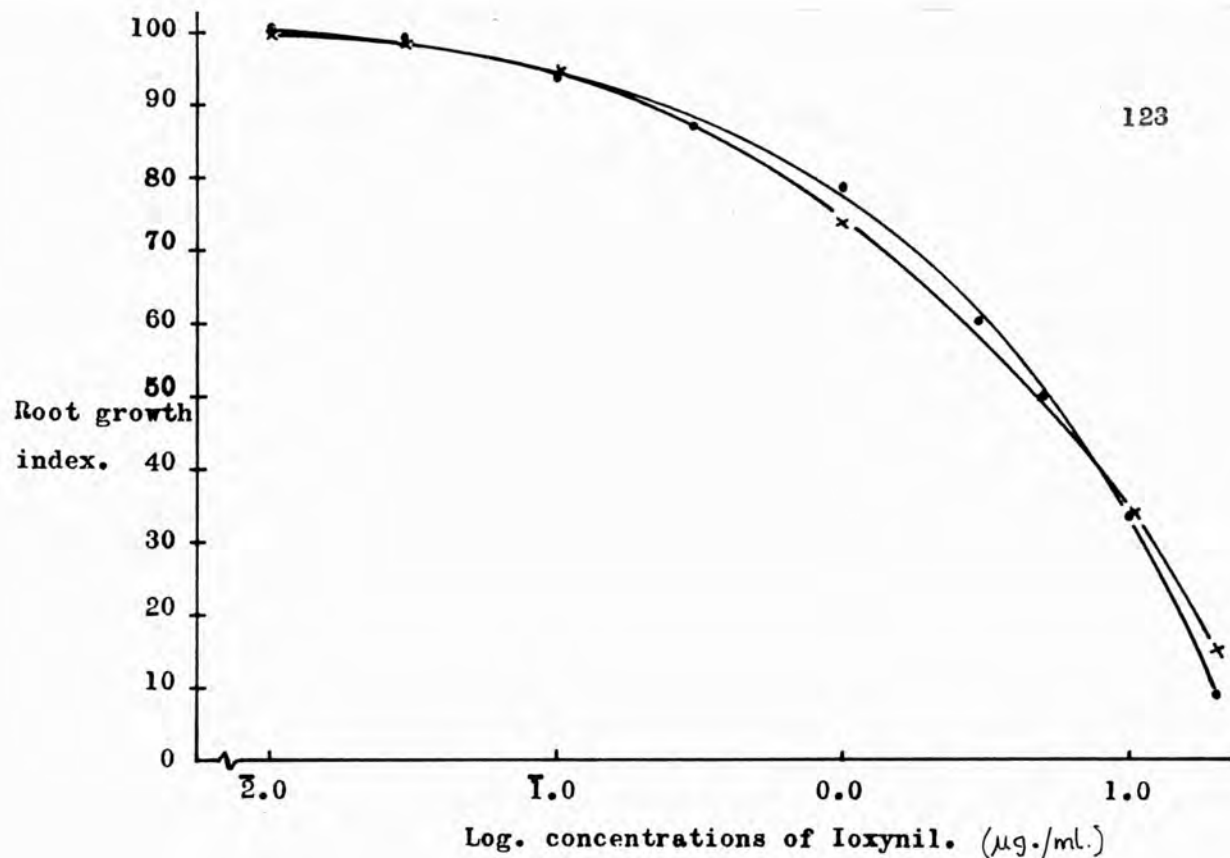


Fig. 40
Cress root growth bioassay calibration curve for the estimation of Ioxynil.

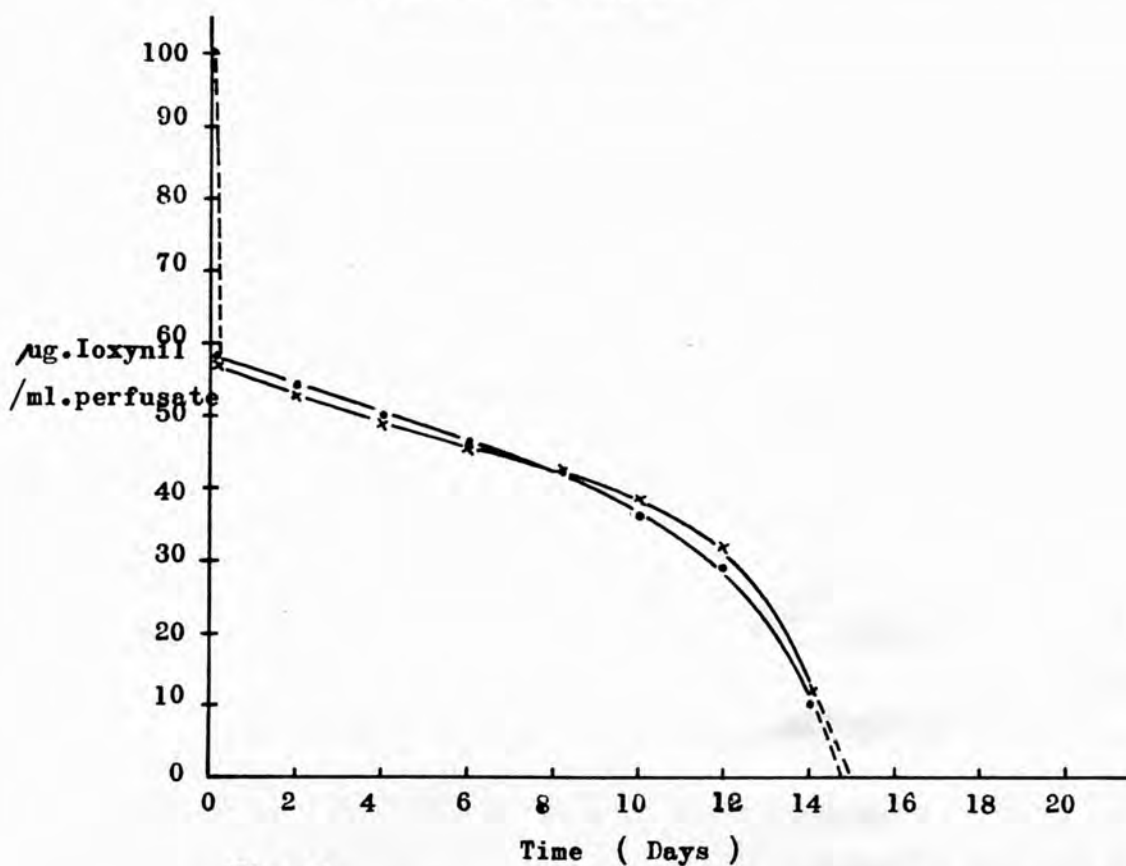


Fig. 41
Time course of detoxication of Ioxynil incorporated into the perfusate of a freshly perfused sample of air dried garden soil at an initial concentration of 100 μg/ml.perfusate

the length of the longest root from each tube (i.e. from a sample of 20 seeds) was measured and the root growth indices were then calculated for each dilution. The concentrations of Ioxynil corresponding to these root growth indices were obtained from the bioassay calibration curve (Fig. 40) and these concentrations were then plotted against time to give the graphic representation (Fig. 41) of the time course of detoxication of Ioxynil in these freshly perfused soil samples under laboratory conditions.

From the results presented in Figure 41 it is evident that when an aqueous solution of Ioxynil, containing 100 μg Ioxynil / ml. solution, was percolated through a well aerated and a water saturated column of air dried garden soil (40 gm.) maintained under laboratory conditions (15-18°C temperature) almost complete detoxication of the added Ioxynil was observed by the fifteenth day of perfusion. This observation could be considered as in close agreement with the findings of Zaki (1966) that Ioxynil was nearly completely broken down by the nineteenth day after application to non sterile soil. The results presented in Figure 41 also show clearly that the zero day concentration of Ioxynil, estimated an hour after the commencement of perfusion was only around 58 p.p.m. although the herbicide was incorporated into the perfusate at an initial concentration of 100 p.p.m. Thus it could be concluded that nearly 45-50% of Ioxynil applied to fresh soil was retained within the soil probably due to adsorption on to the colloidal particles.

Finally the possibility of restoring nitrifying activity to soils previously perfused with herbicides for a certain length of time, then washed thoroughly with several changes of water and reperfused with ammonium sulphate solutions only, were explored. Here the two herbicides Ioxynil and NPH 1320 were used at the usual concentrations (0, 10, 50, 100, 500 and 1000 p.p.m.) tested before and the time course of nitrification (i.e. NO_3^- and NO_2^- - N accumulation) was followed in each of the test soil samples that were previously perfused with the known concentrations of the par-

ticular herbicide in aqueous solution for a period of seven days, then washed and reperfused with ammonium sulphate solutions that did not contain any herbicide. The nitrate and nitrite-nitrogen accumulation data obtained with these 'washed' soil samples (i.e. the soil samples that were previously perfused with Ioxynil or NPH 1320 and then washed) on perfusion with ammonium sulphate solutions are presented in Figures 42 and 43. The maximum rates of accumulation of NO_3^- and $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ calculated from the experimental observations made with these 'washed' soil samples are given in Tables 9 and 10 respectively, while the time lags to attain the maximum rates of accumulation of $\text{NO}_3^- - \text{N}$ and $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ are given in Tables 11 and 12.

From the results presented in Tables 9 and 10, it can be seen that the soil samples which were previously perfused with water only and then reperfused with ammonium sulphate solution (controls) carried out NO_3^- and $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ formations at rates comparable to those observed when fresh soil samples were initially perfused with ammonium sulphate (i.e. the controls in the previous experiments). Soil samples pretreated with 10 p.p.m. and 50 p.p.m. Ioxynil too were seen to attain high rates of formation of NO_3^- and $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ comparable to the corresponding rates in the control soils; with only small amounts of nitrite being accumulated in the perfusates. In fact slight stimulations in the maximum rates of accumulation of $\text{NO}_3^- - \text{N}$ and $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ could be observed in these 'washed' soils pretreated with 10 p.p.m. and 50 p.p.m. concentrations of Ioxynil (Tables 9 and 10). These stimulations could have been due to a preferential inhibition of some other soil organisms, that may be competing with the nitrifiers in soil for similar growth requirements such as proliferation sites, being caused by the pretreatment with low Ioxynil concentrations.

The maximum rate of formation of $\text{NO}_3^- - \text{N}$ (expressed as % of control) observed in the washed soil sample pretreated with 100 p.p.m.

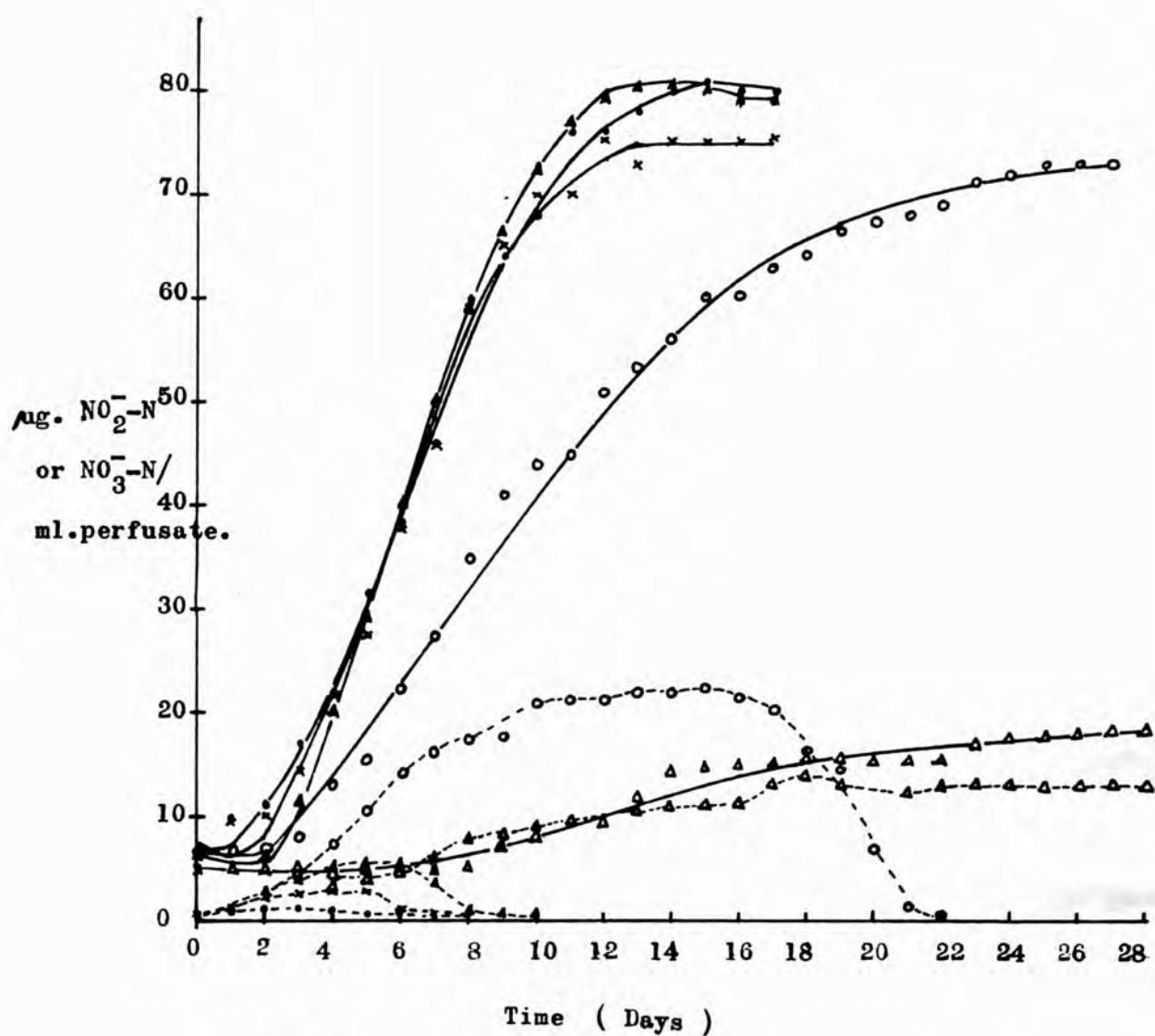


Fig. 42

Time course of accumulation of Nitrate - N (continuous lines) and Nitrite - N (broken lines) in a series of soil samples previously perfused with a range of cocentrations of Ioxynil in water, then washed and later perfused with Ammonium sulphate solutions.

● - control, x - 10 ppm, ▲ - 50 ppm, ○ - 100 ppm, △ - 500 ppm.

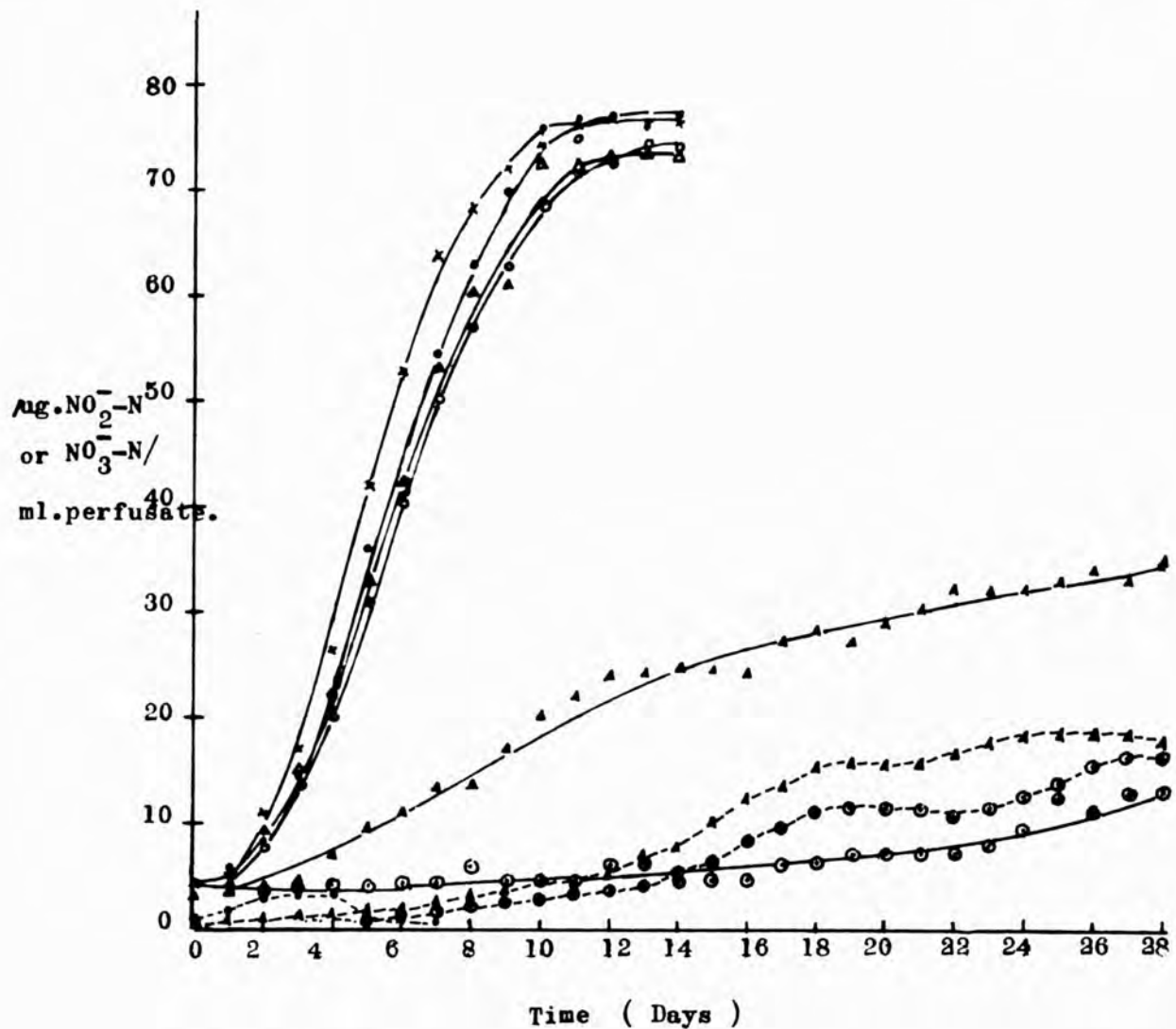


Fig. 43

Time course of accumulation of $\text{NO}_3^- - \text{N}$ (continuous lines) and $\text{NO}_2^- - \text{N}$ (broken lines) in a series of soil samples previously perfused with a range of concentrations of NPH 1320 in water, then washed and reperfused with Ammonium sulphate solutions.

● - control, x - 10 ppm, Δ - 50 ppm, ○ - 100 ppm,
▲ - 500 ppm. ⊙ - 1000 ppm

Table 9

Estimates of the maximum rates of formation of nitrate nitrogen ($\mu\text{g NO}_3^- - \text{N} / \text{ml. perfusate} / \text{day}$) attained in fresh soil perfused with aqueous solutions of herbicides for 7 days then washed and reperfused with ammonium sulphate solutions only.

Herbicide	Herbicide concentration in p.p.m.					
	Control	10	50	100	500	1000
Ioxynil	8.27	8.40	9.56	4.72	1.02	0.28
NPH 1320	9.56	11.06	8.99	8.31	1.83	1.52

Table 10

Estimates of the maximum rates of formation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ ($\mu\text{g NO}_3^- + \text{NO}_2^- - \text{N} / \text{ml. perfusate} / \text{day}$) attained in fresh soil perfused with aqueous solutions of herbicides for 7 days then washed and reperfused with ammonium sulphate solutions only.

Herbicide	Herbicide concentrations in p.p.m.					
	Control	10	50	100	500	1000
Ioxynil	8.43	8.83	10.12	7.62	1.77	0.50
NPH 1320	10.34	11.40	9.49	9.05	2.77	2.43

Table 11

Time lag (days) to reach maximum rates of formation of NO_3^- - N (Nitrobacter saturation) in soils previously perfused with herbicides for 7 days then washed and reperfused with ammonium sulphate solutions only.

Herbicide	Herbicide concentrations in p.p.m.					
	Control	10	50	100	500	1000
Ioxynil	5.6	5.4	5.4	7.6	11.4	∞
NPH 1320	5.2	4.4	4.9	5.4	7.4	∞

Table 12

Time lag (days) to reach maximum rates of formation of $(\text{NO}_3^- + \text{NO}_2^-)$ - N (Nitrosomonas saturation) in soils previously perfused with herbicides for 7 days then washed and reperfused with ammonium sulphate solutions only.

Herbicide	Herbicide concentrations in p.p.m.					
	Control	10	50	100	500	1000
Ioxynil	5.6	5.3	5.6	6.4	9.5	∞
NPH 1320	5.3	4.5	5.1	5.5	9.6	∞

observed by the results presented in Figure 41 which shows that nearly 50% of the Ioxynil incorporated into soil by percolation tends to be retained in soil probably due to adsorption on to the soil colloids.

On the other hand, the maximum rate of formation of $(\text{NO}_3^- + \text{NO}_2^-)$ - N in the perfusate of the washed soil pretreated with 100 p.p.m. Ioxynil solution was found to be high (in accordance with observed excessive NO_3^- - N

solution of Ioxynil was found to be around 57% (Table 9) whereas the maximum rate of formation of $\text{NO}_3^- - \text{N}$ (expressed as % of control) achieved in fresh soil that was continuously perfused with an Ioxynil solution of equivalent concentration was around 50% (Table 5). (These maximum rates of formation of $\text{NO}_3^- - \text{N}$ and the maximum rates of formation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ in the washed soil pretreated with 100 p.p.m. Ioxynil could also be taken as representing the respective maximum sizes of the saturating populations of Nitrobacter and Nitrosomonas spp., because excessive $\text{NO}_2^- - \text{N}$ accumulation could be observed in its perfusate.) The above mentioned comparable values for the maximum rates of formation of $\text{NO}_3^- - \text{N}$ in fresh soil continuously perfused with 100 p.p.m. solution of Ioxynil and in 'washed' soil pretreated with an equivalent concentration of the same herbicide could be due either to:

a) A permanent inhibitory effect, caused by the applied herbicide concentration, on the activities (i.e. on the metabolic rate/cell as well as the capacity to proliferate) of Nitrobacter populations,

or b) A fraction of the initially applied concentration of Ioxynil is being adsorbed and thus retained in soil even after washing, and this residual Ioxynil acts as the fraction effective in causing the observed inhibitions.

The first possibility (a) is most unlikely due to the observed onset of detoxication of Ioxynil with time, as seen from the results presented in Figure 41. But the second possibility (b) would be well substantiated by the results presented in Figure 41 which shows that nearly 50% of the Ioxynil incorporated into soil by percolation tends to be retained in soil probably due to adsorption on to the soil colloids.

On the other hand, the maximum rate of formation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ in the perfusate of the washed soil pretreated with 100 p.p.m. Ioxynil solution was found to be high (in accordance with observed excessive $\text{NO}_2^- - \text{N}$

accumulation in its perfusate, Fig. 42), of the order of 90% of the corresponding control. This high value for the maximum rate of formation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$, in 'washed' soil pretreated with 100 p.p.m. Ioxynil, as opposed to the comparatively low maximum rate of formation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ (equal to 50% of the corresponding control, Fig. 30) observed in the soil sample that was continuously perfused with Ioxynil solution of equivalent concentration does indicate that the Nitrosomonas population in soil had a higher tendency than the Nitrobacter population, to recover their metabolic activities after being under the influence of Ioxynil (100 p.p.m.) for an initial period of seven days. This observed higher tendency for the recovery of Nitrosomonas population growth, in the above washed soil sample under consideration, could be explained as at least being partly due to previous indication that the metabolic activities of Nitrosomonas populations in soil were less sensitive to the toxic effects of many herbicides at specific concentrations including 100 p.p.m. solution of Ioxynil (conclusion from the previous section of this chapter). It may also be that Ioxynil applied to soil at an initial concentration of 100 p.p.m. affected only the metabolic rate per cell and did not reduce the Nitrosomonas cell numbers that were originally present in the soil or inhibit the growth of this Nitrosomonas population during the seven day period of pretreatment, so that the entire original Nitrosomonas population could regain activity once the influence of Ioxynil was removed (at least partly) by washing, and the soil was then reperfused with ammonium sulphate solution. This possibility is supported by the observed fact that the rates of accumulation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ in enriched soil and in fresh soil, treated with 100 p.p.m. solutions of Ioxynil, were closely comparable, the respective rates expressed as percentage values of the corresponding controls being around 53% and 63%.

It is important to be mentioned here that the seven day period for pretreatment was chosen mainly to avoid, as much as possible, the restoration of nitrifying activity to treated soils being influenced by the detoxication

of Ioxynil, as it was previously shown in this chapter that 100 p.p.m. solution of Ioxynil incorporated into soil got detoxicated by about the fifteenth day of perfusion under the experimental conditions used in the present investigation.

It was also interesting to note that the time lag for the rates of accumulation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ to reach maximum values (i.e. the time lag to attain saturation with respect to Nitrosomonas populations) in the 'washed' soil samples that were pretreated with Ioxynil solutions of concentrations ranging up to 100 p.p.m. (i.e. 10, 50 and 100 p.p.m.) were almost equal to the control value (Table 12) indicating again that the viable cell numbers of Nitrosomonas that were originally present in the soil and from which the saturation population is derived on enrichment, were not much affected by the pretreatment of fresh soil samples with these concentrations of Ioxynil. But from Table 11 it is evident that the time lag for the rate of formation of $\text{NO}_3^- - \text{N}$ to reach maximum value in 'washed' soil pretreated with 100 p.p.m. Ioxynil (i.e. the time lag for the establishment of saturating populations of Nitrobacter) was increased by about 35%, with respect to the corresponding control value, indicating that the viable Nitrobacter cell numbers were reduced to some extent by the pretreatment with 100 p.p.m. Ioxynil.

On the other hand, the extremely low values for the maximum rates of formation of $\text{NO}_3^- - \text{N}$ and $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ (Tables 9 and 10) observed in 'washed' soil pretreated with 500 and 1000 p.p.m. solutions of Ioxynil indicated that the initial perfusion with these high concentrations of Ioxynil effectively sterilised the soil samples as regards their nitrifier populations and thus only very low nitrification rates (very little or no growth of nitrifiers) were observed even after 28 days of subsequent perfusion with ammonium sulphate solutions alone.

The progress curves presented in Figure 43 as well as the nitrification rates presented in Tables 9 and 10 clearly indicate that the

normal nitrifying capacities were immediately restored to the soil samples pretreated with 10, 50 and 100 p.p.m. NPH 1320, then washed and subsequently perfused with ammonium sulphate solutions. These observations along with the comparable lengths of the time lag to attain maximum rates of nitrification (Tables 11 and 12) in the control and these pretreated soil samples suggest that the initial perfusion, for a period of seven days, with NPH 1320 at concentrations up to 100 p.p.m. did not affect the cell numbers of Nitrosomonas and Nitrobacter which were originally present in soils and thus the normal nitrifying activities were regained in these soils when perfused with ammonium sulphate solutions after the removal of the inhibitor by washing. This rapid recovery would also indicate that NPH 1320 incorporated into soil could be removed by washing with water unlike the previously discussed herbicide Ioxynil.

The 'washed' soil samples pretreated with 500 p.p.m. NPH 1320 showed moderately high maximum rates of formation of $\text{NO}_3^- - \text{N}$ and $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ (19% and 27% respectively) when compared with the low maximum rates of formation of $\text{NO}_3^- - \text{N}$ and $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ (12% and 21% respectively) observed in 'washed' soil samples pretreated with 500 p.p.m. Ioxynil. This would also mean that the pretreatment with NPH 1320 even at concentrations as high as 500 p.p.m. did not completely sterilise the soil with respect to their nitrifying populations unlike in the case of Ioxynil pretreatment, at equivalent concentrations. Even here the accumulation of nitrite -N observed in the perfusates and the correspondingly high maximum rate of formation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ indicated that the Nitrosomonas population was less sensitive, than the Nitrobacter population, to this pretreatment with 500 p.p.m. NPH 1320. However the fact that the rate of formation of $\text{NO}_3^- - \text{N}$ as well as the rate of accumulation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ did not reach considerably high values in washed soils pretreated with 1000 p.p.m. NPH 1320, even after 28 days of perfusion with ammonium sulphate, could only be due to the viable cell numbers of Nitrobacter and Nitrosomonas

being greatly reduced by pretreatment.

It must be mentioned here that failure of soils to carry out nitrification has very often been found to be essentially a consequence of acidity and therefore the pH of the perfusate at the beginning and at the end of every perfusion experiment (including those dealt with in Chapter 3) was measured by the electrometric method described by Reed & Cummings.⁽¹⁹⁴⁵⁾ It was found that the initial pH of the perfusates was always around 7.2 whereas their final pH values never fell below 6.9. Although it is well known that the nitrifiers in soil work best around neutral pH values of the soil solution, it was assumed that the fluctuations of the pH observed during perfusion experiments were close enough to neutrality so as to allow the observed effects on the nitrification rates in treated soils to be attributed to the herbicides used.

In the present investigation an attempt was made to assess the effects of some herbicides on the respiratory activity of soil animals, enriched with nitrifying organisms by continuous perfusion with nitric sulphate solutions.

Materials and experimental procedures

Soil used in these laboratory experiments was prepared by autoclaving the soil with ammonium sulphate solution during the usual perfusion apparatus as described before, until a relatively high and constant rate

CHAPTER 5

Effect of Herbicides on Oxygen Uptake by Enriched Soils

Introduction

As early as 1932 Smith & Brown made use of a modified Barcroft differential respirometer to measure the activity of micro-organisms in soil. Since then the application of manometric techniques to determine the respiratory activities of soil micro-organisms have received very little attention until 1947 when Webley & Quastel reported the application of Warburg respirometric technique to measure the degree of soil aggregation indirectly by determining the rate of diffusion of oxygen into the soil. Following this Quastel & Scholefield (1949, 1951) have employed the Warburg respirometer in studying the activity of nitrifying organisms while Lees (1949) made use of another type of modified respirometer to follow the general oxidative activity of the soil population. A few years later Gamble et al. (1952) applied respirometry to determine the effects of herbicides on soil organisms. More recently Caseley & Broadbent (1968) have reported on the effects of some fungicides on the overall biological activity in soil as measured by the rate of oxygen uptake in treated soil samples, whereas Hale, Hulcher & Chappell (1957) and Debona (1967) used this same respirometric method to study the effect of several herbicides on nitrification in field soils under laboratory conditions.

In the present investigation an attempt was made to assess the effects of some herbicides on the respiratory activity of soil samples, enriched with nitrifying organisms by continued perfusion with ammonium sulphate solutions.

Materials and experimental procedure

Soil used in these manometric experiments was prepared by percolating the soil with ammonium sulphate solutions (using the same perfusion apparatus as described before) until a relatively high and a constant rate

of nitrification was attained through several changes of percolating solution. At the end of this period, usually around three weeks, the ammonium sulphate solutions in the reservoirs were replaced with distilled water and the enriched soil samples were then washed with several changes of water until no nitrate or ammonium ions could be detected in test samples of the leachates. The tubes carrying the percolating solutions were then disconnected and the excess water in the soil samples were allowed to get drained off for some time before they were removed from the tubes and spread over filter paper for drying. After air drying for half an hour (the final water content being approximately 25%) the soil aggregates were chopped and mixed well with a spatula so as to form small discrete crumbs rather than a mud.

1.5 g. samples of the soil thus prepared were introduced into Warburg manometer flasks, care being taken to prevent any soil entering the centre well or the side arm of these flasks. 1.05 ml. of phosphate buffer, pH 7.8, plus 0.5 ml. of herbicide solution or 0.5 ml. water were added into the main reaction chambers while 0.5 ml. of 50 mM ammonium sulphate solution was placed in the side arm of the individual flasks. 0.2 ml. of 20% KOH solution along with a 2 cm. square filter paper wick was also placed in the centre well before the manometers were set up and shaken in the ordinary way as described by Umbreit et al. (1949). The temperature of the water bath was maintained at 26°C and after the equilibration period of 15-30 minutes the ammonium sulphate solution from the side arm was tipped in. Then the oxygen uptake by the soil samples were measured at 10 minute intervals over a period of 1½ to 2 hours. The contents of a single enriched soil column were used for each experiment carried out with a series of concentrations of a particular herbicide and control experiments were also run, along with each of these series, using soil suspended in water only.

Based on the work reported by Hale et al. (1957) the volume of each 1.5 gm. sample of enriched soil used in these experiments was taken to

be equal to 0.45 c.c. in calculating the flask constants. The final volume of the reactants in each of the flasks was thus 2.5 c.c. and the herbicide solutions were made up to contain five times the desired concentrations of the active ingredients so as to allow for dilution when added into the flasks in 0.5 ml. aliquots. In this part of the present investigation too all the herbicides that have been tested in the previous sections were used with the only exception of Terbacil.

Results

The rates of respiration of the different soil samples, in the presence and in the absence of known concentrations of different herbicides and also the rates of respiration in control soil samples suspended in water only were calculated in terms of μ litres of oxygen consumed / reaction vessel / minute. These calculations were done by means of a linear regression analysis of the oxygen uptake data, obtained with individual soil samples used in the experiments described above, and the rates thus calculated are presented in Table 13 whereas Figure 44 represents a typical set of regression lines fitted for oxygen uptake data obtained with enriched soil samples treated with a range of concentrations of Legurame. All the rates presented in Table 13 were then expressed as percentage values of the corresponding ammonium sulphate controls and plotted against logarithms of the herbicide concentrations used. Figures 45 and 46 show such a graphic representation of the variation of rates of oxygen uptake in soil samples enriched by previous perfusion with ammonium sulphate and later made to respire in the presence of ammonium sulphate (containing known concentrations of herbicides) as the respiratory substrate, with logarithms of the corresponding herbicide concentrations.

Drawbacks associated with this approach

In all the experiments the rates of oxygen uptake by samples of

Table 13

Rates of oxygen uptake (ul. O₂ consumed / vessel / min.) by enriched soil (perfused with Ammonium sulphate during enrichment) supplied with Ammonium sulphate as substrate in the presence of a range of concentrations of different herbicides.

Herbicides used	Herbicide concentrations in p.p.m.							
	Control	10	50	100	250	500	1000	
Ioxynil	0.276	0.258	0.246	0.173				
	0.340				0.158	0.095	0.027	
Totril	0.428	0.373	0.360	0.352	-	0.278	0.183	
Bromoxynil	0.137	0.116	0.106	0.102	0.060	0.046	0.039	
NPH 1320	0.291	0.233	0.218	0.184	-	0.140	0.124	
Legurame	a)	0.223	0.198	0.174	0.163	-	0.138	0.097
	b)	0.256	0.222	0.198	0.182	-	0.153	0.089
Trifluralin	0.253	0.246	0.201	0.173	-	0.118	0.101	
Oxadiazon	0.274	0.263	0.262	0.199	-	0.183	0.144	
Tricamba	0.304	0.288	0.271	0.257	-	0.247	0.194	
Dicamba	0.272	0.263	0.253	0.248	-	0.158	0.138	
M & B 9057 (Asulam)	0.280	0.263	0.243	0.237	-	0.201	0.201	
M & B 9555	0.450	0.483	0.428	0.337	-	0.350	0.272	

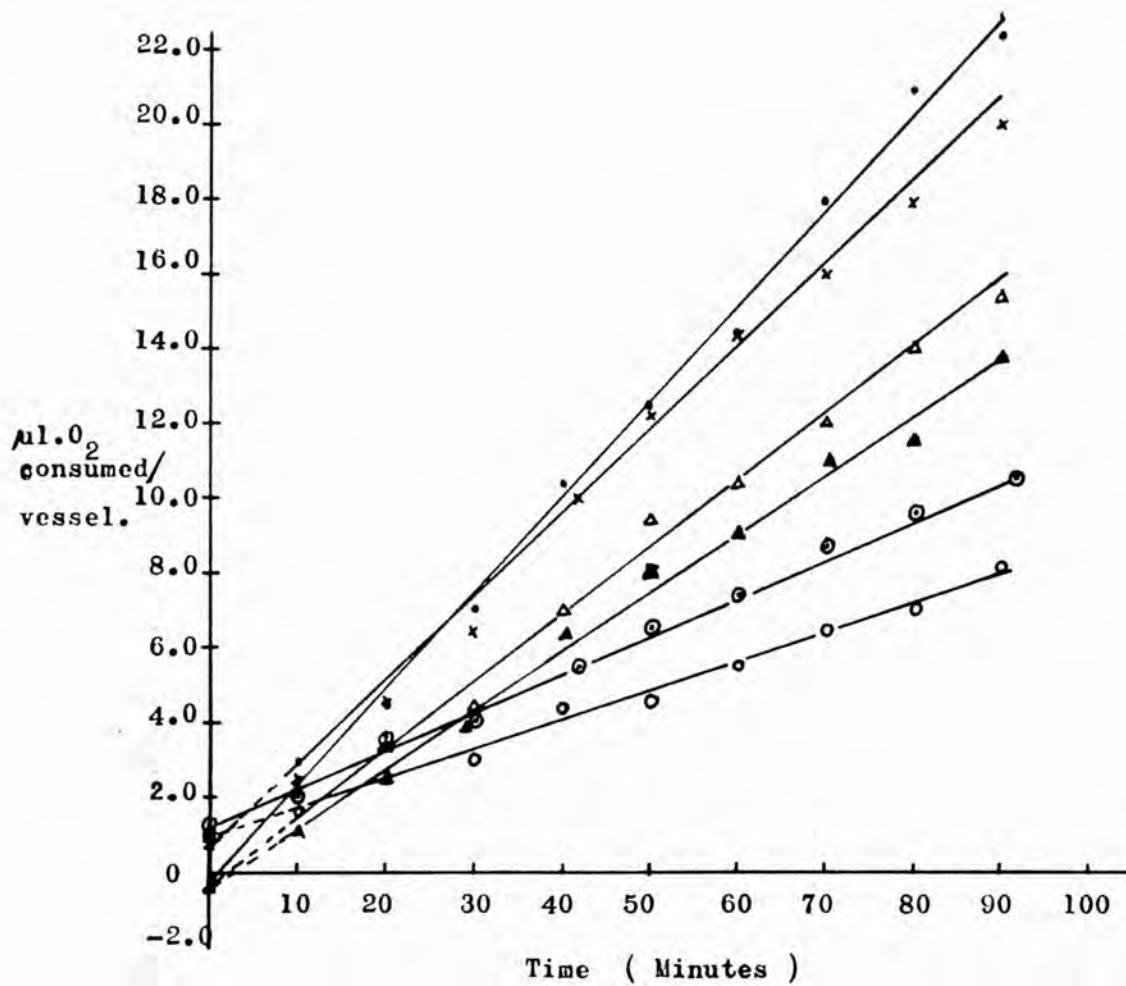


Fig. 44

Rates of Oxygen uptake ($\mu\text{l.O}_2/\text{vessel}/\text{min.}$) by enriched soil (perfused with Ammonium sulphate solutions during enrichment) supplied with 10^{-2}M Ammonium sulphate solutions containing a range of concentrations of Legurame.

• - control, x - 10 ppm, Δ - 100 ppm,
 ▲ - 500 ppm, ○ - 1000 ppm, ⊙ - endogenous respiration.

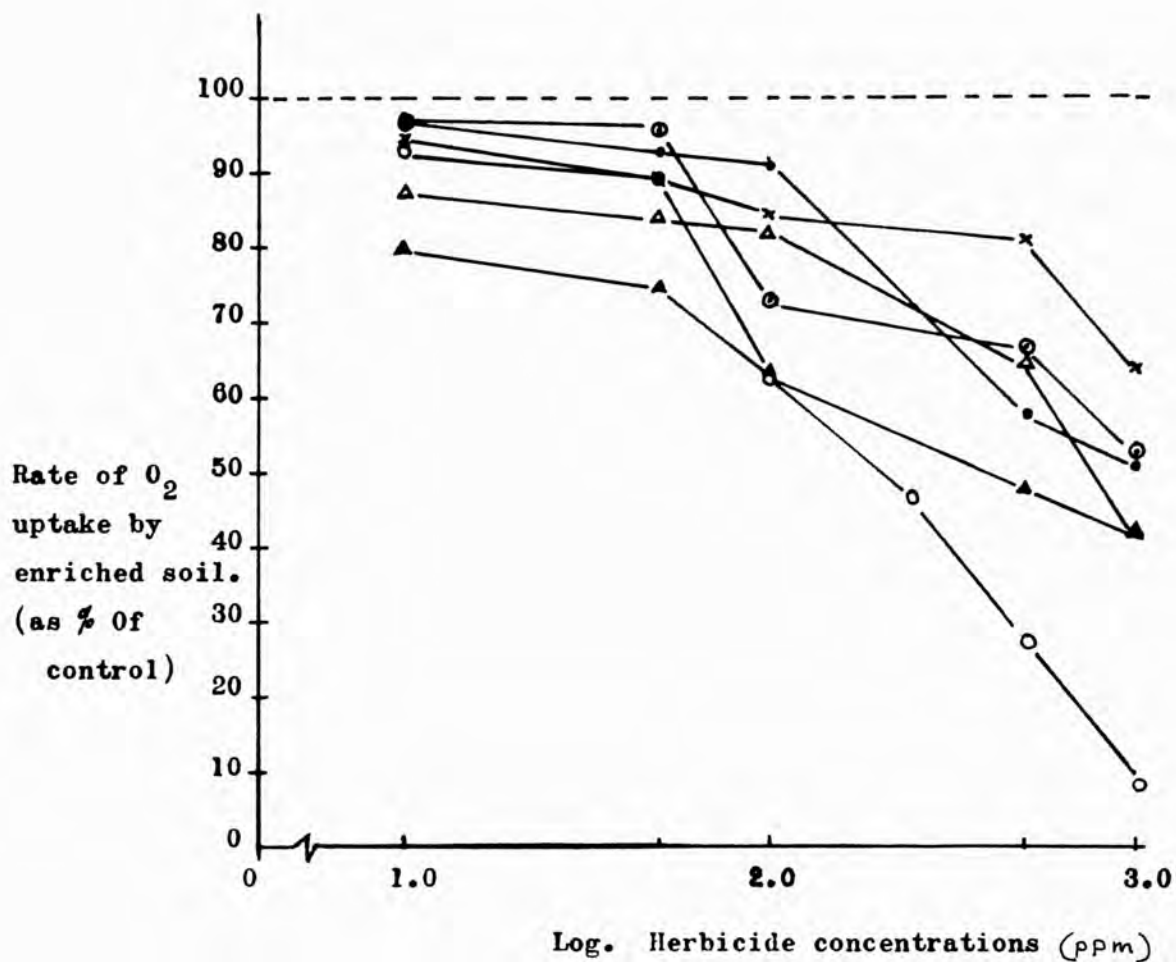


Fig. 45

Rates of Oxygen uptake ($\mu\text{l. O}_2$ consumed/vessel/min.) by enriched soil when supplied with $10 \times 10^{-3} \text{ M}$ Ammonium sulphate solutions containing different herbicides at known concentrations. (All rates expressed as percentage values of the corresponding controls.)

▲ - NPH 1320, Δ - Totril, ○ - Ioxynil,
 ⊙ - Oxadiazon, ● - Dicamba, x - Tricamba.

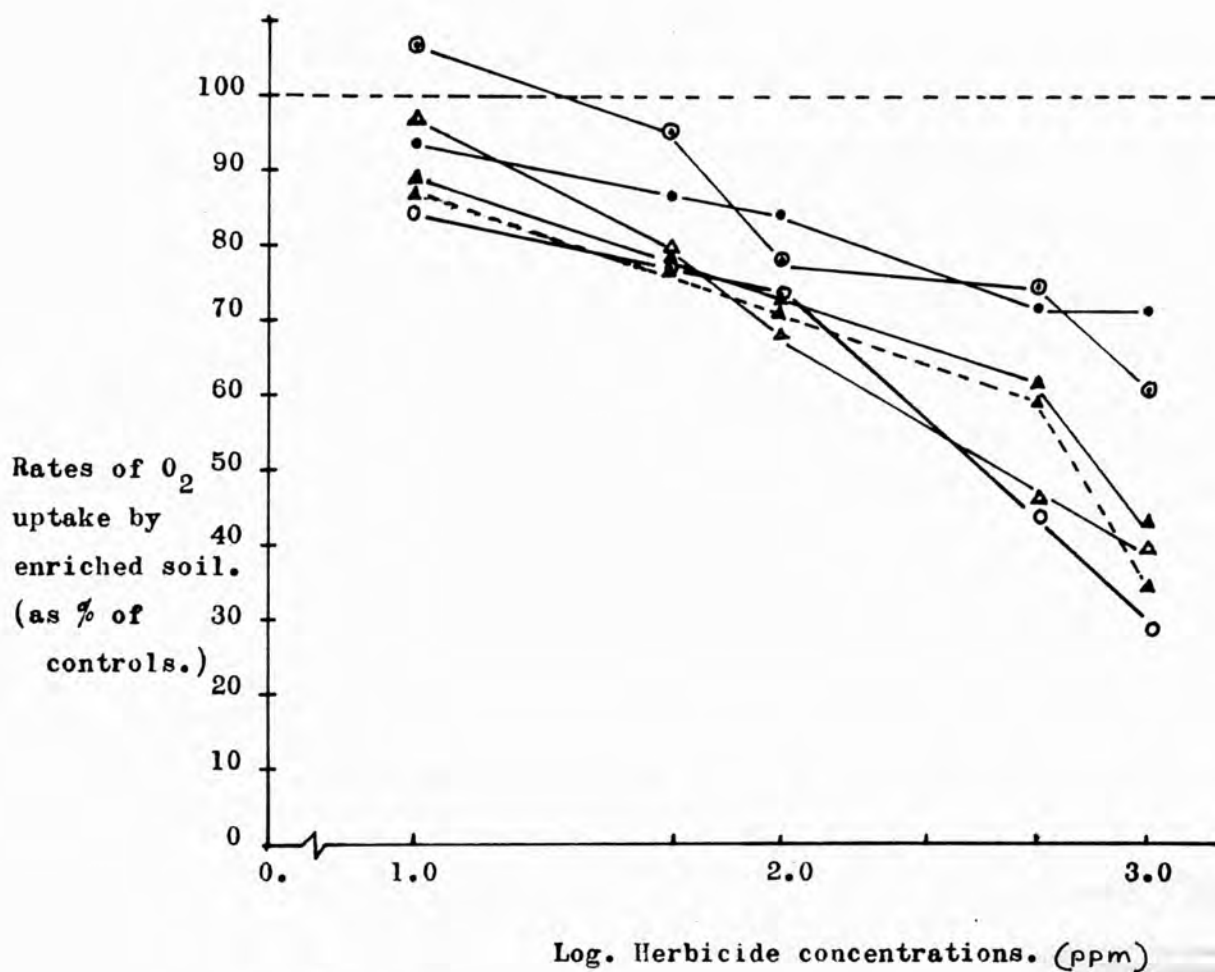


Fig. 46

Rates of Oxygen uptake ($\mu\text{l. O}_2$ consumed/vessel/min.) by enriched soil when supplied with $10 \times 10^{-3} \text{ M}$ Ammonium sulphate solutions containing different herbicides at known concentrations. (All rates expressed as percentage values of the corresponding controls.)

○ - Bromoxynil, ▲ - Legurame (replicate)

△ - Trifluralin, ● - M&B 9057, ⊙ - M&B 9555.

enriched soil* suspended in water only (i.e. in the absence of added ammonium ions which serve the specific inorganic substrate requirement for nitrifiers) greatly exceeded the expected low values for the endogenous rates of respiration. For example it could be seen clearly from Figure 44 that the rate of endogenous respiration in enriched soil suspended in water was around 50% of the respiration rate in control soil sample suspended in ammonium sulphate solution. This same situation was true in the case of all the other soil respiration experiments carried out with enriched soil, the lowest rate of endogenous respiration observed being around 41%. These high rates of endogenous respiration would have been due to many factors namely:

- 1) High rates of endogenous respiration of nitrifiers (formed as a result of enrichment) plus a wide range of other heterotrophic soil micro-organisms that would have been originally present in the soil.
- 2) There could have been enough substrate from natural sources in the soil to sustain a high rate of respiration of the heterotrophic organisms, other than the saturating populations of nitrifiers, thus resulting in overall high rates of respiration even in the absence of any ammonium ion substrate.
- and 3) Although the soil samples were washed with water (after enrichment) until no ammonium or nitrate ions could be detected in the leachate, this leaching process might not have removed all the ammonium ions from these soils that were previously perfused with ammonium sulphate, since NH_4^+ ions have a great tendency to remain adsorbed on to soil colloids and on to the surfaces of soil particles in addition to being retained within the cells of the soil organisms.

Blumenthal, Kaffler & Goldschmidt (1952) working on the effects

* Enriched soil refers to soils enriched by previous perfusion with several changes of ammonium sulphate solutions.

of exogenous substrate oxidations on the rates of endogenous respiration of micro-organisms have shown that correction for endogenous respiration rates is not always valid especially when the endogenous respiration rates are relatively high as compared to their total respiration rates in the presence of a substrate. It must be mentioned here that in the case of the soil respiration experiments reported in the present investigation too, few attempts to make a correction for the endogenous respiration rates resulted in erratic variations which could not be explained.

Based on the above findings and considering the enriched soil samples as complete biological systems having high rates of endogenous respiration (since the endogenous respiration rates were high as discussed before) no corrections for endogenous respiration were made while tabulating the rates of oxygen consumption presented in Table 13. Thus in discussing the results it must be borne in mind that the respiration rates presented in Table 13 give only a measure of the total amount of oxygen consumed by saturating populations of nitrifiers in soil during the oxidation of added exogenous substrate (i.e. ammonium ions) along with the amount of oxygen consumed due to endogenous respiration and also the oxidation of any other substrates, naturally present in these soils, by the heterotrophic organisms.

Conclusions

It is apparent from Table 14 that Ioxynil and Bromoxynil are once again the most toxic herbicides, just as in the two previous cases, and cause 50% inhibition in the rate of oxygen uptake by enriched soil samples at fairly low concentrations of 185 p.p.m. (4.98×10^{-4} M) and 340 p.p.m. (12.19×10^{-4} M) respectively (estimated by probit analysis) when compared with the I_{50} concentrations for all the other herbicides tested here. As seen from Figure 45, Ioxynil was effective in reducing the rates of respiration (expressed as percentage of control) in treated soil samples to very low values of the order of 28% and 8% at respective concentrations

Table 14

The concentrations of different herbicides effective in causing 50% inhibition (I_{50}) in the rate of oxygen uptake by enriched soil supplied with ammonium sulphate as respiratory substrate.

Herbicides used	I_{50} concentrations	
	p.p.m.	molar
Ioxynil	185	4.98×10^{-4}
Bromoxynil	340	12.2×10^{-4}
NPH 1320	* 1000	$\gg 24.7 \times 10^{-4}$
Totril	* 1000	$\gg 20.0 \times 10^{-4}$
Tricamba	* 1000	$\gg 39.4 \times 10^{-4}$
Dicamba	* 1000	$\gg 45.2 \times 10^{-4}$
Oxadiazon	* 1000	$\gg 29.0 \times 10^{-4}$
M & B 9057	* 1000	$\gg 53.8 \times 10^{-4}$
M & B 9555	* 1000	$\gg 43.9 \times 10^{-4}$
Legurame	675	28.6×10^{-4}
Trifluralin	530	15.8×10^{-4}

* These were the maximum concentrations tested.

equivalent to 500 p.p.m. and 1000 p.p.m. However the inhibitions caused by Bromoxynil were comparatively less than those caused by Ioxynil and the rates of oxygen uptake by soil samples treated with Bromoxynil remained around 44% and 28% while being under the influence of concentrations equivalent to 500 and 1000 p.p.m. respectively.

Considering the herbicides tested in the order of their toxic effects it becomes evident from Table 14 that Trifluralin and Legurame having I_{50} concentrations around 530 p.p.m. ($15.8 \times 10^{-4}M$) (estimated by eye) and 675 p.p.m., active ingredient, ($28.6 \times 10^{-4}M$) (estimated by probit analysis) would come next. Even so it can be seen from Figure 46 that the rates of oxygen uptake in soil samples treated with Legurame were considerably high being 61% at 500 p.p.m. and 38% at 1000 p.p.m. concentration.

All the other herbicides tested, namely Totril, NPH 1320, Tricamba, Dicamba, Oxadiazon, M & B 9057 and M & B 9555 were found to have I_{50} values beyond the maximum concentrations (1000 p.p.m.) tested.

As was explained earlier in this chapter, the rates of respiration (expressed as percentage values of corresponding controls) plotted in Figures 45 and 46 are in fact the rates of oxygen uptake by the whole population of micro-organisms present in an enriched soil sample whose main component constitutes the nitrifying organisms. This means that if the herbicide added into the soil samples at a particular concentration is effective only in decreasing or inhibiting the oxygen consumption of a specific fraction of the population then the rates of oxygen uptake by the remaining fraction of the total population would take place unhindered and thus the observed rates of respiration even in the treated soil samples would be considerably high. Therefore it is clear that the specific inhibitors will prove to be less effective in inhibiting the rates of oxygen consumption, by an enriched soil sample, to the same extent as would be caused by any non specific inhibitor of the same effectiveness.

The fact that herbicides such as Totril and NPH 1320 causing

50% inhibition of ammonium oxidations in enriched soils at concentrations around 500 p.p.m. ($10 \times 10^{-4}M$) and 200 p.p.m. ($4.9 \times 10^{-4}M$) (from Table 4) respectively does not reduce the rate of oxygen uptake by these soils, supplied with a similar substrate of ammonium ions, to 50% even when treated with concentrations as high as 1000 p.p.m. could mean that these herbicides possess some degree of selectivity as regards their effects on the nitrifying population in soil. Although this reasoning could be extended to other herbicides such as Legurame, Oxadiazon and Trifluralin which are about two to five times less effective in inhibiting rates of oxygen consumption in enriched soil as compared to the nitrification rates in similarly treated soils, this alone may not be a satisfactory explanation for the observed high values for I_{50} concentrations.

However it is apparent from the results presented in this chapter that the treatment of enriched soil with inhibitory concentrations of different herbicides for short periods, not longer than $2\frac{1}{2}$ hours, did not completely suppress or kill all the organisms present in these soil samples and thus some degree of respiration did occur even when the enriched soil samples were treated with very high concentrations ranging up to 1000 p.p.m. of the herbicides.

CHAPTER 6

Effects of some Herbicides on Nitrification in Pure Cultures of Nitrosomonas and Nitrobacter

Introduction

The original work of Schloesing & Muntz (quoted by Stephenson, 1936) has proved beyond doubt the biological nature of the oxidation of ammonium ions to nitrates in soils. Later many workers have shown that at least in soils of neutral to slightly alkaline reaction the bulk of this biological oxidation resulting in the conversion of ammonium to nitrites and then to nitrates is achieved mainly by the two genera of bacteria Nitrosomonas and Nitrobacter respectively. Thus an interest has been developed over the past ten years or more, to study the biochemical aspects of these chemoautotrophic nitrifying bacteria.

In spite of this growing interest, aerated liquid cultures of these nitrifying organisms have been very infrequently or seldom used as test systems in studying the effects of herbicides on nitrification, the main reason being the difficulties associated with maintaining ~~continuous~~ cultures under completely autotrophic conditions. Another problem associated with pure culture studies is the difficulty in cultivating adequate amounts of cells since the 'generation time' for these bacteria is around 10-11 hours and the yields are not even one hundredth of those for heterotrophic bacteria. Even in continuous cultures fitted with pH stat units to control the pH of the medium at 7.5, especially in growing Nitrosomonas cultures which produces copious amounts of nitrous acid, there has been the danger of a 'wash-out' of cultures due to meagre growth.

Most of the studies dealing with the testing of various pesticides for their effects on nitrification have made use of field trials or determinations using the perfusion unit developed by Lees & Quastel (1946b). But as was discussed in Chapter 1, the drawback in using these methods to assess the effects of herbicides on the nitrifying capacity of the organisms

concerned is that most if not all the herbicides are likely to undergo a number of changes once they come in contact with soil. Whatever these changes are they will in one way or the other affect the activity of the added herbicide. Therefore experiments in which nitrification could be followed in pure cultures of Nitrosomonas and Nitrobacter had to be set up to overcome the above difficulty and get an idea about the precise effect of a particular herbicide on the two main steps involved in this process.

The inhibitory actions of various compounds including few herbicides have been studied by means of experiments in which parallel inoculations of Nitrosomonas or Nitrobacter were made into two batches of culture medium, for the respective organisms, similar in all respects except that one batch contained the herbicide or any other chemical 'X' under investigation while the other did not. After a considerable length of time, usually a number of days or a few weeks, the amount of NO_2^- - N formed in Nitrosomonas cultures and the amount of NO_2^- - N disappeared from Nitrobacter cultures in the presence as well as in the absence of the added inhibitor have been determined in these experiments. If the amount of NO_2^- - N formed or oxidized in the presence of the added substance 'X' was less than in its absence, then the added substance was considered to inhibit nitrification by Nitrosomonas and Nitrobacter in pure cultures. Such experiments however do not give an indication of how 'X' acted. If for instance 'X' inhibited ammonia or nitrite oxidation directly, then less nitrite would be formed or oxidized in the presence of 'X' than in its absence. But if 'X' had no effect on ammonia or nitrite oxidation and yet inhibited cell proliferation in some other way, the neogenesis of ammonia and nitrite oxidising enzymes in Nitrosomonas and Nitrobacter cells respectively, would be less rapid in the presence of 'X' than in its absence.

Since we know that the generation time of these autotrophic nitrifying bacteria is around 10-11 hours, short term nitrification experiments lasting not more than two hours were carried out in the present

investigation using cell suspensions of Nitrosomonas and Nitrobacter separately. During these short term experiments any inhibitions or stimulations observed in the nitrification rates would not be complicated to a great extent by the effects of the herbicides on cell proliferation since in cultures of these organisms, having long generation times, there would be very little proliferation taking place within the experimental periods of two hours. For instance the error involved due to any effects on proliferation during a two-hour period would only be about 15-20% assuming that the generation time for both Nitrosomonas and Nitrobacter populations range from 10 to 11 hours.

At the time of conducting these experiments there was no information available on similar experiments being carried out to determine the effects of herbicides on the nitrifying capacity of Nitrosomonas and Nitrobacter cells grown in liquid media.

Materials and experimental procedures

Pure cultures of Nitrosomonas Europaea and Nitrobacter Winogradski were both obtained from Rothamsted Experimental Station, Harpenden, Herts., and were grown separately in liquid media of the compositions given in Chapter 2. The cells were harvested separately from rapidly growing batch cultures and resuspended in phosphate buffer pH 7.8 as was also discussed in Chapter 2.

a) Effects on conversion of $\text{NH}_4^+ \rightarrow \text{NO}_2^-$ by Nitrosomonas Europaea

2 ml. samples of a Nitrosomonas cell suspension were added to 1 ml. samples of a medium (pH 7.8) similar to that used in cultivating them and incubated at 28°C with a range of concentrations (0, 5, 10, 50, 100, 250, 500 and 1000 p.p.m.) of the herbicides under investigation. The different dilutions of the herbicide solutions were made so as to have a concentration of active ingredient equivalent to four times that needed in a particular reaction mixture and 1 ml. portions of these solutions were added to the appropriate reaction vessel to make the final volume of the

reaction mixtures equal to 4 ml., after all the additions being made. The incubations were carried out in large boiling tubes which were kept plugged with cotton wool to reduce any evaporation during the incubating period.

After 90 minutes of incubation the contents of each of the reaction vessels were diluted to a suitable volume (so that the NO_2^- - N concentration / ml. solution does not exceed 5 $\mu\text{g.}$) and the nitrite -N concentrations in each of these mixtures were estimated using Griess Ilosvay's reagent, as described before in Chapter 2.

b) Effects on conversion of $\text{NO}_2^- \rightarrow \text{NO}_3^-$ by Nitrobacter Winogradski

The Nitrobacter cells were harvested and the cell suspensions were made in exactly the same manner as described before. The experimental details regarding the setting up of the whole series of reaction mixtures were similar to those of the previous case (a) with the only two exceptions being,

- 1) the use of Nitrobacter cell suspensions in place of Nitrosomonas,
- and 2) the medium employed in cultivating Nitrobacter Winogradski and containing $0.65 \times 10^{-3} \text{M NaNO}_2$ was used in place of the medium containing $10^{-3} \text{M (NH}_4)_2\text{SO}_4$ that was added into the reaction mixtures of the previous set up.

After 60 minutes of incubation at 28°C the concentrations of the nitrite -N remaining in each of the reaction mixtures were estimated using the Griess Ilosvay's technique. These concentrations of nitrite estimated for each of the reaction mixtures were subsequently subtracted from the original concentration of nitrite -N added and the differences between these values were taken as indicating the amounts of nitrite oxidised / 60 minutes by the corresponding cell suspensions. Control reaction mixtures, not containing any added herbicide, were always set up in conjunction with each set of reaction mixtures that were under the influence of known concentrations of different herbicides.

Random repeat experiments were carried out with both Nitrosomonas and Nitrobacter cell suspensions to check on the reproducibility of results.

Results

The amounts of nitrite -N formed ($\mu\text{g NO}_2^-$ - N formed / reaction vessel / 90 minutes) by cell suspensions of Nitrosomonas Europaea that were supplied with ammonium ions in the presence and in the absence of each of the fourteen different herbicides under investigation are given in Table 15, whereas the amounts of nitrite -N oxidised ($\mu\text{g NO}_2^-$ - N oxidised / reaction vessel / 60 minutes) by cell suspensions of Nitrobacter Winogradski supplied with sodium nitrite in the presence and in the absence of the same range of herbicides are presented in Table 16.

The experimental values for the rates of nitrification by pure cultures of Nitrosomonas Europaea (from Table 15) and by pure cultures of Nitrobacter Winogradski (from Table 16) were then expressed as percentages of the corresponding controls and plotted against the logarithms of the appropriate herbicide concentrations as shown in Figures 47, 48 and Figures 49, 50 respectively.

It can be seen from the Figures 47, 48, 49 and 50 that the inhibition curves for all the herbicides tested in this section of the present investigation followed more or less sigmoid paths indicating highest nitrification rates, almost equivalent to 100% of the corresponding controls (i.e. very little or no inhibitions) at the lowest herbicide concentration tested (5 p.p.m.) and very little or virtually no nitrification (100% inhibition) taking place at the highest concentrations (1000 p.p.m.) of the more toxic herbicides tested. The rates of nitrification by Nitrosomonas and Nitrobacter cell suspensions (as measured by the rates of formation of nitrite -N and the rates of oxidation of nitrite -N respectively and expressed as percentage values of the corresponding controls) at the intermediate concentrations of the different herbicides were extremely variable depending on the herbicides and their specific toxicities towards the

Table 15

Nitrite formations in washed cell suspensions of Nitrosomonas Europaea incubated for 90 min. at 25°C in the presence of a range of concentrations of different herbicides and $10^{-5}M(NH_4)_2SO_4$

(All estimates are in terms of $\mu g NO_2^- - N$ formed / reaction mixture / 90 minutes and represent the mean of two readings.)

Herbicides		Herbicide concentrations in p.p.m.							
		Control	5	10	50	100	250	500	1000
Ioxynil	P	10.05	10.15	10.05	9.55	7.80	2.65	1.05	0.00
Bromoxynil	P	13.05	12.60	12.15	11.93	11.70	4.20	2.13	0.25
NPH 1320	F	10.20	10.00	9.18	6.45	4.98	2.38	0.52	0.00
Totril	F	10.00	9.70	9.38	7.75	5.50	2.20	0.60	0.00
9057 M & B	P 1)	10.45	9.80	9.60	6.73	4.18	0.93	0.00	0.00
	2)	10.20	9.60	8.70	6.90	3.23	2.23	2.10	-
9555 M & B	P	11.00	11.00	11.75	11.20	9.75	8.15	6.155	4.18
Legurame	F	10.58	10.58	10.13	6.23	3.13	0.33	0.00	0.00
Oxadiazon	F	10.75	10.58	9.23	3.75	0.00	0.00	0.00	0.00
Trifluralin	F	12.53	11.03	10.35	5.15	3.00	0.00	0.00	0.00
Dicamba	P	9.40	9.00	8.80	5.10	3.00	0.10	0.10	0.10
Tricamba	P	9.50	8.75	8.25	4.75	0.19	0.11	0.11	0.11
Chlorbufam	P *	12.00	12.00	11.76	10.15	8.50	4.80	1.40	0.00
Phenmedipham	P *	12.00	12.00	11.78	9.90	7.54	2.90	2.23	1.51
Terbacil	P *	12.00	12.00	11.40	9.54	7.74	3.36	0.66	0.00

* These herbicides with low solubilities were added directly into the reaction mixtures in appropriate quantities so as to form suspensions.

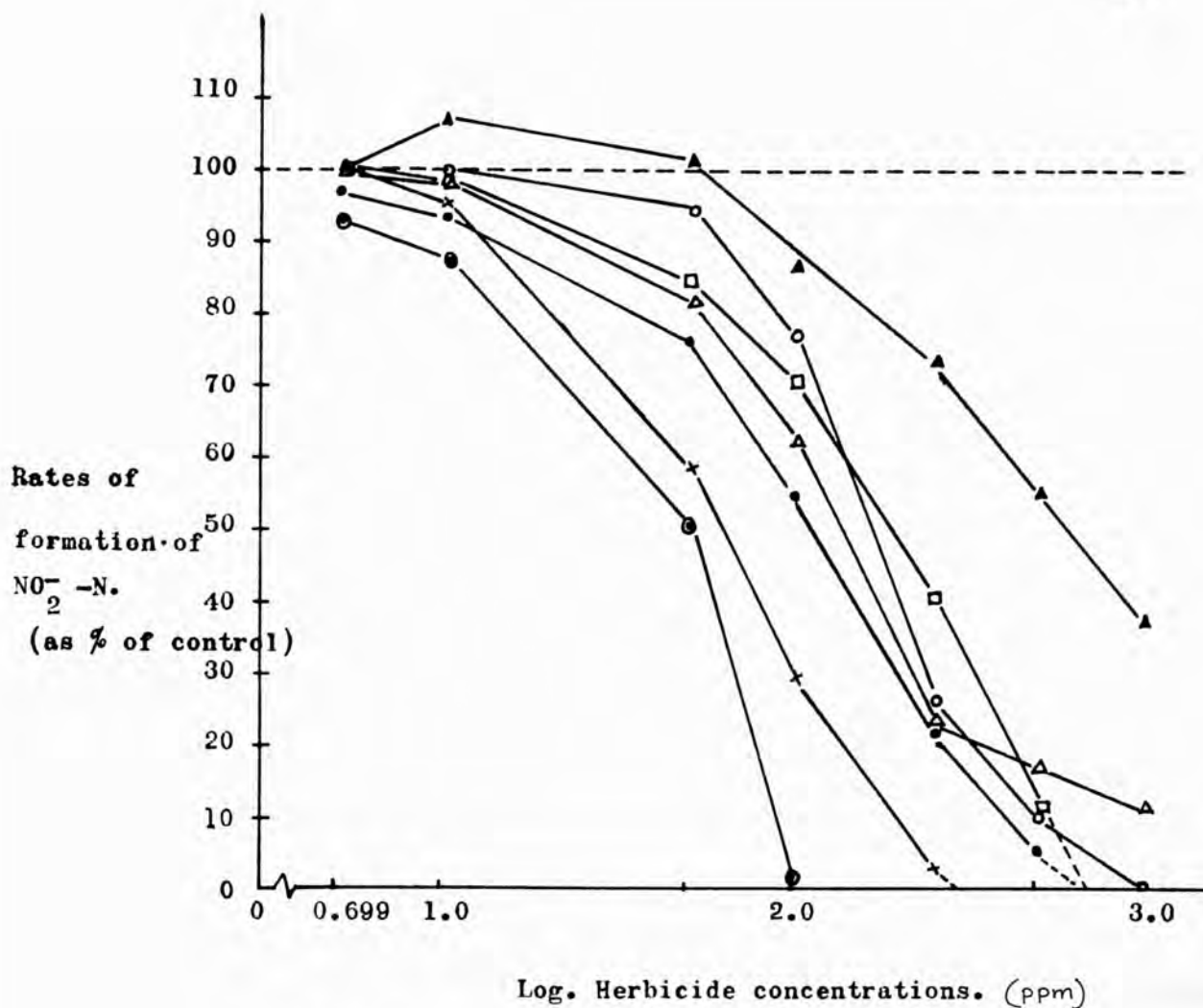


Fig. 47

Rates of formation of NO_2^- ($\mu\text{g. NO}_2^- \text{-N formed/ reaction medium/90 min.}$) in washed cell suspensions of Nitrosomonas Europaea incubated with culture medium containing $10^{-3}\text{M } (\text{NH}_4)_2\text{SO}_4$ and known different herbicide concentrations. (All rates expressed as percentage values of the corresponding controls).

x - Legurame, ● - Totril, Δ - Phenmedipham,
 ○ - Ioxymil, ▲ - M & B 9555, ⊙ - Tricamba,
 ◻ - Chlorbufam.

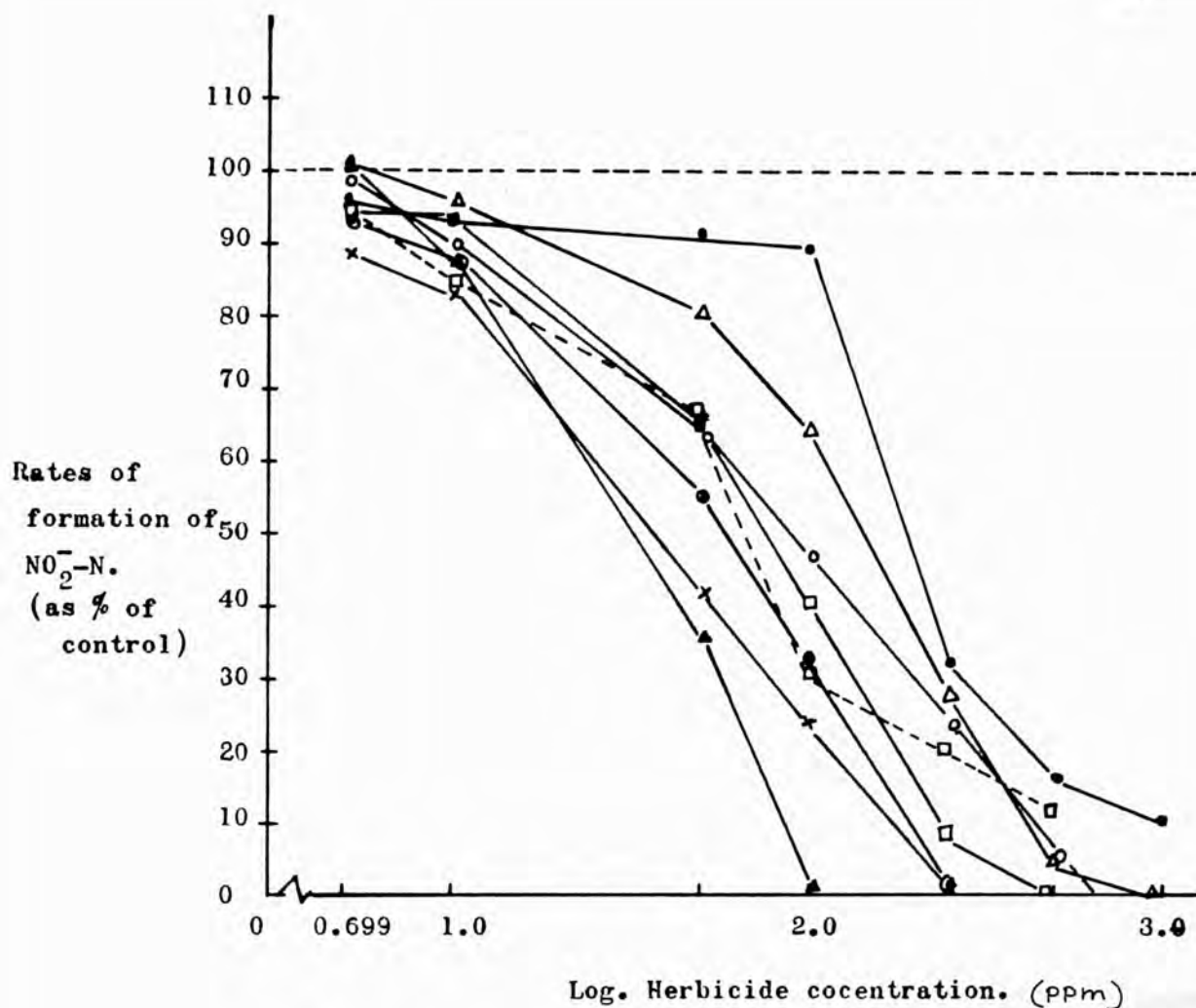


Fig. 48

Rates of formation of Nitrite ($\mu\text{g. NO}_2^- \text{-N formed/}$ reaction medium/90 min.) in washed cell suspensions of *Nitrosomonas Europaea* incubated with culture medium containing $10^{-3} \text{M (NH}_4)_2\text{SO}_4$ and known different herbicide concentrations. (All rates expressed as percentage values of the corresponding controls.)

▲ - Oxadiazon, x - Trifluralin, ○ - NPH 1320,
 ● - Bromoxynil, ⊙ - Dicamba, Δ - Terbacil,
 □ - M & B 9057, □ - M & B 9057 (Replicate).

Table 16

Nitrite oxidations in washed cell suspensions of Nitrobacter Winogradski incubated for 60 minutes at 25°C in the presence of a range of concentrations of different herbicides and $0.65 \times 10^{-3}M$ $NaNO_2$.

(All estimates are in terms of $\mu g NO_2^- - N$ oxidised / reaction mixture / 60 minutes and represent the mean of two readings.)

Herbicides		Herbicide concentrations in p.p.m.							
		Control	5	10	50	100	250	500	1000
Ioxynil	P	11.05	11.00	10.79	9.88	8.13	3.28	0.00	0.00
Bromoxynil	P	11.50	11.42	11.39	10.99	10.20	3.40	0.00	0.00
NPH 1320	F	10.95	9.43	8.20	3.20	1.18	0.00	0.00	0.00
Totril	F	10.83	9.93	7.83	2.17	0.73	0.00	0.00	0.00
9057 M & B	P	9.50	9.50	9.13	7.22	5.95	3.25	1.95	0.85
9555 M & B	P	11.30	11.30	11.30	11.28	9.98	8.73	8.82	7.85
Legurame	F	10.88	10.23	10.10	8.05	6.30	3.55	0.00	0.00
Oxadiazon	F	10.65	9.80	9.43	8.08	5.83	2.65	1.125	0.00
Trifluralin	F	11.20	8.20	7.62	5.55	4.70	4.25	0.70	0.00
Tricamba	P 1)	10.43	10.41	10.36	4.17	2.92	1.76	0.90	0.29
	2)	10.95	10.77	10.72	4.63	2.70	1.54	0.39	0.00
Dicamba	P	10.43	10.41	10.41	3.74	2.99	2.31	2.06	1.49
Chlorbufam	P *	11.05	9.58	8.50	5.20	3.55	1.90	0.60	0.00
Phenmedipham	P *	6.80	6.08	5.75	5.48	5.40	4.95	3.63	0.75
Terbacil	P *	11.05	11.05	10.65	9.39	7.75	2.99	0.45	0.25

* These herbicides with low solubilities were added directly into the reaction mixtures in appropriate quantities so as to form suspensions.

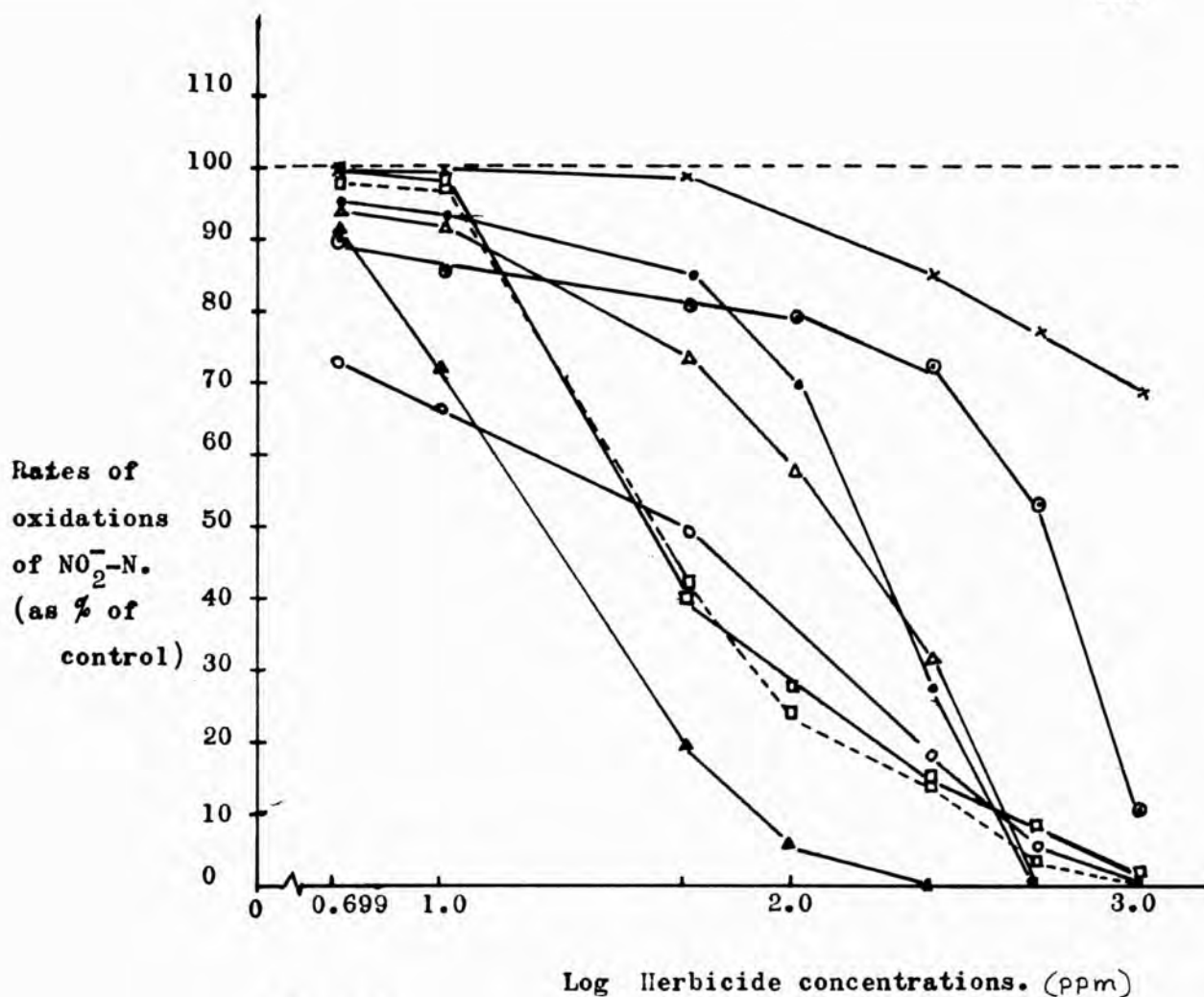


Fig. 49

Rates of oxidation of NO_2^- ($\mu\text{g. NO}_2^-$ -N oxidised/reaction medium/60 min.) in washed cell suspensions of Nitrobacter Winogradski incubated with culture medium containing $0.65 \times 10^{-3} \text{ M NaNO}_2$ and known different herbicide concentrations. (All rates expressed as percentage values of the corresponding controls.)

▲ - Totril, ○ - Trifluralin, Δ - Legurame,
 ● - Ioxynil, × - M & B 9555, ⊙ - Phenmedipham,
 □ - Tricamba, ◻ - Tricamba (Replicate).

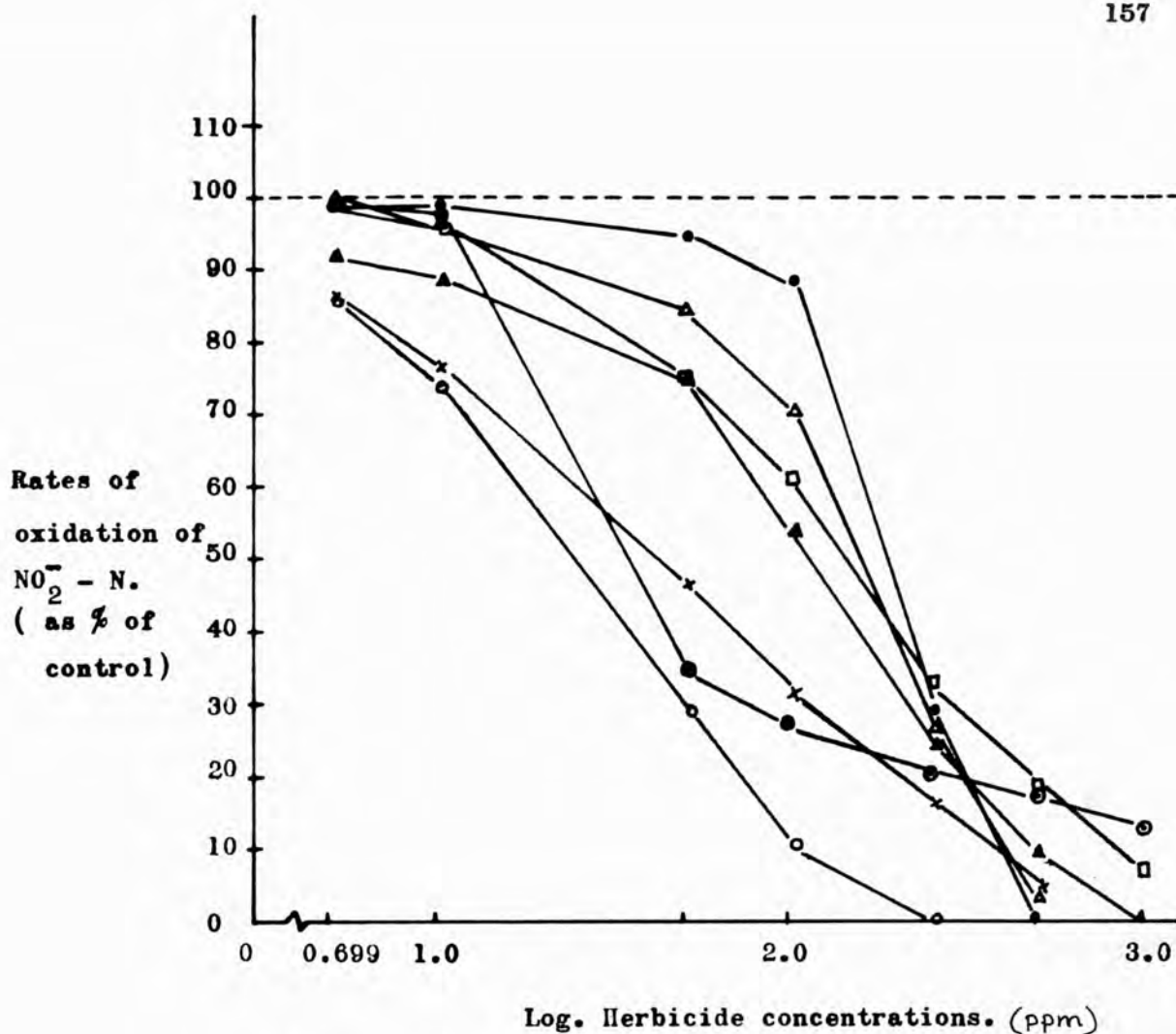


Fig. 50

Rates of oxidation of NO_2^- ($\mu\text{g. NO}_2^-$ -N oxidised/reaction medium/60 min.) in washed cell suspensions of Nitrobacter Winogradski incubated with culture medium containing $0.65 \times 10^{-3} \text{ M NaNO}_2$ and known different herbicide concentrations. (All rates expressed as percentage values of the corresponding controls.)

o - NPH 1320, x - Chlorbufam, ▲ - Oxadiazon,
 △ - Terbacil, ● - Bromoxynil, ⊙ - Dicamba,
 □ - M & B 9057.

metabolic activities of these two organisms.

Considering more closely the toxic levels of the different herbicides tested in this section, it is evident from the I_{50} values presented in Table 17 (all I_{50} values estimated by probit analysis) that almost all these herbicides differ in their concentrations effective in causing 50% inhibition of the nitrification rates of Nitrosomonas cell suspensions as compared with the concentrations effective in causing 50% inhibition of the nitrification rates of Nitrobacter cell suspensions during short term nitrification experiments using pure cultures. For example Ioxynil effective in causing 50% inhibition in the activity of Nitrosomonas at a concentration equivalent to 175 p.p.m. ($4.7 \times 10^{-4}M$) seems to cause similar level of inhibition in Nitrobacter activity at a lower concentration equivalent to 122 p.p.m. ($3.3 \times 10^{-4}M$). This same type of differences were observed with the two formulated herbicides NPH 1320 and Totril, NPH 1320 being effective in causing 50% inhibition of Nitrosomonas activity at a concentration of 82 p.p.m. ($2.0 \times 10^{-4}M$) and 50% inhibition of Nitrobacter activity at a much lower concentration of 22 p.p.m. ($0.5 \times 10^{-4}M$) of the active ingredient Bromoxynil octanoate while the concentration of Totril (25 w/v ioxynil octanoate) causing 50% inhibitions of Nitrosomonas and Nitrobacter activity were 97 p.p.m. ($2.0 \times 10^{-4}M$) and 21 p.p.m. ($0.4 \times 10^{-4}M$) respectively.

All the herbicides did not show higher toxic effects on Nitrobacter in comparison with their effects on Nitrosomonas. Out of the fourteen herbicides tested here only Ioxynil, Bromoxynil, NPH 1320, Totril, Trifluralin and Chlorbufam showed higher toxicities on the activity of Nitrobacter cell suspensions than on the activity of Nitrosomonas cell suspensions, as could be seen from the low values obtained for the concentrations of these herbicides sufficient to bring about 50% inhibition of Nitrobacter activity. Still other herbicides, namely Tricamba, Dicamba, Legurame, Oxadiazon, M & B 9057, M & B 9555 and Phenmedipham showed higher

Table 17

The concentrations of different herbicides effective in causing 50% inhibition (I_{50}) of the rates of nitrification in cell suspensions of Nitrosomonas Europaea and Nitrobacter Winogradski.

Herbicides used	I_{50} concentration for <u>Nitrosomonas</u> activity		I_{50} concentration for <u>Nitrobacter</u> activity	
	p.p.m.	molar	p.p.m.	molar
Ioxynil	175	4.7×10^{-4}	122.5	3.3×10^{-4}
Bromoxynil	200	8.4×10^{-4}	172.5	6.2×10^{-4}
NPH 1320 *	82.5	2.0×10^{-4}	22.0	0.5×10^{-4}
Totril *	97.5	2.0×10^{-4}	21.0	0.4×10^{-4}
Tricamba	33.0	1.3×10^{-4}	a) 67.5 b) 55.0	2.7×10^{-4} 2.2×10^{-4}
Dicamba	53.0	2.4×10^{-4}	80.0	3.9×10^{-4}
Legurame *	32.0	1.4×10^{-4}	100.0	4.2×10^{-4}
Oxadiazon *	60.0	1.7×10^{-4}	87.5	2.5×10^{-4}
Trifluralin *	62.0	1.9×10^{-4}	31.0	0.9×10^{-4}
M & B 9057	a) 60.0 b) 65.0	3.2×10^{-4} 3.9×10^{-4}	155	8.3×10^{-4}
M & B 9555	30.0	1.3×10^{-4}	»1000	» 43.9×10^{-4}
Terbacil	150.0	6.9×10^{-4}	137.5	6.4×10^{-4}
Chlorbufam **	120.0	6.8×10^{-4}	41.0	2.3×10^{-4}
Phenmedipham **	160.0	6.6×10^{-4}	300.0	12.4×10^{-4}

* Used in the form of emulsifiable concentrates.

** Pure compounds in form of fine aqueous suspensions.

toxicities on the nitrifying activity of Nitrosomonas cell suspensions than on the nitrifying activity of Nitrobacter cell suspensions. Terbacil was found to be effective in causing 50% inhibition of the nitrifying activities of both the Nitrosomonas and Nitrobacter cell suspensions at concentrations approximately equal to 150 p.p.m.

The two herbicides Chlorbufam and Phenmedipham which were not tested in the previous sections too were included here and these had to be incorporated into the reaction mixtures in the form of a fine powder since their solubilities were extremely low. Thus the real concentrations of these two herbicides effective in causing 50% inhibition in the nitrifying activities of Nitrosomonas and Nitrobacter cell suspensions might be much less than the concentrations presented in Table 17, because the values in Table 17 represent the I_{50} concentrations in terms of the amount of the herbicides added and not in terms of the amount of these sparingly soluble herbicides that would have actually gone into solution.

The data from the Table 17 show clearly that NPH 1320 (Bromoxynil octanoate) and Totril (Ioxynil octanoate) tested in the form of emulsifiable concentrates (25% w/v ester equivalents) were the most toxic herbicides towards Nitrobacter activity. Since both these emulsifiable concentrates were known to contain a highly aromatic base which by itself would have contributed considerably towards the observed high toxicities it was later decided to check on this point. But due to the extremely low solubilities of these octanoate herbicides, they could only be tested in the form of wettable powders (20% w/w acid equivalents) that did not contain any surfacants, but an inert carrier unlike in the emulsifiable concentrate forms. As shown by the data in Table 18, it was found that in the form of wettable powders Ioxynil and Bromoxynil octanoates were in fact much less toxic towards the nitrite oxidising capacity of Nitrobacter cell suspensions during short term incubations, the respective concentrations of these wettable powder forms effective in causing 50% inhibitions being 135 p.p.m.

Table 18

Nitrite oxidations by washed cell suspensions of Nitrobacter Winogradski incubated for 60 minutes at 25°C in the presence of a range of concentrations of Ioxynil and Bromoxynil octanoates in the form of wettable powders.

(All estimates are in terms of $\mu\text{g NO}_2^-$ - N oxidised / reaction mixture / 60 minutes.)

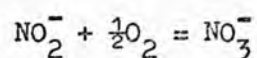
Herbicides	Herbicide concentrations in p.p.m.							
	Control	5	10	50	100	250	500	1000
Bromoxynil octanoate (20% w/w acid equivalent)	14.75	13.35	12.75	10.65	7.65	1.35	0.90	0.00
						I_{50} concentration = 65 p.p.m.		
Ioxynil octanoate (20% w/w acid equivalent)	13.35	12.75	12.75	10.65	7.65	6.15	2.55	0.45
						I_{50} concentration = 135 p.p.m.		

and 65 p.p.m. as against the very low values for I_{50} concentrations obtained when the same compounds were tested in the form of emulsifiable concentrates (21 p.p.m. and 22 p.p.m.). But here again one could not be certain about the exact limits of toxicity since all the active ingredients incorporated into a reaction medium in these wettable powder forms may or may not go into solution, in which form they will be most active.

A few years later Debona (1967), Winsky & San Clemente (1968, 1970) have made use of this same respirometric technique to assess the effects of some herbicides on nitrifying bacteria. The present investigation was aimed at establishing the effects of some newer herbicides, that have not been studied so far, on the respiratory activities of pure cultures of Nitrosomonas europaea and Nitrobacter winogradskyi. In few experiments reported here, a more sophisticated technique of measuring rate of respiration where the rates of oxygen consumption by the cell suspensions of these nitrifying bacteria were measured in terms of the changes in the oxygen tensions in reaction media determined polarographically by means of oxygen electrodes was used instead of the Warburg respirometric technique.

CHAPTER 7Effect of Herbicides on O₂ Consumption by
Nitrosomonas and Nitrobacter Cell SuspensionsIntroduction

The work reported by Lees & Simpson (1956) with special reference to the mechanism of nitrite oxidation by Nitrobacter has shown that the amount of nitrite which is oxidised to nitrate by this organism always agreed, to within 5%, with the amount of oxygen consumed according to the following equation,



Thus it is quite reasonable to assume that the effects of different inhibitors on the nitrifying capacity of this organism could be followed by assessing the degree to which these substances inhibit or stimulate the rates of O₂ consumption. Butt & Lees (1960) made use of the rates of oxygen uptake, measured by the conventional Warburg respirometric technique, as the experimental parameter to assess the inhibitory effects of arsenite, cyanate and nitrate on the nitrite oxidation by Nitrobacter at different oxygen concentrations.

A few years later Debona (1967), Winely & San Clemente (1968, 1970) have made use of this same respirometric technique to assess the effects of some herbicides on nitrifying bacteria. The present investigation was aimed at establishing the effects of some newer herbicides, that have not been studied so far, on the respiratory activities of pure cultures of Nitrosomonas Europaea and Nitrobacter Winogradski. In few experiments reported here, a more sophisticated technique of measuring rate of respiration where the rates of oxygen consumption by the cell suspensions of these nitrifying bacteria were measured in terms of the changes in the oxygen tensions in reaction media determined polarographically by means of oxygen electrodes was used instead of the Warburg respirometric technique.

Winely & San Clemente (1970) have reported the possibility of using small quantities of acetone (10 μ l/flask) to dissolve insoluble herbicides, prior to addition into the Warburg vessels, without seriously affecting the overall results if care is taken to add equal volumes (i.e. 10 μ litre quantities) of acetone to the control reaction vessels too. But in view of the probable undesirable effects that might result from the use of organic solvents like acetone, it was decided to leave out some of the insoluble herbicides from this part of the investigation. It must be mentioned at this point that the formulated herbicides (indicated with F in Table 1) were used in these respiration experiments with no regard to the nature of the solvents and wetting agents they contain, since these commercial preparations are the ones normally used in practical agriculture.

Experimental procedure

Nitrobacter Winogradski and Nitrosomonas Europaea cells were grown and harvested by methods essentially similar to those described in Chapter 2.

The Warburg apparatus (operating at 120 strokes/min.) was used in the manometric measurements of oxygen uptake and the practical details of the standard manometric procedure as described by Umbreit et al. (1949) was followed carefully in setting up the manometers. The observations were also made in accordance with the method described by Umbreit et al. (1949).

Each Warburg flask contained the reaction mixtures having the following main components, 0.5 ml. cell suspension, 1.0 ml. phosphate buffer pH 7.8 (0.1M Na_2HPO_4 + 0.1M KH_2PO_4) and 0.5 ml. of water in the case of the control flasks or 0.5 ml. of the herbicide solution of known concentrations in the case of the test flasks. 0.5 ml. of the substrate (sodium nitrite or ammonium sulphate solution depending on whether the cell suspension used was Nitrobacter or Nitrosomonas) was usually intro-

duced into the side arm of the reaction flask while 0.2 ml. of 20% potassium hydroxide along with a filter paper wick was placed in the centre well of the main chamber.

There is some controversy regarding the optimal substrate concentrations to be used in Nitrobacter respiration studies. Meyerhof (1916) (quoted by Boon & Laudelout 1962) has reported that at concentrations above 20 mM nitrite inhibits the rate of its own oxidation whereas according to Lees & Simpson (1957) the inhibitory effects would be noticeable at concentrations as low as 4 mM nitrite. Later Boon & Laudelout (1962) themselves have shown that the optimal nitrite concentration for Nitrobacter activity is around 20 mM. But in the present investigation the concentration of nitrite used was kept down at 5 mM level since it was observed in preliminary experiments that nitrite concentrations higher than this did tend to decrease the overall respiration rates of the Nitrobacter cell suspensions used here. In experiments carried out with Nitrosomonas cell suspensions 10 mM ammonium sulphate was used as the substrate since this substrate concentration was found to give very satisfactory steady rates of oxygen uptake throughout the experimental period.

After temperature equilibration of the reactants for 15-20 minutes at 26°C the substrates from the side arms were tipped into the main chambers containing the cell suspensions incubated with the herbicides and the oxygen uptake was measured over a period of 90 minutes.

The solutions of the herbicides and the substrates having an initial concentration equal to 5 times that needed in the reaction mixtures were used in 0.5 ml. aliquots per flask so as to give the appropriate dilutions once added to the reaction mixtures (each reaction mixture had a total volume equal to 2.5 ml.).

In all the experiments reported here, the appropriate herbicide solutions were initially added to the cell suspensions and the substrate

solutions were tipped in only after temperature equilibration as mentioned in the above paragraph, since Lees & Simpson⁽¹⁹⁵⁷⁾ working on the inhibitory effects of chlorate on Nitrobacter respiration have shown that precisely similar results were obtained either when the addition of nitrite from the side arm was delayed for one hour after the chlorate solution and Nitrobacter suspension had been mixed in the main vessel or when the chlorate and nitrite solutions were mixed in the side arm and then added to the Nitrobacter suspension in the main vessel.

Controls corresponding to each set of experiments and random repeat experiments were carried out with Nitrosomonas as well as Nitrobacter cell suspensions.

Since some of the herbicides used in the above Warburg respirometric estimations (e.g. NPH 1320, Totril, Legurame etc.) showed very high toxicities even at concentrations as low as 5 p.p.m., it was decided to test at least one or two of these highly toxic herbicides using a more sensitive instrument 'the Oxygen Electrode' which could indicate the rates of oxygen uptake in the bacterial suspensions by polarographic measurement of the oxygen tensions in the appropriate reaction media as was mentioned in the introduction to this chapter.

Here 2 ml. samples of the bacterial cell suspension (either Nitrobacter or Nitrosomonas) were placed in the sample chambers of a Clark Oxygen Electrode (Yellow Springs Incorporation, Ohio, U.S.A.) with 1 ml. of phosphate buffer pH 7.8 (same as used in the Warburg technique) and 1 ml. of the appropriate substrate solution. The contents of the sample chambers were kept equilibrated at the required temperature of 26°C (being kept stirred constantly by means of a magnetic stirrer device attached to the instrument) for 15 minutes in order to saturate the systems with oxygen. The probes were then introduced into the sample chambers, care being taken to exclude all the air bubbles, and the respiration rates were recorded over a period of time lasting at least 10-15 minutes, using the

recorder unit of the same instrument. After completing this measurement, which gives the rate of oxygen uptake by Nitrobacter or Nitrosomonas cell suspensions supplied with the specific substrate in the absence of any herbicides, a small volume (0.2 ml.) of the herbicide solution of known final concentration (i.e. known resulting concentration after the herbicide being diluted in the total volume of the reaction medium) was introduced into the reaction chamber and the rate of oxygen uptake by the same sample of cell suspension in the presence of the added herbicide was recorded in the standard way described above. This procedure was repeated with progressive increases in the concentrations of the herbicide under consideration and a series of measurements were made on the rates of oxygen uptake of the same cell suspension under the influence of known different concentrations of the same herbicide. Thus it follows that the experimental technique described above helps to eliminate undesirable variations in the rates of oxygen uptake due to sample variations. It must also be mentioned here that the volume of the herbicide solution introduced each time into the reaction medium has to be recorded and also kept as low as possible since the final calculation of the rate of oxygen uptake from the recorded gradients (as shown in Fig. 51) makes use of the volume of the reaction mixture and since the maximum volume of the reaction medium that could be used in these experiments is limited by many of the instrumental specifications such as the small size of the sample chambers. A typical set of data recorded in an experiment carried out with an oxygen electrode to evaluate the effects of different concentrations of Totril on the rate of oxygen uptake by a cell suspension of Nitrobacter Winogradski, is presented in Figure 51.

In both the experimental procedures described above, the endogenous rates of respiration of the bacterial cell suspensions used were also determined by setting up one reaction mixture, in every series, containing water in place of the relevant substrates.

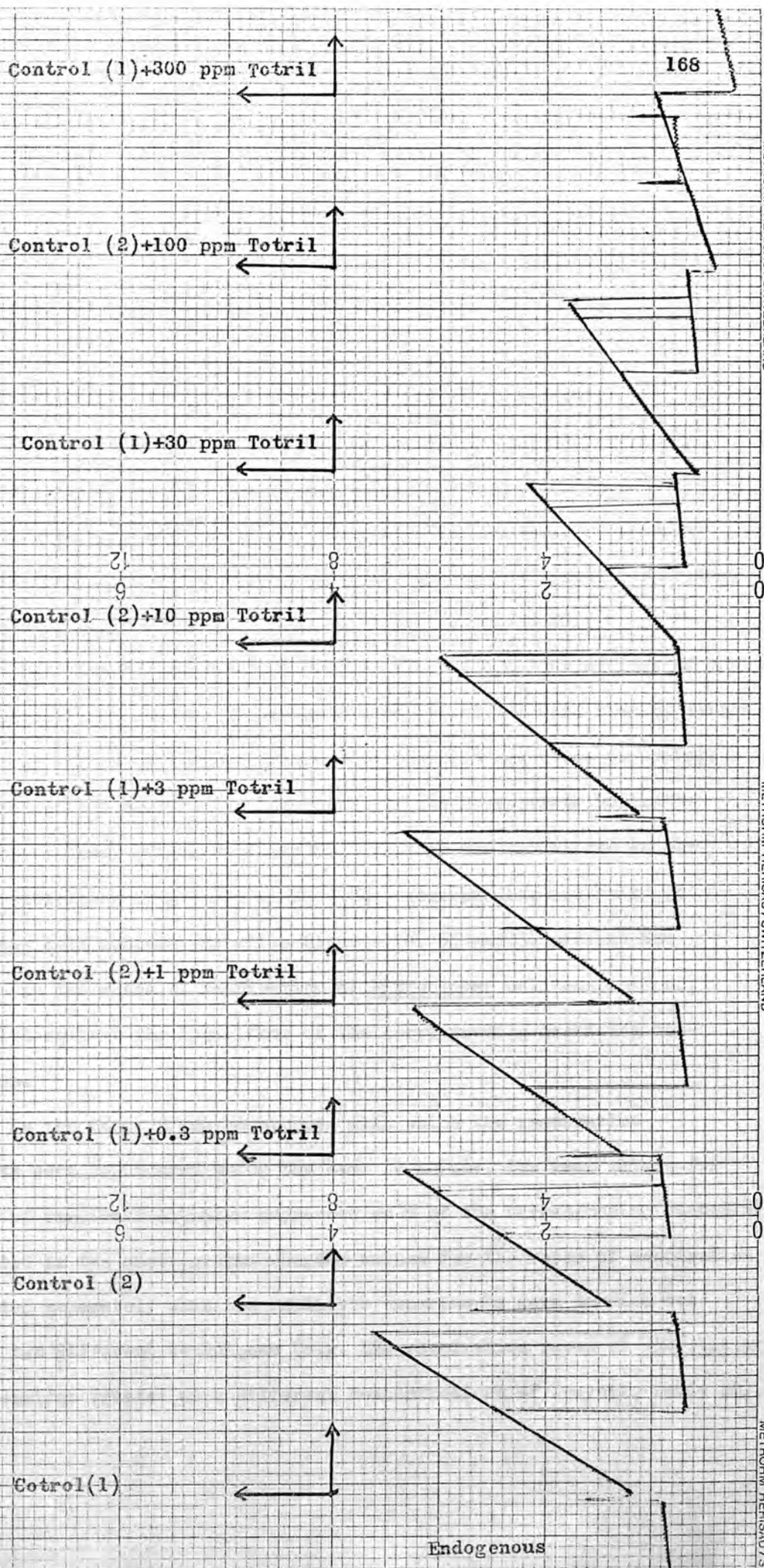


Fig. 51. O_2 Electrode data showing the variations in oxygen tensions in suspensions of *Nitrobacter Winogradski* respiring under the influence of known concentrations of Totril.

Results

The rates of respiration of each of the cell suspensions, in the presence and in the absence of the different herbicide concentrations were calculated in terms of μ litres oxygen consumed / reaction vessel / minute. These rates calculated for cell suspensions of Nitrosomonas Europaea and Nitrobacter Winogradski treated with known concentrations of a range of herbicides are given in Tables 19 and 20 respectively.

Since it was found that the endogenous rates of respiration in the cell suspensions of Nitrosomonas and Nitrobacter used in these experiments were very low and almost negligible (as shown in Figures 14 and 15), no corrections were made to account for the endogenous respiratory rates in evaluating the values presented in Tables 19 and 20.

The rates of oxygen uptake by the treated cell suspensions of Nitrosomonas and Nitrobacter (from the Tables 19 and 20) were then expressed as percentage values of their corresponding controls and these percentage values were plotted against the logarithms of the appropriate herbicide concentrations used. Figures 52 and 53 present the variations in the rates of respiration (expressed as % of the corresponding controls) of Nitrosomonas cell suspensions while Figures 54, 55 and 56 present the variations in the rates of respiration of Nitrobacter cell suspensions, when plotted against the logarithms of the corresponding herbicide concentrations.

In the case of the herbicides with which the respiration experiments were replicated using the same technique, the mean values for the rates of respiration (rates expressed as % of the corresponding controls) were plotted in the above graphs whereas values for the rates of respiration were plotted separately when the replicate experiments were carried out using the two different techniques (e.g. the respiration rates of Nitrobacter cell suspensions treated with different concentrations of Ioxynil, Table 20).

Table 19

Rates of oxygen uptake by *Nitrosomonas Europaea* in the presence of a range of concentrations of different herbicides along with 10^{-2} M $(\text{NH}_4)_2\text{SO}_4$ as substrate.

(All rates expressed in terms of μ litres O_2 consumed / vessel / minute.)

Herbicides	Control	Herbicide concentrations in p.p.m.							
		5	10	50	100	250	500	1000	
Tricamba	a) 0.257	-	0.213	0.211	0.197	-	0.189	0.149	
	b) 0.264	-	0.256	0.217	0.203	-	0.202	0.163	
Dicamba	0.256	-	0.217	0.209	0.196	-	0.175	0.163	
M & B 9057 (Asulam)	0.546	-	0.558	0.528	0.515	-	0.496	0.464	
M & B 9555	a) 0.490	-	0.467	0.462	0.426	0.277	0.161	-	
	b) 0.541	-	-	0.536	0.534	0.293	0.115	-	
Trifluralin	a) 0.781	-	0.644	0.449	0.132	-	0.066	0.039	
	b) 0.840	-	0.649	0.534	0.121	-	0.065	0.046	
	c) 0.991	-	0.836	0.566	0.214	-	0.053	0.040	
Ioxynil	* 4.650	5.325	5.025	4.875	3.400	-	0.750	0.600	
Bromoxynil	* 4.575	5.100	4.925	4.875	3.950	-	1.050	0.225	
	Control	0.3	1	3	10	30	100	300	
NPH 1320	* 3.562	3.126	2.835	2.544	2.036	1.090	0.509	0.009	
Totril	* 2.338	2.192	1.973	1.681	1.608	1.133	0.757	0.365	
	Control	1	2.5	5	10	50	100	250	500
Legurame	a) 0.442	0.358	0.289	0.179					
	0.833				0.284	0.119	0.067	0.066	0.034
	b) 0.442	0.374	0.262	0.210					
	0.866				0.278	0.098	0.076	0.048	0.007
Oxadiazon	a) 0.410	0.399	-	0.379					
	0.635				0.472	0.167	0.078	0.078	0.069
	b) 0.410	0.391	-	0.367					
	0.649				0.408	0.143	0.086	0.066	0.054

* Results of the experiments carried out with Oxygen Electrode.

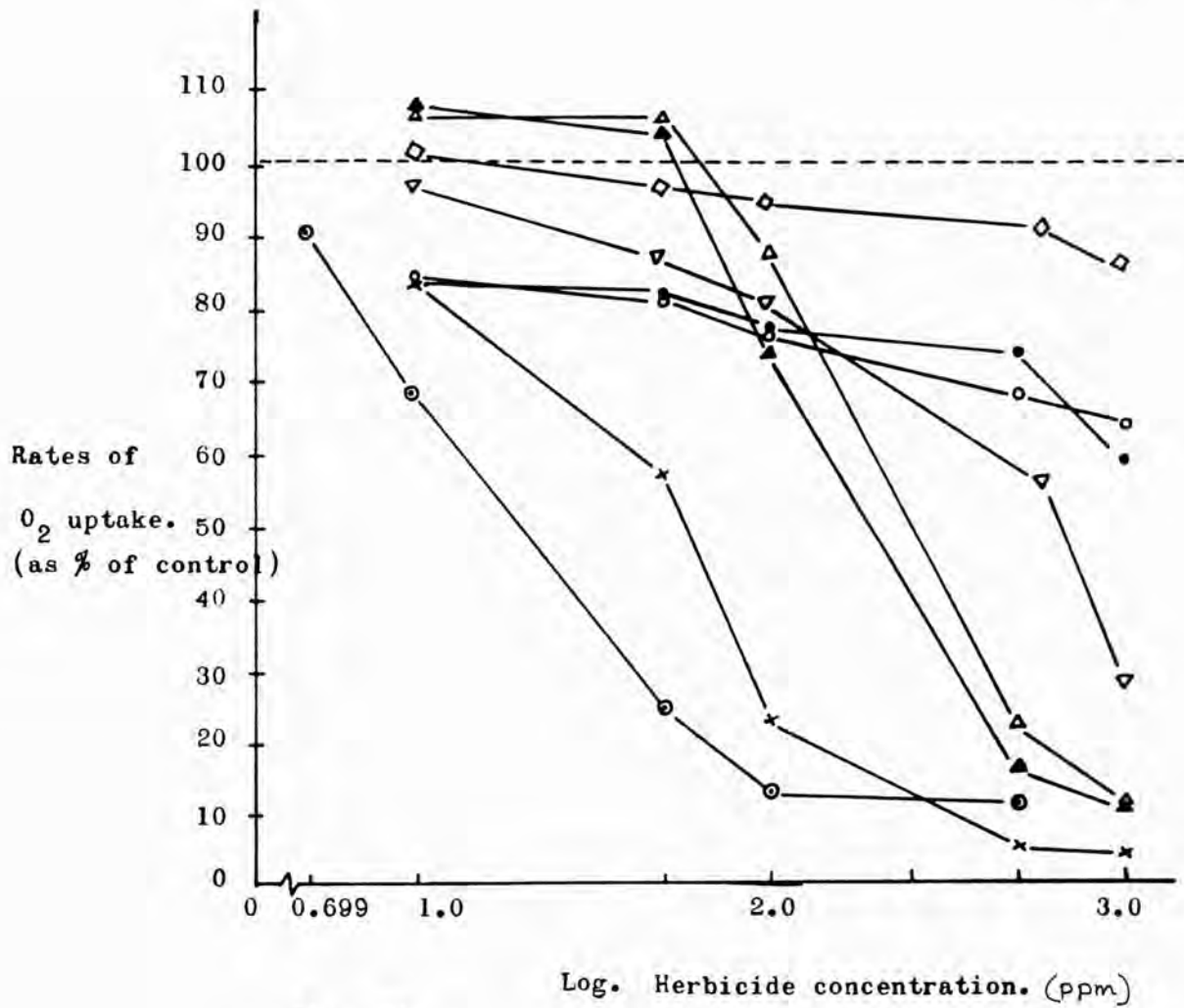


Fig. 52

Rates of Oxygen uptake by washed cell suspensions of *Nitrosomonas Europaea* ($\mu\text{l. O}_2$ consumed/vessel/min.) when supplied with $10 \times 10^{-3} \text{M}$ Ammonium sulphate solutions containing different herbicides at a range of known concentrations. (All rates expressed as percentage values of the corresponding controls.)

○ - Oxadiazon, × - Trifluralin, ▲ - Ioxynil,
 △ - Bromoxynil, ▽ - M & B 9555, ○ - Dicamba,
 ● - Tricamba, □ - M & B 9057.

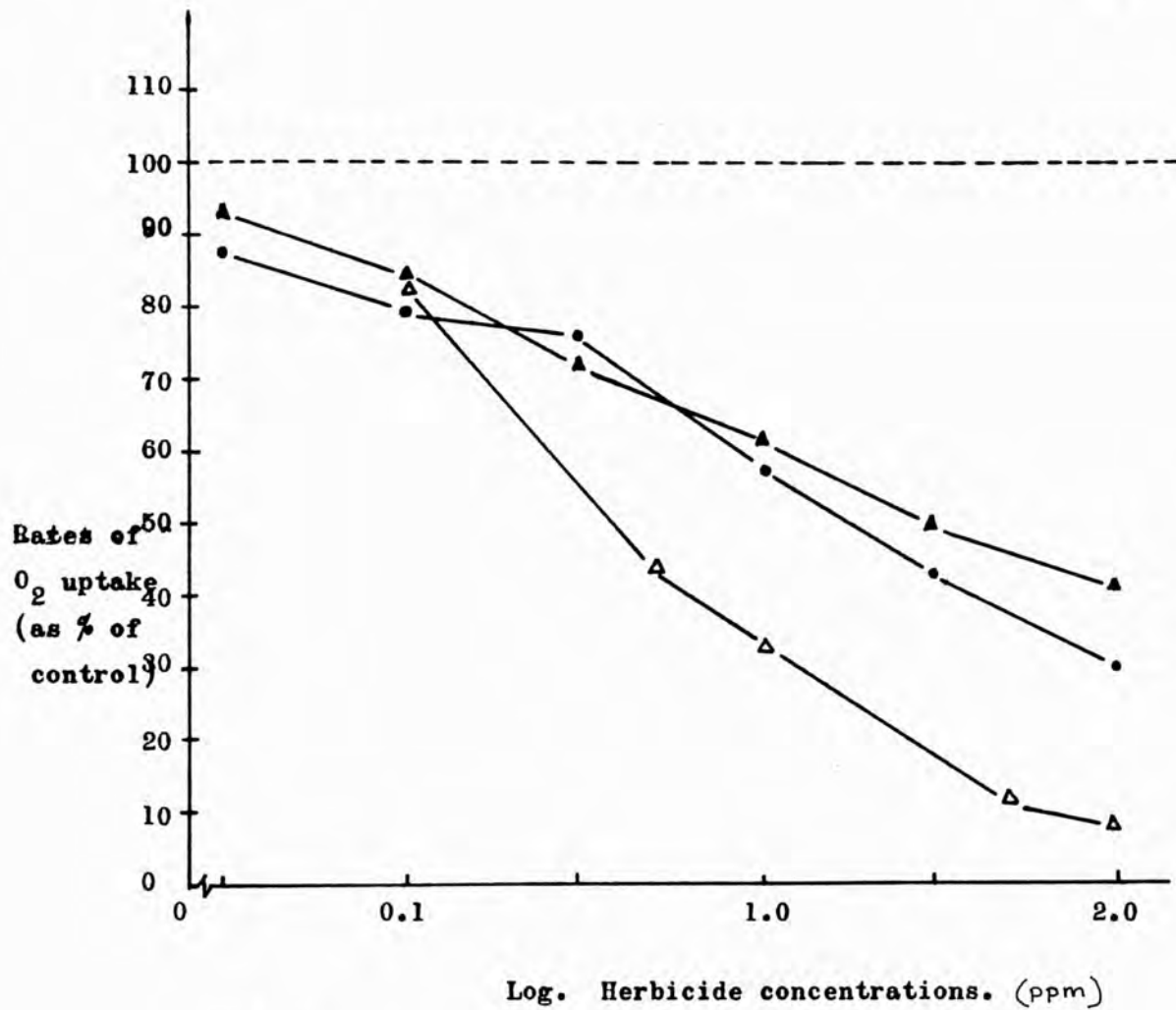


Fig. 53

Rates of Oxygen uptake by washed cell suspensions of Nitrosomonas Europaea ($\mu\text{l. O}_2$ consumed/vessel/min.) when supplied with 10×10^{-3} M Ammonium sulphate solutions containing different herbicides at a range of known concentrations. (All rates expressed as percentage values of the corresponding controls.)

Δ - Legurame, \bullet - NPH 1320, \blacktriangle - Totril.

Table 20

Rates of oxygen uptake by Nitrobacter Winogradski in the presence of a range of concentrations of different herbicides along with $5 \times 10^{-3}M$ $NaNO_2$ as substrate.

(All rates expressed in terms of μ litres O_2 consumed / vessel / minute.)

Herbicides	Herbicide concentrations in p.p.m.								
	Control	5	10	50	100	250	500	1000	
Tricamba	a)	0.124	-	0.117	0.114	0.093	-	0.075	0.063
	b)	0.119	-	0.113	0.111	0.087	-	0.078	0.069
Dicamba		0.169	-	0.178	0.158	0.151	-	0.144	0.139
M & B 9057 (Asulam)		0.139	-	0.124	0.121	0.105	-	0.093	0.073
M & B 9555	a)	0.264	-	0.250	0.244	0.231	0.227	0.208	-
	b)	0.233	-	0.227	0.221	0.204	0.203	0.171	-
Trifluralin	a)	0.232	-	0.193	0.185	0.145	0.128	0.098	-
	b)	0.229	-	0.195	0.168	0.147	0.130	0.106	-
Legurame	a)	0.457	-	0.360	0.317	0.268	0.102	0.038	
	b)	0.434	-	0.367	0.360	0.298	0.156	0.058	
Oxadiazon	a)	0.450	0.459	0.441	0.416	0.282	-	0.024	
	b)	0.445	0.446	0.425	0.410	0.149	-	0.058	
Ioxynil	a)	0.258	-	0.248	0.228	0.221	-	0.051	0.011
	* b)	1.302	1.226	1.149	1.073	0.766	-	0.153	0.077
Bromoxynil		0.226	-	0.206	0.189	0.179	-	0.045	0.027
		Control	0.3	1	3	10	30	100	300
NPH 1320	*	3.236	2.869	2.719	2.207	1.764	1.029	0.736	0.147
Totril	*	2.338	2.192	2.046	1.827	1.462	1.096	0.659	0.292

* Results of the experiments carried out with Oxygen Electrode.

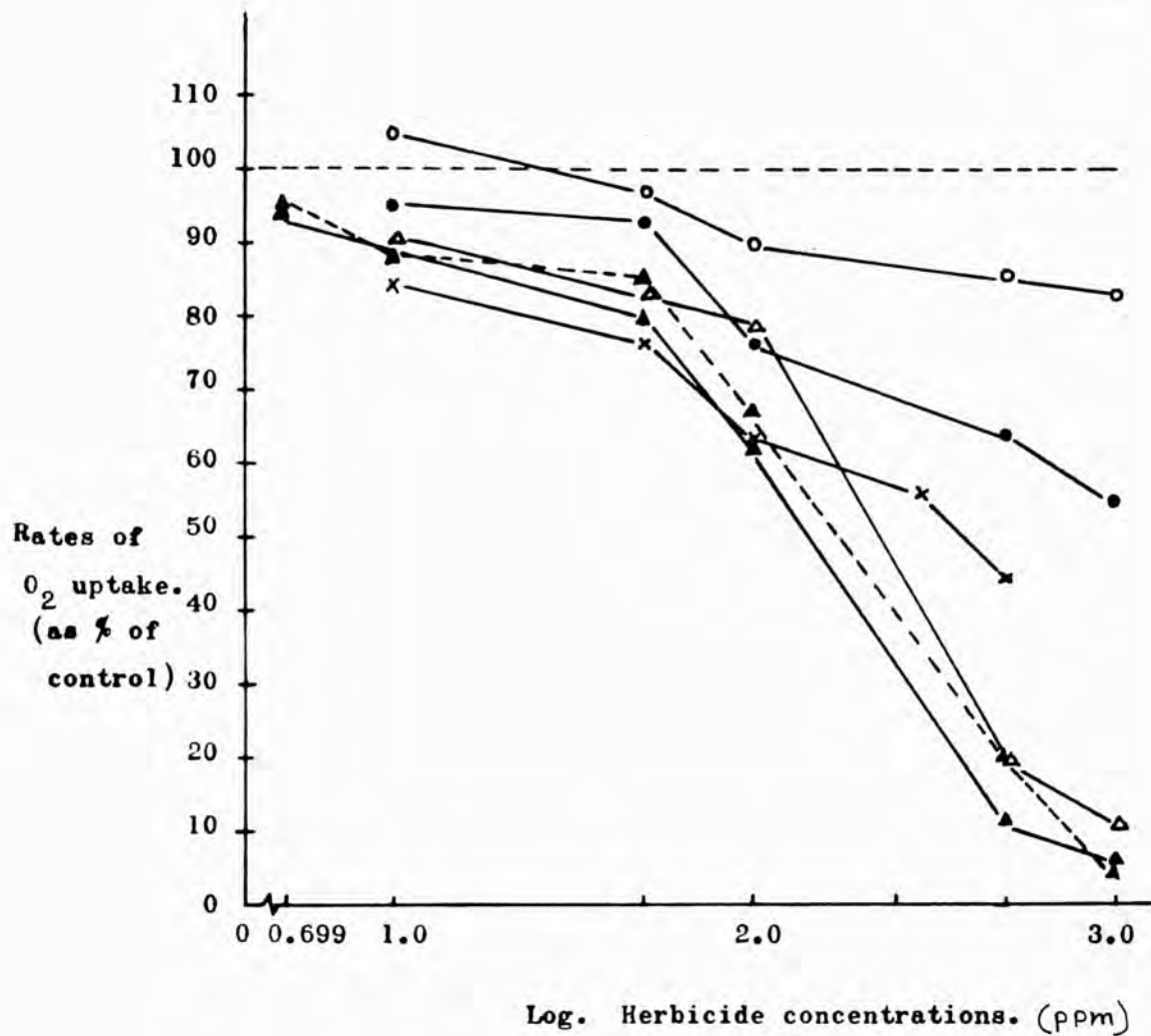


Fig. 54

Rates of Oxygen uptake by washed cell suspensions of Nitrobacter Winogradski (ml. O_2 consumed/vessel/min.) when supplied with $5 \times 10^{-3} M$ Sodium nitrite solutions containing different herbicides at a range of known concentrations. (All rates expressed as percentage values of the corresponding controls.)

Δ—Bromoxynil, x—Trifluralin, ●—Tricamba, o—Dicamba, ▲—Ioxynil (Oxygen Electrode Technique), ▲—Ioxynil (Warburg Technique)

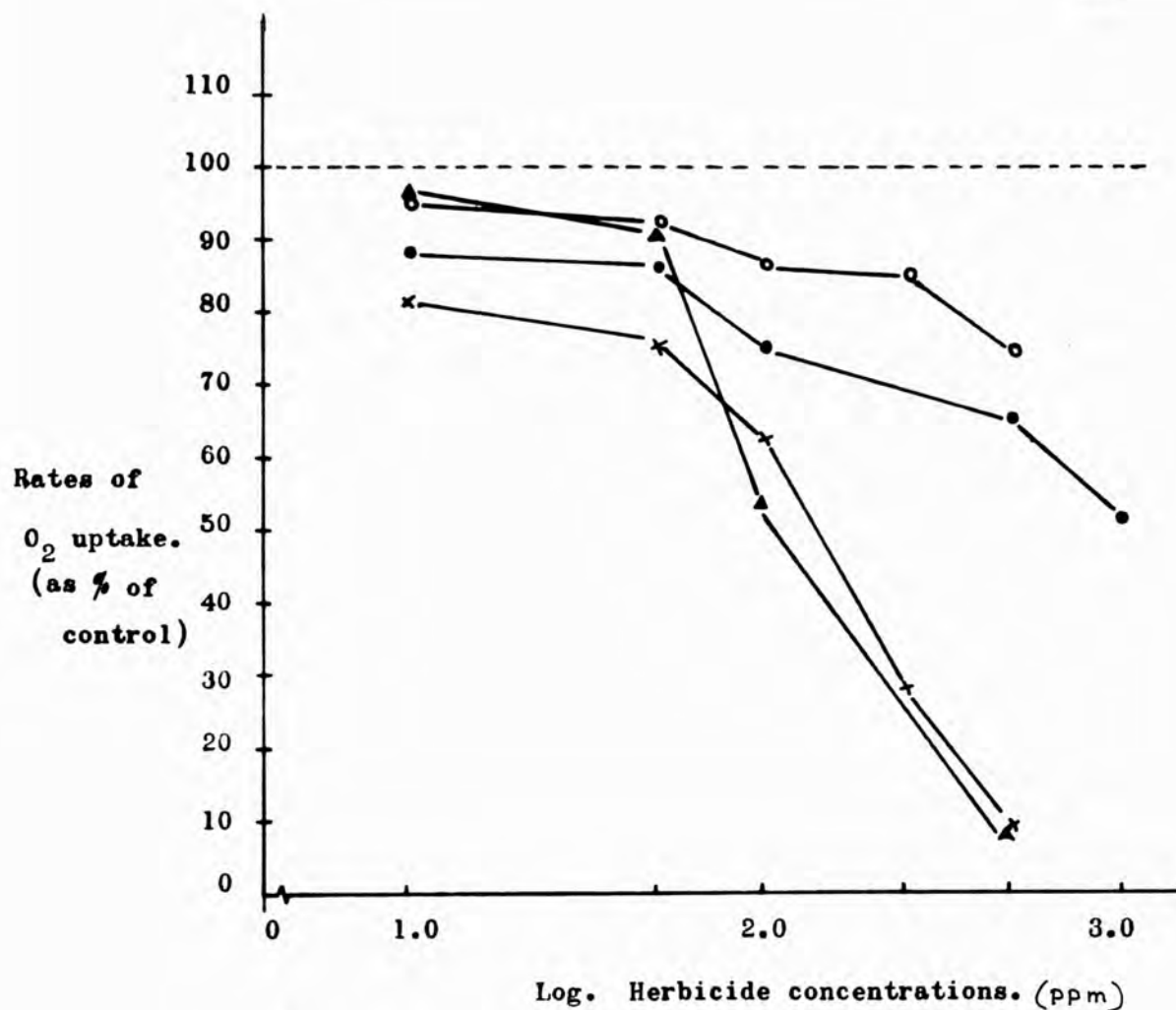


Fig. 55

Rates of Oxygen uptake by washed cell suspensions of Nitrobacter Winogradski (μ l. O_2 consumed/vessel/min.) when supplied with 5×10^{-3} M Sodium nitrite solutions containing different herbicides at a range of known concentrations. (All rates expressed as percentage values of the corresponding controls)

▲ - Oxadiazon, × - Legurame, ● - M & B 9057,
○ - M & B 9555.

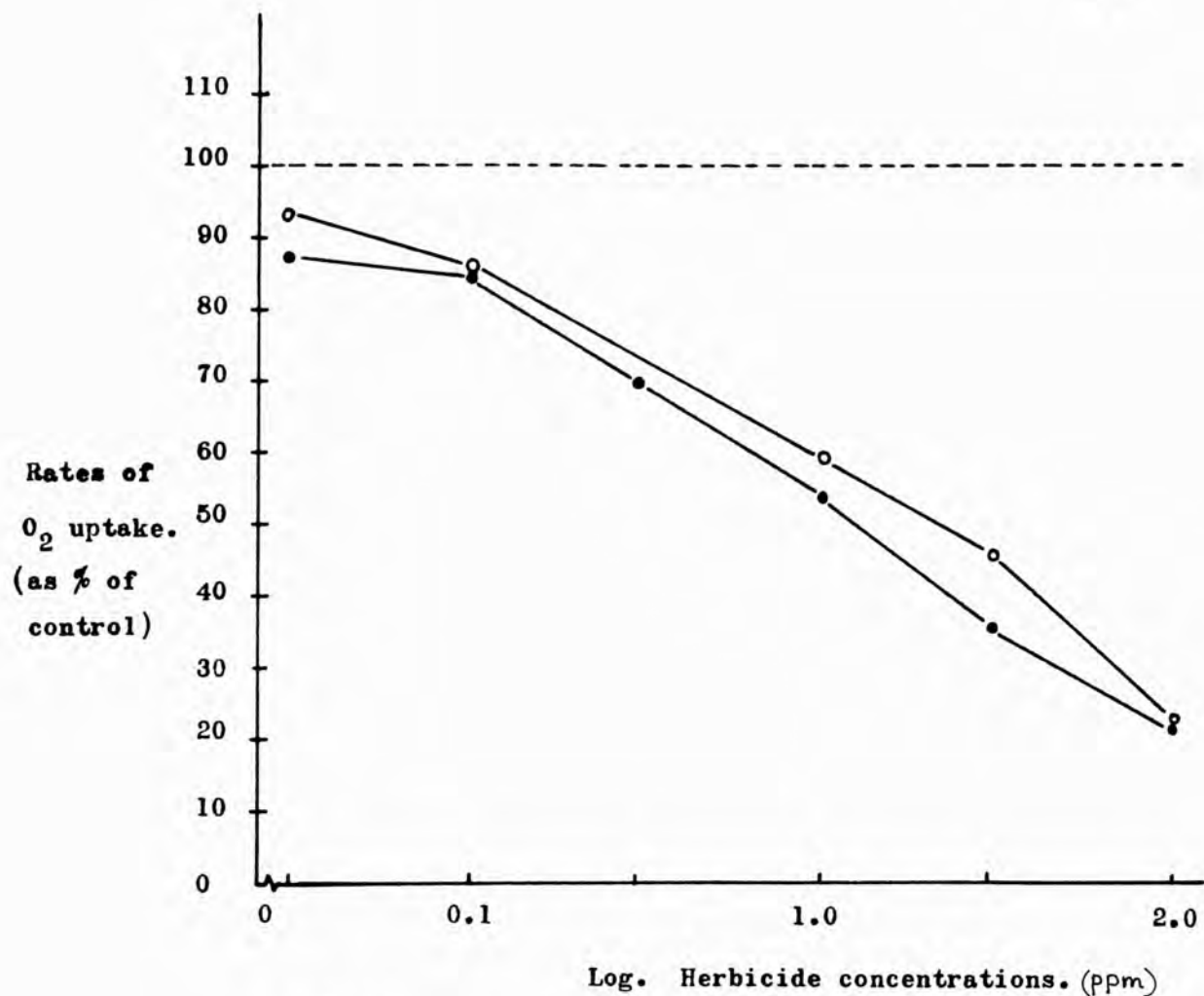


Fig. 56

Rates of Oxygen uptake by washed cell suspensions of *Nitrobacter Winogradski* ($\mu\text{l. O}_2$ consumed/vessel/min.) when supplied with $5 \times 10^{-3} \text{ M}$ Sodium nitrite solutions containing different concentrations of NPH 1320 and Totril. (All rates expressed as percentage values of the corresponding controls.)

• - NPH 1320, ○ - Totril.

Considering the results presented in Figure 52 it can be seen that Ioxynil and Bromoxynil at low concentrations ranging up to 50 p.p.m. caused slight stimulations in the rates of respiration of Nitrosomonas cell suspensions, the respiration rates in the cell suspensions treated with 10 and 50 p.p.m. Ioxynil being 108% and 105% respectively whereas the respective rates of respiration in those cell suspensions treated with 10 p.p.m. and 50 p.p.m. concentrations of Bromoxynil being 107% and 106% of the corresponding controls. On the other hand both these herbicides were found to cause almost complete inhibitions of respiration at concentrations around 1000 p.p.m. It was also evident that these two herbicides being very similar in their molecular structures showed similar inhibition curves. But the I_{50} values from Table 21 show that the molecular concentration of Bromoxynil effective in causing 50% inhibition of the respiratory activity of Nitrosomonas cell suspensions was slightly higher than the I_{50} concentration for Ioxynil. Regarding the effects of Ioxynil and Bromoxynil on the respiratory activities of Nitrobacter, it was found that inhibitory effects could be observed even at the lowest concentration tested (10 p.p.m.), the rates being around 94% and 91% respectively. Concentrations of Ioxynil and Bromoxynil of the order of 500 p.p.m. reduced the respiration rates of Nitrobacter cell suspensions to very low values (12% and 20%) while at concentrations of the order of 1000 p.p.m. the respiratory activities were almost completely inhibited and the rates of oxygen uptake were as low as 6% and 11% respectively. With Nitrobacter cell suspensions too both Ioxynil and Bromoxynil showed similar inhibition curves and as seen from the I_{50} concentrations presented in Table 21, Bromoxynil was found to be slightly less toxic than Ioxynil. Comparing the sensitivities of the two organisms Nitrosomonas and Nitrobacter it was clear that the Nitrobacter cell respiration was much more sensitive to these two herbicides than the respiratory activities of Nitrosomonas cells.

The herbicides NPH 1320, Totril, Legurame, Oxadiazon and Tri-

Table 21

The concentrations of different herbicides effective in causing 50% inhibition (I_{50}) of the rates of oxygen uptake in pure cultures of Nitrosomonas Europaea and Nitrobacter Winogradski supplied with ammonium sulphate and sodium nitrite respectively as the respiratory substrates.

Herbicides used	I_{50} concentrations for <u>Nitrosomonas</u> activity		I_{50} concentrations for <u>Nitrobacter</u> activity	
	p.p.m.	molar	p.p.m.	molar
Ioxynil	235	6.3×10^{-4}	1) 130** 2) 170	3.5×10^{-4} 4.5×10^{-4}
Bromoxynil	1) 300 2) 235	10.8×10^{-4} 8.4×10^{-4}	180	6.5×10^{-4}
NPH 1320 *	20**	0.5×10^{-4}	12.5**	0.3×10^{-4}
Totril *	32**	0.6×10^{-4}	15.0**	0.3×10^{-4}
Tricamba	»1000	» 39.4×10^{-4}	»1000	» 39.4×10^{-4}
Dicamba	»1000	» 45.2×10^{-4}	»1000	» 45.2×10^{-4}
Legurame *	1) 2.6 2) 3.2	0.11×10^{-4} 0.14×10^{-4}	85.0 140.0	3.6×10^{-4} 5.9×10^{-4}
Oxadiazon *	1) 31 2) 25	0.89×10^{-4} 0.72×10^{-4}	145 147.5	4.2×10^{-4} 4.3×10^{-4}
Trifluralin *	1) 50 2) 48 3) 46	1.5×10^{-4} 1.4×10^{-4} 1.4×10^{-4}	340 340	10.1×10^{-4} 10.1×10^{-4}
M & B 9057	»1000	53.8×10^{-4}	»1000	» 53.7×10^{-4}
M & B 9555	560	24.6×10^{-4}	1) »1000 2) »1000	» 43.9×10^{-4} » 43.9×10^{-4}

* Used in the form of emulsifiable concentrates.

** Results of experiments carried out using oxygen electrode.

fluralin which were tested in the form of emulsifiable concentrates were all found to be highly toxic and effective in causing 50% inhibitions of the respiratory activities of both the nitrifying organisms at extremely low concentrations.

NPH 1320 (Bromoxynil octanoate 25% w/v ester equivalents) was found to reduce the rate of respiration in a sample of Nitrobacter cell suspension to about 88% at a concentration as low as 0.3 p.p.m. and caused 50% inhibition of the overall respiratory activity of the same organism, at a concentration equivalent to 12 p.p.m. (0.3×10^{-4} M, estimated by probit analysis) of active ingredient (Fig. 56). The corresponding observations with Nitrosomonas cell suspensions were similar although the I_{50} concentration (estimated by probit analysis) was found to be a little higher and equivalent to 20 p.p.m. (0.5×10^{-4} M) of active ingredient.

Totril (Ioxynil octanoate 25% w/v ester equivalents) too showed inhibition curves similar to those obtained with NPH 1320 and as seen from Figures 53 and 56 this was true regarding the effects of this herbicide on the respiratory activities of both the organisms Nitrosomonas and Nitrobacter. As evident from Table 21 the concentrations of Totril effective in causing 50% inhibition of the rates of oxygen uptake of Nitrobacter and Nitrosomonas cell suspensions were 15 p.p.m. (0.3×10^{-4} M, estimated by eye) and 32 p.p.m. (0.6×10^{-4} M, estimated by probit analysis) respectively. Thus it is clear that NPH 1320 and Totril too were found to be more effective in inhibiting Nitrobacter cell respiration in comparison to their effectiveness in inhibiting Nitrosomonas cell respiration.

Unlike the four herbicides dealt with so far the next three herbicides Legurame, Oxadiazon and Trifluralin which were found to be highly effective in inhibiting the respiratory activities of Nitrosomonas and Nitrobacter cell suspensions showed that they were more toxic to Nitrosomonas cell suspensions as compared with their toxic levels to Nitrobacter cell suspensions. This difference in toxic levels is seen

quite clearly from the I_{50} values presented in Table 21 which shows that Legurame, Oxadiazon and Trifluralin were effective in causing 50% inhibition of the rates of oxygen uptake by Nitrosomonas at concentrations, of active ingredient, equivalent to 3 p.p.m. ($0.14 \times 10^{-4}M$), 30 p.p.m. ($0.8 \times 10^{-4}M$) and 48 p.p.m. ($1.4 \times 10^{-4}M$) respectively while the same three herbicides showed much higher I_{50} concentrations with Nitrobacter cell suspensions, these concentrations being 100 p.p.m. ($5 \times 10^{-4}M$), 145 p.p.m. ($4.2 \times 10^{-4}M$) and 340 p.p.m. ($10 \times 10^{-4}M$) respectively.

The remaining four herbicides tested in this section namely Tricamba, Dicamba, M & B 9057 (Asulam) and M & B 9555 were found to be less effective in inhibiting the rates of oxygen uptake by Nitrobacter cell suspensions and with all the four herbicides, the I_{50} values exceeded the maximum concentration (1000 p.p.m.) tested. Considering the effects of these herbicides on the respiratory activities of Nitrosomonas too, it was found that the concentrations effective in causing 50% inhibition of the rates of respiration of Nitrosomonas cell suspensions exceeded the maximum concentration (1000 p.p.m.) tested with the only exception being M & B 9555 which was effective in causing 50% inhibition of the respiration rates at a concentration equivalent to 560 p.p.m. (estimated by probit analysis).

CHAPTER 8Effects of Herbicides on growth of Nitrosomonas Europaea and Nitrobacter Winogradski in Pure CulturesIntroduction

The two previous chapters 6 and 7 have already dealt with the effects of a range of herbicides on short term nitrification rates and the rates of oxygen consumption by cell suspensions of pure cultures of Nitrosomonas Europaea and Nitrobacter Winogradski. Since both these experiments were short term ones lasting not more than two hours and therefore not involving any growth of the organisms concerned, the results from them do not bear any relation to the effects of the herbicides on the growth of Nitrosomonas and Nitrobacter cells in liquid media. Thus it was decided to carry out some long term growth experiments with cell suspensions of the above organisms and lasting up to 14 days to find out whether at least a few of the herbicides under consideration have similar ranges of toxicities on the growth rates of Nitrosomonas and Nitrobacter cells.

There is only very little information available in literature regarding the effects of herbicides on the growth rates of nitrifying organisms. The earliest work in this field is by Caseley & Luckwill (1965) who reported on the inhibitory effects of some triazine herbicides on pure culture nitrification, during long term experiments lasting for 20 days, by Nitrosomonas Europaea, Nitrobacter agile and also Azotobacter vinelandii. Debona (1967) too has reported on the effects of a range of herbicides including Paraquat, Propanil, Monuron, Picloram etc. on the nitrification by these organisms during long term experiments. Later Winely & San Clemente (1968, 1969) and also Garretson & San Clemente (1968) have reported on extensions of similar work with Nitrobacter agile and several pesticides which were mainly insecticides.

None of the above works has clearly revealed the effects of different pesticides on the growth rates of pure cultures of nitrifiers.

Therefore an attempt was made here to find out whether these herbicidal chemicals in fact have any deleterious effects on the growth rates of Nitrosomonas and Nitrobacter cultures maintained in aerated liquid media.

The most common means of estimating bacterial growth is by 'viable count' or 'total count' techniques, the full critical descriptions of which were given by Wilson (1922). An attempt was made here to estimate, using a haemocytometer slide, the cell numbers in samples of Nitrosomonas and Nitrobacter cultures growing in liquid media containing different herbicides, with the aim of finding out whether the oxidations of ammonia and nitrite ions by growing cultures of the respective organisms were necessarily accompanied by corresponding increases in cell numbers. But this had to be given up in view of the practical difficulties involved in taking large numbers of counts.

Another simple and widely used method for following the growth of micro-organisms is by the measurement of increase in optical density of the growing cultures by means of a nephelometer. The possibility of using this nephelometric technique in the present investigation was also considered and this too had to be ruled out due to the following reasons,

- a) Very thin growth of nitrifying organisms.
- b) Some of the herbicides which were in the form of emulsions caused too much of scattering of light.
- and c) Several of the herbicides used in these growth experiments were coloured thus making it difficult to select a wavelength (for optical density measurements) at which the cells as well as the medium containing these coloured organic herbicidal chemicals do not absorb light appreciably.

Failing to carry out any of the direct methods to make cell counts, a more indirect method involving the assessment of the biochemical activities of these organisms, over the experimental periods, had to be tried in order to get an estimate of their rates of growth in liquid media

containing different known concentrations of the herbicides. In this indirect method, the rates of accumulation of nitrite -N in Nitrosomonas cultures and the rates of oxidation of nitrite -N in Nitrobacter cultures were estimated and the data were analysed as discussed in Chapter 2.

Experimental procedure

0.2 ml. samples of Nitrosomonas and Nitrobacter cell suspensions were inoculated into a series of 250 ml. conical flasks each containing 50 ml. of the appropriate sterile basal medium made up as described in Chapter 2, and having 0.45 gm. of ammonium sulphate / litre as the nitrogen source for Nitrosomonas and 0.45 gm. of sodium nitrite / litre as the nitrogen source for Nitrobacter respectively. The herbicide solutions of known concentrations were prepared in sterile water and later added aseptically into the sterile nutrient solutions (at the time of inoculation) so as to establish the desired serial concentrations of the herbicides in the culture media. The culture media had to be sterilised prior to incorporation of the herbicides since there was no information available regarding the effects of autoclaving on the specific activities of these herbicidal chemicals. The culture flasks were then incubated at 25°-27°C in a horizontal reciprocal shaker to enhance aeration and to ensure that the metabolites did not accumulate around the bacterial cells. All the experiments were carried out in sets using a series of different concentrations of each of the herbicides under consideration, and control experiments were also run along with each of these sets.

The culture media were sampled daily and the amounts of $\text{NO}_2^- - \text{N}$ per 0.1 ml. of each medium ($\mu\text{g NO}_2^- - \text{N} / 0.1 \text{ ml. medium}$) were estimated using the standard Griess Ilosvay's technique described in Chapter 2. The pH of the Nitrosomonas culture media were maintained around 7.8 by the addition of small amounts of sterile 5% potassium carbonate solution. (Trace amounts of phenol red were incorporated, as the pH indicator, into these media.)

The insoluble herbicides used here, such as Phenmedipham and Chlorbufam were added directly into the sterile culture media in fine powder form so as to result in fine suspensions while the other herbicides Legurame, Oxadiazon, Trifluralin, Totril and NPH 1320 were incorporated in the form of emulsions containing the appropriate concentrations of the active ingredients. Ioxynil and Bromoxynil being in the form of soluble salts were incorporated into the culture media in solution form. All the growth experiments reported here were usually carried out over periods of 12-14 days.

Results

In these experiments, the effects of the herbicides (applied at concentrations ranging from 1 to 500 $\mu\text{g/ml.}$) on the growth of nitrifiers were determined by following the degree of oxidation of the nitrogen source supplied to them in their growth media. A typical set of progress curves showing the time course of formation of $\text{NO}_2^- - \text{N}$ by the Nitrosomonas cultures growing in liquid media containing a range of concentrations (0, 1, 10, 50 and 100 p.p.m.) of Totril are shown in Figure 18 while Figure 20 presents a set of progress curves showing the time course of oxidation of $\text{NO}_2^- - \text{N}$ by Nitrobacter cultures growing in liquid media containing a series of concentrations (0, 1, 5, 10 and 50 p.p.m.) of NPH 1320. The rate curves corresponding to the above progress curves are shown in Figures 19 and 21 respectively.

The maximum rates of formation of $\text{NO}_2^- - \text{N}$ (derived from the differential curves) attained by cultures of Nitrosomonas Europaea growing in liquid media each containing a known concentration of a different herbicide, and in the corresponding controls, are given in Table 22 whereas the maximum rates of oxidation of $\text{NO}_2^- - \text{N}$ attained by cultures of Nitrobacter Winogradski growing in liquid media each containing a known herbicide concentration, and in the corresponding controls (these rates being estimated at times when the media had standard concentrations of residual $\text{NO}_2^- - \text{N}$ as

discussed in Chapter 2) are given in Table 23. The rates of formation of $\text{NO}_2^- - \text{N}$ by Nitrosomonas cultures and the rates of oxidation of $\text{NO}_2^- - \text{N}$ by Nitrobacter cultures expressed as percentage values of the appropriate controls were then plotted against the logarithms of the corresponding herbicide concentrations, as shown in Figures 57 and 58 and Figures 59 and 60 respectively.

As previously mentioned in Chapter 2, the rates of formation of $\text{NO}_2^- - \text{N}$ and the rates of oxidation of $\text{NO}_2^- - \text{N}$ by Nitrosomonas and Nitrobacter cultures respectively were considered as indicating the population sizes of these two organisms and in interpreting the results it must be borne in mind that the population sizes measured in this way are usually modified by the effects of the herbicides on the nitrification rates per cell and also due to the possible effects of product accumulation.

From the results presented in Figure 58, it can be seen that the growing cultures of Nitrosomonas Europaea were sensitive to Ioxynil and NPH 1320 applied at concentrations as low as 1 $\mu\text{g./ml.}$ (1 p.p.m.) the respective maximum rates of formation of $\text{NO}_2^- - \text{N}$ being around 80% and 76%. Although these two herbicides exerted similar toxicities when used at low concentrations ranging up to 10 p.p.m. (Fig. 58), the concentration levels effective in causing almost complete inhibition of growth of Nitrosomonas cultures differed significantly and were of the order of 500 p.p.m. and 250 p.p.m. respectively.

On the other hand, the results presented in Table 24 indicate that the time lag to reach the maximum rates of formation of $\text{NO}_2^- - \text{N}$ in growing Nitrosomonas cultures treated with concentrations of Ioxynil ranging up to 50 p.p.m. (i.e. 1, 5, 10 and 50 p.p.m.) were similar to the control value whereas the time lag to attain maximum rate of formation of $\text{NO}_2^- - \text{N}$ in the Nitrosomonas culture growing in the presence of 100 p.p.m. concentration of Ioxynil was increased by 3 times the control value. These observations along with the comparable values obtained for the rates of formation of

Table 22

Maximum rates of formation of NO_2^- - N attained in cultures of Nitrosomonas Europaea growing in liquid media containing different herbicides at known concentrations.

(All rates expressed in terms of $\mu\text{g NO}_2^-$ - N formed / ml. culture / day.)

Herbicides	Herbicide concentrations used in p.p.m.							
	Control	1	5	10	50	100	250	500
Legurame	16.43	16.10	13.34	1.43	0.38	-	-	-
Oxadiazon	15.73	15.03	10.31	1.52	0.04	-	-	-
Trifluralin	16.13	16.04	13.68	11.74	7.02	1.41	-	-
Phenmedipham	25.04	-	24.93	23.72	8.52	0.51	0.23	-
Chlorbufam	16.18	15.30	14.76	15.65	2.56	0.04	-	-
Ioxynil	16.43	12.72	12.66	12.26	12.28	11.54	-	1.58
Totril	13.27	12.69	-	8.06	4.50	3.48	0.31	-
Bromoxynil	13.65	-	13.72	12.95	12.03	8.36	3.62	0.06
NPH 1320	15.24	12.38	12.14	12.06	6.66	2.60	0.40	-

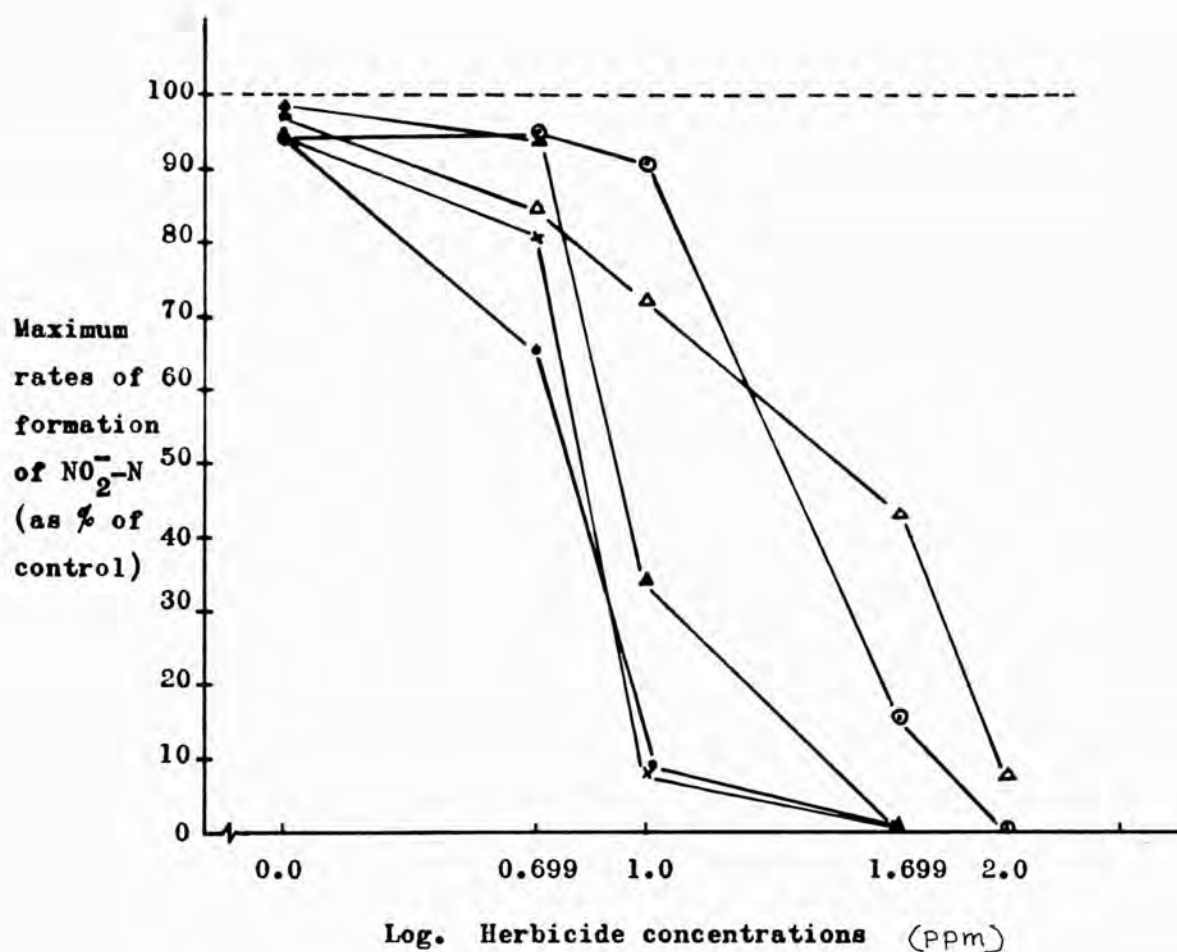


Fig. 57

Maximum rates of formation of $\text{NO}_2^- - \text{N}$ ($\mu\text{g. NO}_2^- - \text{N}$ formed/ml. culture medium/day) attained by pure cultures of Nitrosomonas Europaea growing in liquid media containing different herbicides at known concentrations.

(All rates expressed as percentage values of the corresponding controls)

• - Oxadiazon, x - Legurame, ▲ - Phenmedipham,
 Δ - Trifluralin, ⊙ - Chlorbufam.

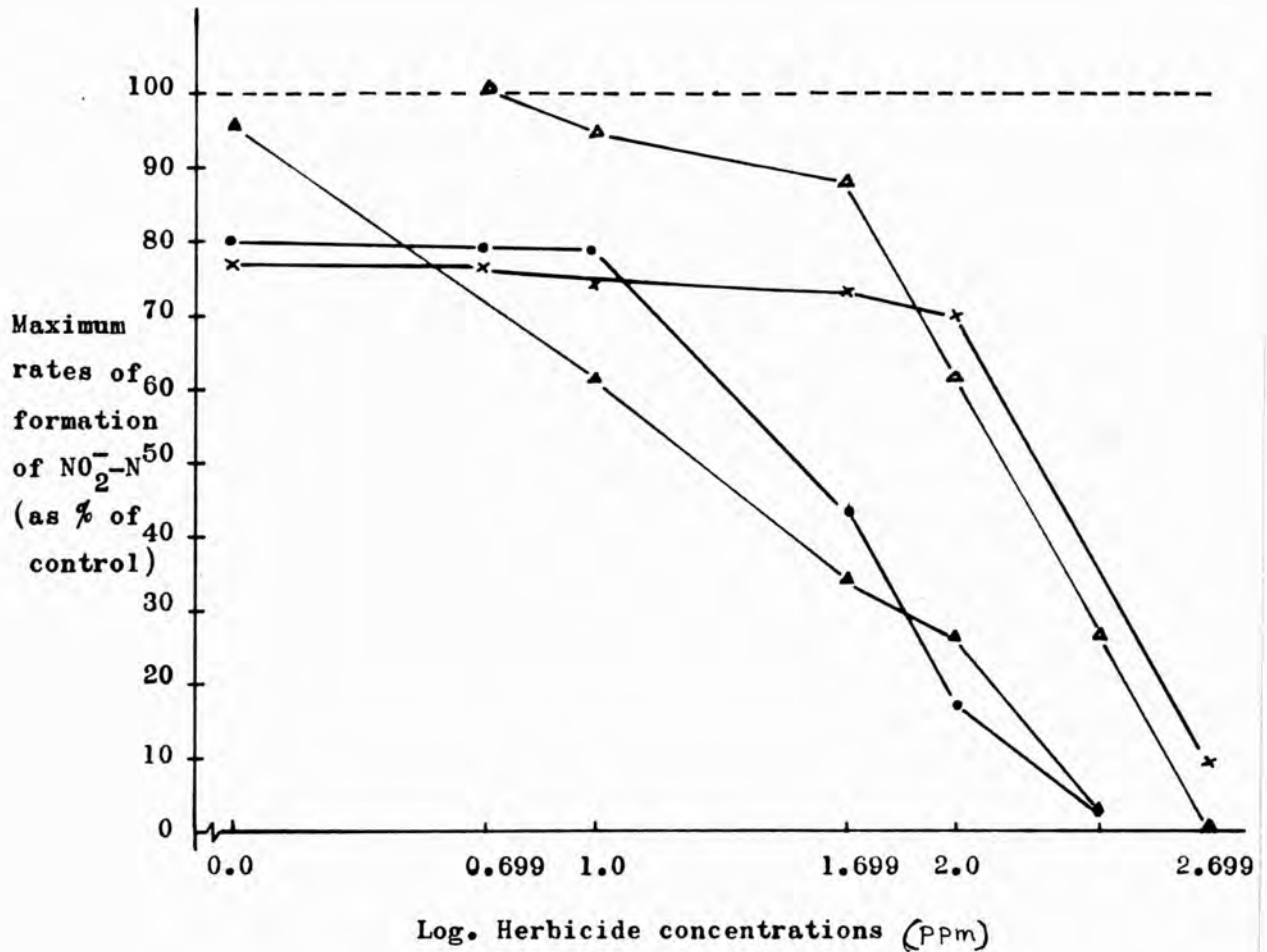


Fig. 58

Maximum rates of formation of $\text{NO}_2^- - \text{N}$ ($\mu\text{g. NO}_2^- - \text{N}$ formed/ml. culture medium/day) attained by pure cultures of Nitrosomonas Europaea growing in liquid media containing different herbicides at known concentrations. (All rates expressed as percentage values of the corresponding controls)

x - Ioxynil, • - NPH 1320, Δ - Bromoxynil,
▲ - Totril.

Table 23

Rate of oxidation of NO_2^- - N by cultures of Nitrobacter Winogradski growing in liquid media containing different herbicides at known concentrations.

(All rates expressed in terms of $\mu\text{g NO}_2^-$ - N oxidised / ml. culture medium / day.)

Herbicides	Herbicide concentrations in p.p.m.							Residual substrate level
	Control	1	5	10	50	100	250	
Ioxynil	63.0	-	67.0	59.0	47.0	25.0	0.0	50 $\mu\text{g/ml}$.
Bromoxynil	67.0	-	68.5	63.0	55.0	35.0	22.0	50 $\mu\text{g/ml}$.
NPH 1320	60.0	41.0	22.0	17.5	0.0	-	-	50 $\mu\text{g/ml}$.
Totril	56.0	40.0	27.0	23.0	0.0	-	-	50 $\mu\text{g/ml}$.
Chlorbufam	50.0	-	44.0	42.0	29.0	21.0	0.0	50 $\mu\text{g/ml}$.
Legurame	72.0	70.0	69.0	51.0	47.0	24.0	0.0	50 $\mu\text{g/ml}$.
Trifluralin	75.5	70.0	59.0	46.0	33.0	30.5	0.0	50 $\mu\text{g/ml}$.
Oxadiazon	80.0	66.0	49.0	34.0	29.0	25.0	0.0	80 $\mu\text{g/ml}$.

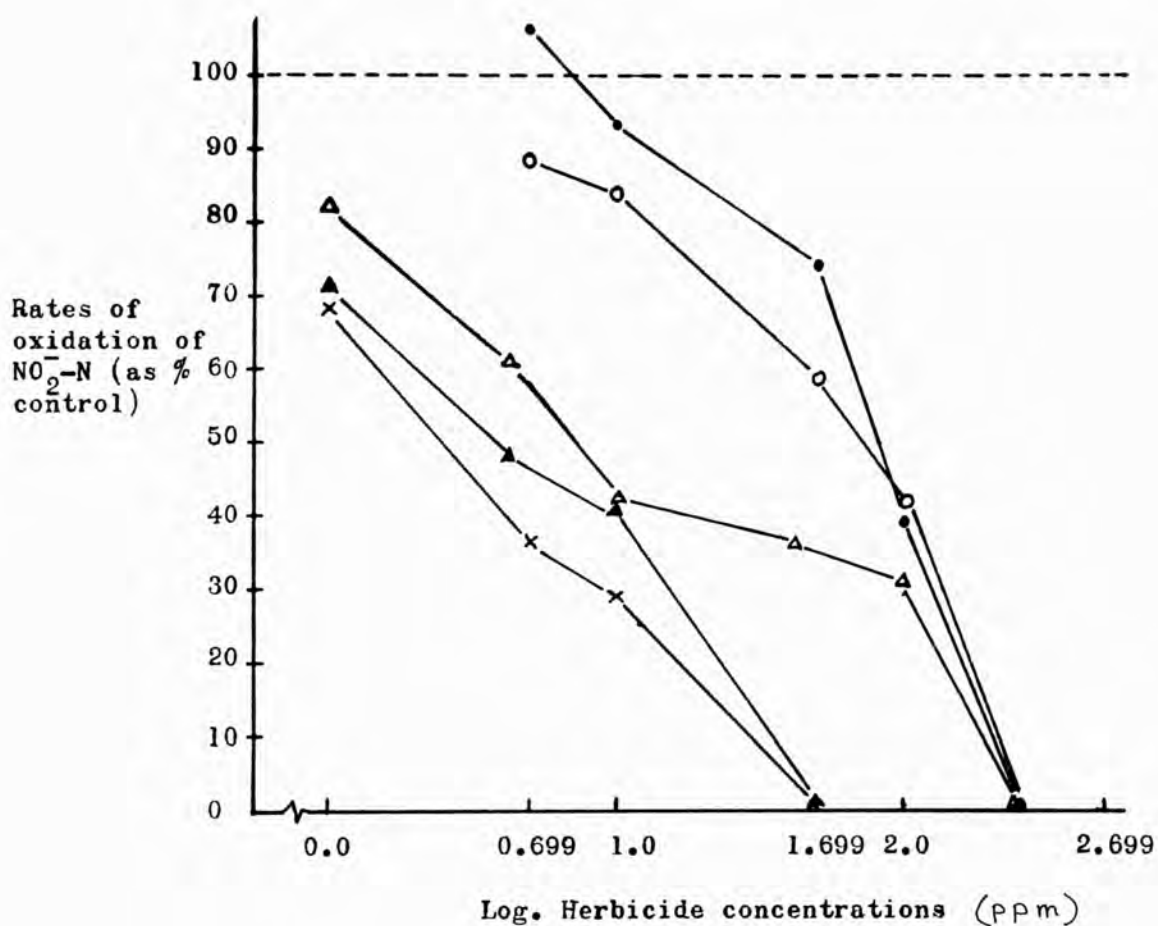


Fig. 59

Rates of oxidation of $\text{NO}_2^- - \text{N}$ ($\mu\text{g. NO}_2^- - \text{N}$ oxidised /ml. culture medium/day) attained by pure cultures of Nitrobacter Winogradski growing in liquid media containing different herbicides at known concentrations. (All rates expressed as percentage values of the corresponding controls)

x - NPH 1320, ▲ - Totril, ● - Ioxynil,
 Δ - Oxadiazon, ○ - Chlorbufam.

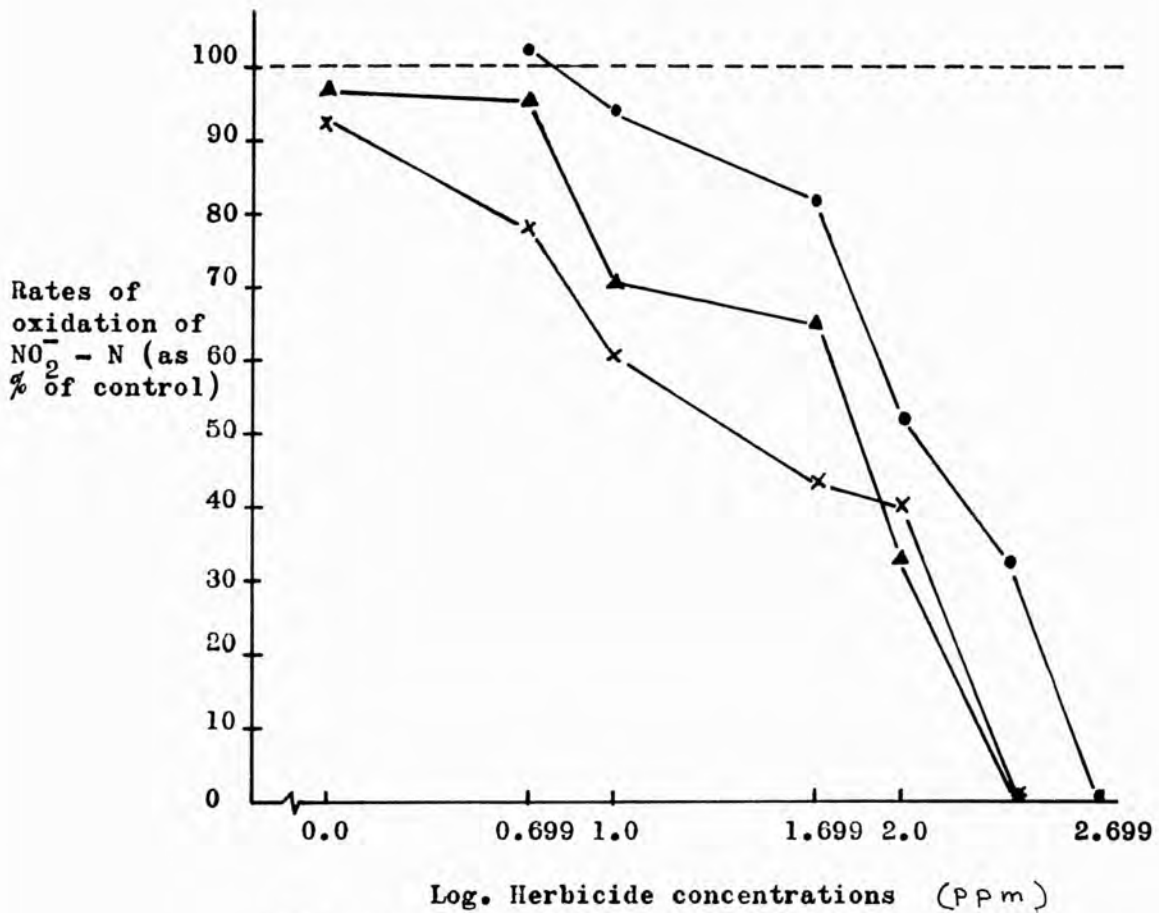


Fig. 60

Rates of oxidation of $\text{NO}_2^- - \text{N}$ ($\mu\text{g. NO}_2^- - \text{N}$ oxidised /ml. culture medium/ day) attained by pure cultures of Nitrobacter Winogradski growing in liquid media containing different herbicides at known concentrations. (All rates expressed as percentage values of the corresponding controls)

● - Bromoxynil, ▲ - Legurame, x - Trifluralin.

Table 24

Time (days) to reach maximum rates of formation of $\text{NO}_2^- - \text{N}$ by pure cultures of *Nitrosomonas Europaea* growing in liquid media containing known concentrations of different herbicides.

Herbicides	Herbicide concentrations used in p.p.m.							
	Control	1	5	10	50	100	250	500
Legurame	3.7	3.6	10.3	9.7	12.0	∞	-	-
Oxadiazon	3.7	3.5	6.0	8.6	12.0	∞	-	-
Trifluralin	2.9	3.3	2.8	3.0	10.0	∞	-	-
Phenmedipham	3.0	-	3.3	3.4	13.0	∞	∞	-
Chlorbufam	3.0	3.4	4.7	5.5	-	11.0	∞	-
Ioxynil	3.7	3.4	3.4	3.5	3.5	11.0	-	∞
Totril	3.1	3.1	-	4.8	6.2	15.0	∞	-
Bromoxynil	3.4	-	3.1	2.9	3.5	7.9	10.5	∞
NPH 1320	3.0	3.0	3.4	5.0	12.0	∞	∞	-

concentrations of Ioxynil (Table 24).

Considering the effects of this same herbicide Ioxynil on the growth of *Nitrosomonas* cultures, it can be seen from Figure 29 that the maximum rates of oxidation of $\text{NO}_2^- - \text{N}$ obtained by cultures of this organism growing in media containing different concentrations of Ioxynil were significantly inhibited, the rates being about 90%, 75% and 60% of concentrations equivalent to 10, 50 and 100 p.p.m. Also, Ioxynil was found to be effective in causing complete inhibition of growth in *Nitrosomonas* cultured at a concentration equivalent to 250 p.p.m. These inhibitions in growth rates were much higher than the inhibitions in the metabolic rates per cell of *Nitrosomonas* cell suspensions (Fig. 40) caused by similar concentrations of Ioxynil indicating that the inhibition of substrate

$\text{NO}_2^- - \text{N}$ (expressed as percentage of the corresponding control rates) by growing cultures of Nitrosomonas and by cell suspensions of Nitrosomonas during short term nitrification experiments (Figures 58 and 47 respectively) treated with different concentrations of Ioxynil ranging up to 50 p.p.m. does indicate that the observed inhibitions, in the maximum rates of formation of $\text{NO}_2^- - \text{N}$ caused by these Ioxynil concentrations were mainly due to the inhibition of metabolic rates per cell rather than due to any inhibition of the growth of this organism. The degree of inhibitions of the growth rates of Nitrosomonas cultures (expressed as percentage of control values) treated with 100 and 500 p.p.m. concentrations of Ioxynil too were almost of the same order (around 70% and 10% respectively) as the degree of inhibitions observed in the rates of formation of $\text{NO}_2^- - \text{N}$ by Nitrosomonas cell suspensions (77% and 10% respectively, Fig. 47) indicating that the inhibitions caused by these higher concentrations were also due to the effects on the metabolic rates per cell and not due to any specific effect on the growth of this organism. But as mentioned before, there was a significant delay in attaining the maximum rates of formation of $\text{NO}_2^- - \text{N}$ in these Nitrosomonas cultures grown in media containing 100 and 500 p.p.m. concentrations of Ioxynil (Table 24).

Considering the effects of this same herbicide Ioxynil on the growth of Nitrobacter cultures, it can be seen from Figure 59 that the maximum rates of oxidation of $\text{NO}_2^- - \text{N}$ attained by cultures of this organism growing in media containing different concentrations of Ioxynil were significantly inhibited, the rates being around 94%, 75% and 40% at concentrations equivalent to 10, 50 and 100 p.p.m. Also, Ioxynil was found to be effective in causing complete inhibition of growth in Nitrobacter cultures at a concentration equivalent to 250 p.p.m. These inhibitions in growth rates were much higher than the inhibitions in the metabolic rates per cell of Nitrobacter cell suspensions (Fig. 49) caused by similar concentrations of Ioxynil indicating that the inhibition of nitrite

oxidase activity might not be the main mode of action of this herbicide. This conclusion seems probable since the concentration of Ioxynil effective in causing 50% inhibition of the NO_2^- - N oxidation by Nitrobacter cell suspensions (122.5 p.p.m. estimated by probit analysis) also worked out to be approximately twice the concentration effective in causing 50% inhibition in growth (concentration of Ioxynil effective in causing 50% inhibition of the growth of Nitrobacter cultures, estimated by probit analysis, being 70 p.p.m.).

As would be expected, the results obtained with Bromoxynil (which is chemically very closely related to Ioxynil) regarding its effects on the growth of Nitrobacter cultures were similar to those discussed above. That is to say that in the case of the growing cultures of Nitrobacter treated with different concentrations of Bromoxynil too, the maximum rates of oxidation of NO_2^- - N (expressed as percentage of the corresponding control) were found to be greatly inhibited (Fig. 59) and these inhibitions were significantly higher than the inhibitions caused by the same concentrations of Bromoxynil, on the rates of oxidation of NO_2^- - N in cell suspensions of Nitrobacter during short term experiments (Fig. 50). But in the case of both these herbicides Ioxynil and Bromoxynil, the time lag to reach the maximum rates of oxidation of NO_2^- - N by growing cultures of Nitrobacter were found to be delayed only when the herbicide concentrations used were equivalent to or greater than 100 p.p.m. (Table 25).

On the other hand, the maximum rates of formation of NO_2^- - N (expressed as % of corresponding control) by growing cultures of Nitrosomonas treated with Bromoxynil concentrations up to 50 p.p.m. were found to be significantly higher than the corresponding rates observed in Nitrosomonas cultures treated with equivalent concentrations of Ioxynil, whereas the rates of formation of NO_2^- - N (as % of control) by Nitrosomonas cultures growing in media containing 100, 250 and 500 p.p.m. concentrations of Bromoxynil were very close to the rates observed in cultures treated

Table 25

Time (days) to reach the rates of oxidation of NO_2^- - N presented in Table 23, by pure cultures of *Nitrobacter Winogradski* growing in liquid media containing known concentrations of different herbicides.

Herbicides	Herbicide concentrations in p.p.m.						
	Control	1	5	10	50	100	250
Ioxynil	2.4	-	2.4	2.4	3.0	9.1	α
Bromoxynil	2.5	-	2.5	2.4	2.7	5.9	9.6
NPH 1320	2.3	4.7	6.4	7.6	α	-	-
Totril	2.3	4.5	6.0	7.0	α	-	-
Chlorbufam	3.0	-	3.4	5.0	5.9	8.6	α
Legurame	1.7	1.8	1.9	2.1	3.0	5.5	α
Trifluralin	2.4	2.7	2.8	2.9	5.4	6.6	α
Oxadiazon	3.4	3.7	3.9	4.7	8.8	10.4	α

toxic effects of NPH 1320 and Totril.

Considering the effects of these same herbicides on the growth of *Nitrobacter* populations, it was found that the different degrees of inhibitions of growth caused by varying concentrations of NPH 1320 and Totril (Fig. 59) were extremely high with complete cessation of growth being caused by 50 p.p.m. concentrations, and that these inhibitions (expressed as percentage of corresponding controls) were much higher than the inhibitions caused by similar concentrations of the same herbicides on the NO_2^- - N oxidation in *Nitrosifying* cell suspensions during short term experiments (Figs. 50 and 49). These observations along with the fact that the concentrations of NPH 1320 and Totril needed to cause 50% inhibition in the rate of NO_2^- - N oxidation by cell suspensions of *Nitrobacter* (32 p.p.m. and 21 p.p.m.) were respectively 11 and 4 times more than the concentrations

with similar concentrations of Ioxynil (Fig. 58).

It could also be seen from Figure 58 that the two closely related herbicides NPH 1320 and Totril exerted fairly high toxic effects on the growth of Nitrosomonas cultures. Comparison of the maximum rates of formation of $\text{NO}_2^- - \text{N}$ observed in these cultures of Nitrosomonas growing in media containing a range of known concentrations of NPH 1320 and Totril, with the rates of formation of $\text{NO}_2^- - \text{N}$ by Nitrosomonas cell suspensions (results from Figures 48 and 47 respectively) indicated that these two herbicides were effective in inhibiting the growth of Nitrosomonas cultures to a much greater extent than their metabolic rates per cell. Since the concentrations of NPH 1320 and Totril effective in causing 50% inhibition of the nitrite formation by Nitrosomonas cell suspensions (82.5 p.p.m. and 97.5 p.p.m. respectively, estimated by probit analysis) were approximately four and five times greater than the concentrations effective in causing 50% inhibition of the growth of Nitrosomonas cultures (21 p.p.m. estimated by probit analysis, and 20.5 p.p.m. estimated by eye, respectively), it could be concluded that growth of this organism was more sensitive to the toxic effects of NPH 1320 and Totril.

Considering the effects of these same herbicides on the growth of Nitrobacter populations, it was found that the different degrees of inhibitions of growth caused by varying concentrations of NPH 1320 and Totril (Fig. 59) were extremely high with complete cessation of growth being caused by 50 p.p.m. concentrations, and that these inhibitions (expressed as percentage of corresponding controls) were much higher than the inhibitions caused by similar concentrations of the same herbicides on the $\text{NO}_2^- - \text{N}$ oxidations in Nitrobacter cell suspensions during short term experiments (Figs. 50 and 49). These observations along with the fact that the concentrations of NPH 1320 and Totril needed to cause 50% inhibition in the rate of $\text{NO}_2^- - \text{N}$ oxidation by cell suspensions of Nitrobacter (22 p.p.m. and 21 p.p.m.) were respectively 11 and 4 times more than the concentrations

Table 26

The concentrations of different herbicides effective in causing 50% inhibition (I_{50}) of the rates of nitrification by growing cultures of Nitrosomonas Europaea and Nitrobacter Winogradski.

Herbicides	I_{50} concentrations for <u>Nitrosomonas</u> activity		I_{50} concentrations for <u>Nitrobacter</u> activity	
	p.p.m.	molar	p.p.m.	molar
Ioxynil	140.0	3.8×10^{-4}	70.0	$1.9 \times 10^{-4} M$
Bromoxynil	120.0	4.3×10^{-4}	115.0	$4.1 \times 10^{-4} M$
*NPH 1320	21.0	0.5×10^{-4}	2.8	$0.07 \times 10^{-4} M$
*Totril	20.5	0.4×10^{-4}	4.8	$0.1 \times 10^{-4} M$
Chlorbufam	21.0	1.2×10^{-4}	48.0	$2.7 \times 10^{-4} M$
*Legurame	6.0	0.3×10^{-4}	60.0	$2.5 \times 10^{-4} M$
*Trifluralin	24.0	0.7×10^{-4}	26.0	$0.8 \times 10^{-4} M$
*Oxadiazon	4.8	0.14×10^{-4}	12.0	$0.35 \times 10^{-4} M$
Phenmedipham	10.0	0.4×10^{-4}	-	-

* These herbicides were tested in the form of emulsifiable concentrates.

effective in causing 50% inhibition of the growth of this organism, in aerated liquid cultures (2.1 p.p.m. and 4.8 p.p.m.), indicate that NPH 1320 and Totril significantly inhibited the growth of Nitrobacter populations too.

The results presented in Figure 57 show very clearly that the remaining five herbicides, tested in this section of the present investigation (i.e. Oxadiazon, Legurame, Phenmedipham, Trifluralin and Chlorbufam) did exert very high toxicities on the growth of Nitrosomonas cultures. It was observed that the maximum sizes attained by the Nitrosomonas populations grown in liquid media containing Oxadiazon, Legurame or Phenmedipham, applied at a concentration equivalent to 10 p.p.m., were reduced to around 10%, 9% and 33% of the controls, respectively, whereas complete cessation of growth of Nitrosomonas cultures were caused by all three of these herbicides when applied at a concentration equivalent to 50 p.p.m. active ingredient (Fig. 57). But the other two herbicides Trifluralin and Chlorbufam were found to be slightly less toxic with the concentrations needed to cause complete cessation of growth being around 100 p.p.m. active ingredient. A comparison of the rates of formation of $\text{NO}_2^- - \text{N}$ (expressed as percentage of corresponding controls) by cell suspensions of Nitrosomonas treated with these herbicides (Figs. 47 and 48), with the maximum rates of formation of $\text{NO}_2^- - \text{N}$ attained by treated growing cultures of Nitrosomonas Europaea (Fig. 57) and a comparison of the concentrations of these same herbicides necessary to cause 50% inhibition of the above mentioned parameters (i.e. the results from Tables 17 and 26) makes it evident that the herbicides Oxadiazon, Legurame, Phenmedipham, Trifluralin and Chlorbufam too, like NPH 1320 and Totril, inhibit growth of Nitrosomonas populations to a much greater extent than the rates of enzymatic oxidation of ammonium ions by fully grown cells during short term experiments.

It could be seen from Table 24 that the time lag to reach the maximum rates of formation of $\text{NO}_2^- - \text{N}$ by growing cultures of Nitrosomonas

(in other words the rates of proliferation of Nitrosomonas cultures) treated with varying concentrations of Oxadiazon, Legurame, Phenmedipham, Chlorbufam and Trifluralin, ranging up to 50 p.p.m. were also significantly delayed, the time lag in treated cultures being increased by about 3 to 4 times the corresponding control values.

Considering the effects of these same herbicides on the proliferation of Nitrobacter cultures, it can be seen from Figure 60 that the maximum population sizes attained by the treated cultures (measured in terms of the maximum rates of oxidation of $\text{NO}_2^- - \text{N}$) were significantly high even when these cultures were growing under the influence of Legurame, Trifluralin or Oxadiazon applied at a concentration equivalent to 50 p.p.m., the respective population sizes attained being of the order of 65%, 44% and 36% of the corresponding controls. Also the concentrations of these herbicides effective in causing complete cessation of growth were approximately 250 p.p.m. and were found to be fairly high when compared with the concentrations effective in causing complete inhibition of the growth of treated Nitrosomonas cultures (discussed earlier in this chapter).

On the other hand, comparison of the derived values for the concentrations effective in causing 50% inhibition of the rates of oxidation of $\text{NO}_2^- - \text{N}$ by cell suspensions of Nitrobacter (Table 17) with the concentrations effective in causing 50% inhibition of the maximum rates of oxidation of $\text{NO}_2^- - \text{N}$ attained by growing cultures of Nitrobacter (Table 26) indicate that Oxadiazon and Legurame inhibited the growth in treated cultures 7 and 2 times, respectively, more than the $\text{NO}_2^- - \text{N}$ oxidation by cell suspensions, during short term experiments (i.e. metabolic rate per cell), whereas Chlorbufam and Trifluralin were found to inhibit both these parameters (i.e. growth and metabolic rates per cell) to equal extents.

Thus it could be concluded that growth of Nitrosomonas cultures were more sensitive than the enzymatic oxidation of ammonium ions by

suspensions of fully grown cells of this organism to the toxic effects of most of the herbicides tested in this section (i.e. NPH 1320, Totril, Legurame, Oxadiazon, Trifluralin, Phenmedipham and Chlorbufam) excepting Ioxynil and Bromoxynil which were found to be effective in causing preferential inhibition of enzymatic oxidation of ammonium ions by fully grown cells. On the other hand Ioxynil, Bromoxynil, NPH 1320, Totril, Legurame and Oxadiazon were effective in inhibiting the growth of Nitrobacter cultures to a much higher degree than their metabolic rates per fully grown cell.

It is evident from the foregoing discussion that the inhibition of enzymatic reactions responsible for the oxidations of ammonium ions and nitrite ions by Nitrosomonas Europaea and Nitrobacter Winogradski cells, respectively, is probably not the main mode of action of many of the tested herbicides. But as mentioned in the introduction to Chapter 6, the possibility remains that these herbicides exert their toxic effects through the inhibition of the neogenesis of the essential enzymes during growth.

CHAPTER 9Discussion

Out of the twelve herbicides that were tested, during the present investigation, for their effects on soil nitrification and on soil nitrifiers, Ioxynil and Bromoxynil were found to exert the highest toxicities on the rates of nitrification in enriched soil as well as in fresh soil. The other herbicides namely NPH 1320, Totrill, Legurame, Oxadiazon, Trifluralin and Terbacil were also found to exert significant toxic effects on soil nitrification whereas Tricamba, Dicamba, M & B 9057 (Asulam) and M & B 9555 were found to exert comparatively low toxicities within the concentration range tested (0 - 1000 p.p.m.).

It was also found that the two main stages of soil nitrification differed in their sensitivities to the toxic effects of the herbicides tested. That is to say that the degree of toxicity exerted by a particular herbicide on the activities of Nitrobacter spp. population in a treated soil usually differed from its effects on the activities of Nitrosomonas population in the same soil. For instance it was evident from the results discussed in Chapter 3 that the metabolic activity per cell of Nitrobacter species in enriched soils were more sensitive than the metabolic activity per cell of their Nitrosomonas populations, to the toxic effects of Ioxynil, NPH 1320, Totrill and Bromoxynil, whereas Legurame, Oxadiazon, Trifluralin and Terbacil were more toxic towards the metabolic activities of Nitrosomonas species than Nitrobacter species. A similar type of differential effects were seen regarding the toxicities exerted by various herbicides on the growing populations of Nitrosomonas and Nitrobacter species in freshly perfused soils (results from Chapter 4).

On the other hand the different degrees of inhibitions (expressed as percentage values of the corresponding controls) caused by many of the herbicide concentrations on the metabolic activities of the saturating

populations of nitrifiers in enriched soils were found to differ from the inhibitions caused by the same herbicide concentrations on the metabolic activities of the growing populations of nitrifiers in fresh soils, as could be seen from the data presented in Table 27.

As shown by Lees & Quastel (1946b) and Quastel & Scholefield (1951) a bacterial saturated or an 'enriched' soil resembles very much a mixed suspension of resting cells (discussed previously in Chapter 2) and therefore the effects of herbicides on the nitrification rates of an enriched soil would give an indication of the direct herbicidal effects on the metabolic rates per cell of the nitrifying organisms. But considering the herbicidal effects on the nitrifying capacity of freshly perfused soils, it becomes evident that an observed effect on the maximum rates of nitrification attained in a treated fresh soil could be due to:-

a) A direct effect on the nitrification rate per cell (effects on enriched soil)

or b) Due to an effect on the proliferation of nitrifiers, in addition to (a).

Both these possibilities could be checked by comparing the effects of the herbicides tested, on the nitrification rates (the nitrification rates due to the activity of Nitrosomonas spp. and Nitrobacter spp. being considered separately) of enriched soils with the effects of the same herbicides on the nitrification rates of freshly perfused soils bearing in mind that the maximum rates of formation of $\text{NO}_3^- - \text{N}$ and $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ (under conditions of excessive NO_2^- - accumulation) in a treated fresh soil would give an indication of the sizes of the saturating populations of Nitrobacter species and Nitrosomonas species, respectively, attained by it being modified by a factor dependent on the effects of herbicides on the metabolic rate per cell - as discussed in Chapter 2. It is clear from the above reasoning that one could not come to any conclusions regarding the effects of herbicides on the growth of nitrifiers in soil by a mere

Table 27

Herbicide effects on soil nitrification: All values expressed as percentage of controls containing no herbicides

(a) Rates of accumulation of $\text{NO}_3^- - \text{N}$ / ml. perfusate / day.

(b) Rates of accumulation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ / ml. perfusate / day.

Herbicides	Rates in previously enriched soil				Maximum rates attained in fresh soil					
	10 ppm	50 ppm	100 ppm	500 ppm	1000 ppm	10 ppm	50 ppm	100 ppm	500 ppm	1000 ppm
Ioxynil	(a) 92.9	57.2	32.3	1.7	0	81.8	62.7	49.9	5.8	0.2
	(b) 96.9	78.7	53.2	13.7	0.5	82.9	66.5	63.2	10.9	2.8
Bromoxynil	(a) 95.2	50.5	32.9	0	0	83.4	77.7	67.7	39.9	1.1
	(b) 86.7	92.9	65.1	16.3	0	84.7	103.8	76.3	45.5	8.8
NPH 1320	(a) 99.2	88.5	64.6	0	-	81.7	72.8	48.2	11.0	7.6
	(b) 98.6	89.9	71.0	21.8	-	69.0	98.9	44.5	11.4	-
Totrill	(a) 95.3	91.1	69.8	7.3	-	88.3	78.2	72.8	17.2	-
	(b) 96.9	91.6	86.7	39.9	-	85.3	107.6	45.9	20.5	-
Legurame	(a) 93.7	71.5	59.9	31.5	3.9	94.3	75.6	71.1	41.4	22.3
	(b) 88.9	74.5	63.5	35.1	4.2	88.8	100.8	69.7	42.8	29.6
Oxadiazon	(a) 98.7	80.1	74.2	42.2	15.0	87.2	84.4	78.5	67.9	53.9
	(b) 98.3	79.1	73.9	40.7	14.5	79.9	82.4	74.5	66.3	52.5
Trifluralin	(a) 93.4	80.1	69.8	39.0	25.8	129.9	148.6	160.1	82.0	40.0
	(b) 102.3	80.3	69.6	67.8	25.3	104.6	115.1	150.2	65.0	40.3
Tricamba	(a) 90.5	80.3	75.3	65.4	56.5	102.2	132.4	178.3	62.9	40.4
	(b) 110.1	98.1	90.2	79.9	65.6	110.9	101.1	82.9	-	50.3
Dicamba	(a) 106.7	103.8	101.5	69.2	56.8	104.8	78.4	75.5	-	62.2
	(b) 108.4	105.8	101.3	84.1	58.4	100.2	78.9	72.2	-	58.7
Terbacil	(a) 68.1	66.3	58.3	34.9	15.3	98.9	78.9	72.2	-	58.9
	(b) 68.1	66.3	58.3	34.9	15.3	84.7	85.1	75.0	58.5	2.9
M & B 9555	(a) 111.3	96.4	95.0	94.5	96.9	79.2	82.8	74.4	68.4	7.29
	(b) 95.6	94.4	96.9	90.4	84.5					
M & B 9057	(a) 87.7	78.2	75.2	69.5	69.3					
	(b) 88.3	75.4	71.8	67.6	66.9					

consideration of the effects these herbicides exert on the nitrification rates of fresh soils although it is known from the work of Seifert (1968) (discussed in Chapter 2) that the degree of nitrification observed in a freshly perfused soil is primarily due to the metabolic activities of the growing cells of nitrifying organisms.

Combining the results from Chapter 3 with those of Chapter 4 and assuming no interfering factors (as discussed in Chapter 2), the following relationship must hold good:

$$\text{i.e. } X_1 N_1 = R_1 \quad \text{————— (1)}$$

where,

X_1 is the rate of nitrification in untreated enriched soil,

N_1 is the maximum number of nitrifying cells (viable nitrifiers)

that would be present in control sample of soil at saturation,

and R_1 is the maximum rate of nitrification attained in fresh soil

perfused with ammonium sulphate in the absence of any herbicides.

But if the rate of nitrification in enriched soil (i.e. rate of nitrification/cell) is reduced to X_2 due to the effect of a particular herbicide,

then,

$$X_2 N_2 = R_2 \quad \text{————— (2)}$$

where, N_2 is the maximum number of nitrifying cells (viable nitrifiers)

that would be present in the treated soil at saturation,

and R_2 is the maximum rate of nitrification attained in fresh soil

treated with an equivalent concentration of the same herbicide as above.

Dividing equation (2) by (1)

$$\frac{X_2 N_2}{X_1 N_1} = \frac{R_2}{R_1} \quad \text{————— (3)}$$

Multiplying each side of equation (3) by 100,

$$\frac{X_2}{X_1} \times 100 \times \frac{N_2}{N_1} = \frac{R_2}{R_1} \times 100 \quad (4)$$

$\frac{X_2}{X_1} \times 100$ = rate of nitrification in treated enriched soil expressed as percentage value of the corresponding control. = Q

$$\text{i.e. } Q \times \frac{N_2}{N_1} = \frac{R_2}{R_1} \times 100 \quad (5)$$

$$\frac{N_2}{N_1} = \frac{R_2}{R_1} \times \frac{100}{Q} \quad (6)$$

Since it was not possible to get actual counts of bacterial cell numbers, during the present investigation, it was necessary to specify the value of $\frac{N_2}{N_1}$ with reference to unity if one need to deduce any conclusions regarding the effects of herbicides on the growth of nitrifiers.

If,

$$\frac{N_2}{N_1} = 1$$

equation (6) can be rewritten as,

$$R_2 = \frac{R_1 Q}{100} \quad (7)$$

It also follows that if $\frac{N_2}{N_1} < 1$,

then

$$R_2 < R_1 Q/100$$

and if

$$\frac{N_2}{N_1} > 1$$

then

$$R_2 > R_1 Q/100.$$

The values of R_2 and R_1 (i.e. the maximum rates of nitrification observed in treated and control samples of fresh soil respectively) and the value of Q being known it was easy to find out how N_2 differed from N_1 (N_2 and N_1 being the cell numbers of nitrifying organisms in herbicide treated and control soils respectively at saturation).

The results from Chapters 3 and 4 were analysed as discussed above and the derived data are presented in Figures 61, 62, 63 and 64 where the 'observed maximum rate of formation' of $\text{NO}_3^- - \text{N}$ or $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ (depending on whether the nitrification rates due to Nitrobacter or Nitrosomonas activities respectively were considered) refers to R_2 and the 'theoretical rates of nitrification' refers to $\frac{R_1 Q}{100}$ in equation (7) above.

Considering the results presented in Figure 61 it could be seen that the 'observed' maximum rates of formation of $\text{NO}_3^- - \text{N}$ in fresh soils treated with varying concentrations of NPH 1320 or Totril (ranging up to 100 p.p.m.) were less than the 'theoretically expected' maximum rates of formation of $\text{NO}_3^- - \text{N}$ under similar experimental conditions. Thus it could be concluded that the population growth of Nitrobacter species in freshly perfused soils treated with NPH 1320 or Totril, at concentrations ranging up to about 100 p.p.m., were more sensitive than the nitrification rates per individual, fully grown cell. But the fact that the 'observed' maximum rates of formation of $\text{NO}_3^- - \text{N}$ in fresh soil samples treated with 500 and 1000 p.p.m. concentrations of NPH 1320 or Totril were slightly higher or almost equal to the 'theoretical' maximum rates of formation of $\text{NO}_3^- - \text{N}$ could either mean:-

- a) that the main mode of action of these high concentrations are probably via the inhibition of the enzymatic systems responsible for the oxidation of nitrite rather than by any inhibition of the proliferation of Nitrobacter species populations;
- or b) that the herbicides Totril and NPH 1320 would have suffered a certain degree of decomposition by the time the observed maximum rates of formation of $\text{NO}_3^- - \text{N}$ were recorded (38 days of perfusion - refer Fig. 10)
- or c) that the growing Nitrobacter populations tend to develop a certain degree of resistance to the herbicide, with time.

However there is no evidence available for the second possibility

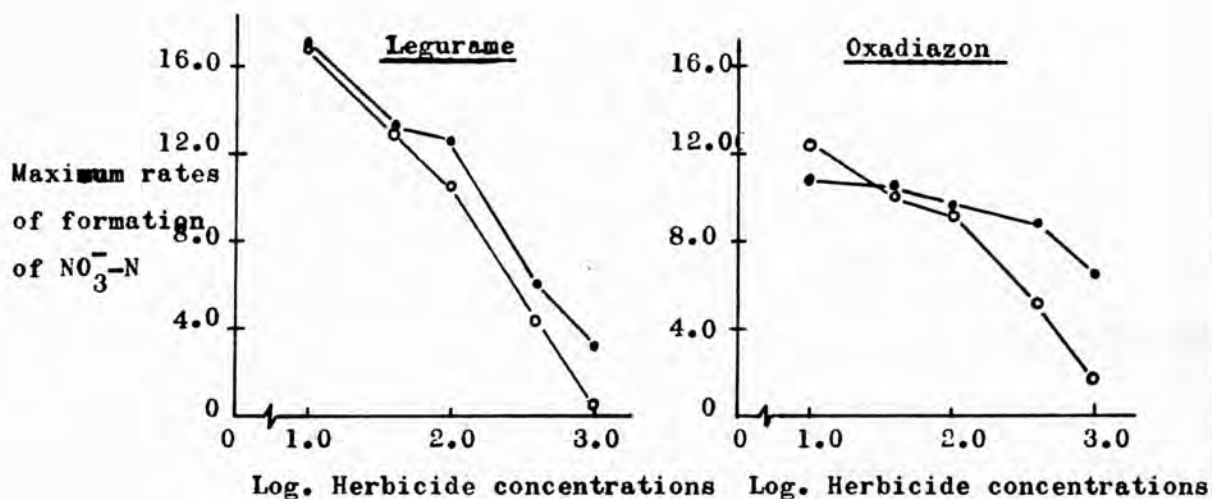
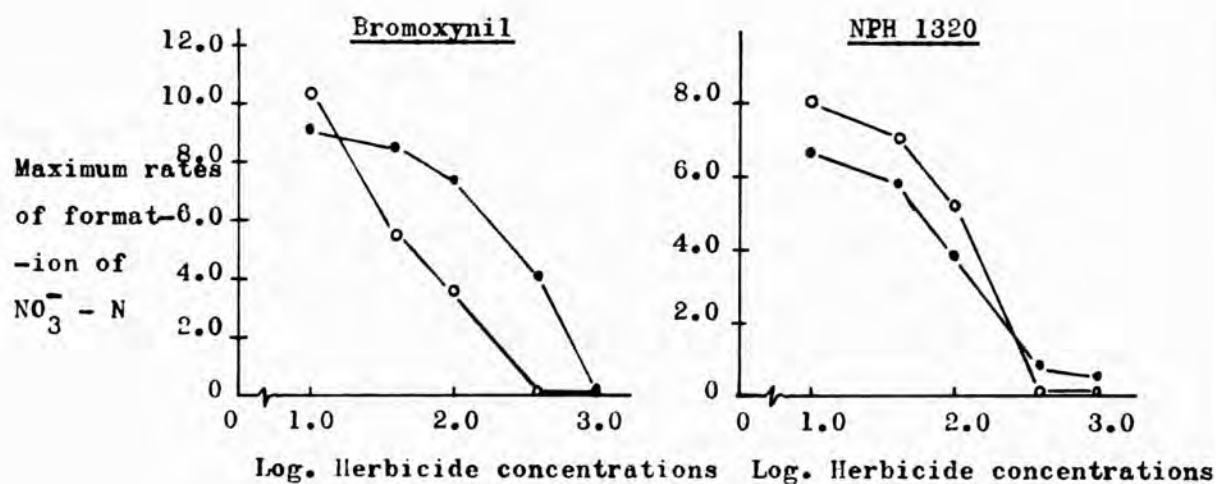
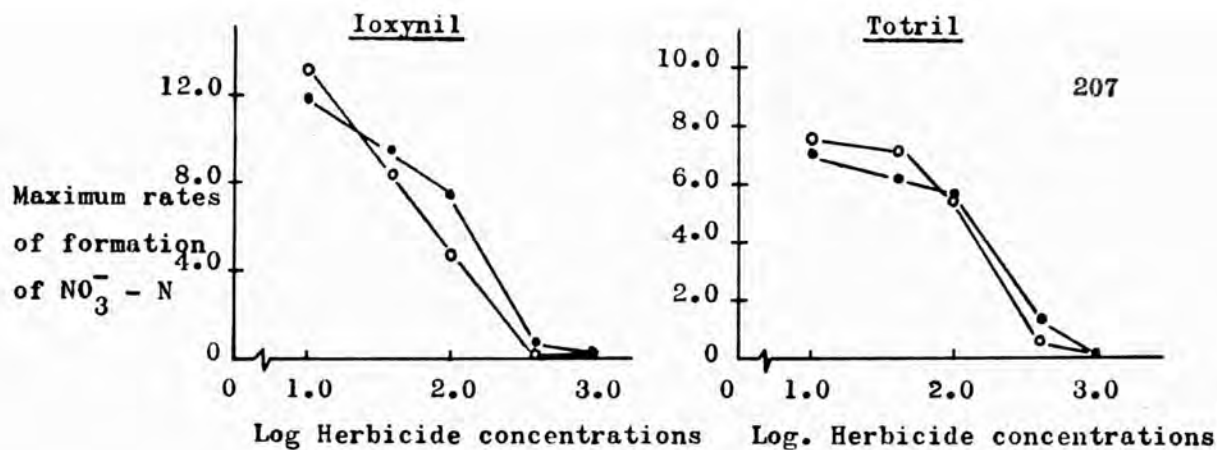


Fig. 61

● - Observed maximum rates of formation of $\text{NO}_3^- \text{-N}$ ($\mu\text{g. NO}_3^- \text{-N}$ formed/ml.perfusate/day) when samples of fresh soil were perfused with Ammonium sulphate solutions containing different herbicide concentrations.

o - Theoretical rates of formation of $\text{NO}_3^- \text{-N}$ in the same soil samples.

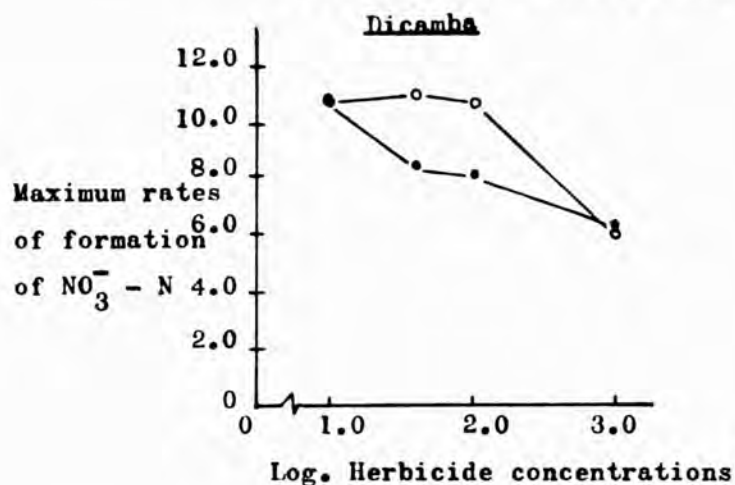
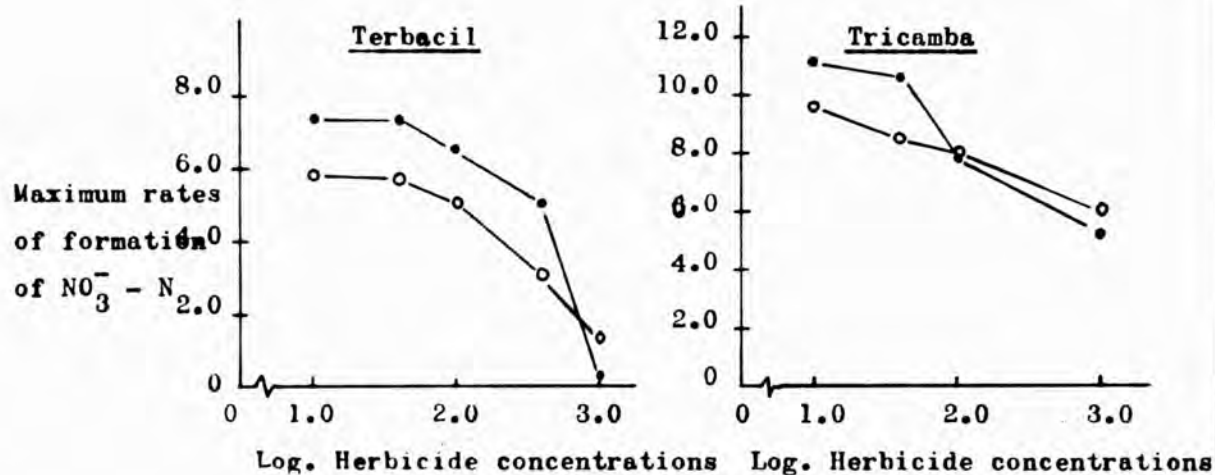
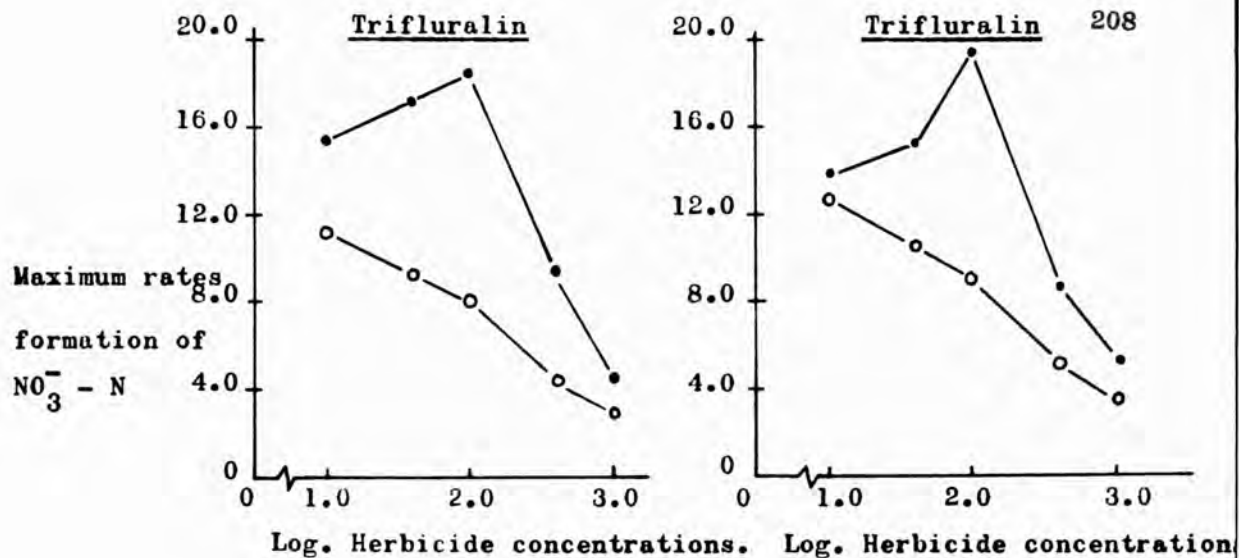


Fig. 62

● - Observed maximum rates of formation of $\text{NO}_3^- - \text{N}$ ($\mu\text{g} \cdot \text{NO}_3^-$ -formed/ml.perfusate/day) when samples of fresh soil were perfused with Ammonium sulphate solutions containing different herbicide concentrations.

○ - Theoretical rates of formation of $\text{NO}_3^- - \text{N}$ in the same soil samples.

(b) although the results from Chapter 4 (Figs. 38 and 39) points towards a possibility of Nitrobacter spp. populations, growing in soil, showing up a certain degree of adaptation with time, to tolerate the toxic effects of high concentrations (500 and 1000 p.p.m.) of NPH 1320.

Considering the parallel set of results obtained with respect to the activities of Nitrosomonas populations in soils treated with Totril and NPH 1320 (Fig. 63) respectively, it becomes evident that the 'observed' maximum rates of accumulation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ were much lower than the 'theoretical' maximum rates of accumulation of $(\text{NO}_3^- + \text{NO}_2^-) - \text{N}$ at most of the test concentrations. These observations allow one to conclude that the two herbicides NPH 1320 and Totril seem to inhibit the proliferation of Nitrosomonas populations in freshly perfused soils to a greater extent than the metabolic activities of saturation populations of Nitrosomonas in enriched soils would be inhibited by them under identical experimental conditions. As expected this conclusion is in line with the observations discussed in Chapter 4, that the nitrifying activities of growing Nitrosomonas populations limited the overall nitrification rates in freshly perfused soils treated with NPH 1320 or Totril at higher concentrations.

Considering the other herbicides namely Ioxynil, Bromoxynil, Legurame, Oxadiazon, Trifluralin, Terbacil and Tricamba, it could be seen from Figures 61 and 62 that the 'observed' maximum rates of formation of $\text{NO}_3^- - \text{N}$ in soils treated with these herbicides were higher than the 'theoretical' rates of formation of $\text{NO}_3^- - \text{N}$ in them. These differences were found to be most significant in the case of Trifluralin and Bromoxynil and could be explained as being probably due to a development of an adaptive mechanism, by the nitrate forming organisms in soil, during the period of exposure to the herbicides concerned. But out of all these herbicides only Ioxynil and Bromoxynil were found to cause significant accumulations of $\text{NO}_2^- - \text{N}$ (as discussed in Chapter 4) and thus the $\text{NO}_3^- - \text{N}$ accumulations in soils treated with Ioxynil and Bromoxynil only, could be considered as

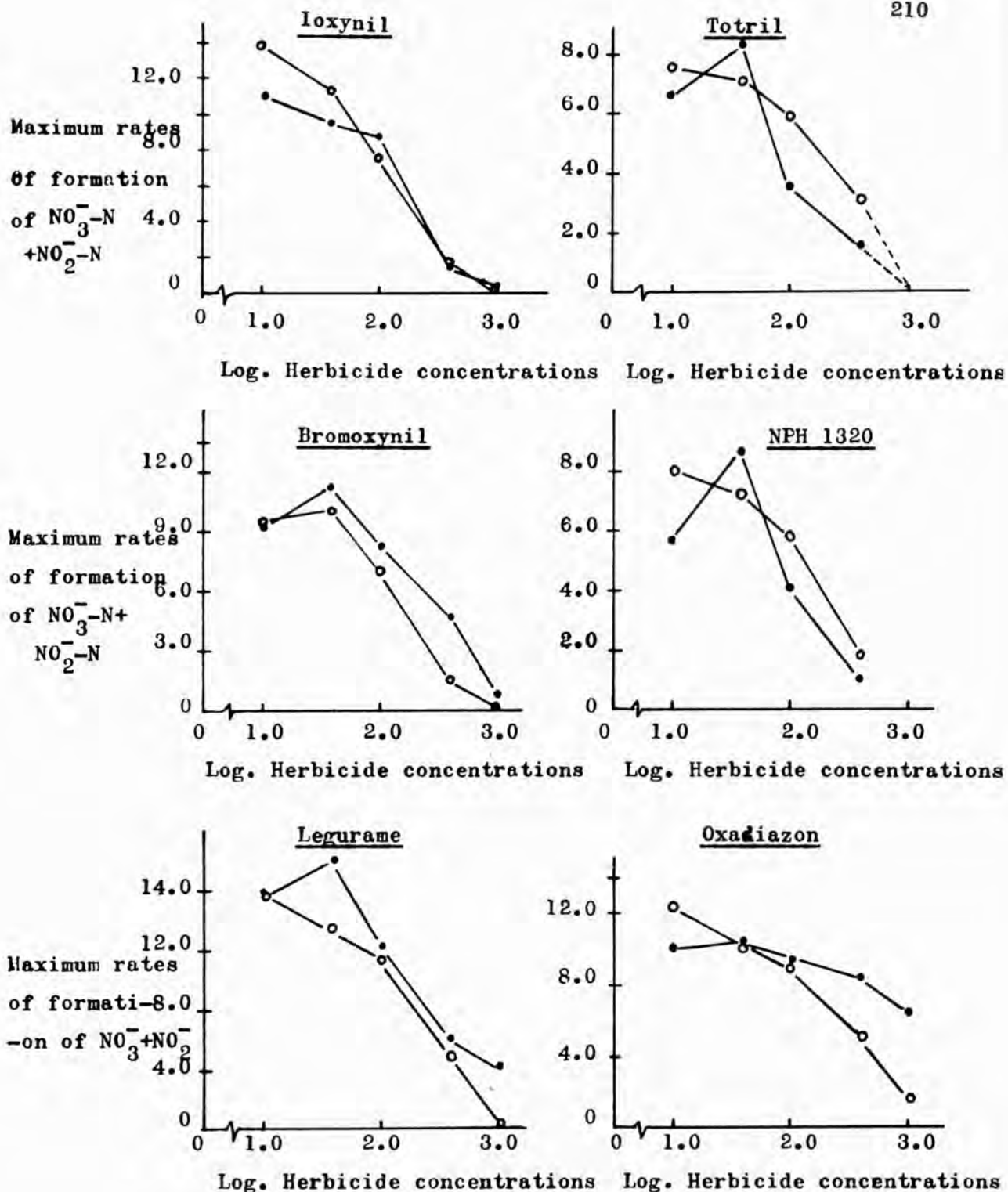


Fig. 63

- - Observed maximum rates of formation of $\text{NO}_3^- + \text{NO}_2^- - \text{N}$ ($\mu\text{g. NO}_3^- + \text{NO}_2^- - \text{N}$ formed/ml.perfusate/day) when samples of fresh soil were perfused with Ammonium sulphate solutions containing different herbicide concentrations
- - Theoretical rates of formation of $\text{NO}_3^- + \text{NO}_2^- - \text{N}$ in the same soil samples.

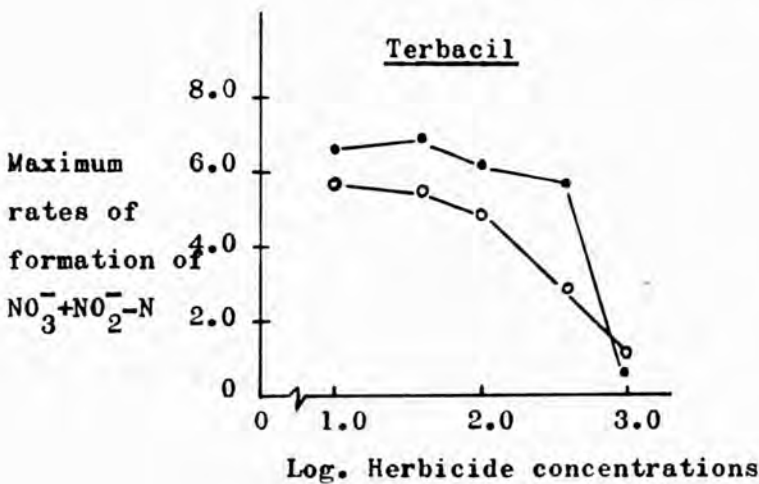
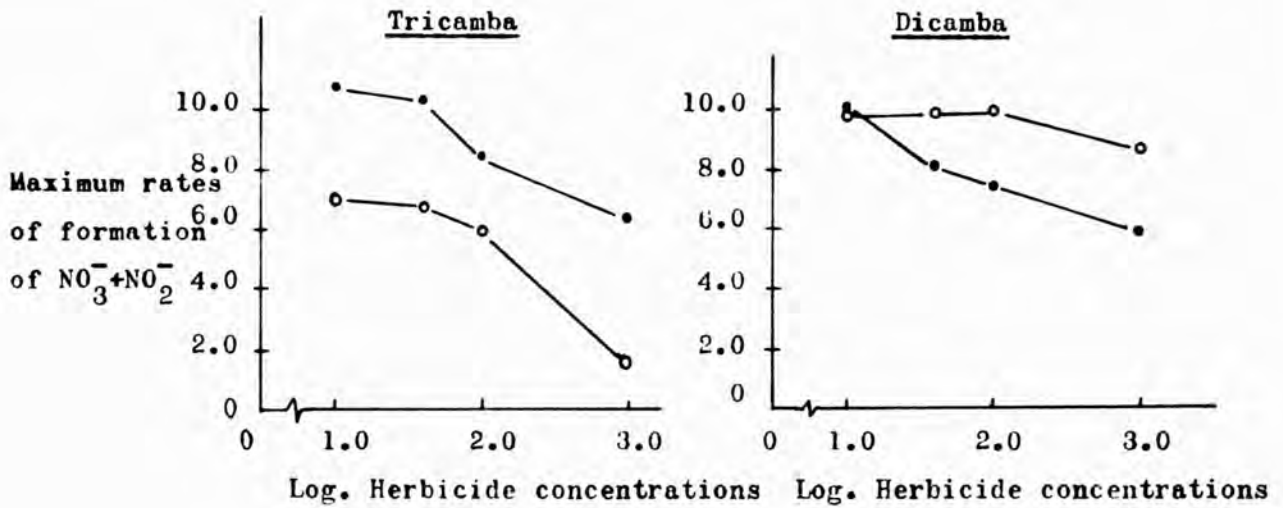
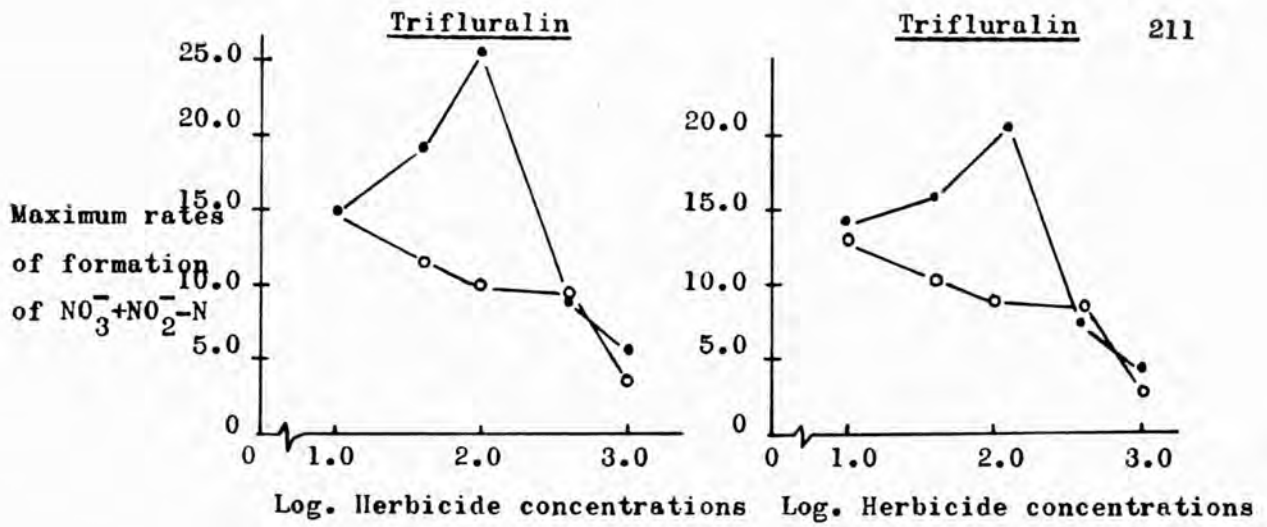


Fig. 64

● - Observed maximum rates of formation of $\text{NO}_3^- + \text{NO}_2^- - \text{N}$ ($\mu\text{g. NO}_3^- + \text{NO}_2^- - \text{N}$ formed/ml.perfusate/day) when samples of fresh soil were perfused with Ammonium sulphate solutions containing different herbicide concentrations.

○ - Theoretical maximum rates of formation of $\text{NO}_3^- + \text{NO}_2^- - \text{N}$ in the same soil samples.

giving an estimate of the size and activities of Nitrobacter populations whereas the rates of accumulation of NO_3^- - N as well as $(\text{NO}_3^- + \text{NO}_2^-)$ - N in the soils treated with the other herbicides would indicate the size and the degree of activities of Nitrosomonas populations of these soils. Therefore it could only be concluded (from the results representing the variations of the 'observed' and 'theoretical' rates of accumulation of both NO_3^- - N and $(\text{NO}_3^- + \text{NO}_2^-)$ - N in soils treated with Legurame, Oxadiazon, Trifluralin, Tricamba and Terbacil - Figs. 61, 62 and 63,64 respectively) that the mode of action of the herbicides Legurame, Oxadiazon, Trifluralin, Tricamba and Terbacil in soil must be via the inhibition of the enzymatic activities of Nitrosomonas cells rather than via the inhibition of their proliferation. However regarding Ioxynil and Bromoxynil it could be concluded that both the nitrifier populations - Nitrobacter and Nitrosomonas - were less sensitive to these two herbicides during their growth phase in soil media and this too might be due to some degree of adaptation developed during initial stages of growth or due to the possible detoxication of these two herbicides with time. (results from Figure 41 have shown that Ioxynil incorporated into the perfusate of a fresh soil sample, at an initial concentration of 100 p.p.m. was almost completely detoxicated by the 15th day of continued perfusion.)

To this end of the discussion dealt with so far, regarding the effects of different herbicide on the two main stages of nitrification in soil, one could add the following general points of conclusion, namely:

- a) The enzymatic activities of fully grown Nitrobacter populations (in treated enriched soils) were more sensitive than the enzymatic activities of similarly grown Nitrosomonas populations in soil, to the toxic effects of the herbicides Ioxynil, Bromoxynil, NPH 1320 and Totril and these could be arranged in a decreasing order of their toxicities towards Nitrobacter activity as Ioxynil > Bromoxynil > Totril = NPH 1320*.

* The 'molar' concentrations of the different herbicides effective in causing 50% inhibition of the nitrifying activities were used whenever the herbicides tested had to be arranged in the order of their toxicities.

- b) The remaining herbicides Legurame, Trifluralin, Oxadiazon, Terbacil, Tricamba, Dicamba, M & B 9057 and M & B 9555 did not show significant differential effects on the metabolic activities of the two main nitrifying populations in enriched soils and thus it was not possible to deduce any conclusions regarding the degree of their toxicities on Nitrobacter population activities in these soils (due to reasons dealt with previously in Chapters 2 and 3).
- c) The herbicides tested in the present investigation could be arranged, in the decreasing order of their toxicities towards the metabolic activities of Nitrosomonas populations in enriched soils, as follows:
 Ioxynil > Bromoxynil > NPH 1320 > Terbacil > Legurame > Oxadiazon > Trifluralin > Totril \gg Tricamba \geq M & B 9555 \geq Dicamba \geq M & B 9057*.
- d) Tricamba, Dicamba, M & B 9057 and M & B 9555 were found to exert extremely low toxicities on the nitrifying activities of Nitrobacter and Nitrosomonas populations in soil and 50% inhibition of the activities of the nitrifiers in soil could not be brought about by these herbicides even at concentrations as high as 1000 p.p.m.
- e) The proliferation of Nitrobacter as well as Nitrosomonas populations in freshly perfused soils treated with NPH 1320 or Totril were found to be inhibited more than the metabolic activities of the fully grown cells of these organisms whereas the metabolic activities of the fully grown populations of the same organisms, under similar experimental conditions, were inhibited more than their growth, by the herbicides Ioxynil and Bromoxynil.
- f) The proliferation of Nitrosomonas populations in freshly perfused soils treated with Legurame, Oxadiazon, Trifluralin, Tricamba or Terbacil were found to be less inhibited than the metabolic activities

* The 'molar' concentrations of the different herbicides effective in causing 50% inhibition of the nitrifying activities were used whenever the herbicides tested had to be arranged in the order of their toxicities.

of the fully grown Nitrosomonas cells in treated enriched soils.

g) All concentrations of NPH 1320, Totril, Ioxynil and Bromoxynil tested, were found to exert significant effects on the lag (i.e. the time taken by the treated soils to attain saturation with respect to the Nitrosomonas and Nitrobacter populations, under the prevailing experimental conditions) whereas the remaining herbicides showed up any significant effects only at the very high concentrations - refer results presented in Tables 7 and 8 and Figures 36 and 37. These observations along with the previous conclusions (that NPH 1320, Totril, Ioxynil and Bromoxynil were effective in causing significant inhibitions in the rates of nitrification even in soils treated with the lowest concentrations while the other herbicides were effectively toxic only at the higher concentrations) suggests that any increase in the lag (suppression of growth rate) was an indirect effect resulting from the inhibitions of the energy flow during nitrification.

Considering next, the effects of the herbicides under investigation, on the pure cultures of nitrifying organisms - specifically Nitrobacter Winogradski and Nitrosomonas Europaea grown in liquid media it was evident that the two organisms differed from each other in their sensitivities to the toxic effects of the various herbicides. For instance it could be seen from the results presented in Table 17 (Chapter 6) that Ioxynil, Bromoxynil, NPH 1320, Totril, Trifluralin and Chlorbufam were more effective in inhibiting the rates of oxidation of NO_2^- - N by fully grown cells of Nitrobacter Winogradski whereas Tricamba, Dicamba, Legurame, Oxadiazon, M & B 9057 (Asulam), M & B 9555 and Phenmedipham were more effective in inhibiting the rates of oxidation of NH_4^+ - N (as measured by rates of formation of NO_2^- - N) by fully grown cells of Nitrosomonas Europaea. All the herbicides tested in this part of the present investigation could be arranged, in the decreasing order of their toxicities towards the enzymatic activities of the treated cell suspensions of Nitrosomonas Europaea as: Tricamba = M & B 9555 > Legurame >

Oxadiazon > Trifluralin > NPH 1320 = Totril > Dicamba > M & B 9057 > Ioxynil > Phenmedipham > Chlorbufam > Terbacil > Bromoxynil. By considering these same herbicides in the decreasing order of their toxicities towards the enzymatic activities of treated cell suspensions of Nitrobacter Winogradski, they could be arranged as Totril > NPH 1320 > Trifluralin > Tricamba > Chlorbufam > Oxadiazon > Ioxynil > Dicamba > Legurame > Bromoxynil > Terbacil > M & B 9057 > Phenmedipham > M & B 9555.

A comparison of the quantitative effects of the different herbicides on the nitrifying activities of cell suspensions of Nitrosomonas and Nitrobacter harvested from artificial liquid media (Table 17, Chapter 6) with the effects of the same herbicides on the nitrifying activities of Nitrosomonas and Nitrobacter species in enriched soils (Table 4, Chapter 3) reveals clearly that all the herbicides with the exception of Ioxynil, Bromoxynil and Terbacil were more effective in inhibiting the metabolic activities of nitrifiers when they were in artificial media than when they were in the natural medium of soil.

This observation, that Ioxynil was more toxic to nitrifiers when they were in soil than when they were in artificial media, is in agreement with a similar observation reported by Debona (1967). This rather interesting observation would probably find an explanation from the work reported by Zaki (1965) which attributes the removal of nearly 50% of the Ioxynil by soil immediately after being incorporated into the soil solution (a situation similar to the results presented in Figure 41) as due to a possible precipitation of this herbicide in the form of a relatively insoluble free phenol rather than due to any adsorption on to soil components. He also predicted the possibility of the production of the amide and then the corresponding acid due to microbial degradation of Ioxynil in soil. Assuming these possibilities to hold good one could explain the higher toxicity of Ioxynil in soil medium as due to this herbicide being more effective in its adsorbed or precipitated form (which is in close agreement with the observations discussed with

reference to the recovery of nitrifying capacity in 'washed' soils pre-treated with Ioxynil - Chapter 4) rather than in an aqueous solution form. Bromoxynil being chemically very closely related to Ioxynil, the above reasoning could probably be extended to Bromoxynil too to explain its herbicidal behaviour which were found to be similar to those of Ioxynil.

On the other hand all the other herbicides tested were found to be more toxic on the metabolic activities of nitrifiers in pure cultures than on the metabolic activities of nitrifiers in soil. In fact Tricamba, Dicamba, M & B 9057 and M & B 9555 which were not effective in causing 50% inhibition of the activities of nitrifiers in enriched soil even when applied at concentrations as high as 1000 p.p.m. were found to be effective in causing 50% inhibition of the metabolic activities of cell suspensions of Nitrosomonas Europaea at concentrations as low as 33 p.p.m., 53 p.p.m., 60 p.p.m. and 30 p.p.m. respectively and 50% inhibition of the metabolic activities of cell suspensions of Nitrobacter Winogradski at concentrations equivalent to 67.5 p.p.m., 80 p.p.m. and 155 p.p.m. respectively.

Although the effects of all the herbicides, on the metabolic activities of nitrifiers in pure cultures differed quantitatively from their effects on nitrifiers in enriched soil, the qualitative effects of at least some of the herbicides were essentially the same irrespective of whether the nitrifiers were in soil or in artificial media. For example the herbicides Ioxynil, Bromoxynil, NPH 1320 and Totril were found to be more toxic on the activities of Nitrobacter, in enriched soil as well as in artificial media whereas Tricamba, Dicamba, Legurame, Oxadiazon, M & B 9057 and M & B 9555 were found to be more toxic on the activities of Nitrosomonas, in enriched soils as well as in artificial media (the metabolic activities of Nitrosomonas populations in enriched soils treated with these herbicides were found to be limiting the overall nitrification rates, Chapter 3). Terbacil, on the other hand, was almost equally toxic towards the activities of Nitrobacter and Nitrosomonas whether in soil or in artificial media.

A consideration of the effects of different herbicides on the growth of pure cultures of Nitrosomonas Europaea in liquid media - dealt with in Chapter 8 - made it clear that the metabolic rates per cell (i.e. rates of nitrification by cell suspensions during short term experiments) and the growth (as measured by the rates of nitrification in growing cultures) of Nitrosomonas Europaea were both equally sensitive to the toxic effects of Ioxynil and Bromoxynil whereas the growth of treated Nitrosomonas cultures were inhibited more than their metabolic rates per cell, by the other herbicides NPH 1320, Totril, Oxadiazon, Legurame, Phenmedipham, Trifluralin and Chlorbufam. It follows from the above discussion that the mode of action of Ioxynil and Bromoxynil on pure cultures of Nitrosomonas Europaea are probably due to an inhibition of the basic enzymatic reactions whereas the mode of action of NPH 1320, Totril, Oxadiazon, Legurame, Phenmedipham, Chlorbufam and Trifluralin seem to be via an inhibition of the neogenesis of these enzymes that take part in the oxidation of ammonium ions by this organism.

On the other hand, the growth of pure cultures of Nitrobacter Winogradski were found to be inhibited more than their metabolic rates per cell, by Ioxynil, Bromoxynil, NPH 1320, Totril, Oxadiazon and Legurame, while Chlorbufam and Trifluralin were effective in inhibiting both the nitrification rate per cell and growth by same levels. One could conclude from these results that the nitrifying capacity of pure cultures of Nitrobacter Winogradski are inhibited by Ioxynil, Bromoxynil, NPH 1320, Totril, Oxadiazon and Legurame mainly via the inhibition caused by them on the neogenesis of the essential enzymes during growth unlike Chlorbufam and Trifluralin which seem to exert their toxic effects via inhibitions of enzymatic activities in fully grown cells.

It is evident from the conclusions arrived at in the two foregoing paragraphs, that it is not possible to make a generalised statement regarding the mode of action of many of the herbicides on soil nitrifiers, since their modes of action seem to differ from one organism to the other.

As far as the effects of different herbicides on growth of nitrifiers are concerned no analogy could be drawn between the results from pure culture experiments and the results from soil experiments mainly because the soil nitrifiers growing under the influence of herbicides in their natural medium were found to show a certain degree of adaptation (as discussed earlier in this chapter) that could be due to

- either a) Possible detoxication of the herbicides in soil.
- or b) Survival of resistant strains.
- or c) Due to both these factors operating together.

Lastly considering the effects of herbicides tested, on the rates of oxygen uptake of cell suspensions of Nitrosomonas Europaea and Nitrobacter Winogradski and also their effects on the rates of oxygen uptake by samples of enriched soil, it could be seen that even here the degree of inhibitions observed were much greater in pure cultures than in soil. Another striking observation noted in considering the concentrations of different herbicides effective in causing 50% inhibition in the rates of oxygen uptake by cell suspensions of Nitrobacter Winogradski and Nitrosomonas Europaea (Table 21, Chapter 7) was that the herbicides used in the form of emulsifiable concentrates (e.g. NPH 1320, Totril, Oxadiazon, Legurame and Trifluralin) exerted highest toxicities on the respiratory rates of these organisms. This observation is in line with the findings of Caseley & Luckwill (1965) who also observed that formulated herbicidal products were far more toxic to Nitrosomonas and Nitrobacter, than were the pure unformulated herbicides. Ivarson & Pramer (1956) have reported that cationic and to a lesser extent the anionic wetting agents inhibited nitrifications in culture solutions, and this may account for the enhanced toxicity of formulated products.

On the other hand the inhibitions caused by different herbicides on the rates of oxygen uptake in samples of enriched soil were extremely low and the concentrations of many herbicides (e.g. NPH 1320, Totril, Tricamba, Dicamba, Oxadiazon, M & B 9057 and M & B 9555) effective in causing 50%

inhibition in the rate of oxygen uptake were well beyond 1000 p.p.m. with the exception of Ioxynil, Bromoxynil, Legurame and Trifluralin. Bearing in mind that the rates of oxygen uptake recorded for enriched soil (Table 13) were in fact the rates of oxygen uptake by the whole microbial populations of those enriched soils (whose main component is considered to be nitrifiers since the soils were enriched by perfusion with ammonium sulphate solutions) and does not represent the rates of oxygen uptake by nitrifiers only (discussed previously in Chapter 5) it could be said that any herbicide causing higher toxicities on the respiratory activities of these enriched soils should be non specific inhibitors (also dealt with in Chapter 5). Based on this reasoning Ioxynil and Bromoxynil which were found to exert similar degrees of toxicities on the respiration rates of pure cultures of nitrifiers and of enriched soil could be considered as having the capacity to inhibit the general soil microbial population whereas the other herbicides namely NPH 1320, Totril, Tricamba, Dicamba, Oxadiazon, M & B 9057 and M & B 9555 could be considered as possessing a certain amount of specificity as regards their inhibitory effects on soil nitrifiers.

Although many workers made use of the effects of several metabolic inhibitors (e.g. Quastel & Scholefield (1951) used urethanes whereas Hale, Hulchur & Chappel (1957) and Debona (1967) used herbicides) on the respiratory rates of enriched soil as the experimental parameter to assess the toxic effects of these inhibitors on soil nitrifiers, in view of the practical difficulties encountered in attempting to account for the very high rates of endogenous respiration (i.e. respiration rates of soil samples in the absence of any added substrate) observed in the present investigation, I strongly feel that the use of respirometric techniques to assess the effects of any inhibitor on a specific fraction of the diverse soil microbial population is not quite appropriate. But it must also be said at this point that these respirometric techniques could be of great help in trying to assess the effects of inhibitors on any soil considering its microbial population as a

whole, in which context this technique was used by Gamble, Mayhew & Chappel (1952).

As far back as 1904 Wimmer (quoted by Lees & Quastel, 1946a) found peptone to be less inhibitory to nitrification process in sand than in solution cultures whereas Stevens & Withers (1910) (quoted by Lees & Quastel, 1946a) found that nitrification in soil was far less inhibited by added organic matter than was nitrification in laboratory media. Also the results presented in this thesis along with the results published by many other workers support the above general conclusion and therefore in trying to interpret the results of the experiments carried out using pure cultures of nitrifiers in artificial media, one must remember that these results could not be extrapolated with certainty to arrive at any conclusions about what quantitative effects these same herbicides would have on nitrification in soil. But as mentioned earlier in this chapter these results might give some idea about the possible qualitative effects of some of these herbicides on soil nitrification process. Also the pure culture studies allow one to look into some of the specific biological effects, these inhibitors exert on nitrifiers and based on the results of pure culture experiments I have in part speculated as to the possible modes of action of some of the herbicides investigated, with respect to their toxic effects on soil nitrification and on nitrifiers grown in artificial liquid media. Although some of these speculations may be unwarranted they would certainly suggest the possible lines of further work.

On the other hand in trying to interpret the results obtained from the soil nitrification experiments reported here it must be borne in mind firstly that these experiments were conducted under controlled laboratory conditions which are bound to be quite different from the actual conditions in the field. For instance it was mentioned in Chapter 2 that the major drawback inherent in the perfusion technique used in all the soil experiments reported in this thesis is that the experiments could be conducted only under conditions of water saturation. Although the activities of soil microbes including

nitrifiers are known to be accelerated at high soil water contents (provided there is no water logging and soils are sufficiently aerated) the soils under ordinary field conditions are not always saturated with water. Also Dubey (1969) has shown that the inherent nitrifying capacity of a soil greatly modify the effects of herbicides on the activities of their nitrifying populations. Therefore all the observations reported here regarding the effects of the several herbicides tested, on the nitrification process in soil used for these experiments might not be applicable so well to another soil of different nitrifying capacity.

According to Fletcher (1960) who considered that one acre of land as having about 3,000,000 lbs. of soil in the top 8-9 inches, worked out that a field application of any herbicide at a rate of 1 lb./acre will result in only a soil concentration of around 2-2.5 p.p.m. assuming no adsorption and complete solubility. On the contrary Garretson and San Clemente (1968) claim that by agronomic convention an acre of land will have 2,000,000 lbs. of mineral soil in its top $6\frac{2}{3}$ inches deep layer and therefore an application of any toxicant at a rate of 10 lb./acre could result in only a soil concentration of 5 p.p.m. From the results of the present investigation it was evident that Ioxynil and Bromoxynil (which were the most toxic of the herbicides tested) had to be applied at a concentration of around 60 p.p.m. to cause 50% inhibition in the soil nitrification rates. But since the herbicide concentrations quoted as being the ones used in perfusion experiments were calculated on the basis of μg herbicide / ml. perfusate and not on the basis of μg herbicides / gm. soil, the actual concentrations of Ioxynil and Bromoxynil needed to cause 50% inhibition in the nitrification rates would be around 300 μg / gm. soil (p.p.m. expressed in terms of w/w to be in line with the calculations of Fletcher and others) the reason being that every 50 gm. sample of soil was perfused with 250 ml. of solution during all the perfusion experiments.

Thus it is clear that Ioxynil and Bromoxynil as well as any other herbicide tested in the present investigation (whose field application rates were never higher than 3 lbs./acre which gives a soil concentration equivalent to about 7.5 p.p.m. according to Fletcher) will not have any deleterious effects on soil nitrification in general although it is possible that at very high concentrations they could cause a certain amount of initial delay.

But again it must be emphasized that one has to take into account the factors such as adsorption, persistence and degradation of herbicides etc. in dealing with practical use of herbicides under field conditions. This seems very important in view of the report by Hoccombe et al. (1966) who have shown that very often the persistence of herbicides under laboratory conditions are greater than in the field. Thus it is clear that the two main problems involved in studying the herbicide effects on any soil process such as soil nitrification, are the study of the initial impact of herbicides on treated soil and a study of the mechanism of degradation of these herbicides.

In the work presented in this thesis I have attempted to look into the initial impact the different herbicides might have on the nitrification process in soil and in pure cultures of nitrifiers under laboratory conditions and probably the results could have been explained better with an adequate knowledge about the mechanisms of degradation of these herbicides that has yet to be explored.

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