THE EFFECT OF SIMAZINE ON NITRIFICATION AND THE DECOMPOSITION OF SIMAZINE BY SOIL MICROORGANISMS

by

Franklin Harris Farmer

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INTRODUCTION

Although various chemical means of weed control have been used for some time, the organic weed killers, which are the ones predominantly in use today, have only been in use for about 20 years. During this time a great number and variety of compounds have been developed, and the amount of organic herbicides used increases every year. An indication of the importance of these chemicals has been expressed by Douros (1958) who reported that government and industry spent 35 million dollars for organic herbicides in 1957.

The addition of any organic compound to soil causes many changes in the quantitative and/or qualitative nature of the soil microflora. Thus the widespread addition of various organic herbicides to soil necessitates an investigation of these changes to determine what they are, and what effects they have on the soil microorganisms which carry out key soil processes. If these effects are harmful to vital reactions of soil organisms, the detrimental influences may outweigh the beneficial economic effects of the herbicides. Several questions on this subject need to be answered. Do any herbicides inhibit the processes which are required to maintain soil fertility? If there is an inhibitory effect, how long does it last? Do the herbicides persist in soil

for any length of time? If they do persist, is their disappearance connected with microbial decomposition or some other mechanism? If they are microbially decomposed, how specific are the organisms which carry out this process and is it beneficial to them?

Many of these questions have been partially answered with regard to several organic herbicides, but much more work remains to be done. Some herbicides have not been investigated at all, while the results from the investigations of some of the other herbicides are so contradictory that no definite conclusions can be made.

Simazine is one of the principal organic herbicides in use today. The investigations into the effect of this herbicide on living systems have only partially answered some of the important questions. It has been established that this herbicide remains in the soil for some time, and that its final disappearance is due principally to the action of soil microorganisms. The effect of this compound on key soil processes such as nitrification have not been thoroughly investigated. Although simazine decomposers have been isolated and in some cases identified, their ability to metabolize simazine has not been clearly established. Also, more information is needed on the biochemical mechanism and sequence of simazine breakdown, and the determination of what parts of the simazine molecule are utilized by the decomposers.

The objectives of this investigation were: (1) to carry out a comprehensive study of the effect of simazine on nitrification, one of the most important soil processes carried out by soil microorganisms, and (2) to establish the unique ability of certain soil microorganisms to metabolize simazine by isolating and studying organisms which could utilize simazine as a sole source of carbon and nitrogen. Although microorganisms capable of decomposing simazine are fairly common in soil, it is probable that the organisms which have the unique ability to utilize the simazine as a sole carbon and nitrogen source are much less common. The nitrification process was selected for study for two reasons: (1) it is the principal method of producing nitrate, the major nitrogen source assimilated by higher plants, found in soil, and (2) the increase in leaf nitrogen and growth in trees treated with simazine reported by Ries et al. (1963) may have been due to the effect of the herbicide on transformations of nitrogen in soil. Although the authors of this report did not draw any final conclusions as to the cause of this increased nitrogen uptake, it would appear to be caused by the effect of simazine on some aspect of nitrogen metabolism.

LITERATURE REVIEW

Simazine

Simazine is the commercial trade name for 2-chloro-4, 6-bis-(ethyl-amino)-s-triazine, the active ingredient in several commonly used herbicides. This compound is almost completely insoluble in water, but is soluble in ethyl alcohol, chloroform, and methyl alcohol. For practical use it is mixed with one or more wetting agents to give it increased solubility. Simazine was first synthesized for use in a commercial herbicide by Gysin and Knusli in the laboratories of Geigy Chemical Corporation, Basle, Switzerland, and was first released for experimental use in 1956. The most common herbicide mixture produced by this company is Simazine 80W, which is a mixture of 80 per cent simazine and 20 per cent inert ingredients, some of which are wetting agents. This commercial mixture is soluble in water up to 5ppm which enables easy application.

Simazine eliminates a large number of grassy and broadleaf annual weeds (Burnside and Behrens, 1961). This herbicide is presently registered as a pre-emergence herbicide in corn, sugar cane, various nursery stock, and certain varieties of strawberries. Seventeen other crops have been shown to be tolerant to low concentrations, while about 20 different weeds seem to be inhibited by similar concentrations (Anonymous, 1960). It is most effective as a pre-emergence herbicide and is applied just prior to, or during, weed emergence. Dosage rates of 1 to 4

pounds active ingredient per acre have been sufficient to control many annual broadleaf and grassy weeds for the entire growing season.

Simazine does not prevent germination of weed seeds, but destroys weed seedlings after gaining entrance through the root system. It has been demonstrated by use of radiotracer techniques that simazine does gain access to the plant through the roots (Sheets, 1961), but does not enter the leaves unless the cuticle is broken (Davis et al., 1959).

The exact mechanism by which simazine causes the death of the susceptable plants is not known. The leaves of susceptable plants which have been treated with simazine become chlorotic and preliminary results show that the herbicide may interfere with some aspect of carbohydrate metabolism (Anonymous, 1960). This hypothesis has been supported by the observation that barley can be kept alive in the presence of otherwise lethal doses of simazine by the addition of glucose through the severed tips of the leaves (Moreland et al., 1959). This work has been confirmed by Allen and Palmer (1963) who found that sucrose was even more effective in reversing the effect of simazine, and that the addition of aspartate could also reverse the inhibition. Allen and Palmer also reported that simazine inhibition, as represented by differences in dry weight, occurs only in the presence of light. These data indicate that some aspect of photosynthesis is being inhibited by simazine. The effect of simazine on the

light reaction of photosynthesis (the Hill reaction) has been investigated in an in vitro system by the use of isolated barley chloroplasts (Moreland, et al., 1959). These investigators found that the reduction of ferricyanide by isolated chloroplasts was inhibited by simazine. Further work by these investigators using this technique has shown that the Hill reaction in the chloroplasts of several other susceptable species was inhibited by simazine (Moreland and Hill, 1962). The dark reaction of photosynthesis (carbon dioxide fixation) has also been shown to be inhibited by simazine (Ashton et al., 1960).

One other theory has been proposed for the mode of action of simazine. It has been observed that sub-lethal concentrations of simazine cause a slight stimulation of growth and seed germination in maize. These results have given rise to the hypothesis that simazine acts as a plant auxin, and thereby causes a stimulation of growth which at higher rates of application becomes fatal to the plant since it cannot assimilate nutrients at a fast enough pace to meet growth requirements (Lorenzoni, 1962).

The selectivity of simazine is another important consideration. All plants thus far examined appeared to contain a mechanism for deactivating simazine, but the susceptable plants were able to assimilate the herbicide more rapidly than the resistant plants (Ragab, 1959). This uptake was so rapid that it exceeded the ability of the deactivating mechanism to deactivate it and thus

phytotoxic concentrations of the herbicide accumulate (Davis et al., 1959; and Roth, 1957). By means of radioactive tracer techniques, it has been shown that under controlled laboratory conditions the carbon from C^{14} labeled simazine eventually appears as carbon dioxide given off from the plant (Ragab and McCollum, 1960). These results have been confirmed by Sheets (1961) who also found that only a small percentage of the C^{14} detected in the roots of oats and cotton 24 hours after treatment with labeled simazine was in the form of unaltered simazine. Other studies with ring labeled simazine (Ragab and McCollum, 1961) have shown that the triazine ring is broken and some of the labeled carbon from the ring is released as carbon dioxide. These workers used paper chromatography techniques to show some unchanged simazine in plant extracts eight days after treatment. However, on the basis of his radiotracer work, Ragab has postulated that eventually all of the simazine in the plant would be totally metabolized.

Physiology of Nitrification

The biological process of converting ammonia to nitrite or nitrate is commonly called nitrification. Nitrosomonas and Nitrobacter are the two principal genera of nitrifying bacteria most frequently isolated from soil or water. The members of Nitrosomonas carry out the conversion of ammonium to nitrite, while the members of Nitrobacter carry out the conversion of nitrite

to nitrate. These nitrifying bacteria are chemoautotrophic in nature and utilize this oxidation as a means of obtaining the energy required to carry out the endogonic reactions of cell metabolism. Nitrosomonas obtains 66kcal of energy per gram atom of nitrogen oxidized, while Nitrobacter obtains only 18kcal of energy per gram atom of nitrogen oxidized in its reaction (Alexander, 1961). These organisms use this energy at about 10 percent efficiency or better to synthesize all required cell components from inorganic salts and carbon dioxide. Although they have extremely simple nutritional requirements, their biosynthetic capabilities are among the most complex and extensive series of reactions found in nature.

Nitrification was first discovered to be a biological process by Schlosesing and Muntz in 1877, and this discovery was verified by Warington in 1878. Although Warington was convinced that the reaction was carried out by microorganisms, it was a young Russian bacteriologist named Winogradsky who actually isolated the causative organisms in 1890. Winogradsky was able to separate the two types of nitrifiers and to determine that they were chemoautotrophs. An interesting résumé of the work of these early investigators can be found in the book by Thimann (1963).

At least 10 independent investigators had, previous to 1951, reported the isolation of pure cultures of Nitrosomonas or Nitrobacter. However, the isolation techniques were extremely tedious

and heterotrophic contaminants were common. It is important to note that the heterotrophic organisms were able to survive in a strictly inorganic medium by utilizing the simple organic compounds given off by the nitrifiers. These contaminants are particularly difficult to eliminate because they are present in greater numbers than the nitrifiers. Through the use of a perculation apparatus, Lees (1951) was able to enrich his nitrifying cultures until there was a sufficient number of cells to permit the isolation of pure cultures. A more recent, and even more efficient method of pure culture isolation has been devised by Lewis and Pramer (1958). These investigators enriched a mixed culture of Nitrosomonas and contaminating heterotrophs in a chemically defined medium with periodic additions of ammonium sulfate while keeping the pH constant at 7.2 with the addition of sterile NaOH. After three weeks this enrichment procedure produced a Nitrosomonas/heterotroph ratio of 100/1 and 35 per cent of the cultures inoculated from this enriched culture by serial dilution were determined to be pure cultures of Nitrosomonas. Thus, although contamination is still fairly frequent, the isolation methods have been greatly improved. In most cases the presence of contamination can be determined by the inoculation of an enriched nutrient broth with some of the nitrifying culture (Engel and Skallau, 1937). Lewis and Pramer (1958) modified this basic procedure to obtain four different methods which can be used to detect contaminating heterotrophic bacteria.

Although much of the first work done with nitrifiers was done in mixed culture, most current investigations are carried out with pure cultures.

The classification of the nitrifying organisms, with the exception of Nitrosomonas spp. and Nitrobacter spp. is a somewhat controvertial subject. The chief point of contention is the existance of some species which have not been isolated since they were initially described. A good discussion of this subject may be found in the review of the nitrifying bacteria by Meiklejohn (1953a).

The biochemistry of the nitrifiers was first studied by Meyerhof in 1916 and later by Kluyver and Donker in 1926, Baas-Becking and Parks (1927) and Bomeke in 1939. A review of the work of these investigators and others prior to 1954 has been compiled by Lees (1954). In spite of the fact that there have been many workers in this area, surprisingly little is known about the biochemistry of these organisms. The main reason for this lack of information is that a large crop of cells is required for biochemical studies and until recently it has been very difficult to obtain such a large number of cells. Recent advances in chemically defined media (Engel and Alexander, 1958), pure culture (Lewis and Pramer, 1958) and cell-free techniques (Aleem and Alexander, 1958) have opened the way for much more comprehensive investigations of the biochemistry of the nitrifying organisms.

Another facet of nitrification is that it is effected by certain chemical and physical factors, including the effect of light, colloids, aeration, acidity, and inorganic and organic chemicals. The last of these factors, particularly the organic herbicides, will be discussed in greater detail below because of their particular significance in relation to this investigation. The effects of the other factors on nitrification are discussed in the review by Meiklejohn (1954), and in the book by Alexander (1961).

Before leaving this subject, it should be mentioned that the autotrophic nitrifying bacteria are not the only micro-organisms which can carry out the process of nitrification. One of the first reports of heterotrophic nitrification was by Cutler and Mukerji (1931). These investigators were able to isolate four species of aerobic non-sporeforming bacteria which converted ammonium to nitrite and which could grow very well on nutrient agar or in peptone broth. However, these organisms were able to produce only 0.1 per cent as much nitrite as produced by Nitrosomonas. Isenberg et al., (1952) and Gunner (1963) were able to isolate an actinomycete and a bacterium respectively, which could be called nitrifiers. The actinomycete could produce small amounts of nitrite from ammonia, while the bacterium could produce small amounts of nitrite and nitrate. A fungus has been isolated by Schmidt (1954) which can produce about 20ppm nitrate nitrogen from organic nitrogen compounds with some accumulation of nitrite.

Further information on the subject of the physiology of nitrification can be found in the reviews by Quastel and Scholefield (1951) and Delwiche (1956).

The Effect of Organic Compounds on Nitrification

The first organic compound reported to have any effect on nitrification was glucose, which Warington in 1891 found to be inhibitory (Thimann, 1963). This inhibition was supported by the findings of Winogradsky and Omeliansky in 1899, who also found that small doses of peptone, asparagine, and sodium butyrate were inhibitory. In a later report, Omeliansky added sodium acetate, urea, and glycerin to the list of nitrification inhibitors. It is important to note that these studies were carried out with concentrations of organic compounds which ranged from 1,000 to 10,000ppm. In 1914, Beijerinck carried out a similar investigation using concentrations of about 500ppm of the organic compounds. He was unable to show that glucose, sucrose, starch, mannitol, sodium and calcium acetate, peptone, tyrosine, or asparagine inhibited the conversion of nitrite to nitrate (Fred and Davenport, 1921). In further studies made by Fred and Davenport (1921), various qualitative concentrations of complex organic substances were used. They reported that no effect was discernable from gelatin, peptone, casein, skimmed milk, and beef infusion, but that beef extract at 1 per cent concentration killed the nitrate formers, and that asparagine

and urea retarded the oxidation. Mixed culture work done by Lees (1948), using the soil perfusion technique, showed no inhibition of nitrification by either 0.1 per cent glucose or starch. The inhibition of nitrification by glucose was closely studied by both Jensen (1950) and Meiklejohn (1951). They were able to show that the inhibition was caused by some substance formed during the sterilization of the glucose by autoclaving. Meiklejohn also found that glucose sterilized by filtration had no appreciable effect on nitrification.

The inhibitory effect of the nitrogenous organic compounds on nitrification has been studied very thoroughly. In 1935, Kingma Boltjes reported that peptone was toxic to nitrification, and he found that the most toxic peptones are those with the greatest concentration of free amino acids (Meiklejohn, 1953a). Jensen (1950) found that 0.1-0.4 per cent concentrations of glycerine, alanine, and asparagine were inhibitory to nitrification, and low concentrations of methionine have also been found to be inhibitory (Quastel and Scholefield, 1951). Meiklejohn (1952) also found peptone to be inhibitory to nitrification. She was able to demonstrate that a 0.2 per cent solution of peptone could inhibit the convertion of nitrite to nitrate in mixed cultures of the nitrifying organisms, but that it required a 1.0 per cent solution to inhibit the convertion of ammonium to nitrite. She also reported that: (1) yeast extract was slightly inhibitory at 0.1 per cent; (2) urine inhibited nitrite oxidation at 10 per

cent; (3) and that thiamin totally inhibited nitrification at a concentration of 0.025 per cent. Furthermore, she was able to show that the plant auxin B-indolacetic acid had no effect on nitrification at a concentration of 0.25 per cent.

The metal-combining organic compounds such as thiorea (Quastel and Scholefield, 1951), allylthiourea (Hofman and Lees, 1953), and the urethanes (Lees and Quastel, 1946) are all inhibitors of nitrification. The mode of action of these compounds is attributed to the surpression of certain metabolic reactions due to the chelation of the iron required by the organisms (Meiklejohn, 1953b). Both Thimann (1963) and Alexander (1961) report the above mechanism as one of the two principal mechanisms by which organic compounds inhibit nitrification. The other mechanism mentioned is the competitive inhibition of the utilization of ammonium by nitrogenous organic compounds. This mechanism may provide a possible explanation for the inhibition of nitrification by peptone and various amino acids. This may also explain the inhibition of Nitrosomonas by root exudates (Molina and Rovira, 1964), since large numbers of amino acids have been reported in root exudates.

Since the addition of large amounts of various pesticides to soil has become common practice, some concern has been expressed as to whether these compounds affect nitrification. This concern has been somewhat justified since many investigators have been able to show that pesticides in soil are able to inhibit the growth

and activity of other soil microorganisms (Kratochvil, 1951; Gamble et al., 1952; Bollen, 1954; Chandra et al., 1960; Chandra and Bollen, 1961; Bollen, 1962; and Chandra, 1964). A more extensive explanation of these effects may be found in the reviews by Newman and Downing, (1953) and Bollen (1961). Recent investigators (Hale et al., 1957; Douros, 1958; Chandra and Bollen, 1961; and Chandra, 1964) have indicated that some herbicides can inhibit nitrification at concentrations slightly higher than field rates, but that some fungicides can inhibit nitrification at normal field rates. However, not all investigators have shown pesticides to be inhibitory to nitrification. For example, Shaw and Robinson (1959) tested three insecticides and one herbicide and were unable to show that these compounds had any effect on nitrification.

It is particularly interesting to note that a compound having a chemical structure similar to that of some commercially used herbicides has been produced specifically as a nitrification inhibitor (Goring, 1962a). This compound, 2-chloro-6(trichloromethyl)pyridine, is used at concentrations approximating those used with commercial herbicides under field conditions. At these concentrations, inhibition of nitrification has been shown in the laboratory in pure culture (Shattuck and Alexander, 1963), in the laboratory using soil incubation techniques, in the greenhouse in potted soil (Goring, 1962b), and in field studies (Swezey and Turner, 1962).

The effects of triazines such as simazine on soil microorganisms have been investigated by several workers. Chandra et al., (1960) were able to show that the addition of 5ppm simazine inhibited the evolution of CO_2 from six out of nine types of Oregon soil. This was interpreted as an indication that the simazine was causing a decrease in the microbial activity of the soils. In an investigation of four French soils, Guillemat et al., (1960) were able to show that normal field concentrations of simazine were able to reduce the bacterial count in two of the four soils. Two other workers in this area were able to find little or no inhibition of microbial activity even at very high concentrations of simazine. Eno (1962) used pure cultures of four fungi and was able to show inhibition of two of these organisms, but only by extremely high concentrations of simazine. He was also able to show inhibition of two of these fungi by 256ppm of atrazine, but in the other two organisms extremely high concentrations were required for inhibition. Burnside et al., (1961) used the evolution of carbon dioxide from soil as an indication of microbial activity and were unable to show any inhibition, even at extremely high concentrations of simazine. However, only one type of soil was used in their study. Several other investigations on the effect of triazines on soil microorganisms are summarized in the review by de Vries (1963).

The inhibition of nitrification by the triazine herbicides has been reported, but the extent of inhibition and the concentration

of herbicide required to produce inhibition seem to vary with the experimental method. Eno (1962) reported inhibition of nitrification by simazine at 512ppm and by atrazine at 16ppm. Burnside et al., (1961) used only one soil type and were unable to find any inhibition of nitrification by simazine in that soil even at concentrations as high as 4096ppm. Guillemat et al., (1960) observed that nitrification was inhibited by 6kg/Ha in two out of the four French soils studied. They reported that the nitrite-forming bacteria were more readily inhibited than the nitrate-forming bacteria. Two other investigations of the effects of triazine herbicides on nitrification have been reviewed by de Vries (1963). Hauck and Stephenson (1964) were able to demonstrate by use of the perfusion technique that two triazines were able to inhibit nitrification at concentrations described as "dilute." Cyanuric acid was found to be more inhibitory than melamine and Nitrobacter seemed to be more affected than Nitrosomonas. The authors attributed this effect to competitive inhibition by the amino groups of melamine and by the ammonium formed by the microbial decomposition of both compounds.

The Decomposition of Herbicides

The first indication that herbicides could be decomposed in the soil was provided by the work of Nutman et al. (1945) on the persistence of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in soil. Further work on this particular herbicide paved the way for investigations into the mode of decomposition of other herbicides.

Newman et al. (1952) were able to show that under field conditions 2,4-D disappeared more quickly than 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and that a population of organisms able to decompose 2,4-D was present in the soils treated with this herbicide. A similar population of decomposers was not evident in soils treated with 2,4,5-T. This work supported the earlier conclusions of Newman and Thomas (1949) and those of Audus (1949, 1950, 1951). The latter investigator used a soil perfusion apparatus to show that 2,4-D was detoxified by microorganisms. He was able to isolate a bacterium which was able to utilize 2,4-D as a sole carbon source. Since it would be almost impossible to cover the studies on the decomposition of all the herbicides, the remainder of this review will be limited to the literature concerning simazine decomposition. Comprehensive reviews by Bollen (1961), Newman and Downing (1958), and Douros (1958) should be consulted for further coverage of the general area of herbicide decomposition.

Several investigators have suggested that soil microorganisms can decompose simazine in soil (Ragab, 1959; Burschel, 1961; Roadhouse and Birk, 1961; and Ragab and McCollum 1961), but only a few workers have been able to isolate and identify any of the responsible organisms (Burnside et al., 1961; Guillemat et al., 1960; Reid, 1960; and Kaufman et al., 1963). Several investigators have been able to show that certain soil microorganisms can utilize simazine as a sole source of carbon or nitrogen. Guillemat et al., (1960) found that although the total fungal

count of the soil was not affected by the addition of simazine, certain fungi were stimulated. He was able to isolate six fungi which were able to use simazine as a sole source of nitrogen. These were identified as Fusarium oxysporum, Fusarium avenaceum, Penicillium cyclopium, Penicillium lanoso-coeruleum, Cylindrocarpon radiciola, and a Stachybotrys spp. He maintained that the carbon molecules of simazine were unavailable to these soil fungi. Reid (1960) isolated a bacterium which could use simazine as its sole carbon source. Kaufman et al., (1963) isolated nine fungi, three streptomycetes, and four bacteria which could utilize simazine as a sole or supplementary carbon source. The principal organism investigated in this study was Aspergillus fumigatus which was able to utilize simazine as a sole source of carbon. These workers were able to isolate three degradation products of simazine produced by the action of this fungus. One of these products was identified as hydroxysimazine.

In other investigations, Waeffler, in 1961 (de Vries, 1963) supported Guillemat's conclusion that the carbon of simazine is not available to soil fungi, and further concluded that the nitrogen of simazine could not be used as a sole source. Eno (1962) found that high concentrations of simazine increased the growth of Aspergillus niger in a complex chemically defined medium, but this organism could not grow in the same medium when simazine was the only carbon source.

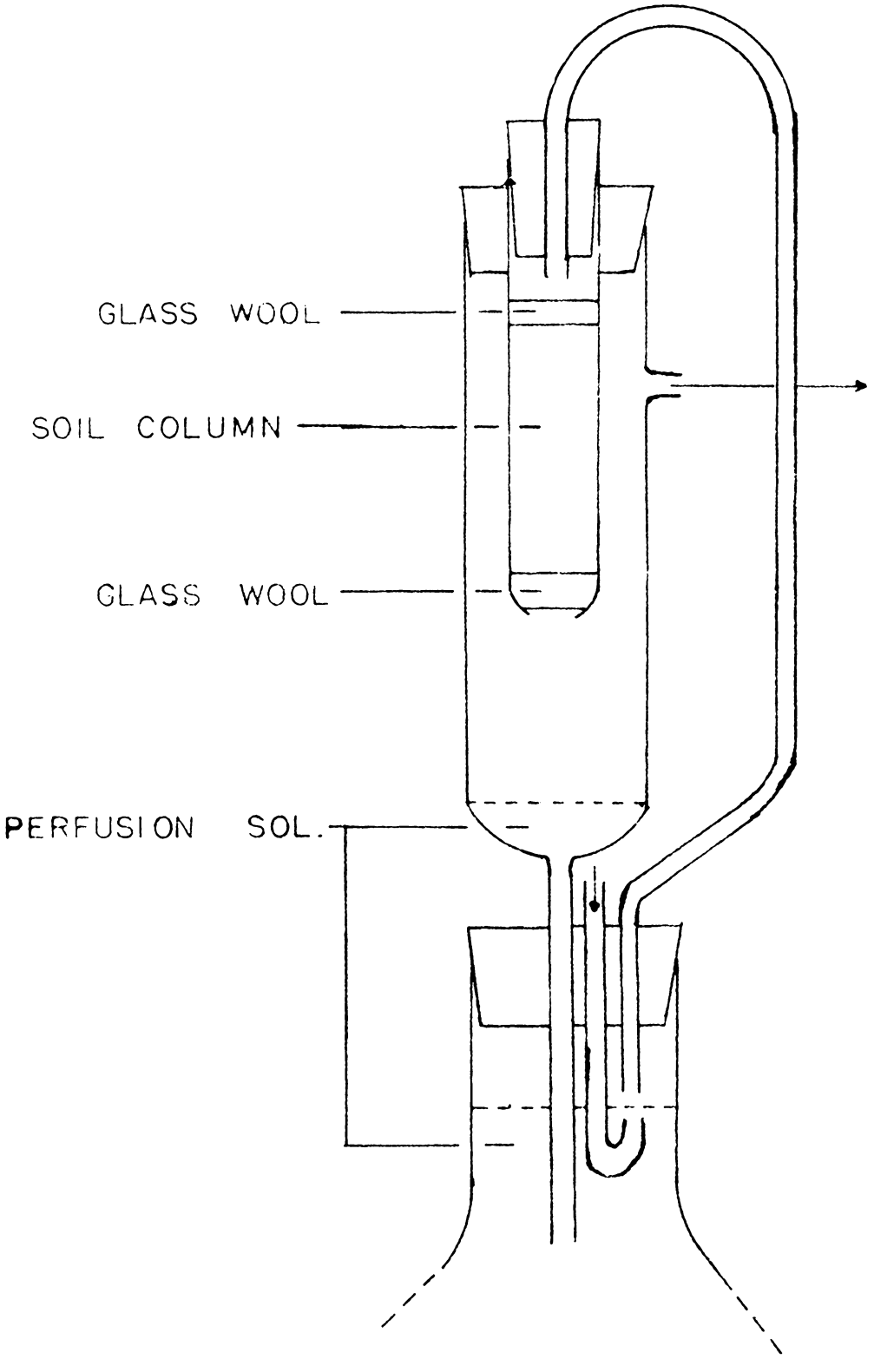
METHODS AND MATERIALS

Perfusion Unit Studies on the Effect of Simazine on Nitrification in Soil

The perfusion units used in this study were those made by Hale *et al.*, (1957) who modified the original unit designed by Lees (Addendum to Lees and Quastel, 1946) for the study of soil nitrification (Figure 1). The soil for these units was obtained from the top three inches of a Lodi loam from an alfalfa plot located on the V.P.I. farm. It was air dried at room temperature, and then sieved to separate the soil aggregates two to four millimeters in diameter from the remainder of the soil. Twenty grams dry weight of these soil aggregates was placed on a layer of glass wool in the soil tube (19 x 150 mm) of the perfusion unit and then covered with another layer of glass wool. The tip of the soil tubes were then placed in a small amount of 0.01M NH_4Cl solution and the soil columns were allowed to adsorb the solution overnight. This procedure was adopted because it allowed the entire column to become wetted via capillary action, thus retarding soil aggregate disintegration. If the solution was added to the top of the dry soil, the surface of the soil became puddled and prevented the solution from perfusing through the column. The soil tubes were then placed in the perfusion units and 0.5g $CaCO_3$ was added to the surface of each soil column to buffer the system at pH 8.3. A 0.01M

FIGURE I

SOIL PERFUSION UNIT



NH_4Cl solution was then added to the perfusion reservoir.

Ten of these units were set up in parallel and a small vacuum pump (225 cubic inches per minute at 2 psi) was used to draw air through the units. The incoming air was washed with distilled water in a sparger in order to saturate the air entering the system, otherwise evaporation in the perfusion units became severe. The units were allowed to perfuse, with periodic changes of the perfusion solution, until the rate of ammonium utilization reached approximately 80ug N/ml for the five-day period immediately following the addition of the fresh perfusion solution. At this point all of the perfusion reservoirs were emptied, washed and immediately refilled with fresh perfusion solution which contained varying concentrations of the simazine. Concentrations of 0, 1, 3, 6, and 9ppm simazine were used in these studies. Samples of four milliliters were taken every five days and analyzed for ammonium, nitrite, and nitrate nitrogen.

The first experiment was carried out for a 20-day period, whereas a second experiment lasted 55 days. Since the control units were able to utilize all of the ammonium in 20 days, it was necessary to add additional ammonium during the course of the 55-day study. Therefore, at 20 and 40 days the ammonium concentration of each unit was increased by 140ug N/ml. This was done by adding an appropriate volume of 1.0M ammonium chloride.

After the completion of an experiment, the perfusion reservoirs were emptied, washed and refilled with fresh perfusion

solution, and another 0.5g $CaCO_3$ was added to the surface of each soil column. After the ammonium utilization reached the prescribed rate in all units, they were used again for the next experiment. If any of the tested units were not able to reach the prescribed ammonium utilization rate, the soil was removed from the soil columns of those units and replaced with fresh soil. These units were then allowed to perfuse fresh ammonium solution until they reached the required level of ammonium utilization.

Determination of Nitrite, Nitrate and Ammonium

Nitrite was determined by the colorimetric sulfanilic acid-α-napthylamine method of Griess (Standard Methods, 1955). The ammonium and nitrate were determined by the distillation method suggested by Bremner (1964).

Pure Culture Studies of the Effect of Simazine on the Nitrifying Organisms

Pure cultures of Nitrosomonas europea and Nitrobacter agilis were obtained from Dr. David Pramer of Rutgers University, and were grown in a chemically defined medium developed by Lewis and Pramer (1958). These cultures were grown in 300ml Erlenmeyer flasks on a reciprocal water bath shaker, which had a shake rate of 70 cycles per minute and a constant temperature of 28°C.

Simazine was added to the flasks from a sterile stock solution of 250ppm in 95 per cent ethanol. Aliquots of the stock

simazine solution were added to the various flasks in order to produce simazine concentrations of 0, 1, 6, 12, and 36ppm, while an identical amount of sterile ethanol was added to each control. All flasks were steamed at 100°C in an autoclave for 15 minutes to drive off the ethanol. The amounts of stock simazine solution used to give these concentrations and the amounts of ethanol used in each control are given in Table 1. In addition to the simazine solution or ethanol each flask contained 18 milliliters of Lewis and Pramer's basic medium, two milliliters of two per cent $NaNO_2$, and 0.1ml of inoculum. The inoculum used in these studies was a culture of the appropriate organism grown in chemically defined media for five days, at which time the organism was in the very beginning of the stationary phase. All experiments contained at least one uninoculated control. The above procedure was followed for each organism with the following exceptions: (1) the Nitrobacter medium used the $NaNO_2$ mentioned above, but the Nitrosomonas medium used two milliliters of two per cent $(NH_4)_2SO_4$; (2) Simazine concentrations of 0, 1, 6, and 12ppm were used in the Nitrobacter studies while concentrations of 0, 1, 6, and 36ppm were used in the Nitrosomonas studies. The Nitrosomonas cultures were sampled for the appearance of nitrite, while the Nitrobacter cultures were sampled for the disappearance of nitrite. Samples of 1.0 and 0.1ml were taken aseptically at 24-hour intervals or greater while the organisms were in the lag phase, at 12-hour intervals

while the organisms were in the log growth phase, or more frequently when deemed necessary.

The duration of these experiments varied according to the organism and the concentration of simazine used. The Nitrosomonas experiments were concluded when the theoretical limit of nitrite production was reached in all flasks, while the Nitrobacter experiments were concluded upon the utilization of all of the nitrite in all flasks. For the former, this period was about five days, while in the latter case it was about 10 days.

An experiment was also carried out to determine the effect of yeast extract on the inhibition of Nitrobacter by simazine. The procedure for this experiment was the same as before with the exception of the addition of an aliquot of one per cent aqueous yeast extract stock solution to the appropriate flasks to give a concentration of 0.1 per cent yeast extract in the media. A control using only yeast extract at 0.1 per cent was used (Table 2).

A study using the Warburg respirometer was made to determine if the inhibition noted in the previous experiments was due to inhibition of the respiration of Nitrobacter. A cell suspension was made by spinning down one liter of a five-day culture of the organisms grown on chemically defined medium at 11,000 rpm in a HR-1 model International High Speed Centrifuge, and then resuspending the centrifuged cells in a 10ml of sterile chemically defined medium minus substrate. A scheme of the

TABLE 1

EXPERIMENTAL PROTOCOL FOR THE ADDITION OF SIMAZINE OR ETHANOL IN PURE CULTURE STUDIES

Flask No.	Simazine Concentration	250ppm Simazine solution*	Ethanol	Inoculum
1-3	0.0ppm	0.00ml	0.08ml	0.1ml
4-6	1.0ppm	0.08ml	0.00ml	0.1ml
7-9	0.0ppm	0.00ml	0.48ml	0.1ml
10-12	6.0ppm	0.48ml	0.00ml	0.1ml
13-15	0.0ppm	0.00ml	0.96ml	0.1ml
16-18	12.0ppm	0.96ml	0.00ml	0.1ml
19-21	0.0ppm	0.00ml	2.88ml	0.1ml
22-24	36.0ppm	2.88ml	0.00ml	0.1ml
25	0.0ppm	0.00ml	0.00ml	0.1ml
26	0.0ppm	0.00ml	0.00ml	0.0ml

*In 95 per cent ethanol

TABLE 2

EXPERIMENTAL PROTOCOL FOR ADDITIONS IN THE NITROBACTER STUDIES USING YEAST EXTRACT

Flask No.	Simazine Concentration	250ppm Simazine solution*	Ethanol	1% Yeast Extract	Water	Inoculum
1-4	0.0ppm	0.00ml	0.00ml	0.00ml	2.0ml	1.0ml
5-8	0.0ppm	0.00ml	0.48ml	0.00ml	2.0ml	1.0ml
9-12	0.0ppm	0.00ml	0.48ml	2.00ml	0.0ml	1.0ml
13-16	6.0ppm	0.48ml	0.00ml	0.00ml	2.0ml	1.0ml
17-20	6.0ppm	0.48ml	0.00ml	2.00ml	0.0ml	1.0ml
21	0.0ppm	0.00ml	0.48ml	0.00ml	2.0ml	0.0ml

*In 95 per cent ethanol

additions to the Warburg flasks used in the experiment is shown in Table 3. The pH 7.0 buffer used in this study was prepared as indicated in Umbreit et al.,(1959). Two aqueous stock solutions of simazine were used: 50ppm for the 3, 6, and 9ppm concentrations, and 500ppm for the 20, 40, and 100ppm concentrations.

Procedures Used for the Isolation and Study of Simazine Utilizing Microorganisms

The isolation of soil microorganisms which could utilize simazine as a sole source of carbon and nitrogen was accomplished by means of the soil enrichment technique. One hundred grams dry weight of Lodi loam, prepared in the same manner as that used for the perfusion studies, was placed in a 500ml beaker and enriched with a suspension containing 350mg of simazine. This soil was kept at field capacity and incubated at room temperature for 45 days. At the end of the incubation period 10gm samples of the soil were added to dilution bottles containing 100ml of a 1000ppm simazine suspension in distilled water and 1.5gm of purified agar which had been maintained at 50°C in a water bath. Ten milliliter portions of this soil-simazine suspension were then transferred to the surface of a solidified simazine plating medium in petri dishes. This medium was the inorganic medium used by Kaufman et al.,(1963), with the exception of the simazine concentration which was increased to 1000ppm. Simazine was the sole source of carbon and nitrogen in this medium.

TABLE 3

PREPARATION SCHEME FOR WARBURG RESPIRATION STUDIES OF THE INHIBITION OF NITROBACTER

Flask No.	Simazine Concentration	Center Well	Main Compartment				Sidearm
		20% KOH	Buffer	0.05M N_aNO_2	Simazine*	H_2O	Cells
2	0ppm	0.0ml	0.0ml	0.0ml	0.00ml	3.50ml	0.0ml
3	0ppm	0.1ml	1.0ml	0.0ml	0.00ml	1.90ml	0.5ml
4-8	0ppm	0.1ml	1.0ml	1.0ml	0.00ml	0.90ml	0.5ml
9-10	3ppm	0.1ml	1.0ml	1.0ml	0.30ml	0.60ml	0.5ml
11-12	6ppm	0.1ml	1.0ml	1.0ml	0.60ml	0.30ml	0.5ml
13-14	9ppm	0.1ml	1.0ml	1.0ml	0.90ml	0.00ml	0.5ml
15-16	20ppm	0.1ml	1.0ml	1.0ml	0.14ml	0.76ml	0.5ml
17-18	40ppm	0.1ml	1.0ml	1.0ml	0.28ml	0.62ml	0.5ml
19-20	100ppm	0.1ml	1.0ml	1.0ml	0.70ml	0.20ml	0.5ml

*50ppm for flasks 9-14 and 500ppm for flasks 15-20.

To eliminate the possibility that these organisms were growing on other substances present in the original soil, the organisms isolated were transferred for seven transfers on the simazine plating medium. Pure cultures of these organisms were obtained by the streak-plate method during the transfer procedures.

Solution culture studies were carried out with several of the organisms that survived the seven transfers. The medium used for these studies was the simazine plating medium minus the agar, with 0.05ml of Tween 80 per liter of medium added to keep the simazine in suspension. The fungi were inoculated by means of a spore suspension in distilled water diluted to five Klett units, while the bacteria were inoculated with an inoculating loop of the organism from a two-week culture on simazine plating medium. Forty milliliters of the simazine medium was placed in each of the 24 250ml Erlenmeyer flasks which were then sterilized in an autoclave for 15 minutes at 15psi at 121°C. After cooling, the flasks were inoculated and placed on shakers. Each organism was inoculated into five flasks, with the remaining flasks serving as uninoculated controls. Due to shaker limitations, two types of shakers were employed in these studies, but both were reciprocal shakers, and the same shake rate (70 cycles per minute) and temperature (28°C) were used with each shaker. Samples were taken at 0, 14, 28, 42, and 60 days by cannibalizing an entire flask for each organism and one uninoculated control. The flasks were dried to complete dryness at 80°C for three days,

and the precipitate was redissolved in 200ml of 95 per cent ethanol, and the flasks were stoppered and stored at room temperature until the end of the experiment. The residual simazine was quantitatively analyzed.

Quantitative Determination of Simazine

The colorimetric Method No. 4 of Ragab (1959) was used for the quantitative simazine determinations.

The Identification of the Simazine Utilizing Microorganisms

The identification of the fungi was determined by use of the keys of Raper and Thom (1949) and Thom and Raper (1945), by observing the culture characteristics of the isolates grown on Czapek's Agar and in one case Difco Tryptic Soy Agar. Further information about structures of these organisms was obtained by the use of growth chambers as suggested by Seeley and VanDemark (1962) and by the preparation of stains with aqueous basic fuscin.

The identification of the bacteria was determined by the use of Skerman's Key (Breed et al., 1957). The principal means of identification were the Gram stain, the culture characteristics on Difco Standard Plate Count Agar and Difco Potato Dextrose Agar, the hanging drop slide for motility, Liefson's Flagella Stain (Liefson, 1951), fermentation of glucose, sucrose and lactose, action on Difco Nutrient Gelatin, and temperature studies.

RESULTS

The Perfusion Studies

In the 20-day perfusion experiment the ammonium was completely oxidized by the mixed population of soil microorganisms in the perfusion units without simazine by the end of the experiment. During this time a significant difference was observed between the rate of ammonium oxidation in these units and in the units containing the 6 and 9ppm concentrations of simazine (Table 4 and Figure 2). The total utilization of ammonium in the units containing 9ppm simazine was only about 50 per cent of that in the units without simazine, while the ammonium utilization in the units containing 6ppm simazine was only about 70 per cent of that in the units without simazine.

A noticeable difference was also observed between the production of nitrate in the units without simazine and in the units containing 6 and 9ppm simazine (Table 5 and Figure 3). Although a complete recovery of the added ammonium as nitrate was not obtained, up to 37ugN per milliliter of nitrate was produced.

In the 55-day perfusion experiment the difference in ammonium utilization between the units without simazine and the units containing 6 and 9ppm simazine was more conspicuous than that shown in the 20-day experiment (Table 6 and Figure 4). A decrease in the rate of ammonium oxidation in all perfusion units occurred in this experiment during the period between 20 and 40 days. This effect was accompanied by a sharp decrease in pH from

TABLE 4

CUMULATIVE UTILIZATION OF AMMONIUM DURING

THE 20-DAY PERFUSION EXPERIMENT

Time (in days)	Simazine Concentration (ppm) 0	1	3	6	9
	Ammonium Utilized (ugN/ml)				
5	81.38	58.63	77.88	53.38	45.50
10	120.75	110.50	119.88	84.88	62.13
15	143.50	135.63	140.88	100.63	77.88
20	153.13	141.75	140.00	112.00	80.50

TABLE 5

PRODUCTION OF NITRATE DURING THE

20-DAY PERFUSION EXPERIMENT

Time (in days)	Simazine Concentration (ppm) 0	1	3	6	9
	Nitrate Concentration (ugN/ml)				
5	11.42	20.99	4.41	15.55	13.80
10	23.16	21.56	4.37	13.12	19.75
15	37.26	49.87	29.87	18.27	17.69
20	28.00	52.50	28.00	21.25	21.22

FIGURE 2

CUMULATIVE UTILIZATION OF AMMONIUM DURING

THE 20-DAY PERFUSION EXPERIMENT

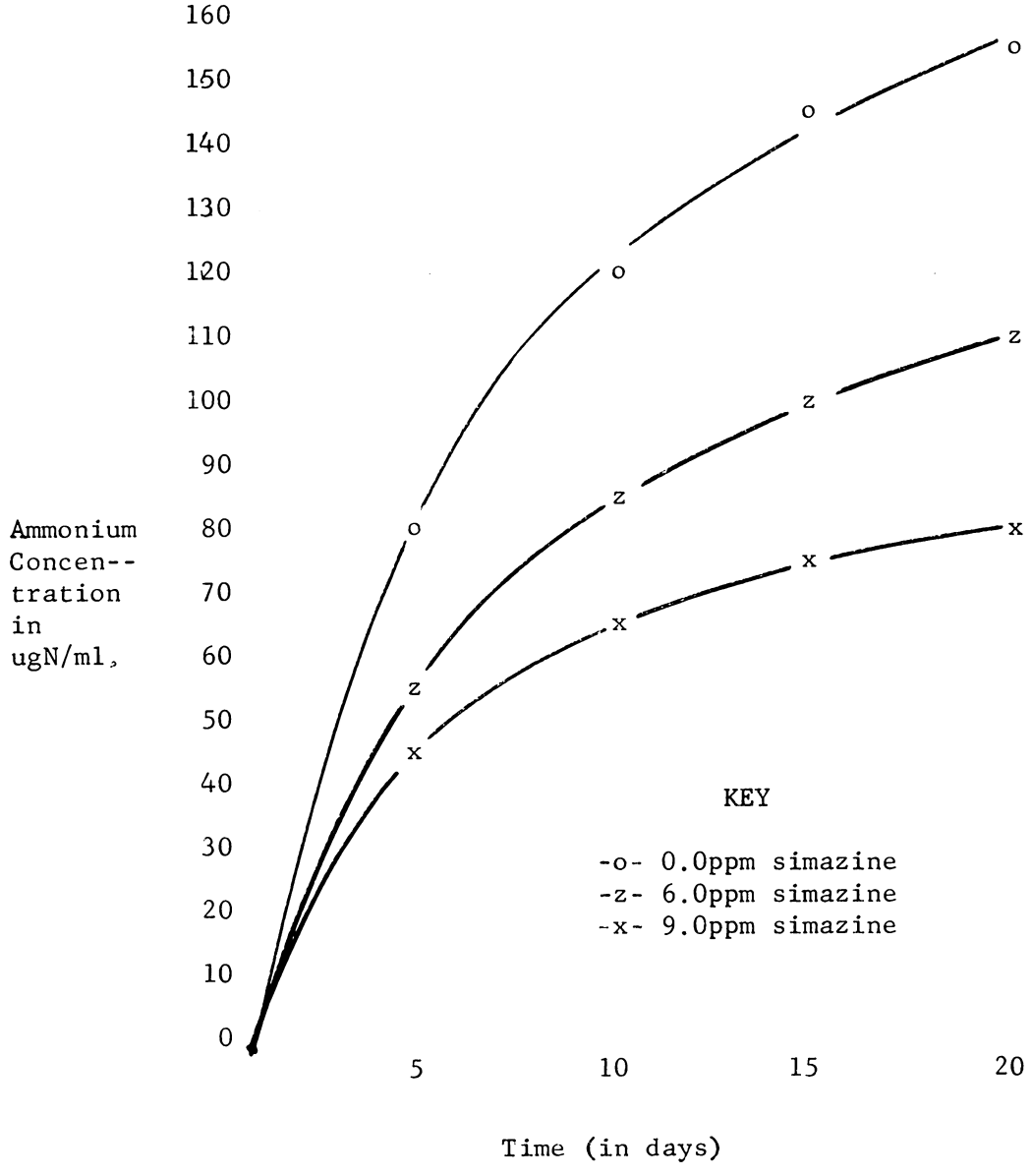


FIGURE 3

PRODUCTION OF NITRATE DURING THE

20-DAY PERFUSION EXPERIMENT

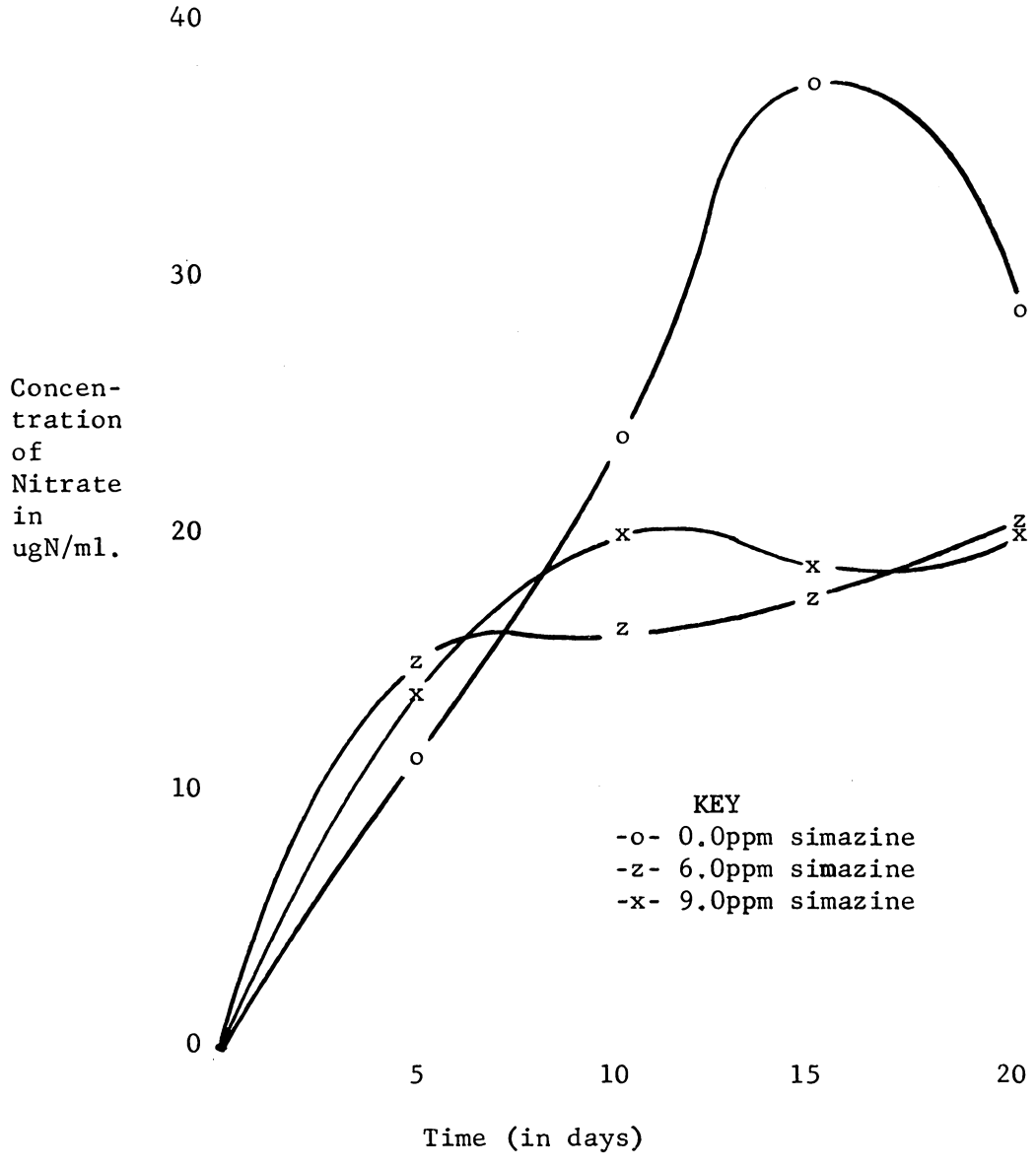


TABLE 6

CUMULATIVE UTILIZATION OF AMMONIUM DURING THE

55-DAY PERFUSION EXPERIMENT

Time (in days)	Simazine Concentration (ppm) 0	1	3	6	9
	Ammonium Utilized (ugN/ml)				
5	81.38	58.63	77.88	53.38	45.50
10	120.75	110.50	119.88	84.88	62.13
15	143.50	135.63	140.88	100.63	77.88
20	153.13	141.75	140.00	112.00	80.50
25	185.51	168.00	162.12	100.62	95.38
30	198.63	180.25	170.62	104.25	102.38
35	196.01	179.38	168.00	102.37	97.13
40	194.26	174.13	167.12	96.25	100.63
45	264.26	225.75	226.62	168.88	149.63
50	305.39	266.88	265.12	226.63	171.60
55	328.62	288.63	270.62	265.51	184.13

TABLE 7

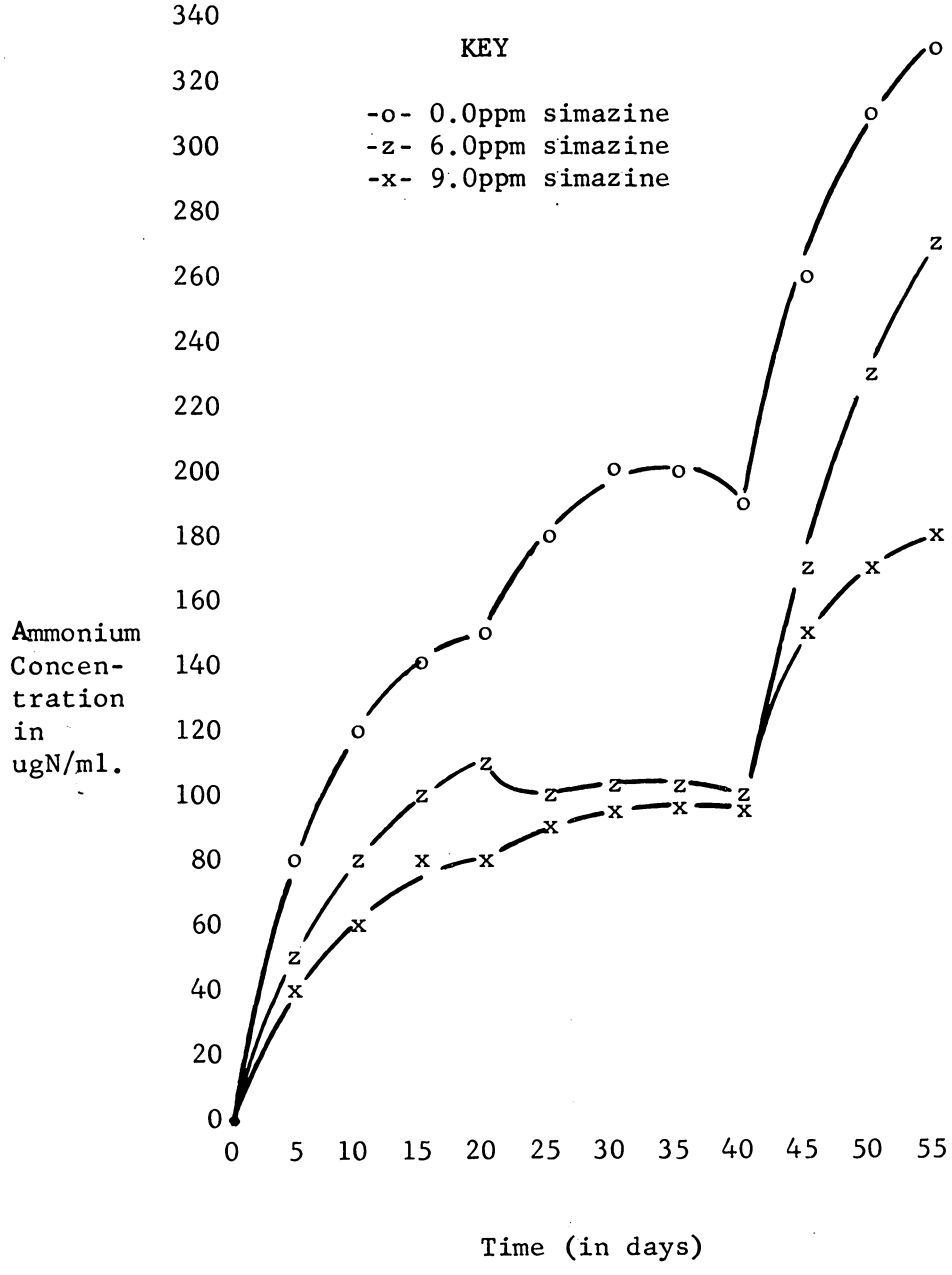
PRODUCTION OF NITRATE DURING THE

55-DAY PERFUSION EXPERIMENT

Time (in days)	Simazine Concentration (ppm) 0	1	3	6	9
	Nitrate Concentration (ugN/ml)				
5	11.42	20.99	4.41	15.55	13.80
10	23.16	21.56	4.37	13.12	19.75
15	37.26	49.87	29.87	18.27	17.69
20	28.00	52.50	28.00	21.25	21.22
25	32.96	35.20	44.18	19.47	29.18
30	60.35	44.62	33.23	17.50	8.69
35	50.75	54.25	55.12	55.12	24.27
40	45.50	54.25	45.50	36.75	20.94
45	90.12	84.87	95.37	82.25	51.42
50	97.45	89.25	97.48	84.87	-
55	64.44	64.22	88.41	92.67	32.77

FIGURE 4

CUMULATIVE UTILIZATION OF AMMONIUM DURING THE 55-DAY PERFUSION EXPERIMENT



8.3 to an average of 5.4. This decrease in pH must be considered when evaluating the difference in ammonium oxidation during this period. This pH condition was corrected by the addition of 0.5 grams of calcium carbonate to each soil column when the additional ammonium was added at 40 days.

In most treatments which contained the 6ppm concentrations of simazine, the inhibitory effect of the simazine decreased as the time of perfusion increased. This recovery effect was noted after 40 days, and the rate of ammonium utilization in these units during this period closely approximated that in the units without simazine. The nitrate production in this experiment (Table 7 and Figure 5) showed a similar inhibitory effect in the first 40 days, and a recovery effect was also noted after this period.

The accumulation of nitrite was very marked during the 55-day experiment (Table 8 and Figure 6), with the units containing 6ppm simazine producing the highest accumulation during the first 20 days and the units containing the 9ppm producing the highest accumulation after this period. In the last 15 days of this experiment, extremely high concentrations of nitrite were observed in the units containing 9ppm simazine.

The 1 and 3ppm concentrations of simazine used in the perfusion studies are not represented in any of the figures because in both of these studies the results with these concentrations closely approximated those with the controls.

FIGURE 5

PRODUCTION OF NITRATE DURING THE

55-DAY PERFUSION EXPERIMENT

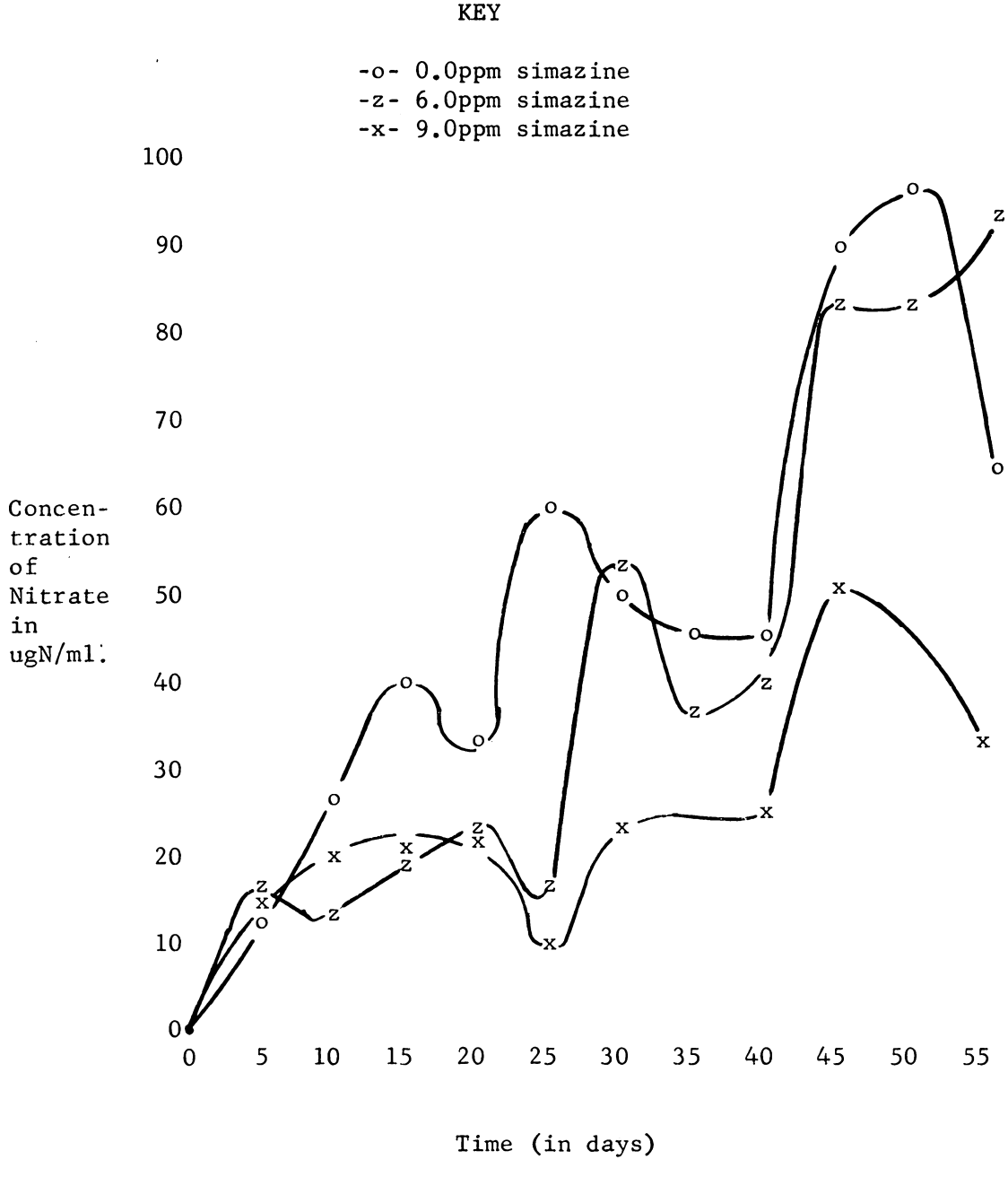


TABLE 8

ACCUMULATION OF NITRITE DURING THE

55-DAY PERFUSION EXPERIMENT

Time (in days)	Simazine Concentration (ppm) 0	1	3	6	9
	Nitrite Concentration (ugN/ml)				
5	3.45	1.25	11.34	2.82	1.95
10	0.46	2.93	18.37	5.25	0.37
15	0.36	0.00	11.25	3.60	0.68
20	0.00	0.00	0.00	2.47	0.65
25	0.29	0.67	0.44	5.52	1.44
30	0.02	0.00	0.02	2.88	7.93
35	0.00	0.00	0.00	0.00	9.85
40	0.00	0.00	0.00	0.00	7.93
45	0.60	1.87	7.52	4.63	19.23
50	0.31	4.90	9.59	3.58	33.72
55	0.00	0.00	0.00	0.00	55.71

TABLE 9

THE EFFECT OF SIMAZINE ON NITRITE PRODUCTION

BY NITROSOMONAS EUROPEA

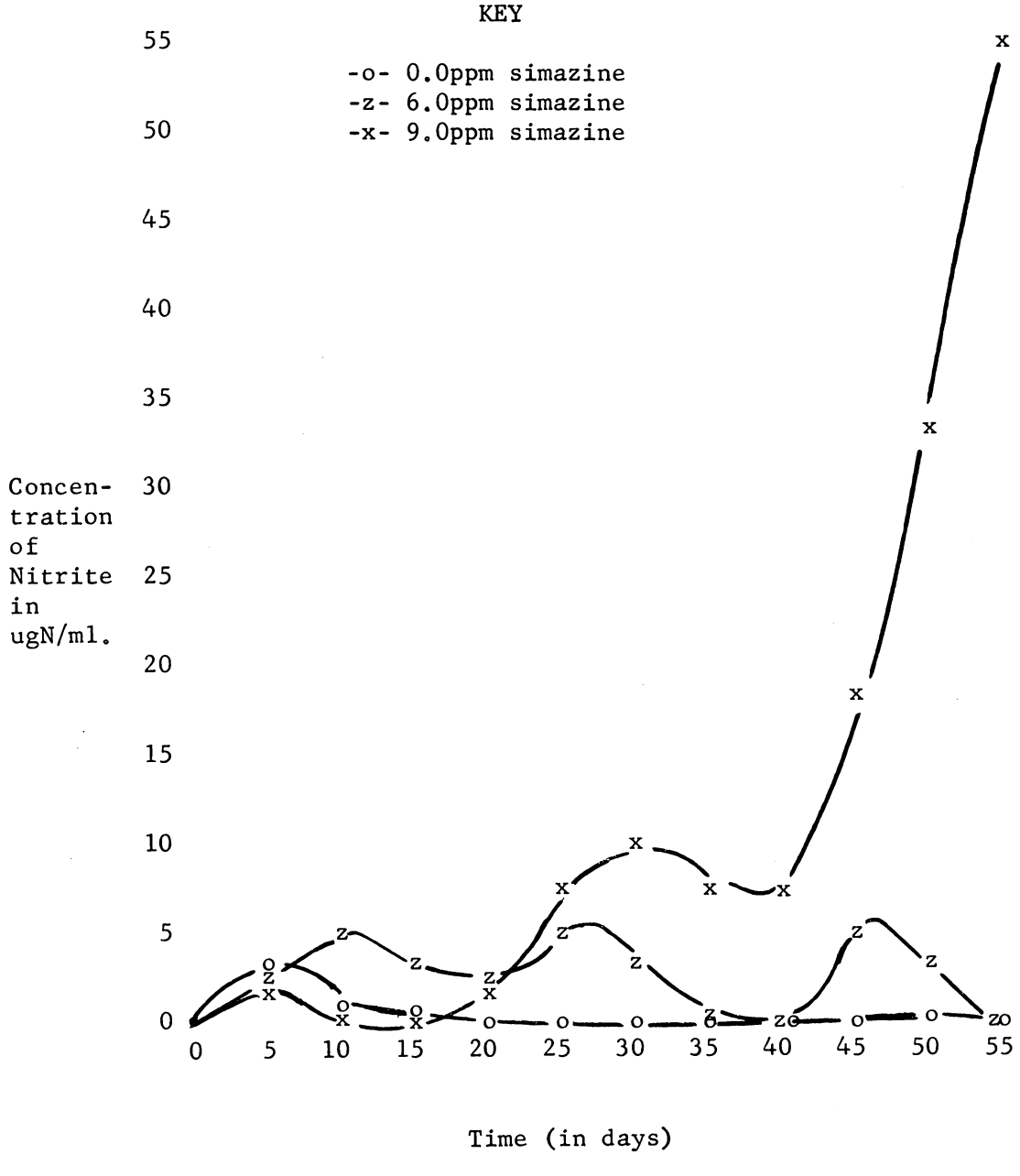
Flasks No.	Simazine Concentration	Time in days 0	3	3½	4	4½	5
		Nitrite (ugN/ml)					
1-3	0.0ppm	0	73	121	208	418	443
4-6	1.0ppm	0	70	115	211	414	443
7-9	0.0ppm	0	65	107	208	359	443
10-12	6.0ppm	0	69	115	203	363	429
13-15	0.0ppm	0	59	107	152	263	443
16-18	36.0ppm	0	64	97	143	250	457
19	0.0ppm	0	80	131	236	443	443
20*	0.0ppm	0	0	0	0	0	0

*Uninoculated control

FIGURE 6

ACCUMULATION OF NITRITE DURING THE

55-DAY PERFUSION EXPERIMENT



The Pure Culture Studies

In the Nitrosomonas pure culture studies, all cultures were able to utilize the ammonium completely in five days. During this period there were no significant differences in nitrite production caused by the addition of simazine (Table 9). The only differences observed where those shown to be caused by the addition of ethanol.

Ethanolic controls were required when it was found that there was some residual ethanol in the simazine treatments after steaming in the autoclave at 121°C for 15 minutes. The higher concentrations of this ethanol had a prolonging effect on the lag growth phase of the nitrifying organisms. At this point it is important to note that there were three different types of treatments in both pure cultures studies: (1) those containing only the basic medium of Lewis and Pramer, (2) those containing the basic medium of Lewis and Pramer and various concentrations of simazine dissolved in ethanol, referred to as simazine treatments, and (3) those containing the basic medium of Lewis and Pramer and the same amount of ethanol added in the corresponding simazine treatments, referred to as the ethanolic controls.

The results obtained in the Nitrobacter pure culture studies were considerably different from those obtained in the Nitrosomonas studies. During the 10-day period required to obtain complete utilization of the nitrite in all flasks, a significant

lag in the utilization of nitrite was noted in the flasks containing 6 and 12ppm concentrations of simazine and in the ethanolic control for the 12ppm simazine concentration (Table 10 and Figure 7). The cultures containing 6ppm simazine lagged 22 hours behind their ethanolic control, and the flasks containing 12ppm simazine lagged 52 hours behind their ethanolic control. There was a slight difference between the flasks containing 1ppm simazine and their control, but it was not considered to be significant. It appears that the addition of simazine increases the length of the lag growth phase, but does not affect the slope of the log growth phase.

In the Nitrobacter pure culture studies in which 0.1 per cent yeast extract was used in the basic medium, the nitrite oxidation in the basic medium was not affected, but the lag in nitrite utilization in the medium containing 6ppm simazine was increased (Table 11). The lag in nitrite utilization caused by the 6ppm concentration of simazine was not as great in this study as in the previous study.

In the Warburg respirometer studies of respiration of Nitrobacter, the presence of simazine resulted in a slight stimulation of the oxygen uptake in the presence of 3 and 6ppm simazine and a slight inhibition of oxygen uptake in the presence of 9, 20, 40, and 100ppm simazine (Table 12). These differences were not considered to be significant.

TABLE 10

THE EFFECT OF SIMAZINE ON NITRITE UTILIZATION

BY NITROBACTER AGILIS

Flasks No.	Simazine Concentration	Time in days 0	4	5	6	7	8	9	9½	10
		Nitrite (ugN/ml)								
1-3	0.0ppm	0	106	221	429	436	436	436	436	436
4-6	1.0ppm	0	84	192	384	429	429	429	429	429
7-9	0.0ppm	0	100	218	424	433	433	433	433	433
10-12	6.0ppm	0	15	84	266	421	429	429	429	429
13-15	0.0ppm	0	0	22	111	269	433	433	433	433
16-18	12.0ppm	0	0	0	8	20	86	223	303	419
19*	0.0ppm	0	0	0	0	0	0	0	0	0

*Uninoculated control

TABLE 11

THE EFFECT OF YEAST EXTRACT ON SIMAZINE

INHIBITION OF NITROBACTER AGILIS

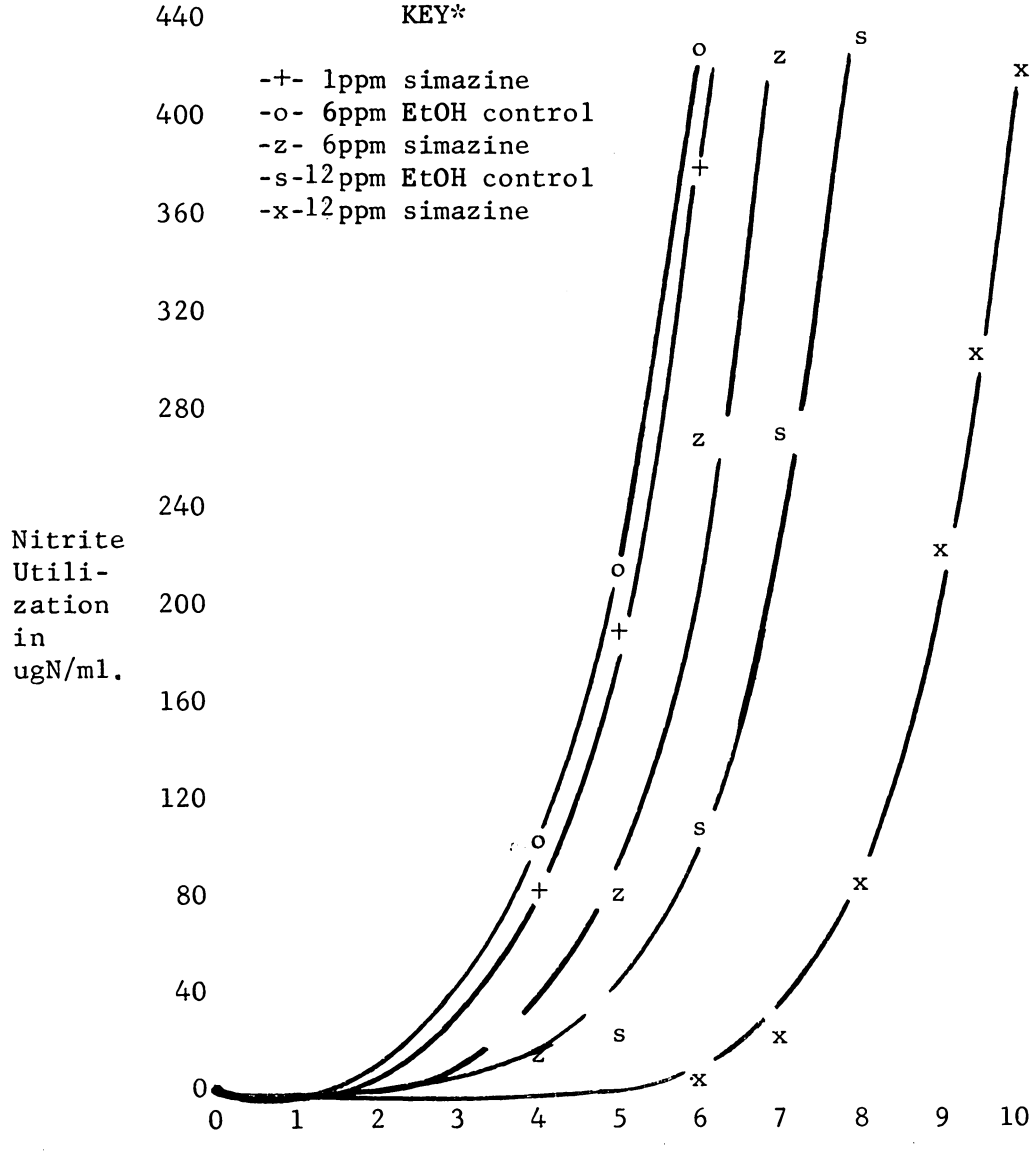
Flasks No.	Simazine Concentration	Time in days 0	1	2	2½	3	3½	4
		Nitrite (ugN/ml)						
1-4	0.0ppm	0	10	96	148	242	337	351
5-8	0.0ppm	0	62	89	137	223	365	386
9-12	0.0ppm	0	37	76	127	230	364	370
13-16	6.0ppm	0	15	58	92	151	284	350
17-20	6.0ppm	0	5	23	48	85	211	323
21*	0.0ppm	0	0	0	0	0	0	0

*Uninoculated control

FIGURE 7

THE EFFECT OF SIMAZINE ON NITRITE UTILIZATION

BY NITROBACTER AGILIS



* The 1 ppm EtOH control was practically identical to the 6 ppm EtOH.

TABLE 12

OXYGEN UPTAKE BY NITROBACTER IN

THE PRESENCE OF SIMAZINE

Simazine Concentration	Time (in minutes) 60	120	180
Control*	76ul	152ul	228ul
3ppm simazine	81ul	163ul	246ul
6ppm simazine	82ul	163ul	245ul
Control**	66ul	144ul	216ul
9ppm simazine	61ul	135ul	202ul
20ppm simazine	51ul	126ul	190ul
40ppm simazine	63ul	139ul	209ul
100ppm simazine	56ul	130ul	196ul

* For 3ppm and 6ppm concentration of simazine
** For 9, 20, 40, and 100ppm concentration of simazine

TABLE 13

DECOMPOSITION OF SIMAZINE BY SOIL MICROORGANISMS

Organism	Residual Simazine After 60 Days*
Fungus #1 (A. syndowi)	680 ppm
Bacterium #3 (unidentified)	1000 ppm
Uninoculated Control for Flasks I and II	810 ppm
Uninoculated Control for Flasks V and VI	820 ppm
Bacterium #1 (Pseudomonas sp.)	930 ppm
Fungus #2 (P. funiculosum)	840 ppm

* Original Simazine Concentration was 1000 ppm.

The Isolation and Study of Simazine Utilizing Microorganisms

Four fungi, two actinomycetes, and three bacteria were isolated from the original soil samples plated-out on simazine plating medium. Only three fungi and two bacteria survived the seven transfers on simazine plating medium. Two of these fungi and the bacteria were those organisms used for solution culture studies. The fungi produced visable growth in solution culture after 14 days, while the two bacteria did not produce visable growth even after a period of 60 days. The results of the quantitative analysis of residual simazine are shown in Table 13. In this table the numerical desigators used for the organisms before identification are used as well as the name of the organism. At the end of the solution culture experiment the first series of samples analysed for residual simazine was the 60 day incubation treatment. After it was discovered that little or no decomposition could be demonstrated after 60 days with the method used, the remainder of the samples were not analyzed.

The Identification of the Simazine Utilizing Microorganisms

Fungus #1 produced more growth on the simazine plating medium than any of the other isolates. Very heavy sporulation occurred and the green spores were macroscopically evident. This organism produced good growth on Czapek's Agar attaining a diameter of 4.5 cm after 14 days at 30°C. The colonies

were dull green in color and surrounded by a very narrow band of white mycelium. The colonies were radially wrinkled, and a light peach color or uncolored on the reverse side of the colony. The conidiophores which rose from the loose mat of mycelium were sparce causing the surface of the colonies to appear rough, and terminated in the rounded conidial heads characteristic of the genus Aspergillus. The heads were globulose, but some small penicillate clusters were observed. The conidia were definitely globulose, thick walled, and rough. Microscopic observation at 970X showed the surface of the conidia to be spinulose. Sexual stages of this organism were not apparent on either Czapek's Solution Agar or Difco Tryptic Soy Agar. On the basis of this information, Fungus #1 was identified as Aspergillus sydowi.

Fungus #2 produced very light spreading growth on the simazine plating medium. The sporulation on this medium was very sparce compared to that of Fungus #1. The production of large yellow diamond shaped crystals in the simazine medium was a striking characteristic of this organism. This fungus produced slow growth on Czapek's Agar attaining a diameter of about 3 cm after 14 days at 30°C. On this medium the colonies were yellow in color and were surrounded by a band of white during the first 6 to 8 days, which became pink after this period. The reverse color was peach in the young colonies, but dull purple in the old colonies. These colonies showed

radial wrinkling with central depressions surrounded by an elevated ridge. The dark purple color of the reverse was slightly soluble and diffused into the medium to some extent. Microscopically the coniophores which rose from the very dense velvety mycelium were shown to be extremely small, few in number, and terminated in characteristic penicilli. The penicilli were strictly biverticulately symetrical, but a few showing no branching occurred. The conidia were subglobulose in old colonies, but elliptical in young colonies, and had thick smooth walls. On the basis of this information Fungus #2 was identified as Penicillium funiculosum.

Fungus #3 was very simular to Penicillium funiculosum and was in fact isolated as a contaminant of this organism. It was able to grow on the simazine plating medium in pure culture, and showed the same type of growth observed for P. funiculosum. The principal differences between these two organisms was in the growth rate and in the production of the purple pigment on the reverse of the colony. Fungus #3 grew slightly s lower on Czapek's Solution Agar than did P. pusillum, the former produced colonies with a diameter of only about 1.5 to 2.0 cm. Fungus #3 did not produce any purple pigment in reverse. The reverse of the young colonies was light tan, while in the older colonies it was brown. On the basis of these

differences, this organism was tentatively identified as Penicillium rugulosum, but it was realized that it could be a mutant or variety of P. funiculosum.

Bacterium #1 produced a very thin colorless colony on the simazine plating medium which spread over much of the plate. The presence of the colony was not macroscopically visable. The bacterium was a short gram negative rod which was motile and the flagella stain showed that in most cases it was lophotrichous. The organism grew well on all complex media used. Growth was observed in nutrient gelatin, but no appreciable liquification occurred. It did not ferment glucose, lactose, or sucrose, did not produce any effect in litmus milk, and grew well in Difco Tryptic Soy Broth at both room temperature (about 25°C) and at 37°C. On the basis of this information plus the culture characteristics on Difco Tryptic Soy Agar and Difco Potato Dextrose Agar this organism was determined to be a member of the family Pseudomonadaceae and is probably a member of the genus Pseudomonas. Further characterization must be undertaken to fully establish the genus and species of this isolate.

Bacterium #3 produced small yellow, fairly smooth round colonies on simazine agar. This organism was shown to be a short gram negative rod which had peritrichous flagellation. Further identification was not possible because it grew so slowly on conventional organic media. This bacterium required 21 days to produce visable growth on Difco Standard Plate Count

or Difco Tryptic Soy Agar and did not produce any growth in liquid media such as Difco Tryptic Soy Broth and Difco Phenol Red Glucose Broth.

It was observed that if, after growing for 60 days on the simazine solution medium, the fungi were again streaked out on simazine plating medium, and then transferred to Czapek's Agar, there were noticeable differences in their culture characteristics from those cultures maintained on complex media. A. sydowi (Fungus #1) produced colonies that were 7.0 cm in diameter after 10 days and which were practically sterile until after 14 days when sporangia were produced in very limited numbers. These were the only differences from the original characteristics observed upon isolation. P. funiculosum (Fungus #2) also showed variations from the original description. After the simazine solution culture studies, this organism produced colonies that were macroscopically similar to the original culture, but which attained an average colony diameter of only 1.5 cm after 14 days.

DISCUSSION

The inhibition of nitrification by simazine at concentrations of six ppm and higher was evident in both the perfusion studies and the pure culture studies using Nitrobacter agilis. The inhibition of ammonium utilization and nitrate formation in the perfusion units, and the inhibition of nitrite utilization in the pure culture experiments are indicative of this effect. In the perfusion units it was clearly demonstrated that although a general inhibition of nitrification occurred, the high nitrite concentrations detected indicate that the organisms which oxidize nitrite to nitrate may be selectively inhibited by the herbicide.

Support for this hypothesis was obtained from the results of pure culture studies with the nitrifying bacteria. The growth of Nitrosomonas europea was not affected by concentrations of simazine up to 36ppm, whereas the growth of N. agilis was inhibited by concentrations of 6 and 12 ppm. The degree of inhibition at the latter concentration was about twice as much as observed in the former.

It was further shown in these studies with N. agilis that simazine increased the length of the lag growth phase, but it did not affect the log growth phase. In fact, the characteristics of the log growth phase were identical to those in the non-herbicide treatment. These data suggest

that simazine may inhibit the biosynthesis of some component(s) of these bacteria, and does not act as a competitive inhibitor in nitrite oxidation. Further evidence for this hypothesis was shown by the fact that respiration of N. agilis was not significantly affected by concentrations of simazine up to 100ppm. However, it was noted in these Warburg studies that the respiration of N. agilis was slightly stimulated by simazine concentrations of 3 and 6 ppm, and there was a slight inhibition of respiration at 9, 20, 40 and 100ppm. It is possible that the inhibition of simazine on nitrite oxidation would be much greater in a cell free extract of N. agilis which contained the nitrite oxidizing system.

It is difficult to postulate the mode of action of this herbicide on the nitrifying bacteria. Since N. europea and N. agilis are closely related organisms, and since there was no response to simazine by N. europea in this investigation, the effect observed with N. agilis may be due to interference with some specific initial step in the biosynthesis of the nitrite oxidizing system. This hypothesis was supported by the fact that if the number of cells in the inoculum was decreased, the inhibitive effect of simazine on N. agilis was increased.

Yeast extract did not reverse the inhibitive action of simazine on N. agilis. This result does not mean that

the proposed mode of action of simazine is incorrect. Several reasons may account for the inactivity of the yeast extract: (1) The cell membrane may not be permeable to the factor(s) which might decrease inhibitor activity, (2) the chemo-autotrophs cannot utilize preformed biochemical constituents, or (3)the yeast extract did not contain the factor(s) required to reverse the inhibition.

Further studies must be conducted to determine if the simazine inhibition observed in these studies can also be observed under field and greenhouse conditions. In the light of the data presented in this thesis it is doubtful that nitrite would accumulate if the recommended field rate applications were used. If rates higher than those recommended were used it is possible that toxic concentrations of nitrite would accumulate. There are several factors at work under natural conditions which might decrease this inhibition and these are graphically illustrated by the recovery effect noted in the perfusion studies. This recovery effect was observed after 40 days in the presence of 6ppm simazine and could be due to several different factors: (1) inactivation or decomposition of the simazine by other organisms present in the soil columns, (2) the adaptation of the bacteria to the inhibitory effect of the herbicide as proposed by Reid (1960), and/or (3) the inactivation of the simazine by the absorption of the

molecules by soil colloids as suggested by Sheets et al. ,(1962).

During the course of the perfusion and the pure culture studies several minor effects occurred which require explanation. These effects were: (1) the production of ammonium in some of the perfusion units during the period of the pH drop, (2) the decreases in nitrate levels at certain times during the perfusion experiments, and (3) the increase in the duration of the lag growth phase of N. agilis caused by the addition of ethanol.

During the period between 30 and 40 days, the decrease in the rate of ammonium utilization in the 55-day perfusion experiment reached the point where the utilization of ammonium had ceased and a net increase in ammonia was detected. This effect was caused by the inhibition of nitrification due to an increase in the hydrogen ion concentration which decreased the pH to approximately 5.4. Ammonium production in the soil is usually due to ammonification, and in some cases denitrification. It is probable that in this particular case the decrease in pH caused the death of a large number of microorganisms which released amino acids from which ammonia was formed by ammonification. Therefore, ammonia would accumulate because ammonification would be proceeding at a faster rate than nitrification.

Significant decreases in the level of nitrate concentration occurred several times during the two perfusion experiments.

These decreases usually corresponded to decreases in the rate of nitrification caused by the depletion of the supply of ammonium, or the effect of a lowered pH. It is probable that the decrease in nitrate was due either to its utilization by other microorganisms as a nitrogen source or the chemical loss of nitrite nitrogen.

It was necessary in the pure culture studies to add simazine to the culture media in an alcohol solution. The accompanying heat treatment used for those flasks which contained 0.48ml of ethanol (corresponding to the 6ppm simazine treatment) allowed the complete removal of the ethanol, but at higher concentrations of ethanol a sufficient amount remained to affect the lag phase of growth. The data indicated that at higher concentrations of alcohol, the alcohol increased the toxic effect of simazine. Therefore, it is possible that a damaged cell may be more sensitive to the toxic action of the herbicide. It was observed that in all ethanol controls, regardless of the concentration of ethanol, all cultures of N. agilis eventually grew. This phenomenon could be attributed to the evaporation of the alcohol, which would remove this antimicrobial agent from the medium and allow normal growth of the organism.

The isolation of five microorganisms which can grow in a medium containing simazine as a sole source of carbon and nitrogen indicates an amazing ability of these organisms

to metabolize this compound. Microorganisms previously isolated by other investigators required some auxiliary source of either carbon or nitrogen for cell growth, as did the other four organisms in this study which were unable to survive the seven transfers on the simazine plating medium. The ability of two of the five isolates to produce significant but limited quantities of cell material in the simazine solution medium is further evidence that simazine can serve as a sole source of carbon and nitrogen for certain microorganisms.

The only organism which was shown to cause significant decrease in the simazine level in the solution cultures was A. sydowi (Fungus #1). The other organisms did not show a significant difference from the controls. Since good growth was obtained in the simazine solution culture with P. funiculosum (Fungus #2) it would be expected that this organism would also show some indication of simazine decomposition. A possible explanation for this effect is to be found in the method used for residual simazine analysis. Ragab's method is based on the presence of a chlorine atom which is attached to the triazine ring of simazine. The ethylamino side chains of simazine do not affect the reaction with the color producing reagents. Thus if the decomposing microorganisms utilized these side chains as their source of carbon and nitrogen, the difference in the quantitative

recovery of simazine would not be detected by the colorimetric analysis. The utilization of these side chains by soil microorganisms was shown to be a primary step in the decompostion of simazine by Kaufman et al., (1963). If this hypothesis is correct then the isolated decomposers studied in the simazine solution culture studies could be utilizing the side chains, but this could not be demonstrated by any means other than the visable growth of the organisms. The decomposition observed by the colorimetric analysis in the case of A. sydowi (Fungus #1) may have been due to either the removal of the chlorine atom from the ring or the structual breakdown of the triazine ring. These possibilities were also mentioned by Kaufman et al., (1963).

The identification of the fungi as members of the Fungi Imperfecti was not unexpected as many of the fungi isolated as simazine decomposers by previous investigators have also been members of this class. However, none of the five organisms which were isolated in this study have been reported previously as simazine decomposers. Also, none of the organisms isolated as simazine decomposers in previous studies have been able to use simazine as a sole source of carbon and nitrogen.

As suggested in the introduction to this thesis, the comparative physiology of these simazine utilizers is of great interest. In this study several metabolic similarities were

noted among the various organisms. The simazine utilizers were all slow growers on conventional isolation media in contrast with the growth rates usually obtained with the majority of soil microorganisms. The fungi isolated were all members of the Fungi Imperfecti. They were all common soil inhabitants as were those isolated by Kaufman et al., (1963). Neither of the bacteria appear to be able to utilize the common carbohydrates. A further investigation of their nutrition might indicate other metabolic similarities.

The physiological and morphological changes to the two fungi used in the simazine solution study are of interest because it was demonstrated that the physiology of these organisms may be affected by simazine treatment. Further investigation of this effect is necessary before any conclusive statements can be made. However, the effects on the organisms could be connected to the utilization of their unique metabolic pathway as a sole means of obtaining carbon and nitrogen for cell growth. There is also the possibility that these changes in their characteristics could be due to a mutation.

This investigation was a preliminary study of the problem of the effect of simazine on nitrification and of the problem of the decomposition and utilization of simazine by soil microorganisms. On the basis of the results reported in this

study, there are a number of questions which arise which warrent further investigation: (1) What is the mechanism of simazine toxicity? (2) Why is N. agilis inhibited by simazine and N. europea is not? (3) What groups of the simazine molecule are used by the isolated organisms as their carbon and nitrogen sources? (4) What, if anything, do the simazine utilizers have in common besides the ability to metabolize this compound? and (5) What is the role of this type of organism in soil systems? The answers to these questions could go a long way toward providing answers to questions regarding the role of pesticides in soil systems.

SUMMARY

Studies of the effect of simazine on the nitrification process were carried out in soil perfusion units and in pure cultures of the two principal nitrifying organisms. Inhibition of nitrification was noted in the perfusion studies at concentrations of simazine of 6ppm and above. A similar effect was noted in the pure culture studies of Nitrobacter agilis, but not with Nitrosomonas europea. This selective inhibition was also apparent in the perfusion studies where an accumulation of nitrite occurred in the units containing inhibitory concentrations of simazine.

In further studies carried out to give some indication of the mechanism of inhibition it was found that 0.1% yeast extract could not reverse the simazine inhibition of N. agilis and that the respiration of this organism in a Warburg respirometer was not appreciably affected by concentrations of simazine up to 100ppm.

The decomposition and utilization of simazine by soil microorganisms was also investigated. In an attempt to establish that this compound is metabolized by soil microorganisms, five organisms which could utilize simazine as a sole source of carbon and nitrogen were isolated by a procedure of soil enrichment with simazine. After 45 days the soil was plated out on a medium containing simazine as the sole source of these

nutrients. Those organisms which were able to survive seven transfers on this medium were considered to be simazine utilizers. They were identified as Aspergillus sydowi, Penicillium funiculosum, P. rugulosum, a Pseudomonas species, and an unidentified gram negative rod-shaped bacterium. These organisms were shown to have several characteristics in common.

Four of these organisms were grown in a simazine solution medium containing no other source of carbon or nitrogen. These data support the hypothesis that these organisms can utilize simazine as sole source of carbon and nitrogen. It appeared that only Aspergillus sydowi could produce a significant decrease in the original level of simazine in the medium. Due to the limitations of the method of simazine analysis, it was not possible to demonstrate decomposition by chemical means, but visual observations of growth showed that at least one of the other three organisms removes and utilizes the side chains of the molecule. The decrease in measureable simazine in the A. sydowi cultures was due to either the removal of the chlorine atom from the triazine ring, in addition to the utilization of the ethyl amino side chains, or the actual breakdown of the triazine ring. The fungi used in the solution culture studies showed slight variations from the original characteristics when observed on complex media after exposure to high concentrations of simazine.

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ABSTRACT

The herbicide simazine (2-chloro-4,6-bis(ethylamino)-1,3,5-triazine) was found to have an inhibitory effect on nitrification in pure and mixed cultures of the nitrifying bacteria. Simazine concentrations of 6 and 9ppm inhibited the rate of nitrification in soil perfusion units, and an abnormally high level of nitrite nitrogen was observed in the herbicide treatments. The herbicide had no effect on the growth of Nitrosomonas europea in shake flask culture, but did inhibit the growth of Nitrobacter agilis. The addition of yeast extract to the culture medium did not reverse the inhibition. Simazine did not inhibit the respiration of N. agilis even at a 100ppm concentration.

Five organisms were isolated which could utilize simazine as a sole source of carbon and nitrogen. Pure cultures of these organisms were made and several were identified.