CULTURAL STUDIES AND EFFECTS OF PESTICIDES IN NITRIFYING SYSTEMS

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ABSTRACT

CULTURAL STUDIES AND EFFECTS OF PESTICIDES IN NITRIFYING SYSTEMS

By

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Physiological characteristics of Nitrosomonas europaea and Nitrobacter agilis in mixed liquid culture and effects of agricultural chemicals on their oxidation of ammonium and nitrite were examined under stationary flask and perfusion conditions.

Persistent heterotrophic contaminants in the mixed culture did not interfere with stoichiometric conversions of ammonium or nitrite unless an organic energy source was added. In the presence of glucose, trichloro-bis-(4-chlorophenyl) ethane [DDT], N, N-dimethyl-2, 2-diphenylacetamide [diphenamid], and 2-chloro-4, 6-bis(ethylamino)-S-triazine [simazine] completely suppressed growth of these contaminants at concentrations which had no effect on ammonium or nitrite oxidation. This suggests a new approach for purifying cultures of Nitrosomonas and Nitrobacter.

Optimum hydrogen ion concentration for nitrification in liquid culture was found to be at pH about 8.0 to 9.0 for Nitrosomonas and 6.0 to 9.0 for Nitrobacter. Disappearance of nitrite at pH below 3.0 was observed and was likely due to chemical reactions of nitrite.

In stationary flask culture high ammonium levels had little or no effect on the rate of ammonium oxidation but nitrate production was restricted to a low, linear rate characteristic for a static or resting population.

This indicates that growth of Nitrobacter rather than nitrite oxidation was suppressed. When ammonium was reduced below about 100 ppm NH_4-N, there was an abrupt increase in rate of nitrate production consistent with resumption of growth. Nitrite accumulated intermediately in amounts and over periods of time which were proportional to the initial concentrations of ammonium. In enriched perfusion systems, no intermediate accumulations of nitrite occurred with concentrations as high as 750 ppm NH_4-N. These observations suggest a bacteriostatic effect of either ammonium or free ammonia on Nitrobacter.

Twenty-seven chemicals were tested for inhibitory effects on nitrification in mixed culture. Nine were highly specific in their action against Nitrosomonas over the entire range of the test concentrations (2.5 to 50 ppm). Two were equally specific in their action against Nitrobacter at 2.5 ppm and five more over the range 10 to 50 ppm. The rest of the chemicals affected both organisms. The concentrations required for complete inhibition of ammonium and/or nitrite oxidation were increased for ten of the chemicals when soil was added to the incubation mixture. Water soluble materials in extracts from the same soil were ineffective in reducing inhibitory effects. This indicates that insoluble organic or inorganic constituents were responsible for inactivation by soil.

At low but biologically active concentrations, in mixed culture of the nitrifying bacteria, 3-amino-1,2,4-triazole [amitrole] combined with 2-chloro-6-(trichloromethyl) pyridine [N-Serve] synergistically inhibited the oxidation of ammonium by Nitrosomonas. The inhibition of Nitrobacter by potassium azide and sodium azide in combination was also synergistic. Isopropyl-N-phenylcarbamate [IPC] and isopropyl-N-(trichlorophenyl) carbamate [CIPC] at low concentrations were antagonistic in their inhibition of both _Nitrosomonas_ and _Nitrobacter_. Both synergistic and antagonistic responses approached additivity as the concentration of one or both chemicals in a combination increased.

In perfusion systems, the inhibition of ammonium and/or nitrite oxidation by amitrole, CIPC, IPC, potassium azide and sodium azide was increased on increasing perfusate volumes and was affected by the nature of column material. The pattern of _Nitrosomonas_ inhibition by amitrole and its residual effects appeared to depend mostly upon mechanisms of its sorption and desorption by column materials. CIPC and IPC inhibited nitrite oxidation by _Nitrobacter_. Living or dead organic materials rather than inorganic column constituents seemed to be responsible for sorption of these two chemicals, and for inhibition carried over into a second perfusate. Potassium and sodium azide prevented nitrite oxidation and none of the chemicals was sorbed by column constituents, which is in agreement with the absence of any residual effect on re-perfusion with untreated medium.

Amitrole concentrations in perfusate were determined chemically. A _Nitrosomonas_ and _Nitrobacter_ bioassay was developed for estimating concentrations of the other chemicals.

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

Page

PART I:

TABLE

PART II:

TABLE

LIST OF TABLES (continued)

LIST OF TABLES (continued)

Page

PART IV:

TABLE

LIST OF FIGURES

Page

PART I:

FIG.

PART IV:

FIG.

LIST OF FIGURES (continued)

GENERAL INTRODUCTION

The application of pesticides to soils and plants to control insects, plant diseases and weeds is a virtual necessity for human survival in our present economic system. Evaluation of the effects of these materials shows that in addition to reducing numbers of specific parasites they may alter the ecological balance of beneficial microbial populations.

Nitrification in soil is considered an important biological process with regard to effective nitrogen nutrition of plants and other organisms living in soil. This process is mainly effected by two groups of aerobic chemolithotrophic bacteria, _Nitrosomonas_ and _Nitrobacter_. Ammonium is oxidized to nitrite by _Nitrosomonas_ and nitrite to nitrate by _Nitrobacter_.

The objectives of this research were (1) to study some physiological characteristics of these two organisms in mixed culture, (2) to look for synergistic or antagonistic interactions among chemicals which inhibit one or both organisms and (3) to investigate the extent to which solid surfaces in stationary flask or perfusion culture may alter the degree to which these organisms are inhibited by several agricultural chemicals.

The research developed logically in four stages. These are the basis for subdivision of the thesis into four parts. Each part has been developed with an independent format to facilitate later publication.

PART I: PHYSIOLOGICAL STUDIES WITH NITROSOMONAS AND NITROBACTER

Introduction

Nitrification is traditionally considered to be the biological conversion of ammonium to nitrate. This is mostly effected in nature in two steps by two highly specialized groups of aerobic chemolithotrophic bacteria. In the first step, ammonium is oxidized to nitrite by the Nitrosomonas group; in the second, nitrite is oxidized to nitrate by Nitrobacter.

The isolation of nitrifying bacteria in pure culture is difficult. However, heterotrophic microorganisms (contaminants) present in liquid culture of Nitrosomonas and Nitrobacter seem to have no effect on the observed rates of ammonium and nitrite oxidation (9,11).

Nitrification under field and laboratory conditions is affected by, among other factors, the hydrogen ion concentration (16,20) and the concentration of ammonium present in the growth medium (5,6,8,19).

Working with mixed liquid cultures of Nitrosomonas and Nitrobacter, this study is concerned with: (a) the selectivity of certain chemicals in preventing heterotrophic growth without influencing nitrification, (b) the effect of hydrogen ion concentration on ammonium and nitrite oxidation and (c) the effect of ammonium levels on the rates of ammonium oxidation and nitrate production under liquid flask culture and perfusion conditions of growth.

Materials and Methods

Biological material. Cultures of Nitrosomonas europaea and Nitrobacter agilis were kindly supplied by Dr. C.L. San Clemente, Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan. A mixed stock culture of these two organisms has been maintained in the dark for more than two years with no change in the apparent rate of ammonium oxidation.

The mixed stock culture was maintained in a mineral medium composed of $(NH_4)_2SO_4$, 0.300 g; $CaCl_2$, 0.136 g; $MgSO_4 \cdot 7H_2O$, 0.175 g; $NaHCO_3$, 0.500 g; Na_2HPO_4, 13.500 g; $KHPO_4$, 0.700 g; $FeSO_4 \cdot 7H_2O$, 5 mg; $NaMoO_4$, 0.375 mg, and one liter of deionized water. The final pH of the medium was adjusted to 8.0-8.3. All media preparations were autoclaved (15 p.s.i., 121C). The culture was incubated with forced aeration at 20-25C in four-liter fermentors and transferred every 10-15 days. An inoculum of 1-5% was used to initiate growth. No turbidity developed in the stock culture.

Perfusion apparatus. Twelve positive pressure soil perfusion percolators (13,18) with cylindrical chambers ($5.25\ cm^2$ x 20.2 cm) and conical reservoirs (250 ml capacity) were used. The chambers (columns) were packed with three different materials (Table I). Four units of each material were employed: (i) Glass beads (1.00-1.05 mm, B. Braun, Apparatebau, Melsungen). (ii) Vermiculite-perlite mixture (commercial sources). (iii) Soil-perlite mixture (air-dried Sims clay loam, 3-5 mm). The cation exchange capacity of the column materials was determined by the method of Chapman (4).

TABLE I

Cation exchange and water holding capacities of perfusion units

Kind and amount of materials	Cation-exchange capacity (me/unit)	Water-holding capacity (g/unit)
Glass beads, 150 g	0.60	30
Vermiculite, 3 g; Perlite, 10 g	4.15	42
Soil, 50 g; Perlite, 4.5 g	15.00	40

The glass beads were boiled first with concentrated H_2SO_4 and then with 10% NaOH for half an hour and washed 10 times with distilled water. Glass wool was placed below each column to prevent movement of fine materials into the reservoir. After packing, all units were autoclaved for half an hour (15 p.s.i., 121C).

Each percolator was connected by way of a manifold system to a pressure pump. The air that entered the apparatus had been passed through 10-20% sulfuric acid and a humidifying chamber. The perfusate was percolated at an approximate rate of 5 ml per minute.

All units were inoculated with the above mixed culture of Nitrosomonas and Nitrobacter and maintained in the described medium but with 0.471 g $(NH_4)_2SO_4$ and 0.3 g KNO_2 to give initial concentration of 100 ppm NH_4-N and 50 ppm NO_2^--N.

In order to stabilize the organisms at a constant rate of substrate oxidation under the described conditions, percolation was conducted for 15-30 days with 200 ml of nutrient solution per unit, changed every 1-2 days.

Suppression of heterotrophic contaminants. The original Nitrobacter culture contained heterotrophic contaminants which had proven extremely difficult to eliminate (9). Heterotrophic contaminants were still present in the mixed stock culture of Nitrosomonas and Nitrobacter after 18 months' continuous culture in the inorganic salt medium. This was readily demonstrated by the rapid development of turbidity when transfers were made into the same medium amended with glucose.

A number of chemicals were tested for specific inhibition of heterotrophic growth in a 50 ml incubation mixture in 250 ml Erlenmeyer flasks.

The mixture comprised: (i) the desired quantity of chemical, transferred to the flask in acetone solution of appropriate concentration and evaporated to dryness at 45-50C, under vacuum in a water bath, (ii) 25 ml nutrient solution with ammonium and nitrite to give a final concentration of 50 ppm in NH_4-N and 50 ppm in NO_2^--N, (iii) 25 ml mixed stock culture which had recently completed oxidation of nitrogen contained in the medium and (iv) either none or 400 ppm glucose-carbon to give C:N=4.

Duplicates were incubated for 6 days in the dark at 20-25C without shaking. Flasks without glucose were analyzed for ammonia, nitrite and nitrate. Those with glucose were visually examined for heterotrophic growth on the basis of turbidity.

<u>pH effects</u>. The pH-values limiting the oxidation of ammonium and nitrite were investigated experimentally. Test mixtures (50 ml in 250 ml flasks) contained: (i) 25 ml nutrient solution with 100 ppm NH_4-N and 100 ppm NO_2^--N and (ii) 25 ml mixed stock culture which had recently oxidized all the nitrogen in the growth medium. The mixture was adjusted with 0.1 N KOH or 0.1 N HCl to pH-values ranging from 3.0 to 11.0. The pH was determined daily and maintained at the initial values by addition of the required amounts of base. After 6 days' incubation in the dark, at 20-25C, without shaking, all flasks were analyzed for ammonia, nitrite and nitrate.

<u>Effects of ammonium levels</u>. The effect of ammonium levels on the rates of ammonium oxidation and nitrate production was studied under liquid culture and perfusion conditions.

Liquid culture. The 50 ml incubation mixture in a 250 ml flask contained: (i) 25 ml nutrient solution with ammonium to give the desired concentration and (ii) 25 ml mixed stock culture which had recently oxidized all the nitrogen contained in the medium. Four flasks for each concentration were incubated in the dark, at 20-25C, without shaking. The pH was adjusted to 8.0-8.3 daily.

Perfusion conditions. 150 ml portions of nutrient solution were used for each ammonium level studied. Between changes in ammonium treatments, all units were maintained under standard conditions for 3-4 days, with daily changes of 200 ml nutrient solution containing 100 ppm NH_4-N and 50 ppm NO_2^--N.

The pH of the perfusate was adjusted to 8.0-8.3 every 4-5 hours with 0.1 KOH solution.

Five-ml perfusate portions were withdrawn through a sampling port kept sterile by immersion in alcohol, and analyzed for ammonia, nitrite and nitrate. Ammonia was determined by Nesslerization (3), nitrite by the modified Griess-Ilosvay method (2) and nitrate by the phenol-disulfonic-acid method (17). All colorimetric measurements were made in a Bausch and Lomb Spectronic 20.

Results and Discussion

Heterotrophic contaminants. From a total of 85 chemicals tested, 14 prevented heterotrophic growth as judged by lack of visually observed turbidity in the growth medium (Table II). Their effective dosages ranged, on the average, between 150 and 250 ppm.

DDT, diphenamid, and simazine, having no effect on ammonium and nitrite oxidation (Table II), were the most suitable for use as inhibitors of heterotrophic microorganisms present in the culture of *Nitrosomonas* and *Nitrobacter*.

All the others which prevented heterotrophic growth also inhibited ammonium and nitrite oxidation within their effective ranges (Table II).

A green color in addition to turbidity appeared in most flasks with glucose after the second day of incubation, suggesting the presence of *Pseudomonas* species. Isolation of heterotrophic species which developed in the cultures was not attempted.

Gram-negative rods isolated from cultures of *Nitrosomonas* and/or *Nitrobacter* are presumed to be *Pseudomonas* (9) but it has not as yet been established whether *Pseudomonas* species are the only persistent contaminants or merely the dominant ones (9,11).

DDT, diphenamid and simazine are specific for the particular case examined here. They may or may not be in isolates of *Nitrosomonas* or *Nitrobacter* from other sources.

pH effects. The effect of hydrogen ion concentration on nitrification in liquid mixed culture is shown in Fig. 1. At the 5 percent level of probability, differences from one pH to another which are greater than 4.7, 8.0 and 7.4 ppm for NH_4-N, NO_2^--N and NO_3^--N are significant. The optimum pH values for ammonium oxidation lay between pH 8.0 and 9.0 and those for nitrite between pH 6.0 and 9.0. Ammonium was not oxidized at pH 6.0 or below, and its oxidation was sharply

TABLE II

Effects of chemicals on Nitrosomonas (Nm), Nitrobacter (Nt) and heterotrophic contaminants (Ht) in mixed culture

Chemical	250 ppm			150 ppm			25 ppm		
	HT	Nm	Nb	Ht	Nm	Nb	Ht	Nm	Nb
DDT	NT	-	-	NT	-	-	T	-	-
Diphenamid	NT	-	-	NT	-	-	T	-	-
2,4-Dichloroaniline	NT	+++	+++	NT	+++	+++	T	+++	-
2,5-Dichloroaniline	NT	+++	+++	NT	+++	+	T	+++	-
3,4-Dichloroaniline	NT	+++	+++	NT	+++	+++	T	+++	-
3,5-Dichloroaniline	NT	+++	+++	NT	+++	+++	T	+++	-
8 - quinolinol	NT	+++	++	NT	+++	++	T	+++	++
IPC	NT	+++	+++	NT	+++	+++	T	-	++
m-chloroaniline	NT	+++	+	T	+++	-	T	+++	-
N-Serve	NT	+++	+++	T	+++	-	T	+++	-
Potassium Azide	NT	+++	+++	T	+++	+++	T	+++	+++
Sevin	NT	+++	++	NT	+++	+	T	+++	+
Simazine	NT	-	-	NT	-	-	T	-	-
Sodium Azide	NT	+++	+++	NT	+++	+++	T	+++	+++

NT=Not Turbid, T=Turbid

-	90-100% of ammonium or nitrite was oxidized at the end of incubation
+	50- 90% of ammonium or nitrite was oxidized at the end of incubation
++	10- 50% of ammonium or nitrite was oxidized at the end of incubation
+++	0- 10% of ammonium or nitrite was oxidized at the end of incubation

limited above pH 9.0. At pH 9.0, 100% of the nitrogen initially present could be accounted for after 6 days. Recoveries at pH 10.0 and 11.0 were 87% and 71%, respectively. Thus volatilization of NH_3, rather than ammonium oxidation, was mainly responsible for decreasing ammonium concentrations in this very alkaline pH range.

The absence of ammonium oxidation in liquid cultures below pH 6.0 has been reported (12,20). In monoculture, pH optima for ammonium oxidation by Nitrosomonas (7) and for nitrite oxidation by Nitrobacter (1) are in the alkaline range and the activity of both organisms drops off sharply below pH 7.0 and above pH 9.0. The data for mixed cultures in Fig. 1 are consistent with these observations made in liquid culture by others. In soil systems, Morril and Dawson (16) observed that the most favorable pH for ammonium oxidation was above pH 7.6 and that for nitrite oxidation was in the range of 6.2 to 7.0.

Chemical instability of nitrite gives rise to gaseous losses of nitrogen from acid media. Gerretson and de Hoop (10) observed such losses below about pH 5.5 and maximum losses occurred at pH 3.0-3.5. In the present study, nitrite disappeared completely at pH 3.0 (Fig. 1). Only 45% of the added N was recovered after 6 days.

Effects of ammonium levels. In liquid mixed culture of Nitrosomonas and Nitrobacter, at pH 8.0-8.3, ammonium was oxidized with average rates of 27, 35, 41, 41 ppm NH_4-N per day for the 400, 500, 700 and 1,000 ppm levels, respectively (Fig. 2A). Within each level of ammonium, the oxidation rate remained approximately constant until depletion levels

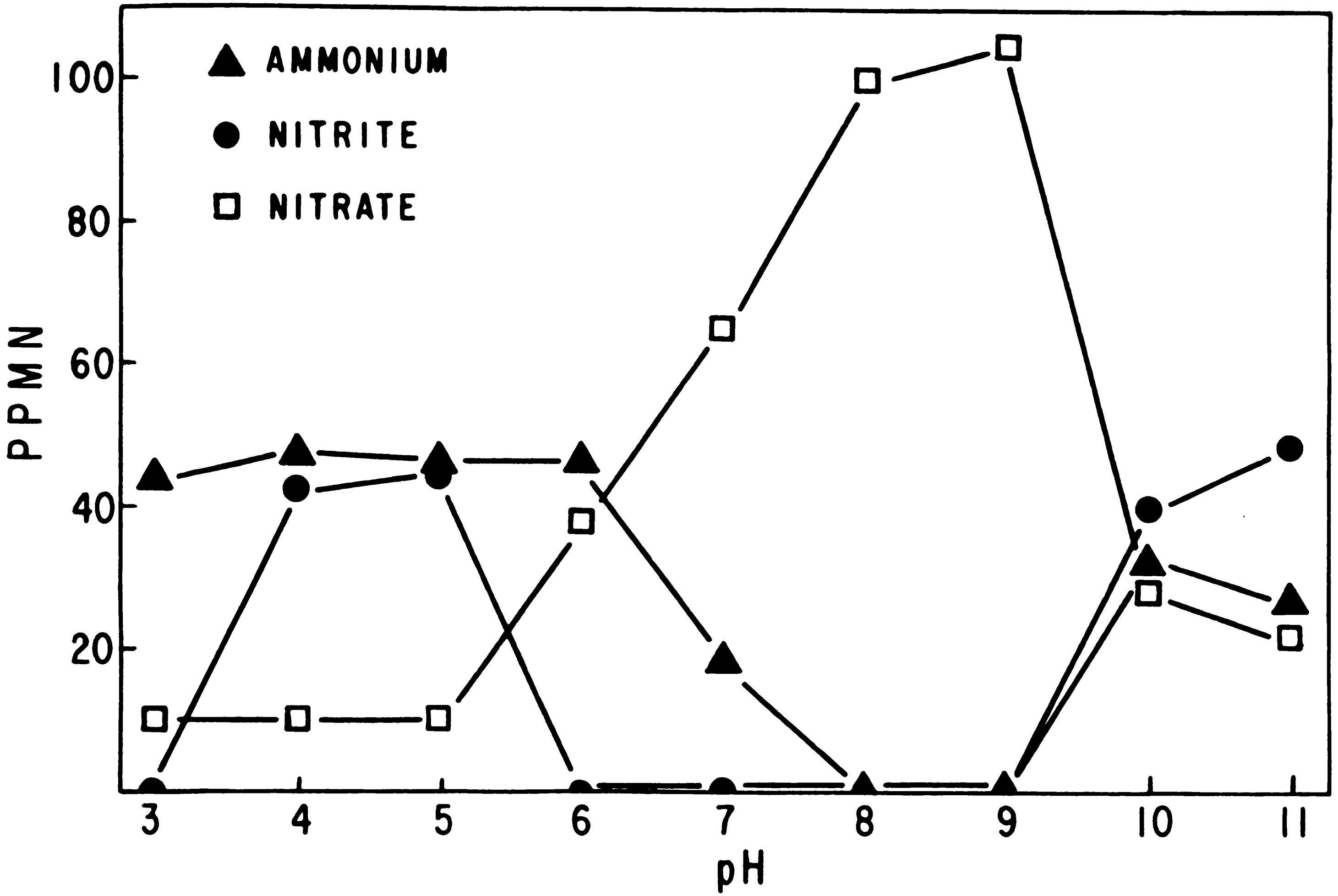

FIG. 1. Effect of pH on nitrification in liquid mixed culture of Nitrosomonas and Nitrobacter. (Incubation for 6 days. Initial nitrogen levels = NH_4-N, 50 ppm; NO_2-N, 50 ppm; NO_3-N, 10 ppm)

were approached (Fig. 2A). There was some evidence in the data for ammonium (Fig. 2A) and for nitrite (Fig. 2B) that ammonium oxidation may have been more rapid after about 18 days when the 1,000 ppm concentration had been reduced to 460 ppm NH_4-N.

Nitrite accumulated initially at all levels at an average rate of 23 ppm NO_2^--N per day. Nitrite continued to accumulate at each level until the ammonium concentration was reduced below about 100 ppm N. The periods of nitrite accumulation and the maximum nitrite concentrations attained (140, 240, 360 and 675 ppm NO_2^--N) were directly related to the initial ammonium concentration.

Nitrate was produced initially at all levels at an average rate of about 10 ppm NO_3^--N per day (Fig. 2C). This low initial rate of nitrate production was maintained at each level until ammonium concentration was reduced into the range of 100 to 200 ppm NH_4-N. At that point, nitrate production increased to 30, 34 and 65 ppm NO_3^--N per day for the first three ammonium levels, respectively. These rates were roughly proportional to the levels of nitrite which had accumulated by this time. These enhanced rates in the first two levels declined later as substrate was depleted.

No enhancement of nitrate production rate occurred at the highest ammonium level but could have been expected to appear if incubation had been continued beyond 23 days.

Levels of ammonium (Fig. 2A) higher than about 100 ppm NH_4-N had negligible effects on ammonium oxidation but did suppress the rate of nitrate production with a resultant accumulation of nitrite. Similar

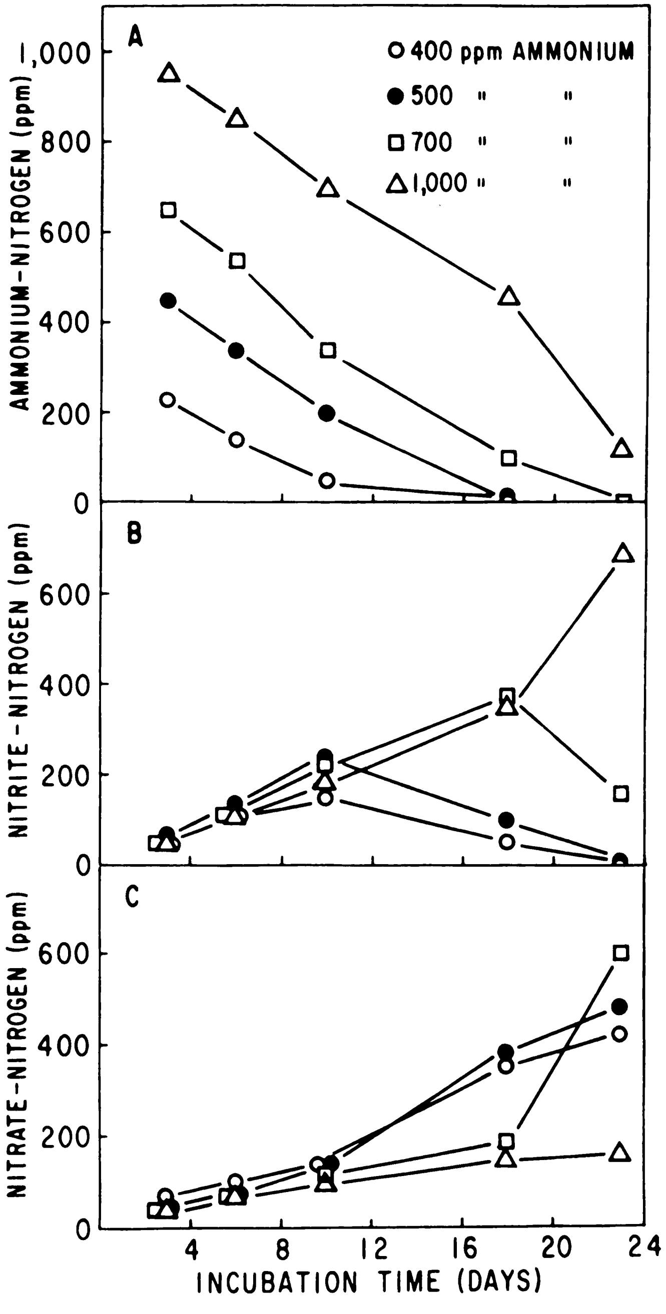

FIG. 2. Effects of ammonium levels on disappearance of ammonium and appearance of nitrite and nitrate in liquid mixed culture of *Nitrosomonas* and *Nitrobacter*. pH = 8.0 - 8.3

accumulations of nitrite have been reported frequently when anhydrous ammonia (8) or ammonium forming fertilizers (6) are introduced into neutral or alkaline soils at levels higher than 300 ppm. Ammonium concentration and pH are both involved. It appears that respiratory processes in Nitrobacter are interfered with by either ammonium or free NH_3 formed as the equilibrium $NH_4^+ \leftrightarrows NH_3 + H^+$ is shifted to the right at alkaline pH (1).

Ammonium or free NH_3 apparently do not specifically inhibit the nitrate oxidizing enzyme itself, since extracts of Nitrobacter oxidized nitrite in the presence of ammonium concentrations which drastically reduced oxygen uptake by whole cells (1). This is indicated also by the data in Fig. 2C which shows that nitrate was produced at a low but steady rate even in the presence of high concentrations of ammonium.

There was little evidence of lag when these high ammonium concentrations were removed, indicating a fully active Nitrobacter population (Fig. 2C). Thus, the effect of excess ammonium or free ammonia in these systems suppressed growth of Nitrobacter rather than nitrite oxidation with a resultant reduction in the observed rate of nitrate formation.

Under perfusion conditions, at pH 8.0-8.3, increasing levels of ammonium had no effect on rates of ammonium oxidation and nitrate production, and nitrite never accumulated during percolation (Table III).

Rates of ammonium oxidation and nitrate production were approximately equal and almost the same for the two perfusion systems. This indicates that the very great difference in cation exchange capacity between glass beads and the vermiculite-perlite system (Table I) had no effect on the oxidizing capacities of these two systems. It would appear that the level of activity in these two systems were determined by other factors involved in their maintenance. The most likely factors are the accessible or colonizable surface area of the column materials, rate of percolation and the maintenance levels of substrate (100 ppm NH_4-N plus 50 ppm NO_2^--N).

In perfusion studies, using soil, Stojanovic and Alexander (19) found that nitrite accumulated when ammonium concentrations in the perfusate exceeded 200 ppm. Their initial population was the indigenous soil inoculum that had survived air-drying. Graphs presented show a slow linear increase in nitrate over periods up to 20 days in the presence of high ammonium concentrations, similar to that observed in the liquid cultures used in this study (Fig. 2C). The linearity of nitrate accumulation indicates a non-proliferating population of Nitrobacter. This restricted Nitrobacter population was inadequate to cope with nitrite produced by the associated Nitrosomonas population which was not suppressed by high ammonium concentrations.

In the enriched perfusion systems used here (Table III) a large and constant Nitrobacter population was maintained by inclusion of 50 ppm NO_2^--N in the maintenance perfusate. Ammonium oxidation and nitrate production rates at all ammonium levels showed the linearity characteristic of a constant population. The nitrifying capacity of these systems was determined by the constant size of Nitrosomonas and Nitrobacter population and was unaffected by ammonium concentrations which could certainly have suppressed growth of *Nitrobacter*. These observations suggest a bacteriostatic effect of either ammonium or free ammonia on *Nitrobacter* similar to that observed with chlorate (14).

TABLE III

Effects of ammonium levels on the rates of ammonium oxidation and nitrate production under perfusion conditions

Column material	Ammonium levels (µg/ml)			
	100	250	420	750
	Ammonium oxidized			
	ppm NH_4-N per hour			
Glass beads	21	20	19	20
Vermiculite-Perlite	22	24	23	24
	Nitrate oxidized			
	ppm NO_3^--N per hour			
Glass beads	23	20	21	20
Vermiculite-Perlite	24	21	25	24

Summary

Trichloro-bis (4-chlorophenyl) ethane [DDT] , N,N-dimethyl-2, 2-diphenylacetamide [diphenamid] and 2-chloro-4, 6-bis (ethylamino)-S-triazine [simazine], prevented growth of heterotrophic microorganisms (contaminants) in culture with the nitrifying bacteria with no effect on ammonium or nitrite oxidation, thus providing a new approach for purifying cultures of <u>Nitrosomonas</u> and <u>Nitrobacter</u>.

(ii). Optimum hydrogen ion concentration for nitrification in liquid culture was found to be at pH about 8.0 to 9.0 and 6.0 to 9.0 for ammonium and nitrite oxidation, respectively. No nitrification was observed at pH below about 6.0 nor above about 9.0. Disappearance of nitrite at pH below 3.0 was observed and was likely due to chemical reactions of nitrite.

(iii). In liquid culture, ammonium levels above about 100 ppm NH_4-N had little or no effect on the rate of ammonium oxidation. Nitrate production was restricted to a low, linear rate characteristic for a static or resting population, indicating that growth of <u>Nitrobacter</u> rather than nitrite oxidation was suppressed. When ammonium was reduced below about 100 ppm NH_4-N, there was an abrupt increase in rate of nitrate production consistent with resumption of growth. Nitrite accumulated intermediately in amounts and over periods of time which were proportional to the initial concentrations of ammonium. In enriched perfusion systems, no intermediate accumulations of nitrite occurred with concentrations as high as 750 ppm NH_4-N.

These observations suggest a bacteriostatic effect of either ammonium or free ammonia on <u>Nitrobacter</u>.

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PART II: EFFECTS OF AGRICULTURAL CHEMICALS ON NITRIFICATION IN LIQUID CULTURES CONTAINING SOIL OR SOIL EXTRACT

Introduction

Chemicals used for pest control in agricultural practice are incorporated into the soil directly or indirectly. Knowledge of their effects on biological processes in soil may help in their proper usage in the future.

Nitrification in soil is affected in varying degrees by different insecticides (3,6), fungicides (12,18) and herbicides (14).

Present evidence indicates that the effect of a pesticide on nitrification and the magnitude of that effect depend upon its chemical properties and the physico-chemical and biological characteristics of the environment. For example, in different studies, chlordane was found to decrease nitrification in soil at 50 ppm (4), to have no effect up to 200 ppm (10,11,16), and to increase nitrification when applied at 10-100 ppm (7). DDD prevented nitrification in liquid culture at 10 ppm (8) but 50 to 100 ppm (4,7) were required under field conditions and it had no effect when used at normal field rates (11). Parathion inhibited nitrate production by Nitrobacter at 10 ppm in liquid culture (8) but high field rates increased numbers of nitrifying bacteria (13). Sevin retarded nitrate formation in soil at about 150 ppm (1) and potassium and sodium azide strongly inhibited nitrification at very low concentrations in soil (15). CIPC at normal field rates had little effect on nitrification (17) but inhibited nitrate production at concentrations of 150 ppm (1,17). N-Serve inhibited ammonium oxidation in the range of 0.05 to 20 ppm (9) depending on the nature of the soil.

Most of the above and other chemicals have been found by K. Sommer and A.R. Wolcott (unpublished data) to prevent ammonium and/or nitrite oxidation in liquid culture of Nitrosomonas and Nitrobacter at concentrations ranging from about 2.5 to 50 ppm. This study examines the relative importance of soluble vs. insoluble soil materials in modifying the influence of these chemicals on nitrification.

Materials and Methods

Biological material. The mixed stock culture of Nitrosomonas europaea and Nitrobacter agilis and the maintenance medium used in these studies have been described.[1]

Pesticides. Twenty-seven chemicals were selected which earlier studies had shown to inhibit Nitrosomonas and/or Nitrobacter in liquid culture at concentrations of 50 ppm or less (K. Sommer and A.R. Wolcott, unpublished data). The chemicals were dissolved in acetone. Appropriate concentrations of each chemical were transferred into 250 ml Erlenmeyer flasks and the solvent was evaporated to dryness, at 45-50C under vacuum in a water bath. Duplicate flasks of each concentration were used to prepare test cultures containing either soil extract or soil as described below.

Soil extract. Air-dry Sims clay loam was used for soil extract preparations (1,000 g of soil plus one liter of tap water, with autoclaving for half an hour). The 50 ml incubation mixture in the 250 ml flask contained (i) the chemical to be tested, (ii) 25 ml of

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[1] Part I, p. 3.

soil extract containing ammonium and nitrite to give a final concentration of 50 ppm in ammonium-nitrogen and 50 ppm in nitrite-nitrogen, and (iii) 25 ml of mixed stock culture which had recently completed the oxidation of all nitrogen contained in the maintenance culture medium.

After 6 days' incubation at 20-25C in the dark without shaking, the material in all flasks was analyzed for ammonia, nitrite and nitrate by methods cited in Part I, p. 7 A 6-day incubation was used since this was the time required for complete oxidation to nitrate in control flasks to which no test chemical was added.

Soil. The soil (Sims clay loam) was incubated before use for 10 days at 20-25C at 75 percent of its water-holding capacity. The incubation mixture in a 250 ml flask contained (i) the chemical to be tested, (ii) one gram of moist soil, (iii) 25 ml nutrient solution with ammonium and nitrite to give a final concentration of 50 ppm ammonium-nitrogen and 50 ppm nitrite-nitrogen and (iv) 25 ml of mixed stock culture which had recently completed the oxidation of the nitrogen contained in the maintenance medium.

All flasks were incubated at 20-25C in the dark, without shaking. Nitrification was slower in the presence of soil than when only soil extract was added, and 10 days was required for complete conversion in control flasks. Ammonia , nitrite and nitrate were determined as before at this time.

Interpretation of data. The use of a mixed culture made it possible to compare inhibitory effects on *Nitrosomonas* and *Nitrobacter* directly in the same environment at each concentration of test chemical. The degree of depression of *Nitrosomonas* activity was estimated from the percentage disappearance of added ammonium-nitrogen (disappearance assumed due to oxidation). Depression of Nitrobacter activity was estimated from percentage conversion of nitrite calculated as follows:

$$\% \text{ nitrite oxidation} = \frac{(\text{Final } NO_3^-\text{-N}) - (\text{Initial } NO_3^-\text{-N}) \times 100}{(\text{Added } NO_2^-\text{-N}) + (NH_4\text{-N oxidized})}$$

The notation in Tables I-V identifies arbitrary ranges of depression based upon the extent to which available substrate was oxidized: Toxic, 0-10%; strongly depressive, 10-50%; depressive, 50-90%; no effect, 90-100%.

Results and Discussion

In Tables I-V, the degree of inhibition shown for cultures to which soil extract was added is the same as that observed by Sommer and Wolcott in the absence of soil extract in the same medium, using inocula from the same stock source (unpublished data). Thus for these chemicals, soluble organic and inorganic compounds in soil extract did not change the concentration needed to depress or prevent ammonium or nitrite oxidation by Nitrosomonas or Nitrobacter.

The addition of 1 gram of pre-incubated soil to the liquid medium, however, reduced the activity of a number of the chemicals, thereby increasing the concentration required for a given degree of inhibition.

Fungicides. From the chemicals in Table I, 8-quinolinol and terrazole were highly specific in their action against Nitrosomonas. Higher concentrations of both were required in the presence of soil to depress ammonium oxidation to the same degree as in the presence of soil extract. Thiram was also more active against Nitrosomonas than

Nitrobacter. The addition of soil materials had no effect either on the degree of specificity or the concentration required for a given degree of inhibition of either organism.

Over this range of concentrations, Dowicide-7 and sodium azide inhibited both organisms about equally in liquid culture. The addition of soil materials had no effect on the activity of sodium azide but the activity of Dowicide-7 was markedly decreased.

TABLE I

Effects of various fungicides on a mixed culture of *Nitrosomonas* (Nm) and *Nitrobacter* (Nb) in a liquid medium with added soil extract or soil

Common or Trade Name	Fungicide concentration (ppm)							
	2.5		10.0		20.0		50.0	
	Nm	Nb	Nm	Nb	Nm	Nb	Nm	Nb
	Soil extract							
Dowicide-7	+++	++	+++	+++	+++	+++	+++	+++
8-quinolinol	++	-	+++	-	+++	-	+++	-
Sodium azide	++	+++	+++	+++	+++	+++	+++	+++
Terrazole	+++	-	+++	-	+++	-	+++	-
Thiram	+++	-	+++	+	+++	++	+++	++
	Soil							
Dowicide-7	-	-	-	+++	-	+++	+++	+++
8-quinolinol	-	-	+	-	++	-	+++	-
Sodium azide	++	+++	+++	+++	+++	+++	+++	+++
Terrazole	-	-	+++	-	+++	-	+++	-
Thiram	+++	-	+++	+	+++	++	+++	++

Toxic, +++; strong depression, ++; depression, +; no effect, -

Insecticides. Among the insecticides in Table II, captan and sevin were highly specific for Nitrosomonas. Nitrite oxidation was unaffected even at 50 ppm. At the lowest concentration, the degree of interference with ammonium oxidation was reduced in the presence of soil materials.

All of the other insecticides in Table II were specifically depressive only on Nitrobacter. Higher concentrations were required for a given level of depression in the presence of soil. The strong depression by Lindane at 20 and 50 ppm was completely eliminated when soil materials were added.

Herbicides. N-Serve, a chemical which is being developed commercially as a nitrification inhibitor, is included with herbicides in Table III. At concentrations up to 50 ppm, its action against Nitrosomonas was highly specific. The same was true for amitrole. The addition of soil did not interfere with the action of either chemical even at the lowest concentration.

The other herbicides were more inhibitory to Nitrobacter than Nitrosomonas. In the case of CIPC, this specificity at 50 ppm was enhanced by the addition of soil. Soil materials had no effect on the activity of IPC but they completely eliminated the toxicity of zytron to Nitrobacter at 10 ppm. Higher concentrations of zytron (20 and 50 ppm) were still toxic after addition of soil.

TABLE II

Effects of various insecticides on a mixed culture of *Nitrosomonas* (Nm) and *Nitrobacter* (Nb) in liquid medium with added soil extract or soil

Common or Trade Name	Insecticide concentration (ppm)							
	2.5		10.0		20.0		50.0	
	Nm	Nb	Nm	Nb	Nm	Nb	Nm	Nb
Soil extract								
Captan	+++	-	+++	-	+++	-	+++	-
Chlordane	-	-	-	++	-	++	-	+++
DDD	-	++	-	+++	-	+++	-	+++
Kelthane	-	++	-	+++	-	+++	-	+++
Lindane	-	-	-	+	-	++	-	++
Parathion	-	-	-	++	-	+++	-	+++
Sevin	++	-	+++	-	+++	-	+++	-
Soil								
Captan	++	-	+++	-	+++	-	+++	-
Chlordane	-	-	-	+	-	++	-	+++
DDD	-	-	-	+++	-	+++	-	+++
Kelthane	-	-	-	+++	-	+++	-	+++
Lindane	-	-	-	-	-	-	-	-
Parathion	-	-	-	-	-	++	-	++
Sevin	-	-	+++	-	+++	-	+++	-

Toxic, +++; strong depression, ++; depression, +; no effect, -

TABLE III

Effects of various herbicides on a mixed culture of *Nitrosomonas* (Nm) and *Nitrobacter* (Nb) in a liquid medium with added soil extract or soil

Common or Trade Name	Herbicide concentration (ppm)							
	2.5		10.0		20.0		50.0	
	Nm	Nb	Nm	Nb	Nm	Nb	Nm	Nb
Soil extract								
Amitrole	+++	-	+++	-	+++	-	+++	-
CIPC	-	-	-	++	-	++	++	+++
IPC	-	-	-	-	-	++	+	+++
Zytron	-	-	-	+++	-	+++	-	+++
N-Serve*	+++	-	+++	-	+++	-	+++	-
Soil								
Amitrole	+++	-	+++	-	+++	-	+++	-
CIPC	-	-	-	++	-	++	-	+++
IPC	-	-	-	-	-	++	+	+++
Zytron	-	-	-	-	-	+++	-	+++
N-Serve *	+++	-	+++	-	+++	-	+++	-

Toxic, +++; strong depression, ++; depression, +; no effect, -

*Not a herbicide

Aniline derivatives. Aniline compounds have been isolated as breakdown products of diuron (5) and other pesticides used in agriculture (2). Such breakdown products may be inhibitory to soil nitrification even though the initial compounds have no such effect.

Most aniline derivatives were toxic to Nitrosomonas in the range from 2.5 to 20 ppm; 2,3,5,6-tetrachloroaniline strongly inhibited Nitrobacter at 10 ppm and only 2,4-dichloroaniline, 2,3,4-, and 2,4,5-trichloroaniline affected both organisms (Table IV).

In the presence of soil, 10 ppm or more were required for the same inhibitory effect on Nitrosomonas with practically no change in the amounts needed for Nitrobacter (Table V).

Significance. Nine of the chemicals in this experiment were highly specific in their inhibitory effect on Nitrosomonas. They interfered with ammonium oxidation, with no effects on Nitrobacter in the same liquid culture, over the entire range of concentrations employed (2.5 to 50 ppm).

Seven chemicals were equally specific in their action against Nitrobacter at concentrations above 10 ppm. Only two of these (DDD and Kelthane) interfered with nitrite oxidation at 2.5 ppm, but none had any effect on ammonium oxidation at concentrations up to 50 ppm.

Other chemicals such as thiram, CIPC, 2,4,6-trichloroaniline, which inhibited both organisms at the higher concentrations, showed marked specificity at lower concentrations. Sodium azide was toxic to both organisms at concentrations of 10 ppm or more, but there was evidence that its specificity against Nitrobacter might be expected to increase at lower concentrations.

TABLE IV

Effects of aniline derivatives on a mixed culture of *Nitrosomonas* (Nm) and *Nitrobacter* (Nb) in a liquid medium with added soil extract

Common or Trade Name	Aniline derivative concentration (ppm)							
	2.5		10.0		20.0		50.0	
	Nm	Nb	Nm	Nb	Nm	Nb	Nm	Nb
2,3-dichloroaniline	+++	-	+++	-	+++	-	+++	-
2,4-dichloroaniline	-	-	+++	+	+++	+	+++	++
2,5-dichloroaniline	++	-	+++	-	+++	-	+++	-
3,5-dichloroaniline	-	-	+++	-	+++	-	+++	-
m-chloroaniline	+++	-	+++	-	+++	-	+++	-
p-chloroaniline	-	-	-	-	-	-	++	-
2,3,5,6-tetrachloroaniline	-	-	-	++	-	++	-	+++
2,3,4-trichloroaniline	-	-	+++	+	+++	+	+++	+++
2,4,5-trichloroaniline	-	-	++	-	+++	-	+++	++
2,4,6-trichloroaniline	-	-	+	-	+++	-	+++	-

Toxic, +++; strong depression, ++; depression, +; no effect, -

TABLE V

Effects of aniline derivatives on a mixed culture of Nitrosomonas (Nm) and Nitrobacter (Nb) in a liquid medium with added soil

Common or Trade Name	Aniline derivative concentration (ppm)							
	2.5		10.0		20.0		50.0	
	Nm	Nb	Nm	Nb	Nm	Nb	Nm	Nb
2,3-dichloroaniline	-	-	+++	-	+++	-	+++	-
2,4-dichloroaniline	-	-	+++	+	+++	+	+++	++
2,5-dichloroaniline	-	-	+++	-	+++	-	+++	-
3,5-dichloroaniline	-	-	+++	-	+++	-	+++	-
m-chloroaniline	-	-	+++	-	+++	-	+++	-
p-chloroaniline	-	-	-	-	-	-	++	-
2,3,5,6-tetrachloroaniline	-	-	-	++	-	++	-	+++
2,3,4-trichloroaniline	-	-	+++	+	+++	+	+++	+++
2,4,5-trichloroaniline	-	-	-	-	+++	-	+++	+++
2,4,6-trichloroaniline	-	-	+	-	+++	-	+++	-

Toxic, +++; strong depression, ++; depression, +; no effect, -

These specific interferences occurred at concentrations which may be expected in the soil solution following normal field applications. The addition of solid soil materials in these experiments increased the concentrations required for interference by a number of chemicals. Such protective action would be expected to increase with increasing soil-to-solution ratio. However, the toxicity to Nitrosomonas of N-Serve, amitrole, and thiram at 2.5 ppm was unaffected and captan at this concentration was still strongly depressive in the presence of soil.

In the field, effects of soil on these chemical interferences with nitrification will vary with the nature of the soil itself and its microbial population, with vegetation, management and climatic conditions. Very different effects of a chemical may actually be observed in different experimental situations.

Thus it has been observed (1,7,13) that nitrification was increased with high field rates of DDD and parathion. In the present study, Nitrobacter was inhibited by both chemicals, even in the presence of soil, after a 10-day incubation period. This result cannot be compared directly with the cited studies where a longer period of time intervened between treatment and observation.

Inhibition of nitrification has been reported when chlordane and lindane were applied at a rate of 50 ppm soil weight bases (4) and when CIPC was applied at a rate of 150 ppm (1,17). The importance of soil chemical and physical properties in modifying effects of exotic chemicals on nitrification can be inferred from the early work on N-Serve (9) where it was found that concentrations effective in reducing nitrification rates varied with soil type from .05 to 20 ppm, increasing generally with organic matter content.

In this study, soil reduced inhibitory effects of test chemicals on nitrification, whereas soil extract was without effect, which suggests that insoluble organic or inorganic soil materials were responsible for inactivation. Since pre-incubated soil was used, the possibility that activities or cell materials of the microflora may have influenced the result cannot be ignored.

From a practical standpoint, if a given pesticide inhibits *Nitrosomonas* specifically, it may exert a useful side-effect by minimizing losses of nitrate or nitrite through leaching or denitrification, thus conserving soil and fertilizer nitrogen. By contrast, if *Nitrobacter* is specifically inhibited, losses of nitrogen through leaching or chemical reactions of nitrite may be enhanced or toxic effects of nitrite on crops may result.

Summary

Twenty-seven chemicals were tested for inhibitory effects on Nitrosomonas and Nitrobacter in mixed liquid culture. Nine were highly specific in their action against Nitrosomonas over the entire range of test concentrations (2.5 to 50 ppm). Two were equally specific in their action against Nitrobacter at 2.5 ppm and five more over the range 10 to 50 ppm. The rest of the chemicals affected both organisms.

The concentrations required for complete inhibition of ammonium and/or nitrite oxidation in liquid culture were increased for ten of the chemicals when soil was added to the incubation mixture. Water soluble materials in extracts from the same soil were ineffective in reducing inhibitory effects. This suggests that insoluble organic or inorganic constituents were responsible for inactivation by soil.

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PART III: SYNERGISTIC AND ANTAGONISTIC INHIBITIONS OF NITRIFICATION BY HERBICIDES

Introduction

Ammonium is oxidized to nitrite mainly by Nitrosomonas and nitrite to nitrate by Nitrobacter. One or both steps may be specifically inhibited by various chemicals.

N-Serve at 10 ppm and amitrole at normal field rates inhibited soil nitrification for four to eight weeks, respectively (1,2). Potassium azide decreased soil nitrification at 50 ppm, and sodium azide is known to be a potent inhibitor of certain microbial oxidases (6). CIPC and IPC retarded nitrate production in soil (7).

The above chemicals have been tested alone for their effects on nitrification. Use of herbicides in various combinations for more efficient weed control is an increasingly important practice (4). Frequently, insecticides, fungicides and herbicides are applied in varying combinations on the same crop. We examine here the effects of combinations of several chemicals on ammonium and/or nitrite oxidation by Nitrosomonas and Nitrobacter in a liquid mixed culture.

Materials and Methods

Biological material. The mixed culture of Nitrosomonas europaea and Nitrobacter agilis and the maintenance medium used in these studies have been described.[1]

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[1] Part I, p.3.

Chemicals. 3-amino-1,2,4-triazole [amitrole], isopropyl-N-(3-chlorophenyl) carbamate [CIPC], isopropyl-N-phenyl carbamate [IPC], 2-chloro-6-(trichloromethyl) pyridine [N-Serve], potassium azide, and sodium azide were used. Amitrole, potassium azide and sodium azide were dissolved in sterile medium at 22C. CIPC, IPC, and N-Serve were dissolved in sterile medium at 40-50C in a water bath.

Assay. The 50 ml incubation mixture in a 250 ml flask contained: (i) 25 ml mineral medium with ammonium (100 ppm NH_4-N) and nitrite (100 ppm NO_2^--N) plus the appropriate concentrations of chemicals to be tested and (ii) 25 ml mixed stock culture which had recently completed the oxidation of nitrogen contained in the growth medium. Duplicate flasks were incubated for 4 days, at 20-25C, in the dark without shaking.

Ammonia was determined by Nesslerization, nitrite by the modified Griess-Ilosvay method, and nitrate by the phenol-disulfonic-acid method.[2] All colorimetric measurements were made in a Bausch and Lomb Spectronic 20.

Interpretation. According to Colby (3) and Gowing (4) the expected additive response to a given combination of two herbicides would be:

$$E = X + \frac{Y(100-X)}{100} \qquad [1]$$

where: E = expected response

X = the percent inhibition of ammonium or nitrite oxidation by herbicide A at m ppm.

Y = the percent inhibition of ammonium or nitrite oxidation by herbicide B at n ppm.

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[2] Part I, p. 7

Equation [1] simplifies to the form used by Limpel et al. (5):

$$E = X + Y - \frac{XY}{100} \qquad [2]$$

When the observed response is greater than expected, the response to the combined chemicals is synergistic; when less than expected, it is antagonistic. If the observed and expected responses are equal, the response to the combinations is additive (3).

Results and Discussion

Effects of amitrole and N-Serve, separately and in combination, on ammonium oxidation by Nitrosomonas are presented in Table I. Amitrole by itself produced inhibitions of 32 and 60 percent at concentrations of 0.5 and 1 ppm. Concentrations of 0.2 and 0.3 ppm N-Serve were less than the 0.4 ppm threshold above which depression of ammonium oxidation by this chemical was detected in this assay system.

By the adopted criteria [eq. 2], the response of Nitrosomonas to these two chemicals in combination would be interpreted as synergistic. Neither had any effect, separately or in combination, on Nitrobacter in the same culture.

In the case of the two azide salts (Table II), concentrations which resulted in less than complete inhibition of Nitrobacter had no effect on ammonium oxidation by Nitrosomonas, regardless of whether the salts were added singly or in combination. Sodium azide by itself was somewhat more inhibitory to Nitrobacter than the potassium salt at equivalent concentrations. The response to the two salts in combination would be interpreted as synergistic.

TABLE I

Combined effects of amitrole and N-Serve on ammonium oxidation by *Nitrosomonas*

Chemical	Dosage ppm	Inhibition (%)		
		Observed	Expected[a]	Difference[b]
Amitrole	0.5	32		
	1.0	60		
N-Serve	0.2	0		
	0.3	0		
N-Serve + Amitrole	0.2+0.5	54	32	+22
	0.2+1.0	72	60	+12
	0.3+0.5	54	32	+22
	0.3+1.0	72	60	+12

[a] Calculated according to equation [2]

[b] Positive difference denotes synergism

The synergisms expressed in Tables I and II have in common the feature that it appears to be the activity of the more potent inhibitor that is enhanced in the presence of the less active chemical. This may be inferred from the fact that, in Table I, the largest synergism occurred when N-Serve at either concentration was combined with the lower concentration of amitrole. In Table II, the largest synergism occurred when potassium azide at either concentration was combined

TABLE II

Combined effect of potassium azide and sodium azide on nitrite oxidation by *Nitrobacter*

Chemical	Dosage ppm	Inhibition (%)		
		Observed	Expected[a]	Difference[b]
Potassium azide	0.2	31		
	0.3	42		
Sodium azide	0.2	34		
	0.3	56		
Potassium azide + Sodium azide	0.2+0.2	74	54	+20
	0.2+0.3	75	70	+ 5
	0.3+0.2	81	62	+19
	0.3+0.3	78	74	+ 4

[a] Calculated according to equation [2]

[b] Positive difference denotes synergism

with the lower concentration of sodium azide. This behavior is analogous to that observed by Limpel et al. (5). They found that the herbicidal effectiveness of dimethyl-tetrachloroterephthalate was enhanced to a greater degree by a number of chemicals when these were combined with the lower dosages of the phthalate.

Data for IPC and CIPC are presented in Table III. At the concentration employed, both chemicals depressed the activity of both *Nitrosomonas* and *Nitrobacter*. Both chemicals were more inhibitory to *Nitrobacter* than to *Nitrosomonas*. At equivalent concentrations, IPC was more inhibitory to *Nitrosomonas* than was CIPC.

At low concentrations, the response to these chemicals in combination was antagonistic. In other words, the observed inhibition was less than would have been expected if they had acted independently of each other to produce an additive response. The antagonism decreased as the concentration of either chemical was increased.

Synergistic, antagonistic or additive responses can be expected when chemicals are applied in combination (3,4,5). However, it is apparent that the extent to which such interactions are expressed is dependent upon concentration and specific activity of each chemical.

TABLE III

Combined effects of CIPC and IPC on ammonium and nitrite oxidation by Nitrosomonas and Nitrobacter

Chemical	Dosage ppm	Inhibition (%)					
		Nitrosomonas			Nitrobacter		
		Observed	Expected[a]	Difference[b]	Observed	Expected	Difference
CIPC	10	4			76		
	20	6			96		
	40	32			100		
IPC	10	26			61		
	20	32			80		
CIPC + IPC	10 + 10	8	30	-22	74	91	-15
	20 + 20	26	36	-10	100	100	0
	40 + 20	50	54	- 4	100	100	0

[a] Calculated according to equation [2]

[b] Negative difference denotes antagonism; zero difference denotes additivity

Summary

At low but biologically active concentrations in liquid culture, 3-amino-1,2,4-triazole [amitrole] combined with 2-chloro-6-(trichloromethyl) pyridine [N-Serve] synergistically inhibited the oxidation of ammonium by *Nitrosomonas*. The inhibition of *Nitrobacter* by potassium azide and sodium azide in combination was also synergistic.

Isopropyl-N-phenyl carbamate [IPC] and isopropyl-N-(trichlorophenyl) carbamate [CIPC] at low concentrations were antagonistic in their inhibition of both *Nitrosomonas* and *Nitrobacter*.

Both synergistic and antagonistic responses approached additivity as the concentration of one or both chemicals in a combination increased.

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PART IV: EFFECTS OF PESTICIDES ON NITRIFICATION IN PERFUSION SYSTEMS

Introduction

Nitrification refers traditionally to the biological oxidation of ammonium to nitrate. In soil this conversion is effected mainly by the aerobic chemolithotrophic bacteria *Nitrosomonas* and *Nitrobacter*, in two steps. In the first step, ammonium is oxidized to nitrite by the *Nitrosomonas* group; in the second nitrite is oxidized to nitrate by *Nitrobacter*.

Pesticides used in agriculture have been reported to inhibit one or both steps in nitrification. Amitrole at 4 ppm inhibited soil nitrification for eight weeks and residual depressive effects were apparent for an additional eight weeks (2). Potassium azide at about 30 ppm inhibited soil nitrification for four weeks (5). In perfusion studies CIPC at 160 ppm almost completely inhibited nitrification (4).

K. Sommer and A.R. Wolcott (unpublished data), using a liquid mixed culture of *Nitrosomonas* and *Nitrobacter*, found that amitrole completely inhibited ammonium oxidation at 5 ppm with no effect on nitrite oxidation up to 250 ppm. CIPC inhibited ammonium oxidation at 150 ppm or less and nitrite oxidation at 23 ppm. IPC completely inhibited ammonium oxidation at 23 ppm and slightly depressed nitrite oxidation at 25 ppm. Potassium azide and sodium azide completely inhibited ammonium oxidation at 23 and 5 ppm and nitrite oxidation at 5 and 1 ppm, respectively. This paper investigates the influence of perfusate volumes and solid matrix materials on the inhibitory effects of these chemicals on a mixed culture of *Nitrosomonas* and *Nitrobacter* in perfusion systems.

Materials and Methods

Biological material. The mixed culture of Nitrosomonas europaea and Nitrobacter agilis and the maintenance medium have been described.[1]

Chemicals. Some pertinent properties of the chemicals used are given in Table I. Amitrole, potassium azide and sodium azide were dissolved in nutrient solution at 25C. CIPC and IPC were dissolved in nutrient solution at 40-45C in a water bath.

Perfusion studies. The perfusion apparatus and the conditions of growth of Nitrosomonas and Nitrobacter on the columns have been described in Part I, p.2. The column materials and their total cation exchange and water-holding capacities are given in Table II.

The experimental medium was the same as the equilibration medium, except for the addition of the desired concentration of test chemical. In a given experimental run, duplicate columns of each matrix material were perfused with 50, 100, 150 or 200 ml of medium containing a given concentration of a test chemical. Fifty and 100 ml were tested in one run followed by 200 and 150 ml in the next, so that after a 2-run cycle each unit had been perfused with a total of 250 ml. A 3 or 4-day equilibration period then followed before the next concentration was tested.

Five-ml aliquots were withdrawn aseptically after 0, 3 and 5 hours in the 50 and 100 ml runs and after 0, 5 and 9 hours in the 200 and 150 ml runs. Ammonia, nitrite and nitrate were determined by methods cited in Part I, p. 3.

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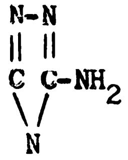
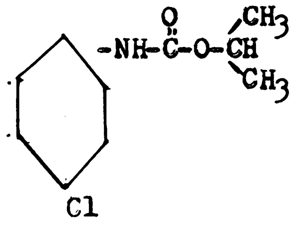
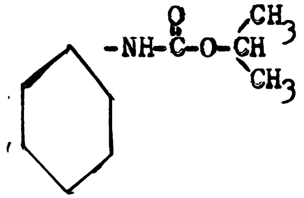
[1] Part I, p. 7.

TABLE I

Structural formulae and properties of chemicals used in the perfusion studies

Compound	Structural formulae	Molecular weight	Melting or boiling point	Water solubility
3-amino-1,2,4-triazole (Amitrole)		84.1	150.0	280,000 ppm (23C)
Isopropyl-N(3-chlorophenyl) carbamate (CIPC)		213.7	43.0	102.3 ppm (25C)
Isopropyl-N-phenyl carbamate (IPC)		179.2	87.0	254.0 ppm (25C)
Potassium Azide	$N{=}N{=}N^{-}K^{+}$	81.0	300.0	Very water soluble
Sodium Azide	$N{=}N{=}N^{-}Na^{+}$	65.0	300.0	Very water soluble

Sorption studies. In several experiments, sorption and desorption of chemicals by the solid matrix was followed by observing changes in concentration or activity of the chemical in the perfusate.

Amitrole in the perfusate was determined according to Zweig (10). An aliquot was treated with nitrous acid coupled with N-(t-naphthyl) ethylenediamine, and the resultant color intensity was measured at 455 mμ.

CIPC, IPC and KN_3 in the perfusate were estimated by the Nitrosomonas and Nitrobacter bioassay described below.

Bioassay. Bioassays have been proposed as useful tools in residue studies with a number of pesticides (1,3).

In preliminary studies CIPC and IPC at 10-20 ppm and potassium azide at 0.3 to 0.5 ppm depressed nitrite oxidation by Nitrobacter in liquid culture. At higher concentrations they were also inhibitory to Nitrosomonas. By utilizing responses of these organisms in mixed culture, it was possible to develop a standardized bioassay for each of these chemicals. Reproducible standard curves were obtained covering the following ranges of concentration: CIPC, 10 to 70 ppm; IPC, 20 to 60 ppm; and KN_3, 0.2 to 0.5 ppm.

A 50 percent inoculum was used from a mixed stock culture which had recently completed the oxidation of all the nitrogen in the maintenance medium. The assay mixture in a 250 ml Erlenmeyer flask comprised (i) 25 ml inoculum, (ii) an aliquot of the perfusate to be tested, (iii) $(NH_4)_2SO_4$ and KNO_2 to give final concentrations of 50 ppm NH_4-N and 50 ppm NO_2^--N and (iv) nutrient solution as needed to give a final volume of 50 ml. The resulting mixtures were incubated, without shaking, for 6 days in the dark at 20-25C.

TABLE II

Cation exchange and water holding capacities of perfusion units

Kind and amount of materials	Cation-Exchange Capacity (me/unit)	Water-holding capacity (g/unit)
Glass beads, 150 g	0.60	30
Vermiculite, 3 g; Perlite, 10 g	4.15	42
Soil, 50 g; Perlite, 4.5 g	15.00	40

Concentration of the tested chemical was estimated graphically, using standard curves with concentration plotted against the ordinate and percent inhibition of nitrite oxidation against the abscissa.

Results and Discussion

<u>Nitrification in control systems</u>. Standard nitrification patterns during control runs are shown in Fig. 1 for the 200 ml perfusate.

Some nitrate was always present at the beginning of percolation. Analyses at zero time for NH_4-N and NO_2^--N were usually quantitative for the concentration desired after dilution by perfusate solution carried over in the column from the previous percolation.

During the first 3 or 4 hours of percolation, ammonium oxidation and nitrate production were essentially linear and occurred at equal rates. The rate of ammonium oxidation declined after 4 hours, but nitrate was produced with little change in rate until both substrates were exhausted at 6 hours.

Nitrification rates during control runs ranged from 20 to 25 ppm N per hour. This is 20 to 25-fold greater than in the stationary flask cultures used in Parts I, II and III. There were no consistent differences in rate between column materials, except as structural deterioration resulted in a gradual decline in the activity of the soil-perlite systems to the point that they were discarded.

In Tables II-V and VII, VIII data taken after 5 hours are presented. Normally at this time, 80 to 90 percent of both substrates had been oxidized in control runs with 200 ml perfusate containing no chemical (Fig 1). In control runs with 150 ml of perfusate, the percentage

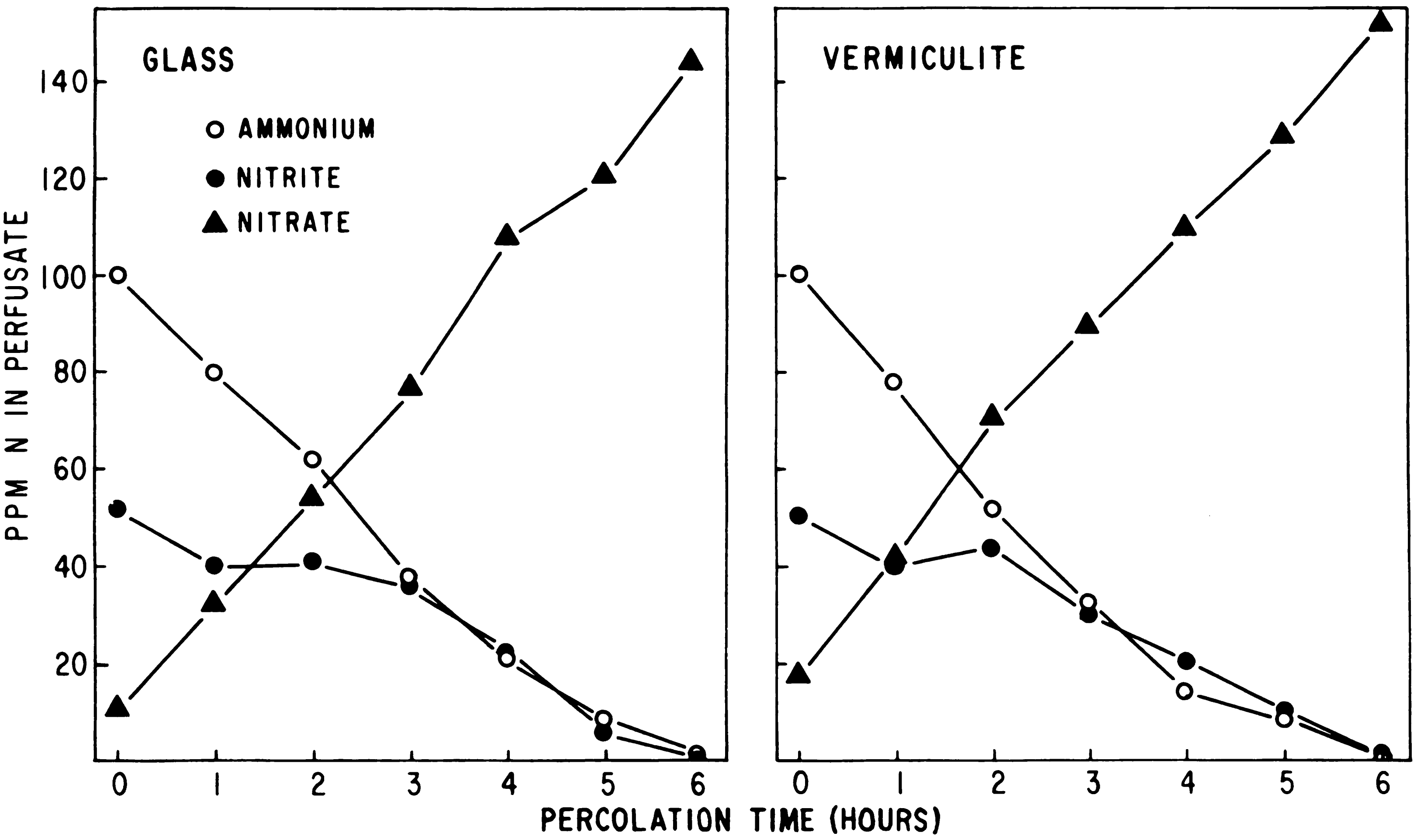


FIG. 1. Nitrification in glass bead columns (left) and vermiculite-perlite (right) inoculated with <u>Nitrosomonas</u> and <u>Nitrobacter</u>. Perfusate volume = 200 ml

conversion was usually closer to 90 than 80 percent. With 50 and 100-ml perfusate volumes, both substrates in control runs were normally oxidized completely prior to the fifth hour.

Control runs were made at each perfusate volume after equilibration preceding each change in concentration. Since there were no consistent differences for column materials, data for all control runs at a given volume were averaged over all perfusion units for the period during which a given chemical was under study. The average total conversion is given in column 2 of Tables III-V and VII, VIII. This value was taken as 100 percent in calculating conversions in the presence of test chemicals to "percent of the control".

Studies with amitrole. In Part II, amitrole had no effect on Nitrobacter at concentrations up to 50 ppm in liquid culture but it caused 90 to 100% inhibition of Nitrosomonas at 2.5 ppm.

A similarly toxic degree of inhibition occurred when glass beads were perfused with 150 or 200 ml of nutrient solution containing 1.7 ppm of amitrole (Table III). The inhibition was substantially reduced when smaller volumes of perfusate containing up to 12 ppm were used. The protection afforded by vermiculite was greater than by glass beads, and that afforded by soil at each concentration and volume was still greater.

Amitrole inhibited Nitrosomonas at extremely low concentrations but its activity was very greatly reduced in the presence of solid matrix materials. It is known that amitrole is strongly adsorbed by soil materials (2,8). Accordingly, an experiment was conducted in which perfusate concentrations of both ammonium and amitrole were followed through two successive percolations. The results are presented in Figs. 2 and 3.

TABLE III

Effects of amitrole on ammonium oxidation as influenced by concentration, perfusate volume and column material

Perfusate volume (ml)	NH_4-N oxidized in the control (µg)	Percent of the control[a]			
		Amitrole concentration (ppm)			
		1.7	4.0	6.0	12.0
		Glass beads			
50	5,500	83	77	58	36
100	10,300	81	51	33	28
150	13,000	2	5	5	ND
200	17,100	7	4	2	ND
		Vermiculite-Perlite			
50	5,500	92	84	80	64
100	10,300	75	59	50	43
150	13,000	25	22	11	ND
200	17,100	22	11	13	ND
		Soil-Perlite			
50	5,500	100	95	94	82
100	10,300	96	93	92	69
150	13,000	56	56	49	ND
200	17,100	51	42	42	ND

[a] Five-hour percolation; ND=not determined

During the first 2 hours of the first percolation, 75 percent of the added amitrole had disappeared from the perfusate in the soil-perlite systems and 85 to 90 percent from the perfusates in the other two systems (Fig. 2). All this sorbed amitrole was later released and recovered in the perfusate. In the glass bead systems, all of the added amitrole was accounted for in the perfusate by the fifth hour of the second percolation (Fig. 3). In the other two systems, complete release of sorbed amitrole was not detected until the last sampling at 18 hours in the second percolation.

Patterns of amitrole sorption and release were clearly related to Nitrosomonas activity. The rate of ammonium oxidation in treated systems decreased abruptly as release of sorbed amitrole was observed in the perfusate. The degree of inhibition was much less during the first 2 or 3 hours of percolation when amitrole was most extensively sorbed.

None of the 0.26 mg of amitrole retained on soil or vermiculite at the end of the first percolation had reappeared five hours later in the second perfusate. After 18 hours, however, 1.2 and 1.3 ppm were found for soil and vermiculite, respectively. The calculated concentration, assuming complete release of 0.26 mg, would have been 1.3 ppm. The calculated 0.25 ppm for glass beads was found in the second perfusate at 5 hours. The perfusate concentrations for soil and vermiculite after 18 hours were well within the inhibitory range indicated in Table III for a 200-ml percolation volume.

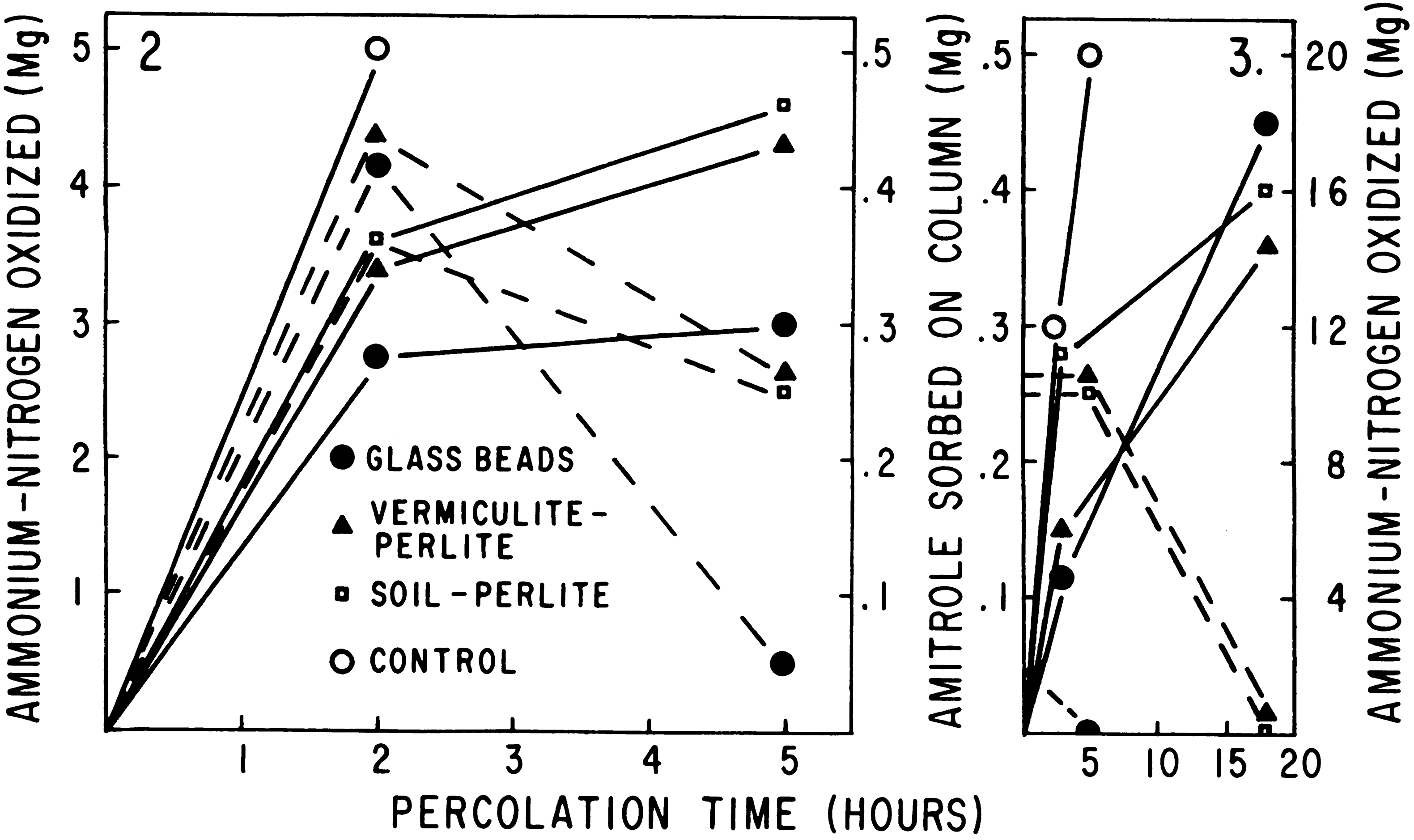


FIG. 2. Ammonium oxidized (————) and changes in sorbed amitrole (—— ——) during perfusion with 50 ml nutrient solution containing, initially, 10 ppm amitrole

FIG. 3. Ammonium oxidized and changes in sorbed amitrole after replacing the perfusate in Fig. 2 with 200 ml untreated nutrient solution

These amitrole concentrations in the second perfusate were related to the observed inhibition in ammonium oxidation after about 3 hours (Fig. 3). Thus, the glass bead system which had suffered the greatest inhibition during the first percolation oxidized more ammonium during the second percolation (even though less than the control) because negligible amounts of amitrole had been left on the columns (Fig. 2).

The data in Fig. 2 and 3 and in Table III show that amitrole inhibits _Nitrosomonas_ at extremely low solution concentrations, and that the protective effect of solid matrix materials involves sorption of amitrole from solution. In the case of the glass beads, the sorption must be due to living cells and cellular debris, since clean glass beads did not adsorb any amitrole. In the soil and vermiculite systems, mineral surfaces as well as dead and living organic materials must be involved.

Infra-red studies (8) have shown that sorption of amitrole by NH_4-montmorillonite involves the formation of the aminotriazolium cation by the following reaction:

$$R\text{-}NH_2 + NH_4^+\text{-clay} \longrightarrow R\text{-}NH_3^+\text{-clay} + NH_3 \qquad [1]$$

In the experiments of Fig. 2 and 3, this reaction at exchange sites on clay or organic materials would have been shifted to the right by the high NH_4-N concentration of the fresh nutrient solution. This would account for the extensive sorption of amitrole observed in the first hours of percolation (Fig. 2).

Subsequent desorption and increasing inhibition by amitrole in Figs. 2 and 3 appears to be related to activities of the nitrifiers themselves. The mechanisms for displacement of sorbed aminotriazolium are not clear.

The data suggests that interference with nitrite formation by Nitrosomonas may involve competition between the aminotriazolium cation and NH_4-N or NH_3 for uptake sites or for sites on the oxidizing enzyme.

The extensive sorption and later release in an active form which is suggested by these data would explain the residual inhibition observed by Chandra (2).

Studies with carbamates. Both CIPC and IPC reduced the amounts of nitrate formed in 5 hours (Tables IV, V). At 80 ppm, CIPC was somewhat more inhibitory than IPC. With both chemicals, inhibition increased with increasing concentration and perfusate volume. Nitrate production was somewhat retarded in 50 ml volumes at 2 hours but this was no longer apparent at 5 hours.

The inhibition increased with increasing concentration or volume to a greater extent with glass beads than with vermiculite-perlite. However, even at the highest concentration and volume, complete inhibition was never attained. This is in contrast with the complete inhibition of Nitrobacter observed in Part II of this thesis in stationary flask cultures with 50 ppm of CIPC and IPC, respectively. On the other hand Hale et al. (4) reported a 90 percent inhibition of nitrification in enriched soil perfusion systems with 160 ppm CIPC. This compares favorably with the results obtained here using the 200 ml perfusate volume. It is apparent that inhibitory concentrations will vary with size and activity of the population and the experimental conditions under which the chemicals are tested.

TABLE IV

Effects of CIPC on nitrate formation as influenced by concentration, perfusate volume and column material

Perfusate volume (ml)	NO_3^--N produced in the control (µg)	Percent of the control[a]		
		CIPC concentration (ppm)		
		40	80	140
		Glass beads		
50	6,180	100	100	100
100	12,350	84	82	65
150	18,500	93	29	31
200	23,800	85	20	22
		Vermiculite-Perlite		
50	6,180	100	100	100
100	12,350	82	97	80
150	18,500	100	51	38
200	23,800	97	52	29

[a] Five-hour percolation

At the highest concentration of CIPC used here (140 ppm), the rate of ammonium oxidation was somewhat reduced (Fig. 4). However, both chemicals were otherwise specific in their action against Nitrobacter, with the result that nitrite accumulated intermediately (Fig. 5).

TABLE V

Effects of IPC on nitrate formation as influenced by concentration, perfusate volume and column material

Perfusate volume (ml)	NO_3^--N produced in the control (μg)	Percent of the control[a]		
		IPC concentration (ppm)		
		40	60	80
		Glass beads		
50	9,770	100	100	100
100	16,410	100	100	92
150	20,250	93	70	62
200	23,800	78	61	62
		Vermiculite-Perlite		
50	9,770	100	100	100
100	16,410	100	100	100
150	20,250	92	89	80
200	23,800	96	85	78

[a] Five-hour percolation

High nitrite concentrations were maintained over a longer period of time with CIPC than with IPC because ammonium oxidation was retarded and continued over a longer period of time. The more rapid oxidation of ammonium in the vermiculite system than in the glass beads when perfused with CIPC (Fig. 4) served to maintain a higher level of nitrite in the CIPC-vermiculite system (Fig. 5).

After 9 hours, the initial perfusates in Fig. 5 were replaced with 200 ml fresh nutrient solution containing no test chemical. Some residual delay in nitrite oxidation was apparent, notably with CIPC and in the glass bead systems. However, the retardation occurred during the first 3 hours of percolation, after which nitrite disappeared as rapidly as in the controls.

The Nitrosomonas-Nitrobacter bioassay was used to estimate the extent to which the chemicals had been inactivated or removed from the initial perfusate at 9 hours. Approximately 30 percent (24 ppm) IPC (Table VI) and 50 percent (80 ppm) of CIPC (data not given) had disappeared according to this assay.

With both chemicals, the indicated removals were the same for glass bead columns as for vermiculite-perlite columns. Thus there was no evidence that the very great difference in cation exchange capacity of inorganic surfaces in the two matrix materials had influenced the activity of these chemicals. Neither was adsorbed by clean glass beads, so inactivation in the nitrifying glass bead systems must be ascribed to effects of living cells or cellular debris adhering to the surfaces of the beads.

The possibility that some degradation of the carbamates by heterotrophic contaminants might have occurred was not investigated. However, the accumulation of neutral pesticide molecules by sorption on living and dead microbial tissues in soil has been reported by Ko and Lockwood (6). Mortland et al. (7) found that a more stable complex was formed

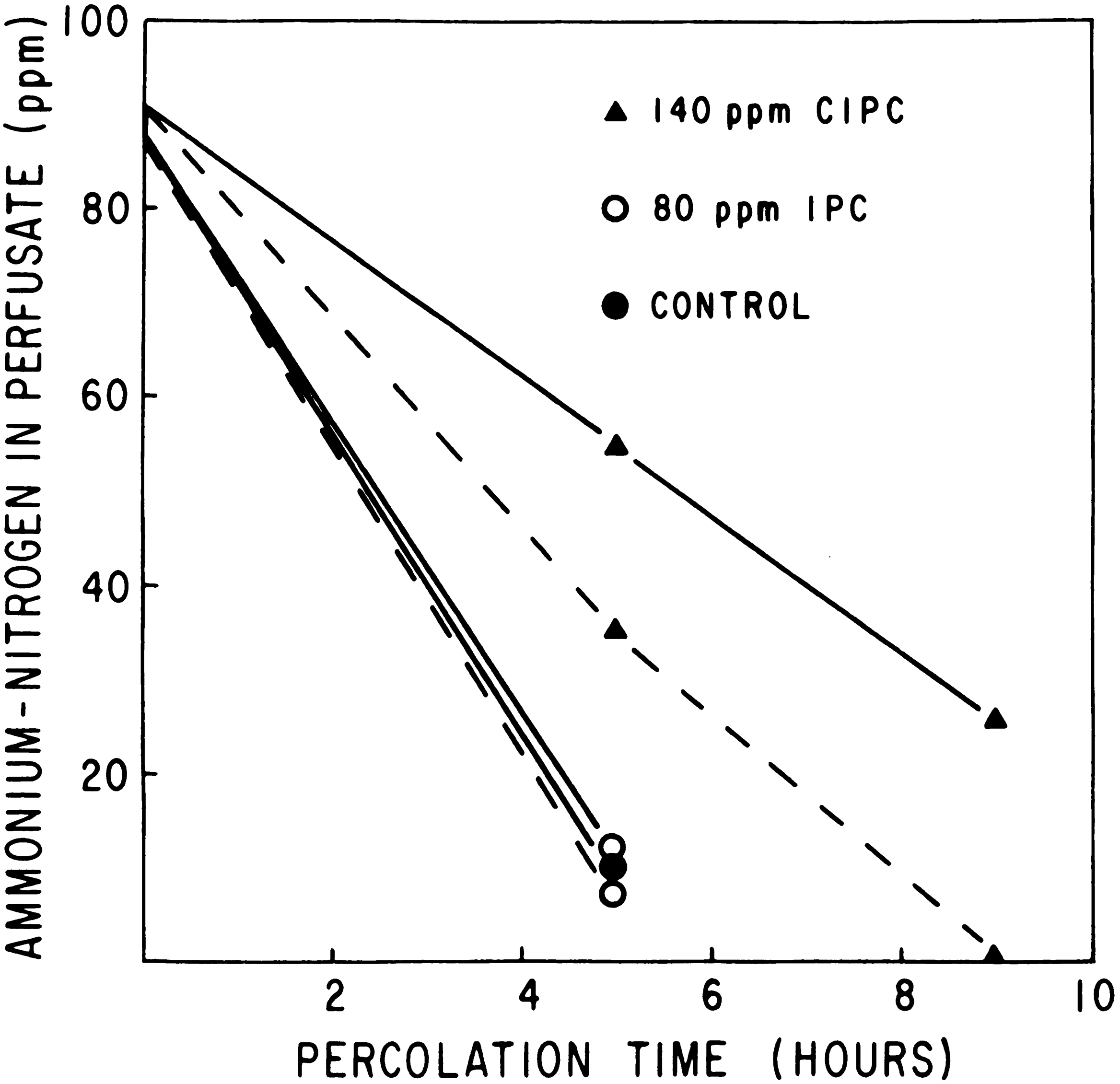


FIG. 4. Effects of CIPC and IPC on ammonium oxidation during perfusion through glass beads (————————) and vermiculite - perlite (— — —). (Perfusate volume = 200 ml)

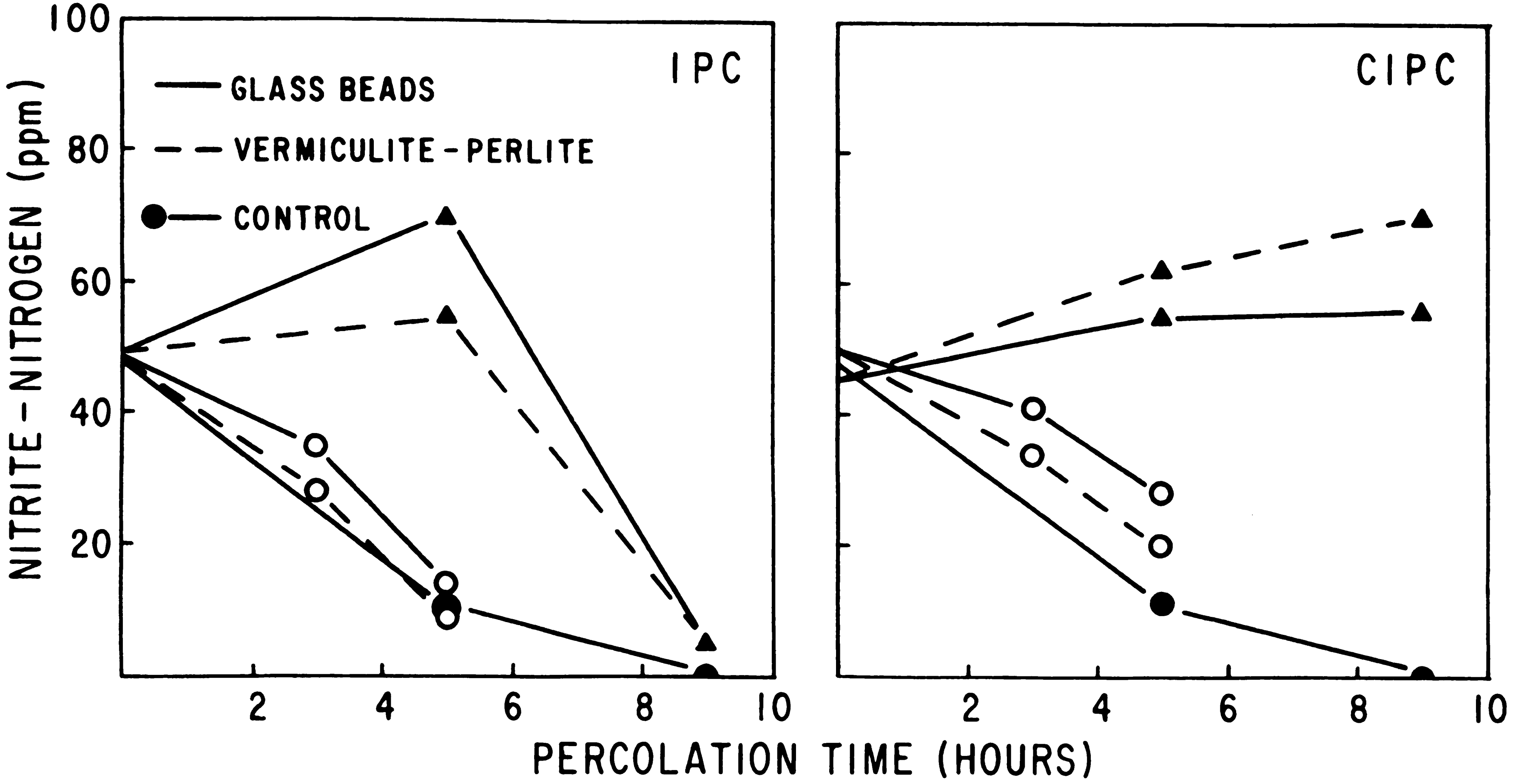


FIG. 5. Nitrite found during initial perfusion (▲) with 200 ml nutrient solution containing 80 ppm IPC or 140 ppm CIPC and during subsequent perfusion (○) with 200 ml untreated nutrient solution

between a carbamate and montmorillonite when the clay was first saturated with an organic cation (pyridinium) rather than with metal cations. Scott et al. (9) found that the phytotoxicity of CIPC was reduced by addition of organic soil to the growth medium but not by addition of Montmorillonite or Kaolinite clays.

These observations by others support the inference made here that disappearance of IPC and CIPC activity from the initial perfusate was due largely to sorption by living or dead organic matter. Both the glass beads and the vermiculite-perlite were devoid of organic matter prior to enrichment with the nitrifying cultures. A similar high rate of nitrifying activity had been maintained for months in both systems before these experiments were conducted.

Similar quantities of organic materials with apparently equal sorptive capacities would reasonably have accumulated. The reductions in activity observed by bioassay (Table VI) are consistent with the inference that sorption by organic materials was mainly responsible.

If all of the initially sorbed chemicals had been released again into the second perfusate (Fig. 5), the effective concentrations would have been about 70 ppm CIPC and 20 ppm IPC. According to Tables IV and V, 70 ppm CIPC in 200 ml perfusate would be inhibitory, whereas negligible inhibition would be expected with 20 ppm IPC. The residual retardations observed during the second percolation in Fig. 5 were about what was expected if the sorbed chemicals had equilibrated with the fresh nutrient solution. No assays were made at this time, but release of sorbed CIPC and IPC into the fresh solution was expected since water competes effectively with carbamates in the H-bonding interactions involved in their sorption by clays (7).

TABLE VI

Amounts of IPC removed from the initial 9-hour perfusate as estimated by the *Nitrosomonas-Nitrobacter* bioassay

Column materials	Perfusate volume (ml)	IPC concent. in perfusate (ppm) at 0 hours	IPC concent. in perfusate (ppm) after 9 hours	IPC removed (%)
Glass	150	67[a]	44	34
beads	200	70	48	31
Vermiculite-	150	63	44	30
Perlite	200	66	44	33

[a] Corrected for water-holding capacity of each column material

Although the bioassay detected no difference between glass beads and vermiculite-perlite, the residual protection afforded by the latter system was distinctly greater.

Studies with azides. Both the potassium and the sodium salts reduced nitrate production in the same manner as the carbamates with respect to effects of perfusate volume and column materials (Tables VII, VIII). Sodium azide appears to be more inhibitory to *Nitrobacter* than potassium azide.

At higher concentrations both salts also inhibit Nitrosomonas (Parts II and III of this thesis). However, at the concentrations used here, only KN_3 at 1.5 ppm interfered with ammonium oxidation (Fig. 6). Otherwise, both salts were highly specific in their action against Nitrobacter. As a result, nitrite accumulated intermediately (Fig. 7) as was the case with the carbamates.

The concentrations in both chemicals needed for complete inhibition are in the range of those found effective in liquid culture in Parts II and III of this thesis. At the lower concentrations and larger volumes in Tables VII and VIII inhibitory effects of both salts were less in the vermiculite-perlite systems than in the glass beads. At the highest concentrations, and volumes, however, the difference between column materials were negligible. This interaction with column materials was of lesser magnitude than in the case of amitrole or the carbamates.

The Nitrosomonas-Nitrobacter bioassay was used again to estimate the degree of removal or inactivation of KN_3 during the first 9-hour percolation in Fig. 7. The results of the bioassay in Table IX indicate that negligible sorption or inactivation occurred. There is a suggestion that the perfusate concentration in the 50 ml perfusate may have been increased by negative sorption of the anion. However, it must be recognized that the accuracy of the bioassay is qualitative rather than quantitative.

The bioassay was not performed on the 200 ml perfusates used in the experiments of Fig. 7. Nevertheless, the evidence in Table IX that azide was not sorbed by column materials to any extent is consistent

TABLE VII

Effects of potassium azide (KN_3) on nitrate formation as influenced by concentration, perfusate volume and column material

Perfusate volume (ml)	NO_3^--N produced in the control (µg)	Percent of the control[a]		
		KN_3 concentration (ppm)		
		0.5	1.5	2.5
		Glass beads		
50	7,830	100	100	100
100	15,120	68	45	40
150	20,250	42	19	16
200	23,800	27	10	9
		Vermiculite-Perlite		
50	7,830	100	100	100
100	15,120	80	69	64
150	20,250	70	36	17
200	23,800	52	24	10

[a] Five-hour percolation

with the immediate recovery in the rate of nitrite oxidation when the perfusates containing these salts were replaced with untreated nutrient solution (Fig. 7). Some interaction with solid matrix materials must

TABLE VIII

Effects of sodium azide (NaN_3) on nitrate formation as influenced by concentration, perfusate volume and column material

Perfusate volume (ml)	NO_3^--N produced in the control (µg)	Percent of the control[a]		
		NaN_3 concentration (ppm)		
		0.1	0.5	1.0
		Glass beads		
50	7,600	100	100	100
100	16,400	91	58	42
150	20,250	38	24	13
200	23,800	27	16	11
		Vermiculite-Perlite		
50	7,600	100	100	100
100	16,400	89	72	65
150	20,250	33	32	22
200	23,800	35	24	18

[a] Five-hour percolation

have occurred, however, to have given the larger intermediate accumulation of nitrite in the glass beads during the initial percolation. The nature of this interaction is not clear.

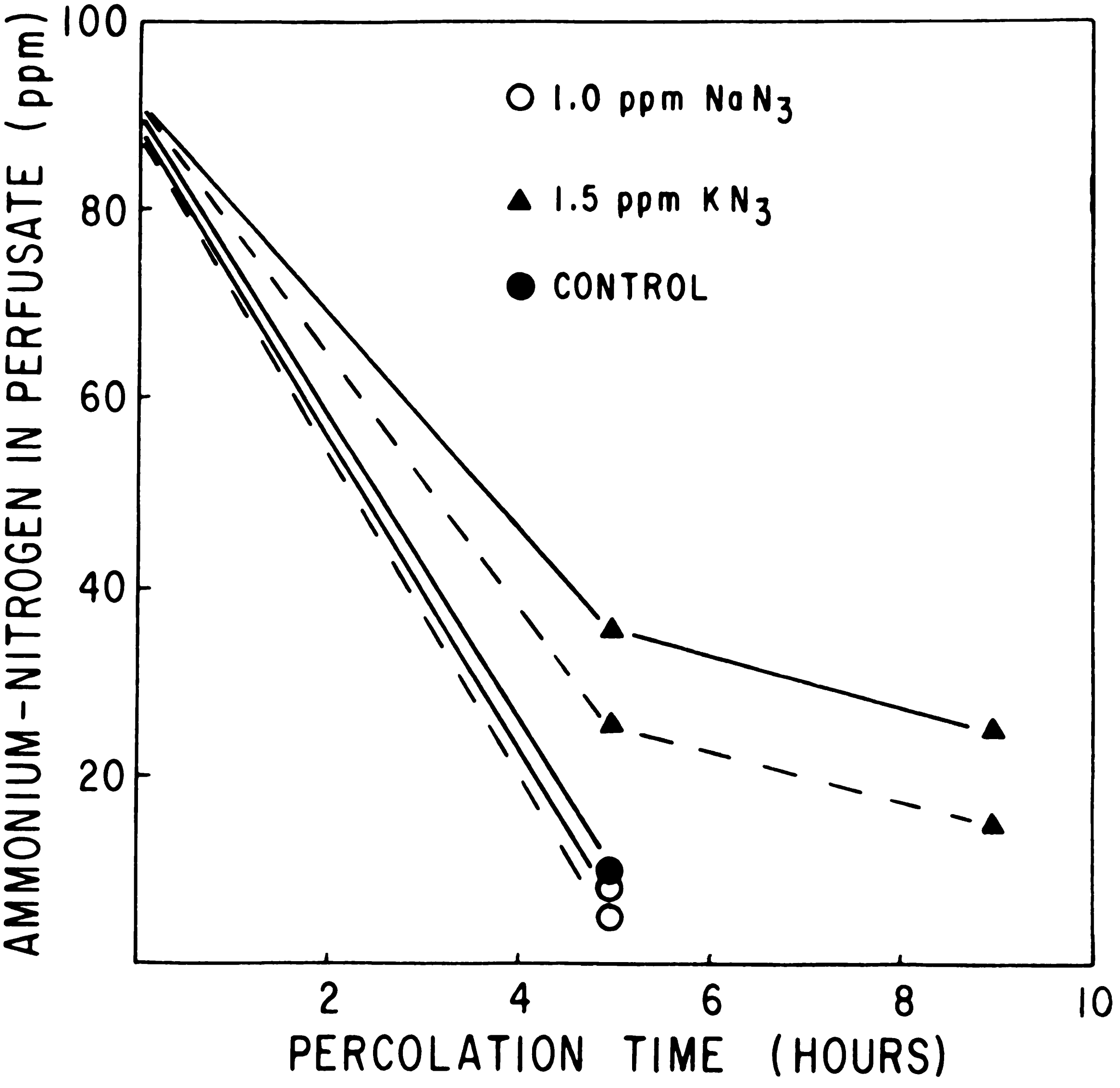


FIG. 6. Effects of sodium (NaN_3) and potassium (KN_3) azide on ammonium oxidation during perfusion through glass beads (————) and vermiculite-perlite (— - - —) (Perfusate volume = 200 ml)

TABLE IX

Amounts of potassium azide (KN_3) removed from the initial 9-hour perfusate as estimated by the *Nitrosomonas-Nitrobacter* bioassay

Column materials	Perfusate volume (ml)	KN_3 concent. in perfusate (ppm) at 0 hours	KN_3 concent. in perfusate (ppm) after 5 hours	KN_3 removed (%)
Glass beads	50	1.50[a]	1.90	(-21.5)
	100	1.90	1.80	5.2
Vermiculite-Perlite	50	1.35	1.60	(-15.5)
	100	1.75	1.50	8.5

[a] Corrected for water-holding capacity of each column material

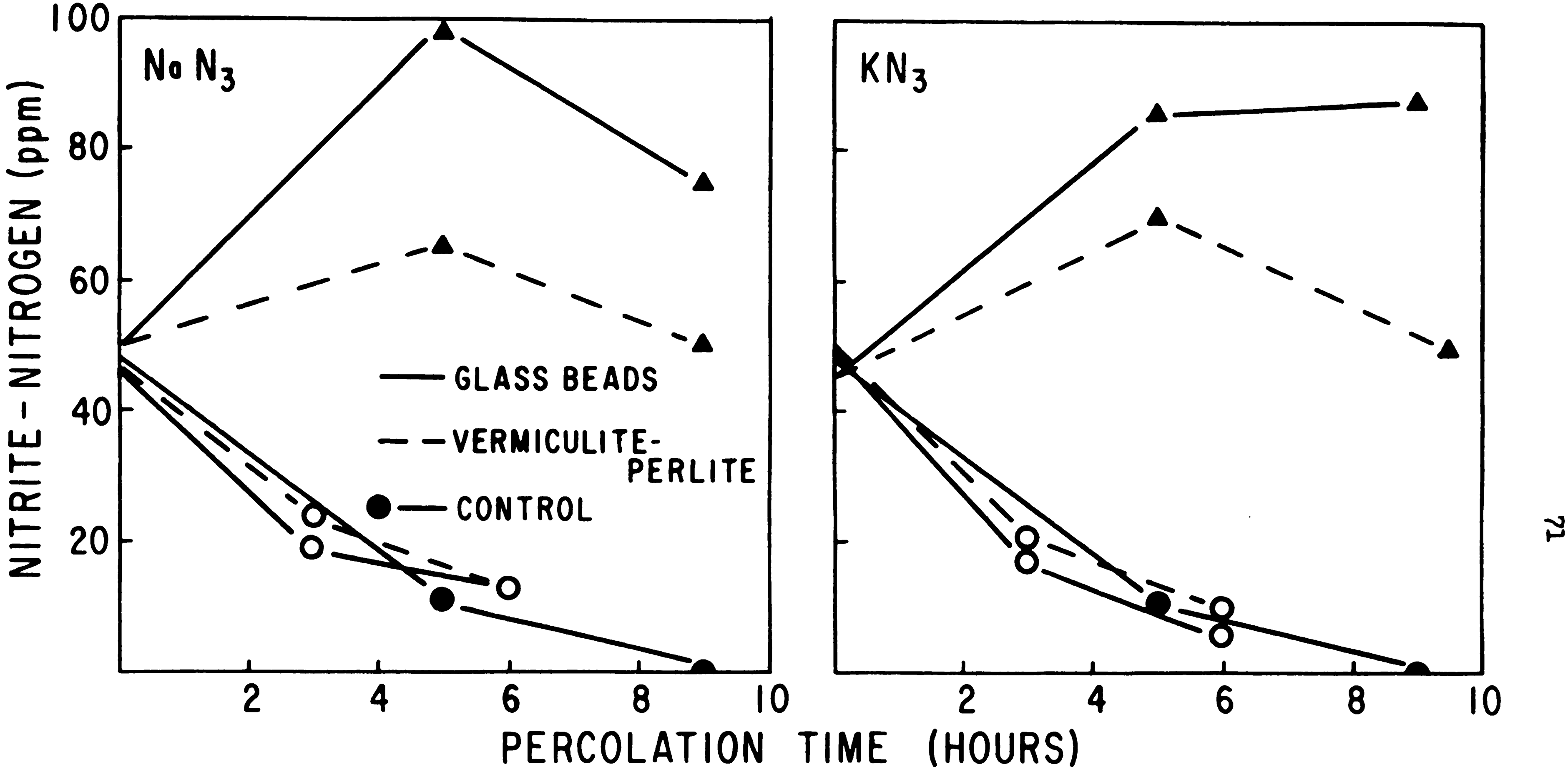


FIG. 7. Nitrite found during initial perfusion (▲) with 200 ml nutrient solution containing 1.0 ppm NaN_3 or 1.5 ppm KN_3 and during subsequent perfusion (○) with 200 ml untreated nutrient solution

Summary

The inhibition of ammonium and/or nitrite oxidation by 3-amino-1,2,4-triazole [amitrole], isopropyl-N(3-chlorophenyl) carbamate [CIPC], isopropyl-N-phenyl carbamate [IPC], potassium azide [KN_3] and sodium azide [NaN_3] was increased on increasing perfusate volumes and was affected by the nature of the column material.

The pattern of *Nitrosomonas* inhibition by amitrole and its residual effects appeared to depend mostly upon mechanisms of its sorption and desorption by column materials.

CIPC and IPC inhibited nitrite oxidation by *Nitrobacter*. Living or dead organic materials rather than inorganic column constituents seemed to be responsible for sorption of these chemicals, and for the inhibition carried over into a second perfusate.

Potassium and sodium azide prevented nitrite oxidation and none of the chemicals was sorbed by column constituents, which is in agreement with the absence of any residual effect on re-perfusion with untreated medium.

Amitrole concentrations in perfusate were determined chemically. A *Nitrosomonas* and *Nitrobacter* bioassay was developed for estimating concentrations of the other chemicals.

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APPENDICES

APPENDIX A

A listing of the trade or common and chemical names and formulae of compounds

Name		Formula
Trade or Common	Chemical	
	Insecticides	
Captan	N-trichloromethylthio tetrahydrophthalimide	$C_9H_8O_2SCl_3$
Chlordane	1,2,3,4,5,6,7,8,8-Octachloro-4,7-methano-3a,4,7,7a-tetrahydroindane	$C_{10}H_6Cl_8$
DDD	2,2 bis-(p-chlorophenyl)-1, 1-dichloroethane	$(C_6H_4Cl)_2CHCHCl_2$
DDT	Trichloro-bis-(4-chlorophenyl) ethane	$C_{14}H_9Cl_5$
Kelthane	1,1-bis-(p-chlorophenyl)-2,2,2-trichloroethanol	$C_{14}H_9OCl_5$
Lindane	Benzene hexachloride	$C_6H_6Cl_6$
Parathion	0,0-Diethyl-0-paranitrophenyl thiophosphate	$C_{10}H_{14}NO_5PS$
Sevin	1-naphthyl methylcarbamate	$C_{12}H_{11}NO_2$
	Fungicides	
Dowicide-7	Pentachlorophenol	C_6Cl_5OH
8-quinolinol	8-hydroxyquinoline	C_9H_7ON

APPENDIX A (continued)

Name		Formula
Trade or Common	Chemical	
	Fungicides	
Potassium Azide	Potassium Azide	KN_3
Sodium Azide	Sodium Azide	NaN_3
Terrazole	5-ethoxy-3-trichloromethyl-1,4-thiadiazole	$C_5H_5OCl_3N_2S$
Thiram	Tetramethylthiuramdisulfide	
	Herbicides	
Amitrole	3-amino-1,2,4-triazole	$C_2H_4N_4$
CIPC	Isoproprpyl N-(3-chlorophenyl) carbamate	$C_{10}H_{12}ClNO_2$
Diphenamid	N,N-dimethyl-2,2-diphenylacetamide	$C_{15}H_{17}NO$
IPC	Isopropyl N-phenylcarbamate	$C_{10}H_{13}NO_2$
Simazine	2-chloro-4, 6-bis(ethylamino)-S-triazine	$C_7H_{12}ClN_5$
Zytron	O-(2,4-dichlorophenyl)-methyl isopropylphosphoramidothioate	$C_{10}H_{14}O_2Cl_2NPS$
	Others	
N-Serve	2-chloro-6-(trichloromethyl)pyridine	$C_6H_3NCl_4$

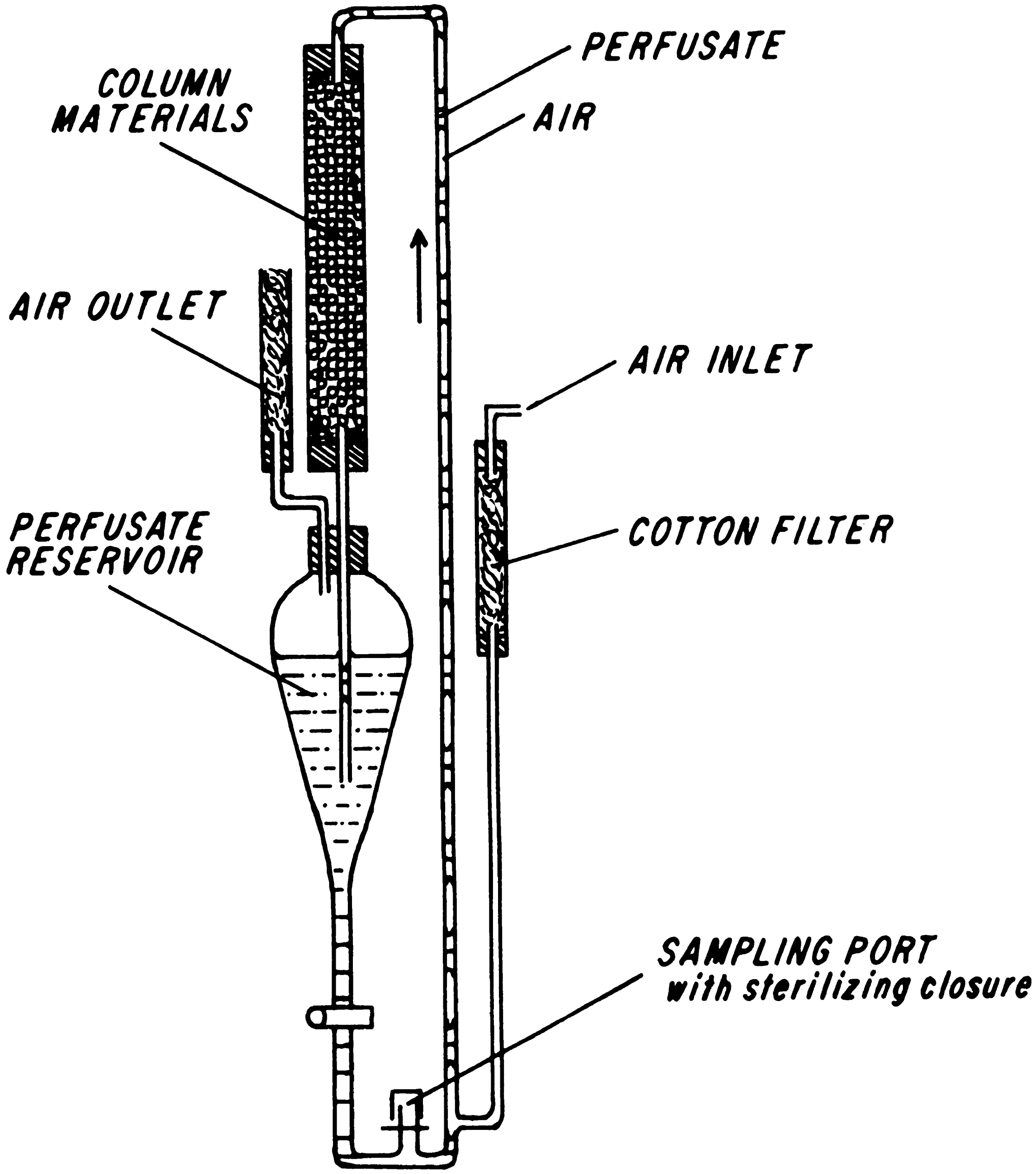
APPENDIX B

Chemical names and formulae of aniline derivatives

Chemical name	Formula
2,3-dichloroaniline	$C_6H_5Cl_2N$
2,4-dichloroaniline	$C_6H_5Cl_2N$
2,5-dichloroaniline	$C_6H_5Cl_2N$
3,4-dichloroaniline	$C_6H_5Cl_2N$
3,5-dichloroaniline	$C_6H_5Cl_2N$
m-chloroaniline	C_6H_6ClN
p-chloroaniline	C_6H_6ClN
2,3,5,6-tetrachloroaniline	$C_6H_3Cl_4N$
2,3,4-trichloroaniline	$C_6H_4Cl_3N$
2,4,5-trichloroaniline	$C_6H_4Cl_3N$
2,4,6-trichloroaniline	$C_6H_4Cl_3N$

APPENDIX C

PERFUSION APPARATUS



APPENDIX D

CULTURE FERMENTOR