1974

Pesticide Effects in a Simulated Soil Ecosystem

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PESTICIDE EFFECTS IN A SIMULATED SOIL ECOSYSTEM

(TITLE)

BY

RAYMOND J. SAMP

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF SCIENCE

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

1974
YEAR

I HEREBY RECOMMEND THIS THESIS BE ACCEPTED AS FULFILLING
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ACKNOWLEDGMENT

The author wishes to express his most sincere appreciation to Dr. William Weiler, whose direction, constructive criticism, and unending encouragement were instrumental to the completion of this research project.

Further thanks are extended to the members of the author's graduate committee, Dr. U. Douglas Zimmerman, Dr. Patrick Docter, and Dr. William Scott, for their involvement and guidance in this study.

The DuPont and Dow Chemical Companies also deserve recognition for their gracious gifts of the pesticides used.

ABSTRACT

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Pesticide effects in a simulated soil ecosystem

Major Professor: Dr. William Weiler

The screening of pesticides to determine which were most inhibitory to bacteria was accomplished by testing the effects of 10 pesticides on 9 different organisms using the disk assay method. Results showed that Gram positive bacteria were more sensitive to all pesticides tested than were the Gram negative bacteria. In addition, the hormone herbicides were found to be the most inhibitory to these bacteria and 2,4-D (2,4-dichlorophenoxyacetic acid), a hormone herbicide, was chosen for subsequent in vivo studies.

A soil perfusion apparatus was used to determine the effects of 2,4-D in a typical soil ecosystem. Bacterial plate counts and nutrient analyses were used to determine the effects of the chemical on the soil bacteria and on their metabolic processes. Results showed that 2,4-D significantly reduced bacterial populations and nitrate production within 2 weeks of application. Nitrate production was reduced to the point where none of the soil nitrogen found in the soil perfusate was present as nitrate.

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INTRODUCTION

The indiscriminate use of pesticides for agricultural gain was common until some serious side effects were realized. The persistance of pesticides in soils is recognized as one serious side effect of the chemical treatment of plants and insects and consequently the soil. In the case of the more stable pesticides which may have a half life of years, researchers note their tendancy to accumulate in the food chains of higher organisms. A substantial amount of damage to wildlife, particularly birds and fish, has occurred because of the accumulation of these toxic materials and their metabolites in their bodies. Whole ecosystems have been altered because of the effects of these dangerous chemicals.

All is not lost however. New pesticides are constantly being developed that do not persist in nature as long as the earlier chemicals. The new pesticides may have half lives of weeks or even days due to their unstable chemical structure and the degradative properties of many soil microorganisms. This adjustment has reduced some of the danger that was involved with pesticide usage.

Another less obvious problem that pesticide usage presents is the effects of pesticides on the soil microflora. Most experimentation involving pesticides in the soil have been directed toward tracing the pesticide's accumulation and longevity in the soil, than toward determining what effects the pesticides have on the microflora and on the chemistry of the soil. Soil microorganisms play such an

important role in the recycling of inorganic minerals and nutrients in the soil that they cannot be overlooked. It is this aspect of pesticide action that will be examined in this study.

HISTORICAL

Pesticides are substances designed to control or eradicate specific pests of economic plants (Alexander, 1961). Herbicides, insecticides, fungicides, and nematocides are the broad groups of chemicals that are included by the general term pesticide. Of these four, herbicides and insecticides are, by and large, the economically more important pesticides.

The Advent of Pesticides

Magic, superstition, and sorcery were the main line of defense against pests of crop plants for hundreds of years. The idea that substances which were distasteful to humans were also distasteful to insects became a popular belief during the middle ages. Mixtures of substances such as dung, ashes, urine, and scraps were recommended for warding off undesireable pests. Little scientific experimentation was endeavored and therefore few effective pesticides were discovered. As early as 1690 useful pesticides finally came into use when tobacco water and tobacco powder were used in Europe. The active agent in these mixtures was the alcaloid nicotine. This substance is still used today in some pesticides. Rotenone, another insecticide, was discovered as having pesticidal properties by Chinese gardeners in Malaya before 1848 (Hough, 1951). Rotenone is a root extract of plants of the genera Derris and Lanchocarpus. Still another insecticide, pyrethrum, a flower extract of the plant Chrysanthemum cineraraefolium,

was discovered at an unknown date by the natives of Asia Minor (Pimentel, 1971). In about 1868 someone discovered that Paris Green, an arsenical insecticide, would protect the potato plant from the potato beetle which at the time was ravaging the potato crop of the western United States (Hough, 1951).

New pesticide discovery continued when in 1896 Bonnet, a French grapegrower, discovered that the Bordeaux mixture (composed of copper sulfate and lime) applied to his vines to protect against downy mildew also turned the leaves of yellow charlock (Sinapsis arvensis) black suggesting its potential use as an herbicide. After the Bordeaux mixture, kerosene-soap emulsions, lime-sulfur, lead arsenate, ammonium sulfate, cupric nitrate, calcium cyansmide, and other inorganic insecticides were used (Audus, 1969).

In 1932, the first synthetic organic chemical herbicide, 2-methyl-4,6-dinitrophenol (DNOC), was discovered. From this beginning, a long line of organic pesticides followed which were much more efficient and more specific than the primitive substances already in use for years. In 1939, the insecticidal properties of DDT (dichloro diphenyl trichloroethane), a chlorinated hydrocarbon, was discovered in Switzerland. This remarkable insect killer became known around the world and led to a tremendous amount of research in the area of pest control.

During the 1930's a great deal of work was done concerning plant hormones. It was during this period that indole acetic acid (auxin) was first recognized as a potential herbicide. Experimentation showed that it was selective for broad leaved plants as opposed to grasses. Further testing showed that 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 2,4-dichlorophenoxyacetic acid (2,4-D) were much

more efficient and effective than auxin (Audus, 1969).

Subsequent research led to other organic herbicides. The benzoic and phenylacetic acids were found to be specific for grasses (Audus, 1969). The substituted ureas, 3-(p-chlorophenyl)-1,1-dimethylurea (monuron) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron), which were discovered in 1951 by Bucha and Todd and the uracil herbicides, 5-bromo-3-sec-butyl-6-methyluracil (bromocil) and 3-tert-butyl-5-chloro-6-methyluracil (terbacil), were found to be of considerable value in controlling annual and perennial grasses along with other deep rooted plants. Triazines were produced as nonselective weed killers, but were found to be effective in low concentrations as selective weed killers in corn fields. The most common of the triazines is 2-chloro-4,6-bis(diethylamino)-1,3,5,-triazine (simazine) (Audus, 1969).

A few of the different types of insecticides that were produced in this era were the carbamates and the organo-phosphates. The carbamates, particularly zectran (4-dimethylamino-3,5-xylyl methylcarbamate), are used as contact poisons on insects. One of the most important groups of insecticides to be discovered in this era of pesticide production were the organo-phosphates. They were discovered during World War II by German scientists researching war gases and have since become some of the more outstanding insecticides yet developed for use on crop plants (Hough, 1951).

Mode of Action and Persistance of Selected Pesticides

Since the list of pesticides is continually being added to, it is of interest to discuss some of the physiological attributes of the major groups. The groups to be discussed are the carbamate and

organo-phosphate insecticides and the urea, uracil, and hormone herbicides.

The carbamates and organo-phosphates are categorized as insecticides, both exhibiting similar actions on their target organisms. These compounds are known for their potential lethal effects on higher organisms. Solutions of the pesticides can be absorbed through the skin of higher organisms including humans. The organo-phosphates are highly soluble compounds that are effective in small doses making overkill very easy. Unlike their counterparts, the chlorinated hydrocarbons (DDT), the organo-phosphates have short half lives which make them desireable since they persist in the soil for only a matter of days or weeks due to microbial activity and the effects of the environment (Menzie, 1969). The mode of action of the carbamates and the organo-phosphates is the same. They both act as cholinesterase inhibitors which kill insects by tying up nerve synapses. The carbamates act primarily as contact poisons whereas the organo-phosphates act as stomach poisons, contact poisons, or as respiratory poisons (Hough, 1951 and White-Stevens, 1971).

The urea and uracil herbicides elicit similar physiological and biochemical behavior in target plants. They are used as selective herbicides to eliminate annual and perennial grasses. Because of this, they are used extensively to kill weeds in cotton, citrus, and pineapple fields (Technical Data Sheets, 1972). These relatively water insoluble herbicides are absorbed into the roots of susceptible plants along with ground water and are transported via the xylem to the leaves of the plant. Once in the leaves, the compound interferes with the Hill reaction and prevents photosynthesis, killing the plant by taking away

its ability to synthesize food (Tahori, 1972). Because of their water insolubility the ureas and uracils cannot be applied in extremely high concentrations. Persistance of the urea and uracil herbicides in the soil is relatively short, being degraded to inactive end products within three to six months (Pimentel, 1971). Microbial degradation plays an all-important role in eliminating these herbicides from the soil. Recent studies (Bartha and Pramer, 1970) show that ureas are microbially demethylated in a step-wise fashion and degraded to aniline derivatives, the aniline derivatives themselves being non-toxic.

The hormone herbicides (auxin analogs) are probably among the most widely used herbicides. Of the hormone herbicides, 2,4-D, 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), silvex (2-(2,4,5-trichlorophenoxy) propionic acid), and MCPA are most commonly used. These act as auxin simulators and cause uncontrollable growth in receptive plants. The hormone herbicides are used for the destruction of broad leaved weeds, as a preemergent, or as a contact spray. A much lower concentration of 2,4-D is necessary when used as a preemergent weed killer than as a contact spray, since the embryonic roots of weeds are more sensitive than are mature roots and stems. In mature target plants, the compound is absorbed at the point of application (usually leaves or stems) and may be transported to other parts of the plant where interference with normal growth patterns takes place. In this way, application to leaves may result in rapid translocation to roots or underground stems resulting in altered growth which can kill the plant (Audus, 1959). Several symptoms are exhibited by mature plants poisoned with 2,4D. Certain cells swell, roots stop growing, leaves develop a thickened mesophyll, and photosynthesis is impaired (Walker, 1973). Detoxification of hormone

herbicides, in particular 2,4-D, in soil is due entirely to microbiological activity (Audus, 1949).

Non-Target Effects of Pesticides

The development and often excessive use of chemical pesticides soon brought forth the question of how pesticides and pesticide residues affect the environment. Though initially, a few people asked this question, most didn't bother to research the matter as long as increased crop yields were an end result. As time passed however, environmental awareness took hold and more and more people became interested in the toxic effects of pesticides on non-target organisms.

Investigations of the chlorinated hydrocarbon insecticides (DDT, aldrin, dieldrin) began in the early 1960's. These studies showed that the persistance of these compounds is very high and that they have dangerous toxic effects on higher organisms. It is not clear how long DDT persists in ecosystems. Fifty per cent of the DDT sprayed in a single treatment may still be found in a field ten years later. However, this does not mean that the other fifty per cent has been degraded to biologically inactive molecules since it may only have gone elsewhere with runoff. DDT probably has an average half life of much more than a decade and DDE, the biologically active breakdown product of DDT, may be virtually immortal (Ehrlich and Ehrlich, 1970).

Several studies have demonstrated that different types of animals are adversely effected by the continued persistence of DDT. It has been shown that DDT tends to accumulate in food chains presenting great danger to the secondary and tertiary consumers of an ecosystem. Birds, fish, and mammals have all exhibited the toxic effects of DDT. Birds

have been especially affected by this compound, the main symptom being decreased egg production. Some instances of complete reproductive failure have been demonstrated because of the effects of DDT on egg shell formation. Shells have been shown to be up to 34% thinner than shells produced before exposure to DDT (Pimentel, 1971).

The destructive effects of DDT have implanted a sense of caution in the minds of men concerned with pesticide usage and the balance of nature. Since the intense studies of chlorinated hydrocarbons, many more studies have been initiated to test the effects of other pesticides on the environment.

Most of the research on the more commonly used pesticides has been done within the last decade. The carbamate insecticides have been demonstrated to be toxic to some birds and fish at low concentrations (Pimentel, 1971). The organo-phosphate insecticides have been shown to be dangerous to mammals. Since it can be used as a contact or stomach poison, it is doubly dangerous to herbivores and small mammals that may be present in the fields at the time of application. These compounds have also been found to be toxic to birds and fish at low concentrations. The redeeming characteristic of the organo-phosphate insecticides is that they have a short half life and only persist in the environment for a matter of days or weeks. Because of this, the danger to animals is only present for a short period of time and any sublethal dose ingested by any organism would also be neutralized in a short period of time. Research has also demonstrated that the organo-phosphates have no observable toxic effect on plants, due to their relative specificity (White-Stevens, 1971).

The urea and uracil herbicides have been shown to be toxic to fish, and to a lesser degree, birds. (There is less danger for birds than for fish since the herbicides are more subject to degradation in the terrestrial environment.) Trees and shrubs whose roots are in contact with treated water were found to be susceptible to the compounds. Other non-target plants, the algae, have been found to be very sensitive to low concentrations of urea herbicides (Pimentel, 1971).

The hormone herbicides have also been shown to have some unintended effects on the environment. Birds are effected by the hormone herbicides only in high concentrations while a fish kill can be accomplished with a relatively low concentration of 2,4-D. 2,4-D can cause some toxic effects on various mammals either directly or indirectly. The compound itself will kill if ingested in high concentrations by herbivores foraging for food (Pimentel, 1971). At the same time, the damage that it causes to the flora of an area can also cause a tremendous reduction in population of many animals since significant portions of the foliar environment are removed by the application of 2,4-D. An example of this was the defoliation policy that the United States assumed in the Vietnam war. Many habitats and food sources were destroyed therefore eliminating all wildlife from the applied area. This, however, is more concerned with man's abuse of the herbicide rather than complications that accompany the directed use of the compound (Ehrlich and Ehrlich, 1971).

2,4-D is used to eliminate broad-leaved weeds from crop fields, but careless use may cause problems. Fults and Payne (1947) noted that in spraying a field with 2,4-D, adjacent fields can also be effected and broad-leaved crops can be destroyed. Shaw and Robinson (1960) indicated that 2,4-D had lethal effects on tomato plants at all concentrations

from 10 to 100 pounds per acre, probably by severely retarding root growth (Nutman and Thornton, 1945). Legumes such as peas, beans, red clover, and alfalfa are particularly inhibited by salts of 2,4-D. In addition, according to Carlyle and Thorpe (1947) and Fults and Payne (1947), even residual forms of 2,4-D restricted germination, limited growth, and practically inhibited nodulation on these leguminous plants.

Since a considerable amount of research has indicated that pesticides have lethal effects on higher plants and animals, one might ask, what effects do pesticides have on the microflora of the soil. Soil bacteria are extremely important organisms in spite of their microscopic size. They are instrumental in keeping the essential elements of life in constant circulation by decomposing dead organic matter. This process allows living plants and animals to utilize these nutrients for their own life processes. Not only do soil microorganisms recycle minerals, they are also responsible for nitrogen fixation in soils (Waksman and Starkey, 1931). Indeed the bacteria are very important to the soil and should be considered in pesticide research, but most studies to date have been concerned with bacterial effects on pesticides (detoxification) rather than the effects of pesticides on the soil microflora. This may possibly be due to the justifiable concern with the persistence of pesticides in soil and accumulation in higher plants and animals. Nonetheless, the bactericidal tendancies of pesticides must not be overlooked.

Little work has been done with the carbamate insecticides. Clark and Wright (1970) observed that Arthrobacter and Achromobacter were able to degrade IPC (isoprophyl N-phenyl carbamate). The only data showing the effects of carbamates on the soil bacteria was that of

Bartha et al. (1967) who demonstrated that these pesticides depressed nitrate and carbon dioxide levels in treated soils.

The organo-phosphate insecticides, as one might expect from their mode of action, are not very inhibitory to soil bacteria and were, in fact, found to increase bacterial populations occasionally (Bollen, 1961).

The urea and uracil herbicides seem to have few inhibitory tendancies toward the soil bacteria and are subject to the microbial activities of the soil. Engelhardt et al. (1971) isolated a linuron inducible enzyme from Bacillus sphaericus which suggests that the compound is broken down by this microbe and is possibly used as a nutrient. Ulasevych et al. (1970) and Breazeale and Camper (1972) discovered that the application of urea herbicides on soil did not effect the growth rate of the gross microbial populations of the soil. However, Geller and Khariton (1961) and Babek (1968) showed that the use of urea herbicides reduced the numbers of ammonifying and nitrifying bacteria, especially members of the genera Azotobacter and Clostridium. This apparently conflicting data shows that although superficial testing may exhibit no inhibitory tendancies of a herbicide there may be an ecological imbalance that would indeed decrease soil fertility.

The hormone herbicides have also been shown to have bactericidal properties. Audus (1969) states that the herbicide 2,4-D is almost entirely broken down by microbial action. Organisms of the genera Bacillus (Audus, 1949), Achromobacter (Bell, 1960), Pseudomonas, Azotobacter (Bollen, 1961), Flavobacterium (Rogoff, 1961), Streptomyces (Bounds and Colmer, 1965), and Arthrobacter (Sharpee et al., 1973) are all able to degrade 2,4-D. Other hormone herbicides such as MCPA and 2,4,5-T follow similar degradative pathways with a more or less long

lag period of bacterial growth followed by rapid detoxification of the compound (Audus, 1949). Yet, during the time that these compounds are present in the soil, they apparently have some toxic effect on some of the soil microflora. Kliuchnikov and Petrova (1960) noted that bacterial populations were depressed in the presence of 2,4-D. Breazeale and Camper (1970) corroborated this finding and in addition, pointed out that fungal populations were also depressed and that actinomycete populations increased. They suggested that this may have been due to the lack of competition for food and nutrients for actinomycetes. It can therefore be said that 2,4-D may cause significant changes in the micro-ecology of the soil (Worth and McCabe, 1948). Besides decreasing bacterial populations it would seem that mineral production by the 2,4-D inhibited organisms would also decrease because of the influence of the pesticide. However, there are contrasting views on this aspect of pesticide action. Van Schreven et al. (1971) indicated that MCPA depressed the rate of nitrification in soils, and Newman (1947) and Bollen (1961) obtained similar results with the use of 2,4-D. On the other hand, Jones (1948) found that 2,4-D added to soils at a relatively high rate (25 pounds per acre) had little or no influence on nitrate levels. Substancially more research must be done in this area before absolute conclusions can be reached.

Micro-Ecological Methodology

The methods used in previous studies to determine the effects of pesticides on soil bacteria have varied. Bartha et al. (1967, 1970) used carbon dioxide evolution to determine an increase or decrease in microbial populations. This method suffers by its non-specificity.

Bacteria, protozoans, fungi, and all other soil inhabitants are all contributing to the gross carbon dioxide evolution. Breazeale and Camper (1970) eliminated this problem by using plate count methods for enumerating bacteria, molds, and actinomycetes in treated and untreated soils. This method, however, does not take into consideration the different microbial micro-ecosystems that may be present in different portions of soil tested, the normal effect of environmental changes on the microflora, nor the introduction of new organisms. Breazeale and Camper (1972) again tried to eliminate these variables by growing soil organisms in a closed environment. They used prepared liquid growth media, inoculated with typical soil organisms in mixed culture, and incubated in an oscillating water bath. To the culture they added 2,4-D to determine its effect on the population by measuring changes in the optical density of the medium with a spectrophotometer. This process eliminated some of the undesireable variables, but took away the advantage of operating <u>in vivo</u>. The method that appears to be best for testing the effects of pesticides (2,4-D) on the natural microflora of the soil is the soil perfusion apparatus as described by Audus (1946) and Lees and Quastel (1946). This apparatus consists of a glass column of selected soil with water or any desired solution being percolated through and collected as an effluent. The effluent is tested to determine microbial population levels and inorganic chemical production by the microbes inside the column. This technique allowed them to test the organisms <u>in vitro</u>, but at the same time in a simulated, controlled soil environment. All variables to be considered were relatively easily controlled and bacterial populations were determined by running standard

plate counts with selective media. This method appears to satisfy most of the conditions that are required to properly test the reactions of soil bacteria to pesticide application.

PART I. ANTIBACTERIAL ACTIVITY OF SELECTED PESTICIDES

Materials and Methods

Bacterial Stock Cultures

Nine species of seven bacterial genera were used to determine the inhibitory tendancies of ten pesticides. Those chosen were comparatively common organisms having different morphological and physiological characteristics in an attempt to survey a cross-section of soil inhabitants. Species used and characteristics of the organisms can be found in Table 1.

The nine bacterial species were reisolated from oil stock cultures (Hartsell, 1953) by streaking onto Plate Count Agar (PCA, Difco, 1953) to obtain isolated colonies. Cells from typical colonies were Gram stained and observed under a Bausch and Lomb oil emersion microscope to determine purity, transferred to PCA slants, incubated 24 hours for growth, and refrigerated. These became the stock cultures for the experiment. Once in the course of the experiment, a contamination was noted and all cultures were reisolated and transferred to new stock slants.

Pesticide Preparation

Ten pesticides of four major types were used in the experiment to determine the ones which were most bactericidal or bacteristatic. Names, descriptions, and properties of these chemicals can be found in Table 2.

TABLE 1.

CHARACTERISTICS OF TEST ORGANISMS

ORGANISM	GRAM REACTION	MORPHOLOGY	INCUBATION TEMP.
Achromobacter liquefaciens	Gram -	short rods	30°C.
Escherichia coli	Gram -	short rods	37°C.
Bacillus cereus	Gram +	spore forming rods	30°C.
Bacillus megaterium	Gram +	spore forming rods	37°C.
Micrococcus luteus	Gram +	cocci	30°C.
Micrococcus lysodeikticus	Gram +	cocci	37°C.
Proteus vulgaris	Gram -	short rods	37°C.
Pseudomonas aeruginosa	Gram -	short rods	37°C.
Staphylococcus aureus	Gram +	cocci	37°C.

TABLE 2.

COMMON NAME	linuron
GROUPING	urea herbicide
SOURCE	DuPont Chemical Company
CHEMICAL NAME	3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea
EMPIRICAL FORMULA	$C_9H_{10}Cl_2N_2O_2$
SOLUBILITY	75 ppm in water, soluble in acetone
SOLVENT USED	ethanol
COMMON NAME	siduron
GROUPING	urea herbicide
SOURCE	DuPont Chemical Company
CHEMICAL NAME	1-(2-methylcyclohexyl)-3-phenylurea
EMPIRICAL FORMULA	$C_{14}H_{20}N_2O$
SOLUBILITY	18 ppm in water, soluble in ethanol
SOLVENT USED	methanol
COMMON NAME	bromocil
GROUPING	uracil herbicide
SOURCE	DuPont Chemical Company
CHEMICAL NAME	5-bromo-3-sec-butyl-6-methyluracil
EMPIRICAL FORMULA	$C_9H_{13}BrN_2O_2$
SOLUBILITY	815 ppm in water, moderately soluble in ethanol
SOLVENT USED	ethanol
COMMON NAME	terbacil
GROUPING	uracil herbicide
SOURCE	DuPont Chemical Company
CHEMICAL NAME	3-tert-butyl-5-chloro-6-methyluracil
EMPIRICAL FORMULA	$C_9H_{13}ClN_2O_2$
SOLUBILITY	710 ppm in water
SOLVENT USED	ethanol
COMMON NAME	MCPA
GROUPING	hormonal herbicide
SOURCE	DuPont Chemical Company
CHEMICAL NAME	4-chloro-2-methylphenoxyacetic acid
EMPIRICAL FORMULA	$C_9H_9ClO_3$
SOLUBILITY	good solubility in most organic solvents
SOLVENT USED	ethanol
COMMON NAME	silvex
GROUPING	hormonal herbicide
SOURCE	DuPont Chemical Company
CHEMICAL NAME	2-(2,4,5-trichlorophenoxy) propionic acid
EMPIRICAL FORMULA	$C_9H_7Cl_3O_3$
SOLUBILITY	slightly soluble in water, soluble in methanol
SOLVENT USED	ethanol

COMMON NAME	2,4-D
GROUPING	hormonal herbicide
SOURCE	E.I.U.
CHEMICAL NAME	2,4-dichlorophenoxyacetic acid
EMPIRICAL FORMULA	$C_8H_6Cl_2O_3$
SOLUBILITY	slightly soluble in water, soluble in ethanol
SOLVENT USED	ethanol

COMMON NAME	reulene
GROUPING	organo-phosphate insecticide
SOURCE	Dow Chemical Company
CHEMICAL NAME	4-tert-butyl-2-chlorophenyl methyl phosphoroamidate
EMPIRICAL FORMULA	$C_{12}H_{19}ClNO_3P$
SOLUBILITY	slightly soluble in water, soluble in acetone
SOLVENT USED	acetone

COMMON NAME	ronnel
GROUPING	organo-phosphate insecticide
SOURCE	Dow Chemical Company
CHEMICAL NAME	O,O-dimethyl-O-2,4,5-trichlorophenylphosphorothioate
EMPIRICAL FORMULA	$C_8H_8Cl_3O_3PS$
SOLUBILITY	almost insoluble in water, soluble in acetone
SOLVENT USED	acetone

COMMON NAME	zectran
GROUPING	organo-phosphate insecticide
SOURCE	Dow Chemical Company
CHEMICAL NAME	4-dimethylamino-3,5-xylyl methylcarbamate
EMPIRICAL FORMULA	$C_{12}H_{18}N_2O_2$
SOLUBILITY	almost insoluble in water, soluble in acetone
SOLVENT USED	acetone

Eight mm disks were cut by punching holes in number 470 S & S (Schleicher and Schuell) filter paper with a paper hole punch. These disks were soaked in pesticide solutions at various concentrations. The total amount of pesticide present in each disk was determined by weighing dried disks at a specific concentration on a milligram scale and comparing this weight to that of an equal number of disks soaked only in the solvent and dried. The difference was divided by the number of disks weighed to give the total amount of pesticide present in each disk. The pesticide-solution concentrations and the average amount of pesticide (dry weight) in each disk can be found in Table 3. After weighing, all disks were refrigerated until use.

Assay Procedure

The assay procedure was begun by inoculating 10 ml m-PCB (0.5X) tubes (Difco, 1953) with the nine cultures and incubating the cultures at their optimum temperatures for 24 hours. A base layer of 15 ml PCA was poured into sterile Petri plates and allowed to solidify. Four drops of the 24 hour broth culture was aseptically added to five ml of previously melted and equilibrated (50C) 0.5X m-PCB and one per cent agar. The tubes were then shaken vigorously to distribute the cells evenly and were poured over the PCA base layer. The top layer is referred to as the "seed" layer. After solidification of the seed layer, six disks were placed equidistant from each other on the surface of the seed layer. Five different concentrations of each pesticide were tested on each plate. A control disk, saturated in solvent only and dried, was also applied. The plates were incubated at the optimum temperature for growth. Results were read by measuring the diameter of the clear zone of inhibition (if any) imposed on the cells of the seed layer by the pesticide in the disk after 12, 24, 36, and 48 hours of incubation.

TABLE 3. Dry weight of pesticide in disks.

PESTICIDE	SOLUTION CONCENTRATION (mg/l)	mg/DISK
Reulene	10^5[a]	1.6
	10^4[b]	trace
Ronnel	10^5[a]	2.4
	10^4[b]	0.3
Zectran	10^5[a]	3.4
	10^4[b]	0.2
Linuron	6.7×10^4[a]	1.3
	6.7×10^3[b]	0.1
Siduron	6.7×10^4[a]	2.1
	6.7×10^3[b]	0.5
Bromocil	5×10^4[a]	1.6
	5×10^3[b]	trace
Terbacil	6.7×10^4[a]	2.4
	6.7×10^3[b]	0.7
2,4-D	10^5[a]	3.2
	10^4[b]	0.7
MCPA	10^5[a]	3.4
	10^4[b]	0.6
Silvex	4×10^4[a]	1.5
	4×10^3[b]	0.6

[a]Hereafter referred to as the high concentration.
[b]Hereafter referred to as the low concentration.

RESULTS AND DISCUSSION

The results of this experiment showed that some pesticides have inhibitory tendancies toward soil bacteria. Only a minority of them, however, produced significant zones of inhibition and then only at the highest concentrations used in the experiment. It must be remembered, though, that even the lowest concentration of pesticide which resulted in inhibition was substantially greater than normal field application rates (generally under 100 parts per million) (Technical data sheets, 1972).

In addition, the use of the disk assay method to determine bactericidal tendancies of pesticides necessitates the consideration of the molecular weight and water solubility of the compounds before assessing final results (Food and Drug Administration, Pesticide Reference Standards). Compounds with lower molecular weights diffuse through the medium farther than compounds with higher molecular weights, thereby producing larger zones of inhibition. Similarly, the more water soluble a compound is the more it will diffuse through the water based growth medium to assert its influence on the organism. Table 4 shows that silvex produced a smaller zone of inhibition than either 2,4-D or MCPA in all cases. This may be due to the lower concentration of herbicide in the disk or to the higher molecular weight of silvex. On the other hand, one might expect MCPA to be more inhibitory than 2,4-D on the basis of its (MCPA) lower molecular weight and higher water solubility. Such was not the case. For all organisms 2,4-D was about as inhibitory as

TABLE 4. Influence of water solubility and molecular weight of the pesticides on the zone of inhibition.

HORMONE HERBICIDE	M.W.	H_2O-SOL.	DISK CONC.	Diameter of Zones of Inhibition (in mm)				
				B. megaterium	M. lysodeikticus	B. cereus	P. vulgaris	P. aeruginosa
MCPA	200	sol.	3.4 mg	17	27	20	13	9
2,4-D	221	sl. sol.	3.2 mg	18	29	22	16	10
SILVEX	270	sl. sol.	1.5 mg	13	22	15	10	-

MCPA suggesting greater inhibitory tendancies for 2,4-D. The subsequent discussion of bacterial inhibition by these pesticides must then take these factors into consideration before arriving at any firm conclusions.

Because only the disks with the two highest concentrations of pesticide showed inhibition, only the results of those concentrations are presented in Table 5. They will be referred to as the "high" and "low" concentrations.

Definite inhibition was shown by certain pesticides on a few organisms. Most of the inhibition was evidenced in the Gram positive genera Micrococcus, Staphylococcus, and Bacillus. For some reason these cell types are more susceptible to the pesticides than the Gram negative genera (Newman and Downing, 1958). For example, linuron and zectran showed inhibition only to Gram positive species and none at all to Gram negative species. Some of the more inhibitory pesticides imposed much larger zones of inhibition on Gram positive species than on Gram negative species. MCPA produced zones of inhibition ranging from 17 to 27 mm on the Gram positive cultures whereas the corresponding ranges for the Gram negative cultures were from 0 to 13 mm. This trend was common to all organisms and pesticides tested. These results suggest that bacterial cellwall-membrane permeability may result in resistance or sensitivity to a specific pesticide. It is possible that the lipoprotein and lipopolysaccharide layers of the Gram negative cell walls may be impermeable to these compounds (Sekiguchi and Iida, 1967; Rupnow, 1973).

The different pesticides exhibited different degrees of bactericidal or bacteristatic activity. All the pesticides, except for the hormone herbicides, were only mildly inhibitory to the test organisms. The organo-phosphate insecticide ronnel was not inhibitory at all and the

TABLE 5. Bacterial inhibition by pesticides as determined by disk assay.

ORGANISM	2,4-D		MCPA		Silvex		Zectran	
	High	Low	High	Low	High	Low	High	Low
Gram Positives								
Bacillus megaterium	18[a]	9	17	9	13	8.5	-[b]	-
Bacillus cereus	22	11	20	9	15	8.5	-	-
Micrococcus luteus	26	17	27	13	23	11	8.5	-
Micrococcus lysodeikticus	29	14	27	13	22	8.5	15	12
Staphylococcus aureus	23	10	22	11	18	11	-	-
Gram Negatives								
Achromobacter liquefaciens	9	-	11	-	-	-	-	-
Escherichia coli	-	-	-	-	-	-	-	-
Proteus vulgaris	16	8.5	13	-	10	-	-	-
Pseudomonas aeruginosa	10	-	9	-	-	-	-	-

[a] Figures represent the diameter in mm of the zone of inhibition after 24 hours incubation.
[b] - represents no observable zone of inhibition surrounding the 8 mm disk.

ORGANISM	Reulene		Linuron		Bromocil	
	High	Low	High	Low	High	Low
Gram Positives						
Bacillus megaterium	10	9	10	8.5	-	-
Bacillus cereus	12	9	9	8.5	-	-
Micrococcus luteus	12	10	-	-	-	-
Micrococcus lysodeikticus	-	-	9	-	15	-
Staphylococcus aureus	-	-	11	9	-	-
Gram Negatives						
Achromobacter liquefaciens	-	-	-	-	-	-
Escherichia coli	9	-	-	-	-	-
Proteus vulgaris	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-

The pesticides siduron, ronnel, and terbacil had no inhibitory tendancies toward any of the organisms tested.

other organo-phosphate, reulene, showed only slight inhibition to a few cultures. (Five organisms were not affected at all.) The carbamate insecticide, zectran, was mildly inhibitory to only two organisms, both Gram positive.

The two uracil herbicides, bromocil and terbacil, exhibited no inhibitory tendancies toward any organism at any concentration. The urea herbicides were found to be weakly inhibitory to the test organisms. Siduron was absolutely harmless at all concentrations tested whereas linuron was slightly inhibitory to only four of the nine organisms.

Perhaps the most important result of the study was the effect of the hormone herbicides on the bacteria. These herbicides, 2,4-D, MCPA, and silvex, were by far the most inhibitory of all the chemicals tested. They produced zones of inhibition within the range of 13 to 29 mm at the high concentration when tested on Gram positive organisms and a range between 0 to 16 mm when tested on Gram negative organisms. Evidently these compounds have a detrimental effect on the microflora of the soil as well as on the macroflora.

In addition to this data it was also noted that as incubation time progressed, the zones of inhibition nearly always tended to diminish in size. Table 6 shows how the zone of inhibition caused by the hormone herbicide MCPA tended to decrease in diameter as incubation time increased. Possible explanations for this are that 1. the pesticide may be bacteristatic rather than bactericidal 2. gradual dissimilation of the pesticide by the bacteria may be occurring 3. development of heartier cells in the culture through natural selection may be occurring. In any case, the removal of pesticide by biodegradation or leaching would tend to return population and variation of organisms to normalcy.

TABLE 6. The effect of the length of time of incubation on the diameter of the zone of inhibition.

ORGANISM	Hours of Incubation 12	24	36	48
Gram Positives				
Bacillus megaterium	23[a]	17	16	15
Bacillus cereus	18	20	20	19
Micrococcus luteus	-[b]	27	25	22
Micrococcus lysodeikticus	-[b]	27	25	16
Staphylococcus aureus	22	22	20	18
Gram Negatives				
Achromobacter liquefaciens	14	11	11	10
Escherichia coli	-[c]	-	-	-
Proteus vulgaris	17	13	13	12
Pseudomonas aeruginosa	10	9	9	8.5

[a]Figures represent diameter of zone of inhibition at the highest MCPA concentration.
[b]Represents no growth.
[c]Represents no inhibition.

PART II. 2,4-D EFFECTS ON SOIL MICROFLORA IN A MODEL ECOSYSTEM

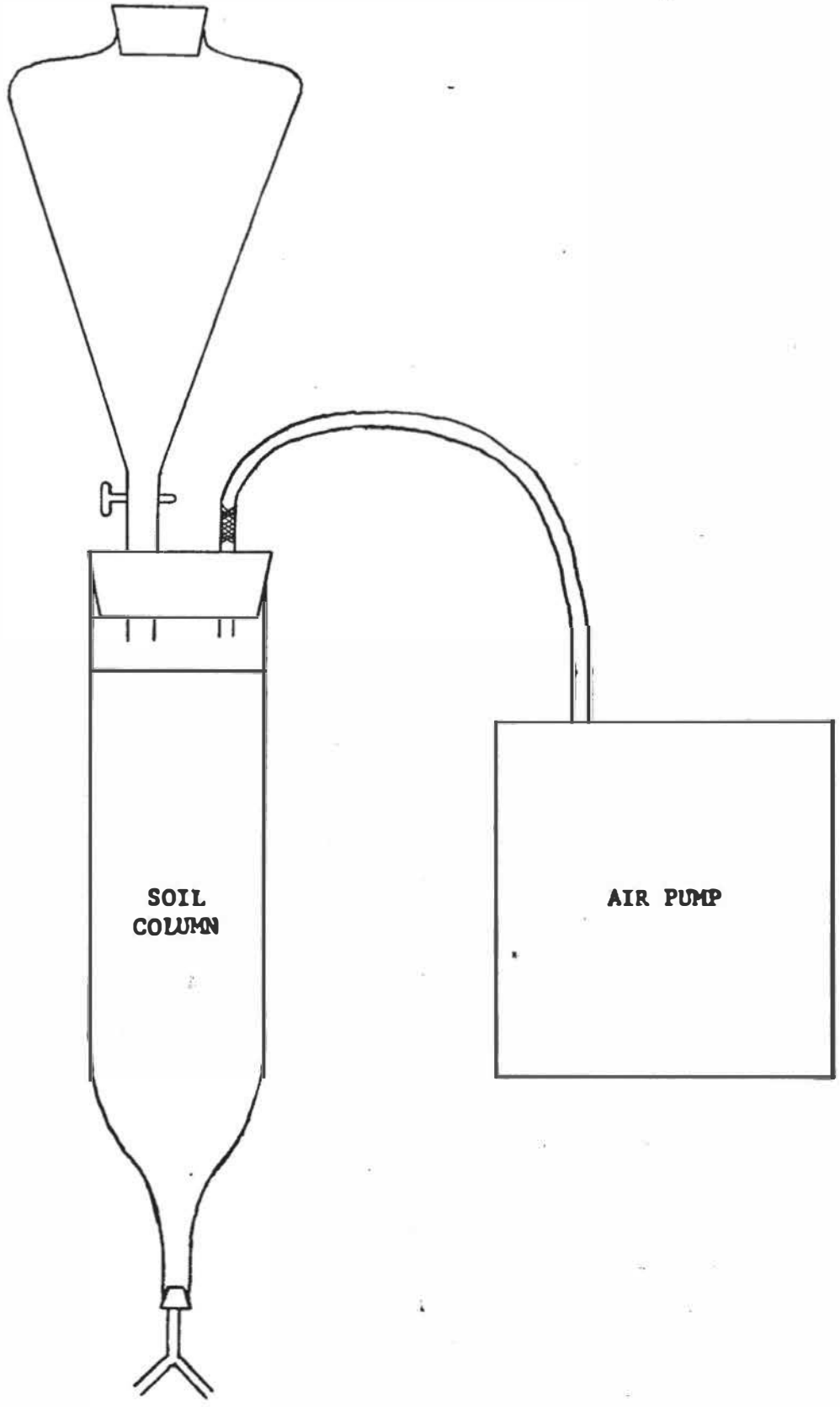
Materials and Methods

Column Preparation

The soil perfusion apparatus (Figure 1) used during the experiment consisted of a 500 ml separatory funnel at the top which emptied into a glass column filled with soil. The column was 36 cm long and 5.5 cm in diameter, tapered to 1.5 cm at the bottom, and covered with black tape up to the soil surface to protect against unnatural algal growth. The top of the column was fitted with a rubber stopper which had two holes bored through it. One hole was fitted with a glass tube to pump filtered air through the column to aerate the soil. The other was fitted with the drain tube of the separatory funnel so that the liquid added to the funnel would drip directly onto the surface of the soil. The bottom of the soil column was fitted with a release valve which could be opened or closed at will to facilitate the collection of the effluent. The entire apparatus was kept in a constant temperature room (26C) to eliminate variability due to temperature change. Once during the course of experimentation, the temperature rose to 28C.

This apparatus differed from that described by Audus (1946) and Lees and Quastel (1946) in that the nutrient solution was added to the apparatus on a weekly basis whereas the original apparatus kept the solution in constant circulation through the column. An attempt at the latter method was terminated because of technical difficulties and

FIGURE 1. Soil perfusion apparatus.



because of a desire for a more realistic method of applying the liquid to the soil. The change was made so that use of a pump was eliminated and so that weekly tests could be made on the effluent, simulating rainfall.

The column of soil was prepared by layering (bottom to top) large and small stones, sand, and soil, thus insuring good percolation of the solution through the soil. The bottom 6 cm of the 24 cm soil column was composed of large stones and glass wool. Next came a 6 cm layer of small stones and gravel followed by a 6 cm layer of sand. The next 3 cm was composed of a mixture of 50% sand and 50% field yellow-gray silt loam soil. The top 3 cm was composed entirely of loam soil. All materials used inside the column, excluding the soil itself, were sterilized so that only typical soil bacteria would be present in the column.

Column Operation

Weekly operation of the column involved first soaking the release valve at the base of the column with 70% ethanol to kill all external bacteria that may have been present which might invalidate plate counts. Five hundred ml of diluted (1-25) inorganic plant nutrient solution (Nicholson, 1970, Table 7) was added to the separatory funnel. Dilute nutrient solution was used in place of water since repeated applications of water might tend to leach all available nutrients out of the soil and present a limiting factor for bacterial growth. The concentration of the nutrients in the solution was controlled so that nutrients collected over and above the amount present in the nutrient solution could be attributed to the microbes of the soil or to normal leaching. The solution was allowed to drip onto the soil from the separatory

TABLE 7. Components of nutrient solution diluted with one liter of water.

$Ca(NO_3)_2 \cdot H_2O$	0.708g
KH_2PO_4	0.272g
KNO_3	0.202g
$MgSO_4 \cdot 7H_2O$	0.493g
$FeCl_3 \cdot 6H_2O$	0.024g
EDTA	0.10g
H_3BO_3	0.005g
$MnCl_2 \cdot 4H_2O$	0.003g
$ZnCl_2$	0.0002g
$CuCl_2 \cdot 2H_2O$	0.0001g
MoO_3	0.00008g

funnel at a rate of about 40 ml per minute. The rate of flow was such that solution rarely accumulated at the soil surface. The first 75 ml collected at the release valve was discarded. Discarding this portion of the effluent assured the collection of organisms picked up from the soil and not the organisms that may have settled or grown at the base of the column. The next 400 ml (or so) of effluent was collected in a sterile flask.

Microbiological Plate Counts

An estimation of the viable numbers of bacteria in the effluent was accomplished by using standardized plate counts. The effluent solution was diluted by pipetting one ml of the well-mixed effluent into a 99 ml distilled, deionized water dilution blank with a one ml pipette. One ml of this dilution was then placed into each of ten sterile Petri plates using a 5 ml pipette (1-100 dilution plating). One to a thousand dilutions were similarly made by depositing 0.1 ml of the soil effluent dilution into each of ten sterile plates using a one ml pipette calibrated to 0.01 ml. Twenty ml nutrient agar (Difco, 1953) melted and equilibrated at 50C was added to five plates at each dilution and twenty ml half strength nutrient agar (1.5% agar) was added to the remaining plates. Full and half strength media were used to minimize the selective effects of high peptone media on the soil bacteria (Salle, 1967). Colony counts were made at 4X magnification on a Quebec Darkfield Colony Counter after 24 and 48 hours of incubation at 30C.

Nutrient agar was chosen because it appeared to be the least specific, yet compositionally-controlled, growth medium available. It was chosen over PCA, another available enumeration medium, because

nutrient agar produced higher or equivalent plate counts in early experimentation. It is conceded that nutrient agar is not the perfect growth medium for culturing soil bacteria, but it was used because of the lack of a better one. Soil extract agar (Salle, 1967) has been suggested specifically for the purpose of enumerating soil bacteria, but was not used because of the inconsistancy of constituents associated with it and the difficulty in its preparation (Salle, 1967).

Nutrient Analysis

The concentration of inorganic chemical nutrients present in the soil effluent was determined by the Hach method for water analysis. Reagents were supplied by the Hach Chemical Company (Ames, Iowa). Procedures outlined by Hach Chemical Company were followed for nitrate, nitrite, ammonia, sulfate, and hydrogen sulfide tests. pH was determined on a Fisher Accumet pH meter. Nutrient production levels were determined on a DR-2 spectrophotometer and were compared with those obtained from testing the diluted nutrient solution directly. The difference reflected the amount of each nutrient produced within the column and leached into the effluent.

Control and Pesticide Application

Six months of testing demonstrated the idiocyncracies of the soil perfusion apparatus. The system was modified several times to assure relatively constant bacterial populations and nutrient production each week. Experimental protocol was followed each week so that natural variability in plate counts and nutrient concentrations could be held to a minimum. This was necessary so that when the pesticide was finally added, any change in the microbial population or nutrient concentration could be attributed to the presence of the pesticide.

A ten week control period following experimental protocol was followed by the application of a hormone herbicide. 2,4-D (2,4-dichlorophenoxyacetic acid) was added to the column at a level that would simulate an application of four pounds per acre (Breazeale and Camper, 1970). After the addition of 2,4-D, nutrient and bacterial levels were followed for four weeks to trace the effect of the herbicide in the soil. Herbicide application and subsequent effluent analysis occurred on three successive occasions.

RESULTS AND DISCUSSION

In order to assure reproducibility of the results of these experiments, the procedure involving the operation of the soil perfusion apparatus was meticulously controlled. All environmental conditions were kept constant and testing procedures were made as accurately as equipment permitted. To demonstrate the accuracy of the bacterial plating-counting technique, early results were sampled randomly to obtain an indication of the expected deviation in plate counts. Table 8 shows the efficiency of the plating procedure to be an average of $\pm$12% with range of $\pm$5 to $\pm$25%.

Two types of commercially available growth media (nutrient agar 1X and 0.5X) were used and both yielded different bacterial counts after 24 and 48 hours of incubation. Table 9 shows that full strength nutrient agar in most cases yielded the highest numbers of colonies. Thus, plate count data presented in this study was that from full strength nutrient agar after 48 hours of incubation.

With plate count procedure standardized, environmental variability had to be eliminated to further reduce variability in the column. Early testing showed that changes in temperature had definite effects on the bacterial population of the column so the column was maintained in a dark, constant temperature room. Figure 2 shows the temperature of the column and pH readings on the effluent throughout the experiment. Both variables were kept under control throughout the experiment except for

TABLE 8. Efficiency of plating.

Plate counts of ten different weeks in quintuplicate.

	I	II	III	IV	V	VI	VII	VIII	IX	X
	289	168	155	282	165	396	131	234	260	898
	292	194	151	280	180	360	130	257	227	982
	284	163	148	250	199	406	104	254	204	894
	310	157	139	296	236	432	124	374	278	942
	272	156	146	256	189	472	127	273	242	968
$\bar{X}$ =	289	168	148	273	194	413	123	278	242	937
R =	38	38	16	46	71	112	27	140	74	88
$\frac{R}{2}$ =	19	19	8	23	36	56	14	70	37	44
±% =	7	11	5	8	19	14	11	25	15	5

Average deviation in mean counts (range 100 to 1000) is ± 12.0%

TABLE 9. A comparison of plating media and incubation time on bacterial counts of the soil perfusion effluent.

	Hours of Incubation at 30C			
	24		48	
Sampling	0.5X N.A.[a]	1X N.A.	0.5X N.A.	1X N.A.
1	1.25	1.58	4.31	4.46
2	0.85	0.94	4.15	4.06
3	1.69	1.67	3.68	3.84
4	1.64	1.65	3.07	3.14
5	0.76	0.84	3.05	3.08
6	0.82	0.88	3.08	3.02
7	1.05	1.15	3.87	3.92
8	0.19	0.27	1.40	1.37
9	1.04	1.12	3.93	4.04
10	0.74	0.66	2.86	3.00

[a] "Standard plate counts" ($X10^4$) of effluent diluted in distilled water. Counts reported were made from plates containing 40 to 400 colonies.

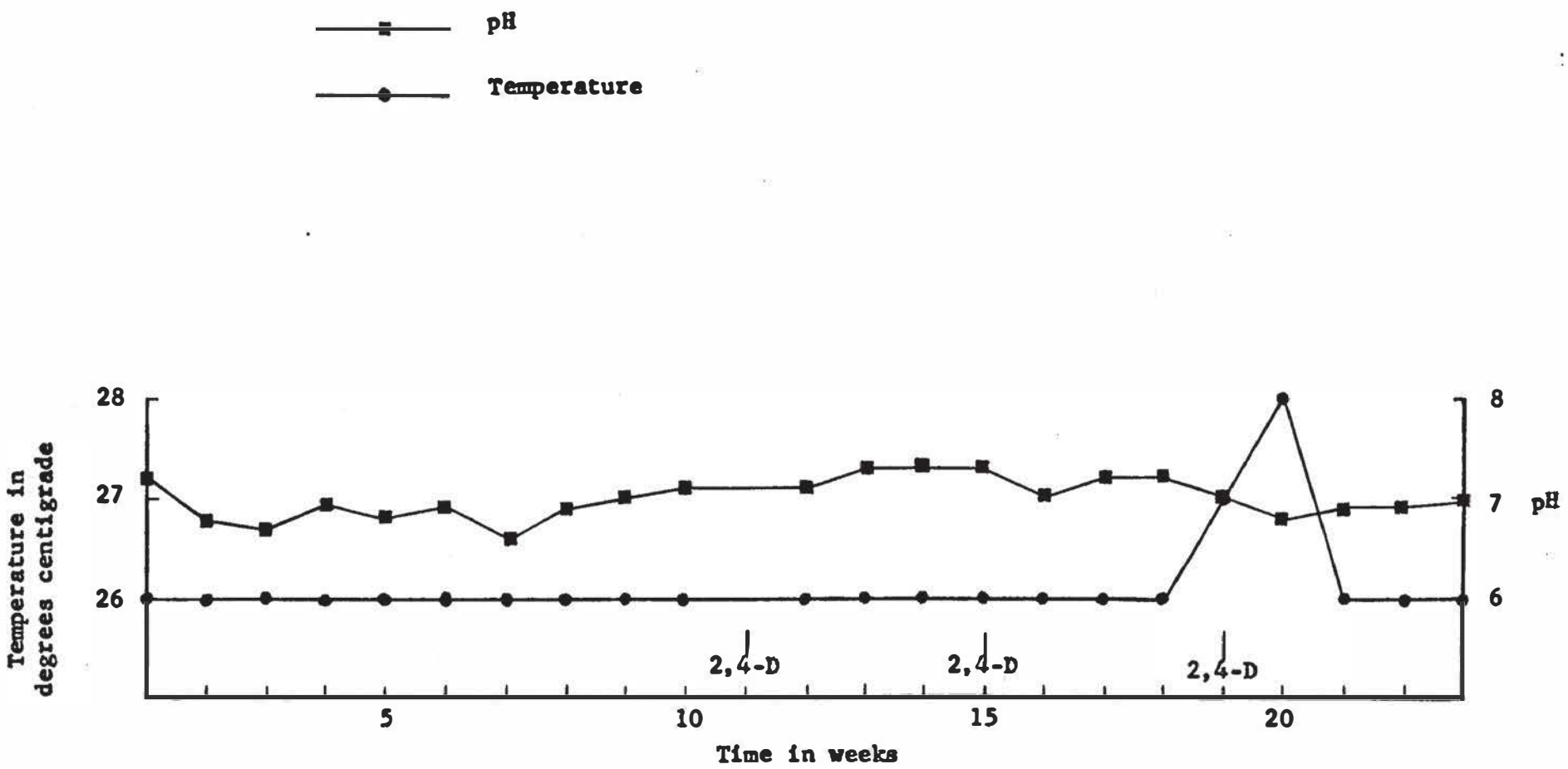


FIGURE 2. Variation in temperature of the column and pH of the effluent.

the temperatures recorded at weeks 19 and 20. A malfunction in the thermostat caused this increase.

With the establishment of procedural protocol and the control of environmental variation, the effects of 2,4-D on the soil ecosystem can be inspected. Figure 3 shows the "standard plate counts" of the soil effluent during the course of the experiment. The ten week trial period (weeks 1-10) yielded relatively constant plate counts exhibiting a range from 1.4 to 4.4 X 10^4 viable cells per ml. The addition of 2,4-D at normal field rates (weeks 11, 15, and 19) created wide weekly variation in counts ranging from 1.4 to 11.9 X 10^4 cells per ml. The trend seemed to indicate an immediate (within one week) decrease in bacterial population followed by a drastic increase in counts and then a return to more normal levels. This sequence of events occurred following the first and second addition of 2,4-D. The third addition of 2,4-D appeared to cause the population to increase, but this may have been due to the increase in temperature which occurred at this time. These results suggest that the herbicide 2,4-D has bactericidal and/or bacteristatic effects on the heterotrophic bacterial population of the soil when applied at normal field rates.

These data demonstrate that 2,4-D does indeed have detrimental effects on the bacterial populations of the soil, but what organisms does it affect and how does it affect inorganic mineral production? Sulfur in the forms of hydrogen sulfide and sulfate in the effluent was followed weekly. Figure 4 shows the molar concentration of hydrogen sulfide and sulfate. (In essence, all sulfur was present as sulfate.) Great variability was present throughout the stabilization period (weeks 1-10) and little predictable change was noted upon the

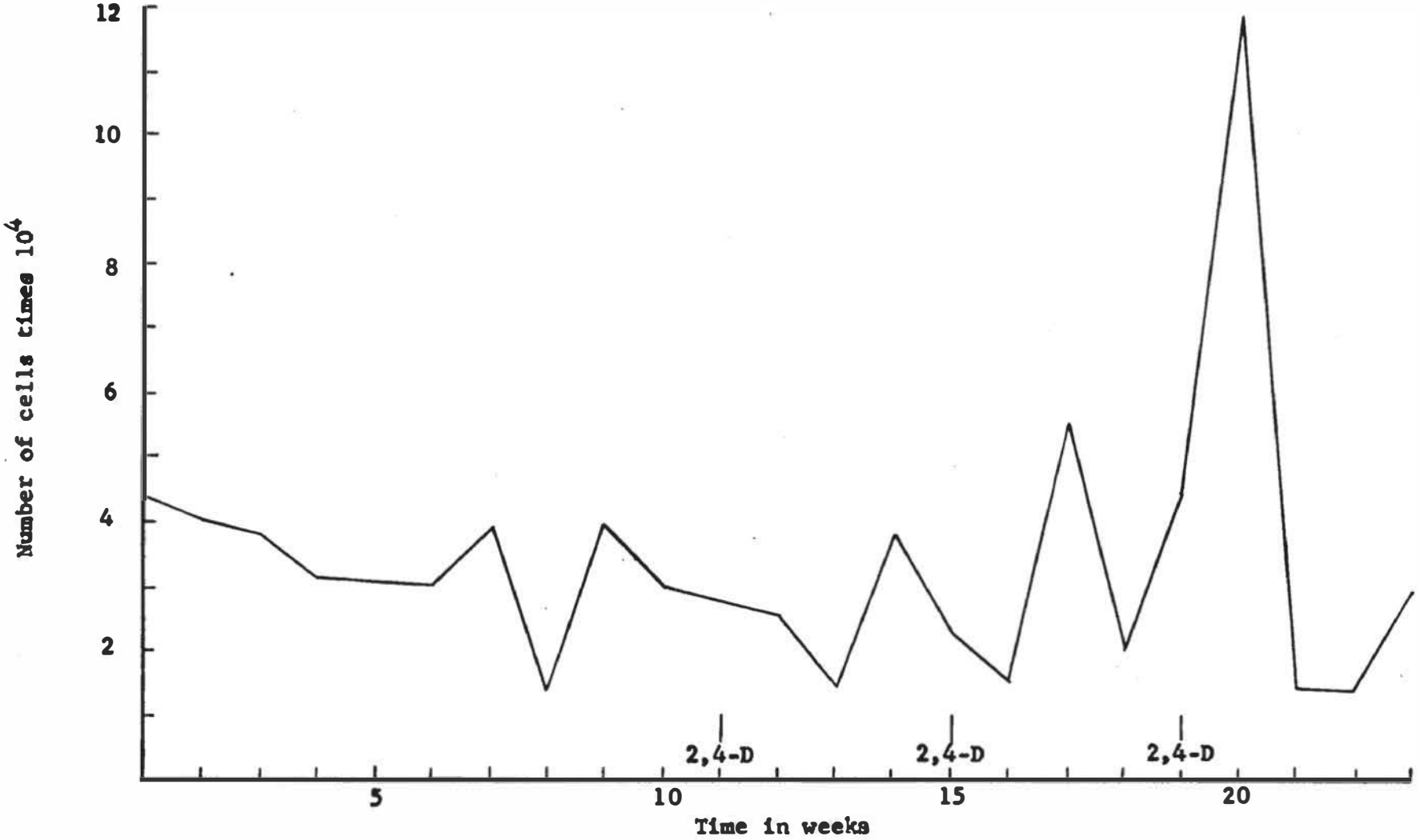


FIGURE 3. "Standard plate counts" of soil effluent. (Incubated 30C/48 hours, 1X nutrient agar)

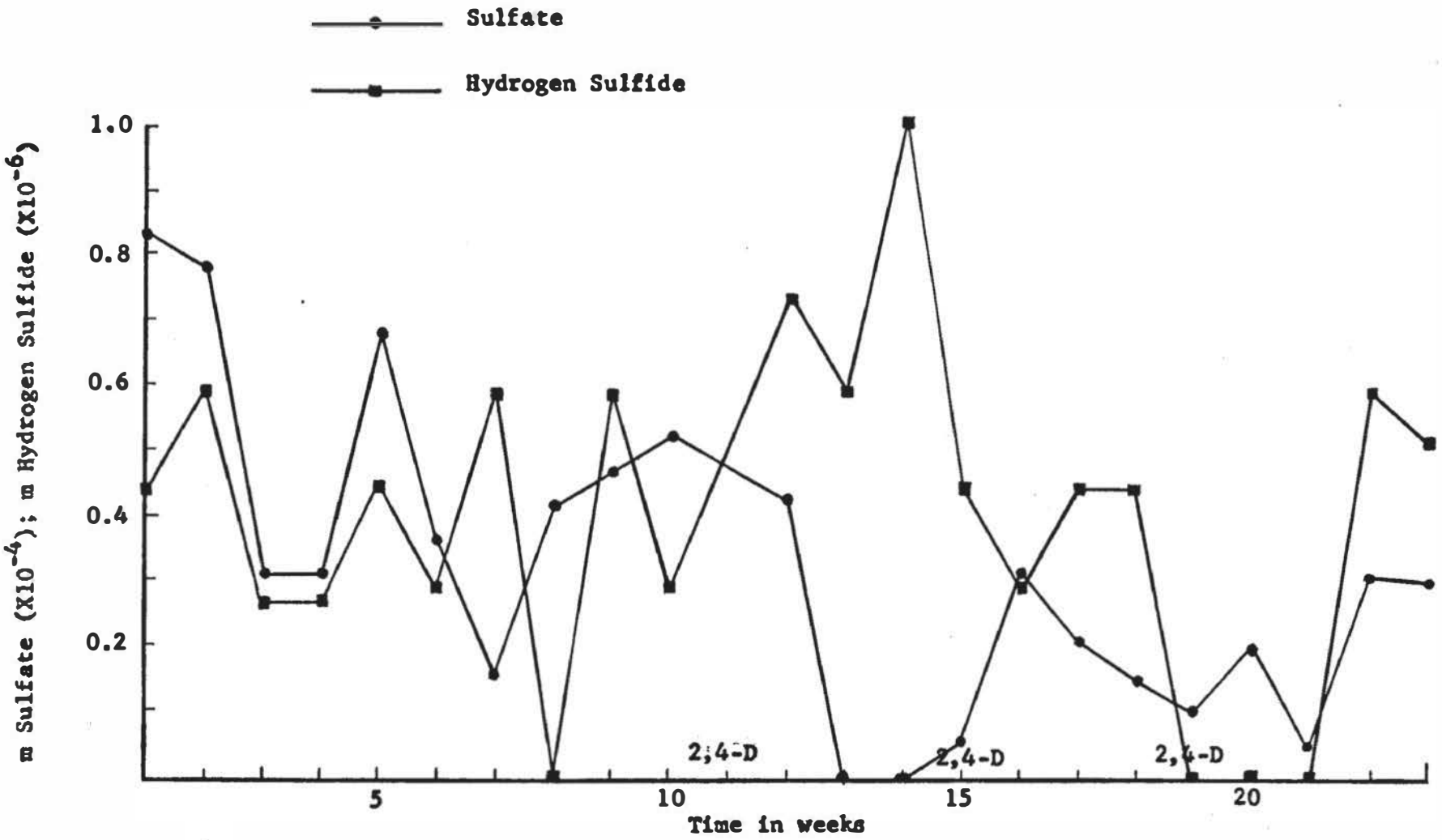


FIGURE 4. Inorganic forms of sulfur in soil effluent.
(Production of hydrogen sulfide and sulfate above that of the perfusion solution)

addition of 2,4-D. It did appear, however, that 2,4-D may have inhibited the oxidative processes of some sulfur oxidizing chemoautotrophs since sulfate concentrations always decreased after the application of 2,4-D. This can be considered no more than speculation since there was no consistency at any time during the stabilization period and since there appears to be no apparent correlation between sulfate and/or hydrogen sulfide concentration and the bacterial population (Figure 3).

Nitrogen (as ammonia, nitrite, and nitrate) in the effluent was monitored weekly. Figure 5 shows the variation in moles of ammonia, nitrate, and total moles of nitrogen (ammonia+nitrate) produced in the column throughout the course of the experiment. Nitrite was not included in this figure since virtually the same amount of nitrite was produced every week and did not appreciably alter the total nitrogen levels. The nitrogen levels during the ten week stabilization period appeared to be much more consistant than the sulfur readings for the same period. A high level of nitrogen occurred at week 8 when a significant decrease in bacterial population was noted. When 2,4-D was added at weeks 11, 15, and 19 the results show a definite decrease in nitrate and total nitrogen a week or two after application, suggesting a decrease in soil nitrification. This discovery was also noted by Slepecky and Beck (1950) when they found that the conversion of ammonia nitrogen to nitrate nitrogen was completely inhibited by solutions containing 50 parts per million 2,4-D. However, continuous percolation of the 2,4-D solution through the soil resulted in a reappearance of nitrification. Although continuous percolation was not used in this experiment similar results were observed. When nitrogen levels were correlated with bacterial population changes, it appeared that as the

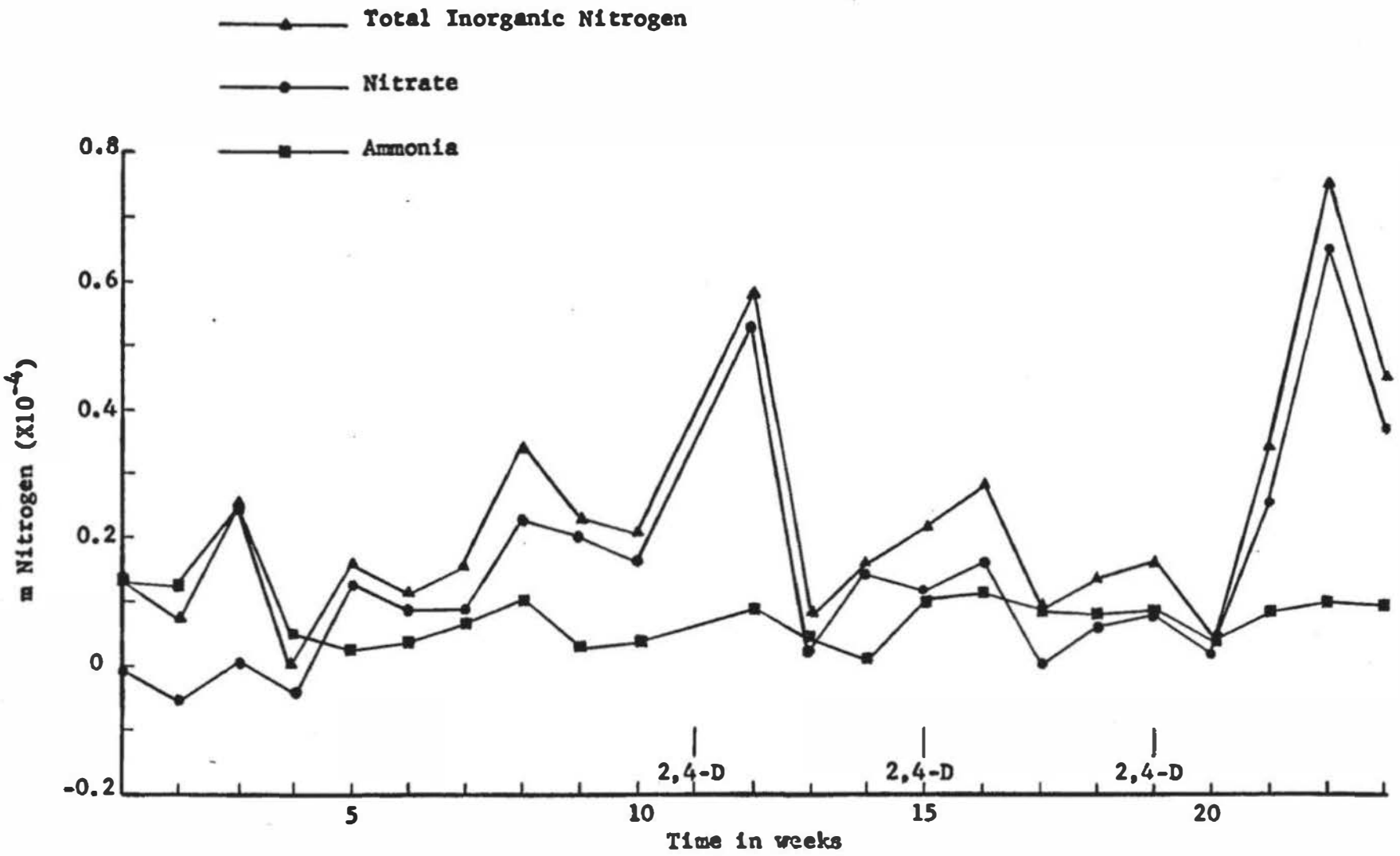
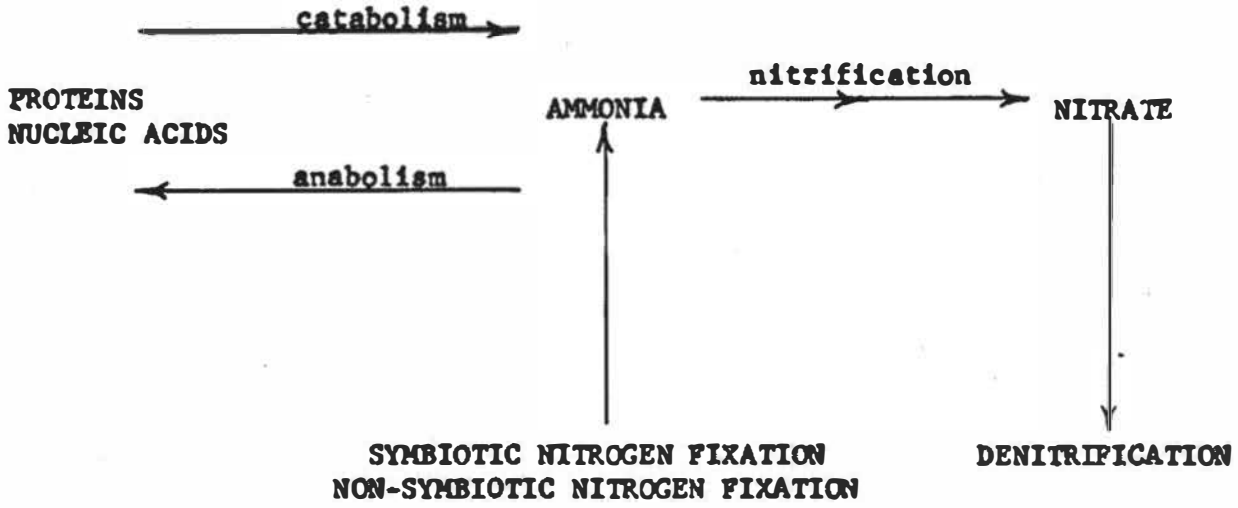


FIGURE 5. Inorganic forms of nitrogen in soil effluent.
(Production of ammonia and nitrate above that of the perfusion solution)

bacterial population decreased (one week after 2,4-D application), nitrate and total nitrogen levels tended to increase. Thereafter, the bacterial population increased and the nitrate and total nitrogen levels tended to decrease to a point where all soil nitrogen was present as ammonia (weeks 13, 17, and 20). The reason for this phenomenon is not clear. Lees and Quastel (1946) demonstrated that the rate of nitrification is influenced by temperature, ammonia concentration, and the base-exchange capacity of the surface soil. An unmentioned factor that may affect the level of nitrates in soils is the composition of the microbial population. Although the chemoautotrophic nitrifiers do not compete directly with the heterotrophs of the soil ecosystem for carbon sources, they may compete with one another for their nitrogen source and/or microhabitat. Thus cause and effect relationships are difficult to discern from the data collected in this experiment. For example, is the decrease in nitrate levels and total nitrogen a result of inhibition of the nitrifiers or a result of an increase in ammonia and nitrate utilizing heterotrophs?

Consider what may be happening in the soil column (Figure 6). We can eliminate from consideration symbiotic nitrogen fixation by Rhizobium species and non-symbiotic nitrogen fixation by the anaerobic genus Clostridium due to soil conditions. In addition, denitrification (an anaerobic process) can be disregarded. Thus decreases in ammonia may be due to increases in biosynthesis of nitrogenous organic compounds or to increases in nitrification. Increases in nitrate levels may be the result of increases in nitrification and/or increases in ammonia levels due to the increase in degradation of organic nitrogen-containing compounds.

FIGURE 6. The nitrogen cycle.



In essence, there are two possible series of events that could explain the data collected. They are described as follows:

1. a. At 2,4-D application population and nitrogen levels are normal.
   b. After one week, 2,4-D reduces bacterial population slightly and at the same time total nitrogen and nitrate levels increase since the nitrifiers are more resistant to 2,4-D than most of their competition. Also with decreased populations more ammonia can be channeled toward nitrification since the rate of transformation of ammonia to cellular parts has decreased.
   c. After two weeks much of the 2,4-D has been decomposed or leached out resulting in increased heterotrophic populations. As a result, the nitrifying bacteria encounter increased competition for ammonia by heterotrophic populations. Thus, most ammonia in the column is preferentially channeled toward biosynthesis of cell parts.
   d. After three weeks both bacterial populations and nitrogen levels return to normal.

2. a. At 2,4-D application population and nitrogen levels are normal.
   b. After one week 2,4-D reduces bacterial population somewhat and may stimulate nitrate production.
   c. After two weeks bacterial populations drastically increase and nitrate levels decrease due to the delayed effects of 2,4-D on the nitrification process.
   d. After three weeks both bacterial populations and nitrogen levels return to normal.

Both of these theories are worthy of consideration. The inversely correlated nitrogen levels and bacterial population levels tend to support theory one, but the fact that the nitrate level decreased to zero two weeks after application while there was still free ammonia present in the column supports theory two (Figure 5).

In general, these data show that nitrification of soils is inhibited by field rate applications of 2,4-D either by an altered flow of available ammonia molecules due to changes in the bacterial population or because of blockage of the normal nitrification process.

DISCUSSION AND CONCLUSIONS

From the data received from these experiments certain conclusions can be made.

1. Of the pesticides tested the hormone herbicides are the most inhibitory to bacteria.

2. Gram positive bacteria are more sensitive to pesticides than Gram negative bacteria.

3. The inhibitory tendancies of the pesticides may be only temporary (i.e., bacteristasis).

4. At normal field application rates, 2,4-D decreases bacterial populations as well as the amount of nitrification.

5. The decreased nitrification may be due either to the alteration of bacterial populations in the soil or to the inhibition of the nitrification process directly.

Until now there have been conflicting reports concerning the effects of 2,4-D on the soil bacteria and their metabolic processes. The data presented in this paper definitely shows that bacterial populations and nitrification are adversely effected by 2,4-D. What remains to be done is to determine how the pesticide inhibits bacterial growth and what causes the rate of nitrification to decrease following 2,4-D application.

Because of the success experienced with this method of testing the effects of chemicals on soil microflora, it seems that, in the interest of conservation as well as agriculture, similar tests should be performed on all agronomic chemicals to determine their potential harmful properties to the soil ecosystem.

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