THE EFFECT OF SELECTED HERBICIDES ON ANAEROBIC NITRATE AND NITRITE REDUCTION

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By

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TO GAIL

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ABSTRACT

Herbicides are known to affect a variety of microbial processes. This study examined the effect of diclofop-methyl and trifluralin, and their respective commercial formulations, HoeGrass and Treflan™, on anaerobic nitrate and nitrite reduction in a shaken flask system containing 20 g of a sandy or loamy soil. The study also examined the effects of the herbicides in soil amended with 100 μg glucose g^{-1}. To minimize the differences in the soil microbial populations between the soils, a stock microbial culture, isolated from fresh soil, was used as an inoculum. The maximum rates of anaerobic nitrate and nitrite reduction were determined by measuring the concentration of nitrate and nitrite in the soil solution at various times by high performance liquid chromatography.

The maximum nitrate reduction rates ranged from 1.0 ± 0.1 μg NO_3-N g^{-1} h^{-1} in the sand to 23.8 ± 2.2 μg NO_3-N g^{-1} h^{-1} in the glucose-amended loam. Biphasic anaerobic nitrate reduction also occurred in the sand; the initial rates were approximately 1/2 the final rates. Diclofop-methyl and HoeGrass™ had a variable effect on the nitrate reduction rates. In both soils, nitrate reduction was increased by the 10 μg g^{-1} diclofop-methyl treatments. The 100 μg g^{-1} HoeGrass™ treatments decreased the nitrate reduction rates in the sand, in the absence and presence of glucose. Nitrate reduction rates were generally not affected by both treatments of trifluralin or Treflan™. The time for the complete disappearance of nitrate was generally increased for the 100 μg g^{1} treatments; the largest increase was produced in the loam with HoeGrass™ treatment.

Nitrite reduction rates ranged from 0.86 ± 0.03 to 4.03 ± 0.70 μg NO_2-N g^{-1} h^{-1}. Nitrite reduction rates were not affected by the 10 μg g^{-1} diclofop-methyl or HoeGrass treatments. At the highest treatment level, 100 μg g^{-1}, the nitrite reduction rates decreased for all soils except the glucose-amended sand. HoeGrass™ appeared to have a greater effect than diclofop-methyl at reducing the rate of nitrite reduction. Nitrite reduction rates were variously affected by all trifluralin and Treflan™ treatments. For the 10 μg g^{-1} treatments, the time for the complete disappearance of nitrite was variously affected by diclofop-methyl or HoeGrass™ and was increased in both non-amended soils by trifluralin and Treflan™. At 100 μg g^{-1} all herbicide treatments increased the disappearance times, with the greatest increases usually observed for the Hoegrass™ treatments.

An examination of the reduction rates and disappearance times for nitrate and nitrite showed that 1) nitrite reduction was more sensitive than nitrate reduction to the presence of the herbicides, 2) any inhibition of nitrate and nitrite reduction by the presence of the herbicides could often be alleviated by glucose amendment, and 3) the technical and commercial formulation often produced different effects.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURES (cont'd) PAGE

FIGURES (cont'd)

LIST OF TABLES

TABLES (cont'd) PAGE

HISTORICAL

I. Effects of Herbicides on Denitrification

The importance of the effects of pesticides on microorganisms is shown by the large number of publications available, and the effects are thoroughly outlined in numerous reviews and texts such as Kaiser *et al.* 1970, Cullimore 1971, Parr 1974, Tu and Miles 1976, Anderson 1978, Wainwright 1978, McCann and Cullimore 1979, Lal and Saxena 1980, Rodriguez-Kabana and Curl 1980, Anderson 1983, Goring and Laskowski 1982, Smith 1982, and Domsch *et al.* 1983. The pervasive attitude in the literature is that pesticides applied at recommended field rates, have little or no effect on the activities of the soil microflora (Anderson 1978). However, the contributions of the microflora to soil fertility cannot be precisely assessed and quantified (Grossbard 1976) and therefore it is difficult to quantify the effects of pesticides on soil fertility or the microbial population (Greaves and Malkomes 1980).

Owing to the immense variation possible in both the microbial population and the physical and chemical characteristics of the soil, it is not uncommon to find apparently contradictory information in the literature. In fact, Marsh and Davies (1981) were unable to replicate one of their experiments and speculated that it was due to differences in the characteristics of the soil, namely the moisture content at the time of sampling. Probably one of the greatest obstacles in obtaining consistent or pertinent data is also the unavailability of reliable testing procedures (Greaves and Malkomes 1980, Smith 1982).

The effects of pesticides on denitrification has not been studied as extensively as other microbial activities and probably reflects the

difficulty in studying the denitrification process (Firestone 1982, Goring and Laskowski 1982). Anderson (1978) reviewed approximately 500 publications published between 1964-76, on the effect of pesticides on non-target soil microorganisms. Only 23 publications, detailing some 40 herbicides, were reviewed on the effects of herbicides on denitrification in soil. He concluded that herbicides generally increased denitrifying populations and/or activity. There were insufficient publications on the effects of insecticides and fungicides to warrant any conclusions. The author also provided a detailed tabulated summary of the publications, listing the herbicide, soil types, and effects on denitrification.

In recent years, the denitrification method favored by the majority of investigators, i.e., the few investigators studying pesticide effects on denitrification, has been the direct measurement of the gaseous products evolved during denitrification by gas chromatography (Bollag and Henninger 1976, Mills 1984, Yeomans and Bremner 1985). In these laboratory studies, approximately 20 g of soil is treated with the pesticide and incubated, non-shaken, in a helium atmosphere, usually with a final soil/water ratio of 2:1. Grant and Payne (1982) utilized the acetylene inhibition technique (Balderston *et al.* 1976, Yoshinari and Knowles 1976) to study denitrification in salt marsh sediments. Acetylene inhibits the reduction of nitrous oxide to dinitrogen, and the amount of nitrous oxide evolved can be used as a measure of denitrification. In place of acetylene, a bacterium which produces nitrous oxide as the end-product of denitrification can also be used (Bollag and Nash 1974, Henninger and Bollag 1976).

Other techniques involving the enumeration of denitrifiers, either plate counts or most probable number (MPN) method (Grant and Payne 1982), are not popular. Plate counts are only suitable for following population trends of a singular culture. The MPN method can provide an index of the denitrifying capacity of the microbial population but the technique is time consuming, requires a large number of tubes for precise estimates (Alexander 1982), and is very sensitive to variations in incubation conditions (Davidson *et al.* 1985).

Although not classified as herbicides, some nitrification inhibitors have been found to be potent inhibitors of denitrification. Henninger and Bollag (1976) studied the effect of the nitrification inhibitors 2-amino-4-chloro-6-methyl-pyrimidine, 4-amino-1,2,4-triazole, 2-chloro-6-(trichloromethyl)-pyridine (N-Serve), 2,5 dichloroaniline, phenylmercuric acetate, potassium azide, and 2-sulfanilamidothiazole on denitrification in soil and by a bacterial soil isolate, identified as a *Pseudomonas* sp. The disappearance of nitrate and accumulation of nitrous oxide were used as indicators of denitrification. The authors found that 25 $\mu g\ g^{-1}$ N-Serve inhibited denitrification by the *Pseudomonas* sp. for the first 4 days and at 50 $\mu g\ g^{-1}$, inhibition was still observed after 8 days. An accumulation of nitrite in the culture medium was observed in the presence of 50 $\mu g\ g^{-1}$ 2,5-dichloroaniline. Except for potassium azide, the nitrification inhibitors had no effect in a silt loam. Potassium azide, which had no effect on the nitrous oxide producing *Pseudomonas* sp., inhibited the reduction of nitrous oxide to nitrogen in the silt loam.

After 14 d of incubation, nitrapyrin at 0.2-20 $\mu g\ g^{-1}$ has been found to reduce nitrogen and nitrous oxide evolution and to maintain high levels of nitrate in a liquid medium inoculated with soil (McElhannon and Mills 1981). After 24 h of incubation in a liquid nutrient medium, 2 $\mu g\ mL^{-1}$ nitrapyrin reduced the amount of N_2 and N_2O evolved by 7.5% and 88.7% respectively (Mills 1984). In a 50:50 sand-soil mixture grown to *Zea mays* (corn) for 56 days, a 2 $\mu g\ g^{-1}$ application of nitrapyrin increased soil nitrate and total plant nitrogen. In another soil culture study, after 72 h, 2.0 $\mu g\ g^{-1}$ of nitrapyrin reduced nitrous oxide evolution by a factor of 4 (McElhannon *et al.* 1984). In greenhouse studies, 0.5 $\mu g\ g^{-1}$ of nitrapyrin increased soil nitrate 8.4 fold and slightly increased total plant nitrogen. Mills and McElhannon (1984) also found that terrazole at 0.2 to 200 $\mu g\ g^{-1}$ suppressed nitrogen and nitrous oxide evolution in a liquid medium. In a greenhouse study, a clay soil treated with 0.5 and 2.0 $\mu g\ g^{-1}$ of terrazole had increased levels of nitrate.

The degradation product can have a different effect on the soil microbial population than the parent compound. Chlordimeform (insecticide) concentrations up to 100 $\mu g\ g^{-1}$ have been found to have no effect on denitrification (Bollag and Kurek 1980). At 50 $\mu g\ g^{-1}$, the degradation product, *N*-formyl-4-chloro-o-toluidine, also did not affect denitrification. Another degradation product, 4-chloro-o-toluidine, produced a 75% decrease in the amount of nitrate reduced after 36 h and resulted in an accumulation of 4% nitrite and 36% nitrous oxide. Nitrite accumulation was markedly enhanced by increasing the sand content from 15 to 50% or by the addition of 5%

montmorillonite. Since the anilines bind to the soil organic matter, it was hypothesized that with the addition of sand, less organic matter would be available to bind the aniline, thus making it more available to the microorganisms. At 100 $\mu g\ g^{-1}$, metolachlor was found to slightly inhibit denitrification in a silt loam, resulting in an accumulation of nitrite (Bollag and Kurek 1980). The aniline derivatives of chlordimeform, fluometuron, and metolachlor were also more potent inhibitors of denitrification and caused a greater accumulation of nitrite than the respective herbicides.

Bollag and Nash (1974) examined the effect of phenylureas and anilines on denitrification. Nitrous oxide and carbon dioxide evolution from a liquid medium, containing a soil isolate deficient in nitrous oxide reductase, was examined. Chlorbromuron, diuron, fluometuron, linuron, neburon, and siduron, at 50, 100, and 200 $\mu g\ g^{-1}$, retarded nitrous oxide production and slightly increased carbon dioxide production. Fenuron, metobromuron, and monuron produced no inhibition at any of the levels tested. Metoxuron inhibition increased with increasing concentrations studied. Bollag and Nash (1974) found that the inhibition of nitrate reduction was influenced by the number of halogen substituents on the aromatic ring; the inhibition generally increased with increased substitution.

At 1.5-3.0 kg ha^{-1}, fluometuron and methurin (*n*-hydroxy-*n*-methyl-*n*-phenylurea) has been found to decrease denitrifier populations in a serozem-meadow soil (Tulabaev 1972). Fluometuron at 100 $\mu g\ g^{-1}$ inhibited denitrification and enhanced the accumulation of nitrite in a silt loam after 35 h at 30°C (Bollag and Kurek 1980). Torstensson

(1974) found no effect of 2 or 20 kg ha^{-1} of linuron in a sandy or silty clay.

Bollag and Henninger (1976) studied the effect of ametryne, atrazine, 2,4-D, diuron, endothall, hydroxysimazine, linuron, propham, siduron, and simazine on denitrification in both a liquid medium, using a nitrous oxide reductase deficient soil bacterial isolate, and a silt loam (1.8% O.C.). At 100 $\mu g\ g^{-1}$ of diuron, linuron, or siduron, high levels of nitrate remained in Giltay medium after 2 days of incubation at 30°C. In contrast, no effect was observed in the silt loam after 5 d.

Yeomans and Bremner (1985) studied the commercial formulations of alachlor, atrazine, butylate, chloramben, chlorpropham, cyanazine, dalapon, dicamba, dinoseb, diuron, EPTC, linuron, metribuzin, monuron, propham, siduron, simazine, trifluralin, and 2,4-D amine and ester in 3 soils. Except for the increased $N_2/_N20$ ratio, by dinoseb, none of the herbicides, applied at 10 $\mu g\ g^{-1}$, affected denitrification after 8 days of incubation. Siduron and monuron at 50 $\mu g\ g^{-1}$ enhanced denitrification, i.e., increased the total amount of N_2 and N_2O evolved and/or increased the N_2/N_2O ratio, but the effects depended upon the soil studied. Linuron at 50 $\mu g\ g^{-1}$ inhibited denitrification and the inhibition was also soil specific. Diuron had no effect on denitrification. In contrast, Rolston and Cervelli (1980) reported that 30 $\mu g\ g^{-1}$ diuron inhibited denitrification in soil. Propham at 100 $\mu g\ g^{-1}$ has been found to inhibit denitrification in culture but not in a silt loam (1.8% O.C.) (Bollag and Henninger 1976).

At 10 $\mu g\ g^{-1}$, formulated dinoseb has been found to increase the N_2/N_2O ratio in all 3 soils studied (Yeomans and Bremner 1985). The authors also found that in the 3 soils, 50 $\mu g\ g^{-1}$ of formulated alachlor, chloropropham, dicamba, or propham either enhanced denitrification or had no significant effect on denitrification, but increased the N_2/N_2O ratio. Inhibition of nitrite reduction was found to only occur in the soil with the lowest organic carbon content (0.5%); from 14-43% of the NO_3-N lost was recovered as NO_2-N for the 50 $\mu g\ g^{-1}$ treatments of metribuzin, propham, trifluralin, diuron, monuron, linuron, and chlorpropham.

At 100 $\mu g\ g^{-1}$ 2,4-D, approximately 29% of the initial nitrate remained in a silt loam (1.8% O.C.) after 5 days of incubation, whereas in a pure culture study approximately 16% remained after 2 d (Bollag and Henninger 1976). Manometrically measured gas production by liquid cultures, with nitrate as the electron acceptor, has been found to be completely inhibited by 7.5 μmoles mL^{-1} of 2,4-D (Hart and Larson 1966). No inhibition of gas production occurred with a nitrite medium. After 65 h of incubation, 10 $\mu g\ g^{-1}$ of the commercial formulation of 2,4-D, reduced nitrous oxide production by 36% in salt marsh sediment at the 65 h sampling, but the inhibition disappeared at the 72 h sampling (Grant and Payne 1982). Concentrations of 100 and 1000 $\mu g\ g^{-1}$ were able to inhibit denitrification at both the 65 and 72 h sampling.

Yeomans and Bremner (1985) found that with 50 $\mu g\ g^{-1}$ of commercially formulated 2,4-D amine or ester, denitrification was slightly enhanced and the ratio of N_2 to N_2O increased in a silty clay (6.6% O.C.) and a silty clay loam (0.5% O.C.), but no effects were

observed in a clay loam (3.3% O.C.). Enhancement of denitrification has been also observed in a low organic matter chernozem soil with 1.5 kg ha^{-1} (Tyagny-Ryadno 1967) and in a liquid medium with 25 $\mu g\ g^{-1}$ of 2,4-D (Sethunathan 1970).

Grant and Payne (1982) studied the commercial formulation of dalapon. After 65 h, 10 $\mu g\ mL^{-1}$ of dalapon produced a 94% decrease in the amount of nitrous oxide produced in a salt marsh sediment. At 100 $\mu g\ mL^{-1}$, dalapon was completely inhibitory after 72 h. A loamy soil treated with 10 $\mu g\ g^{-1}$ of Dowpon™ (dalapon) was shown to have 75.0% more nitrate present after 6 weeks and 81.8% more after 12 weeks (Weeraratna 1980). In contrast, Yeomans and Bremner (1985) found no effects on denitrification in 3 soils by 10 or 100 $\mu g\ g^{-1}$ of formulated dalapon.

Grant and Payne (1982) studied the effect of commercial dalapon and the active ingredient, 2,2-dichloropropionic acid, on nitrate and nitrite reduction by *P. perfectomarinus* in a liquid medium. At 100 $\mu g\ mL^{-1}$, the rates and disappearance times of nitrate were not affected, but nitrite concentrations were greater and nitrite presisted longer in the 2,2-dichloropropionic acid treated cultures. No effect was observed in the dalapon treated soil. At 250 $\mu g\ mL^{-1}$, the commercial formulation retarded nitrate and nitrite reductions, but the dichlorinated acid was still more effective.

The effects of atrazine on denitrification has been extensively studied, and the documented results demonstrate the discord sometimes present in the literature. Atrazine concentrations from 10 to 100 $\mu g\ g^{-1}$ were found to have no effect on denitrification in various soils

(Bollag and Henninger 1976, Yeomans and Bremner 1985). Commercially formulated atrazine at 10 $\mu g\ mL^{-1}$ has been found to increase nitrous oxide concentrations by 51% in salt marsh sediment after 65 h (Grant and Payne 1982). After 72 h, the amount of nitrous oxide evolved by 100 and 1000 $\mu g\ mL^{-1}$ atrazine treated sediments was equivalent to the control sediments. Cervelli and Rolston (1983) studied denitrification in soil columns and found that 3 $\mu g\ g^{-1}$ atrazine also stimulated nitrate reduction about 10% in a silt loam and < 5% in a loam. With atrazine treatment, there was an increase in the total amount of nitrogen and nitrous oxide evolved but a decrease in the N_2/N_2O ratio.

On the other hand, Kuryndina (1965) observed that a 3 y application of 10 $kg\ ha^{-1}$ atrazine depressed the denitrifying bacteria, mold fungi, and ammonifiers. Mills (1984) found that in a liquid nutrient medium, 2 $\mu g\ mL^{-1}$ atrazine or simazine reduced the amount of N_2 and N_2O evolved after 24 h of incubation. In a 50:50 sand-soil mixture grown to *Zea mays*, a 2 $\mu g\ g^{-1}$ application of atrazine or simazine increased soil nitrate and total plant nitrogen after 56 days. McElhannon *et al.* (1984) that found 2.0 $\mu g\ g^{-1}$ atrazine or simazine reduced nitrous oxide evolution in liquid cultures after 72 h, but had no effect on nitrogen evolution. In greenhouse studies, 0.5 $\mu g\ g^{-1}$ of atrazine or simazine increased soil nitrate by 20.5% and 15.3% respectively. Total plant nitrogen was also increased approximately 17% by 0.5 $\mu g\ g^{-1}$ atrazine.

Simazine has been found to have no effects at 2-100 $\mu g\ g^{-1}$ in soil (Peshakov *et al.* 1969, Kuzyakina 1971, Bollag and Henninger 1976, Yeomans and Bremner 1985) and in culture medium (Bollag and Henninger

1976). In contrast denitrification populations have been found to increase, especially at harvest time, with an application of 1.5 kg ha^{-1} to a low organic matter chernozem (Tyagny-Ryadno 1967). An increase in the denitrifying population was also found with 2 and 20 kg ha^{-1} applications to a sandy clay and a silty clay (Torstensson 1974). Simazine decreased the number of denitrifying bacteria with 10 (Kuryndina 1965) and 100 kg ha^{-1} (Kuzyakina 1971) applications. For equivalent simazine concentrations, increases in populations or activities usually appear to be documented in sandy soils rather than the heavier texture soils.

Grant and Payne (1982) studied the commercial formulation of paraquat in salt marsh sediments. At the 65 and 72 h sampling 10 μg mL^{-1} of commercial paraquat increased nitrous oxide evolution approximately 7%. No effect was observed with 100 μg mL^{-1}. The 1000 μg mL^{-1} treatment decreased nitrous oxide evolution ca. 84 and 29% at the 65 and 72 h samplings respectively.

Considering the large number of registered herbicides and the importance of denitrification, the number of publications documenting the effects of herbicides on denitrification or denitrifying bacteria is small. Including the 23 publications reviewed by Anderson (1978), there appear to be less than 50 publications. Of the approximately 185 herbicides listed in Hartley and Kidd (1983), apparently only ca. 43 herbicides have been examined between 1960 and 1986 (Table 1). The majority of the herbicides have been examined or re-examined within the last 10 years, probably as a result of the advances in the methodology for denitrification studies. The conflicting results documented for

Table 1: Herbicides studied from 1960-86 as to their effects on denitrification.

- Acid amide:
  - alachlor
  - metolachlor
  - propanil*
- Aliphatic-carboxylic:
  - dalapon
  - TCA*
- Aromatic-carboxylic:
  - Benzoic acid:
    - chloramben
    - dicamba
  - Phenoxy-carboxylic:
    - 2,4-D
    - 2,4,5-T*
    - MCPA*
  - Phthalic acid:
    - chlorthal-dimethyl*
- Bipyridylium:
  - diquat*
  - paraquat
- Carbamate:
  - Phenylcarbamate:
    - chlorpropham
    - propham
  - Thiocarbamate:
    - butylate
    - EPTC
- Diazines:
  - maleic hydrazide*
  - pyrazon*
- Dinitroaniline:
  - trifluralin
- Phenol:
  - dinoseb
  - PCP*
- Triazine:
  - ametryne
  - atrazine
  - cyanazine
  - hydroxysimazine
  - metribuzin
  - prometryne*
  - simazine
- Ureas:
  - chlorbromuron
  - DCU (dichloralurea)
  - diuron
  - fenuron
  - fluometuron
  - linuron
  - methurin
  - metobromuron
  - metoxuron
  - monolinuron
  - monuron
  - neburon
  - siduron
- Others:
  - sodium chlorate*
  - endothall

* reviewed by Anderson (1978)

some herbicides, appear to be due to numerous factors, such as the use of different soils, the differences in environmental conditions in field studies, and the differences in microbial isolates in laboratory studies. It is therefore difficult to definitively state the effect that a herbicide would have on the denitrification process or the denitrifying population.

II. Effects of Diclofop-Methyl and Trifluralin on Microbial Processes

HoeGrass™ (active ingredient: diclofop-methyl), introduced in 1975 (Spencer 1981), is a post-emergence herbicide used in western Canada for the control of wild oats and other annual grasses. Treflan™ (active ingredient: trifluralin), introduced in 1960 (Spencer 1982), is a pre-emergence herbicide used for the control of grasses and broadleaf weeds in both dicotyledonous and cereal crops. Since the appearance of trifluralin 26 years ago, it has been extensively examined and, except for the denitrification process, the effect of this herbicide on microbial populations and processes has been repeatedly studied. A review of the publications between 1964-76 on the effects of trifluralin/Treflan™ can be found in Anderson (1978). Assuming an unbiased review, nitrification appears to be the microbially mediated activity most frequently studied, probably reflecting the ease of studying nitrification and the importance of nitrogen in agriculture. On the other hand, reported effects of diclofop-methyl/HoeGrass™ on microbial populations or activities are scarce.

Although trifluralin can be degraded under anaerobic conditions by the soil microflora (Parr and Smith 1973), the actual role of trifluralin in microbial metabolism is still questionable. It is unclear whether trifluralin in degraded by metabolic or co-metabolic processes, although the evidence for the latter process is stronger. Using trifluralin labelled in the trifluoromethyl ring, Jacobsen *et al.* (1980) studied the co-metabolism of trifluralin in sewage under aerobic and discontinuous anaerobic conditions. Using thin layer

chromatography, 49% of the ^{14}C was recovered as unknown trifluralin products after 88 d of aerobic incubation; 91% was recovered under discontinuous anaerobic incubation. Since the bacterial nucleoside pool was found to contain none of the radioactivity and trifluralin was stoichiometrically transformed to the organic products, it was assumed that trifluralin was co-metabolized. Using ^{14}C-glucose and the fungi *F. oxysporum* and *T. viride*, Zayed *et al.* (1982) found that the amount of $^{14}CO_2$ increased with increasing concentrations of trifluralin. Zeyer and Kearney (1983) found mixed soil cultures degraded less than 1.6% of the added 0.15 mM (50 μg mL^{-1}) trifluralin to carbon dioxide within 3 d. Trifluralin enrichment procedures with mixed cultures did not result in an increase in trifluralin degradation; however, a third of the 180 isolated strains grown on complex medium were found to show good activity by enrichment, liberating between 1.5 and 12% $^{14}CO_2$ within 21 d. Spain and Veld (1983) also attributed the slow degradation of trifluralin under "natural" water and sediment conditions to abiotic or nonspecific co-metabolic processes. No adaptation of the microbial community was observed after an 8 d exposure of soil cores to 1.08 μM trifluralin.

The effect of trifluralin on bacterial soil isolates was studied by Carter and Camper (1975). Seventy two bacterial isolates were selectively isolated from trifluralin-treated soil by incorporating 200 mg L^{-1} of trifluralin into a simple salts medium. Of 8 randomly selected bacterial isolates, all were tentatively identified as *Pseudomonas* sp. Bacteria from the genera *Pseudomonas* and *Alcaligenes* are the most commonly isolated denitrifying organisms from soil samples

(Gamble *et al.* 1977). Growth was monitored by counts of colony forming units (CFU) or by measuring absorbancy at 600 nm. For the 2 isolates chosen for further studies, 50 mg L^{-1} of trifluralin produced better growth than 25 or 100 mg L^{-1}. It was hypothesized that the lower concentration was insufficient to stimulate growth and the higher concentration was inhibitory. Successive culture transfers resulted in enhanced bacterial growth in the presence of 50 mg L^{-1} of trifluralin; the first and second transfers resulted in an 25-200% increase and the third transfer produced a 1150-1400% increase.

It therefore appears that the presence of trifluralin may stimulate the growth of selective soil microorganisms and may be degraded by both metabolic and co-metabolic processes. Environmental differences, such as species composition and nutrient availability, may determine the pathway of trifluralin degradation. Additionally, it should be mentioned that trifluralin is degraded by abiotic methods (Helling 1976). Southwick and Willis (1979) found that trifluralin rapidly degraded in sterile flooded soil when the redox potential was < +100 mV. The redox potential is poised at about +200 mV during the reduction of nitrate and at approximately +180 mV during nitrite reduction (Bailey and Beauchamp 1973). At redox potentials below approximately +100 mV, denitrification is essentially complete.

Diclofop-methyl also undergoes both abiotic and biological degradation. Non-biological degradation of diclofop-methyl to diclofop occurs rapidly; depending the soil type, up to 35% of the diclofop-methyl has been found to hydrolyze within 3 h of its application to soil (Smith 1977, Martens 1978, Karanth *et al.* 1984).

Under aerobic conditions, diclofop has been found to have a half life of 10-38 days (Martens 1978, Karanth *et al.* 1984) and under anaerobic conditions, the half-life was approximately 150 days (Martens1978). The maximum amount of $^{14}CO_2$ recovered from ^{14}C-labelled diclofop-methyl, ranges from approximately 3.5% after 30 d (Martens 1978) to 25% after 96 d (Karanth *et al.* 1984), under aerobic incubation. Martens (1978) found no $^{14}CO_2$ was evolved under anaerobic conditions. The anaerobic dissipation of diclofop in soil may be a function of the conversion of the herbicide to soil-bound residues, highly resistant to extraction (Smith 1977, Smith 1979, Karanth *et al.* 1984), rather than microbial degradation of the herbicide.

Although the effects of trifluralin/Treflan™ on microorganisms have been studied extensively, the reported effects differ substantially (Anderson 1978). Reports of both increased (Rankov and Elenkov 1970) and decreased (Breazeale and Camper 1970, Kondratenko *et al.* 1981) microbial populations are common. For example, Carter and Camper (1973) applied formulated trifluralin over a 3 y period and found that the bacterial population decreased while the actinomycete and fungal populations increased. A 5 y annual application of 1.1 kg ha^{-1} formulated trifluralin was also found to increase the fungal and actinomycete populations (Breazeale and Camper 1970).

In a laboratory experiment, Stojanovic *et al.* (1972) examined the effect of an 11,227 kg ha^{-1} application of analytical or formulated trifluralin to a calcareous loam. This high treatment level was chosen to simulate the disposal of unused pesticides, pesticide wastes, and pesticide containers in soil. After 56 d trifluralin produced an 84%

decrease in the bacterial population, while formulated trifluralin produced a 34% decrease. The streptomycete and fungal populations were respectively increased 114 and 33% by trifluralin and 1100 and 36% by formulated trifluralin. A mixture of formulated trifluralin, 2,4,5-T, and malathion had no effect on the bacterial population and increased the streptomycetes and fungi by ca. 600 and 382% respectively.

At recommended field application rates (ca. 1.0 kg ha^{-1}), Treflan™ EC was found to slightly stimulate the actinomycete and bacterial populations for the first two weeks after application to a clay loam and a loam (Olson *et al.* 1984). In a growth chamber study, the fungi appeared to be more sensitive to Treflan™ EC than the bacteria and actinomycetes.

In contrast, a 7 year application of 2 kg ha^{-1} of commercial trifluralin, to a medium pale grey loam, reduced the actinomycete and bacterial populations during the first one-half of the growing season; however, the microbial populations recovered before harvest (Tolkachev and Solyanova 1979). Kondratenko *et al.* (1981) studied the effect of formulated trifluralin in a grey-brown weakly calcareous soil at 60% field capacity. The fungi were reduced for the first 7 d after trifluralin application and the bacteria were reduced for 3 d. The actinomycetes were found to be intermediate in sensitivity to trifluralin. Mordaleva *et al.* (1981) found that Treflan™ at 2 kg a.i. ha^{-1} slightly decreased the microbial activity in a chernozem soil for the first 30 days, after which time activity increased.

Solbakken *et al.* (1982) studied the effect of 1 and 5 kg ha^{-1} applications of trifluralin to 2 Norwegian field soils over a 2 year

period. With increasing concentrations of trifluralin, microbial numbers tended to increase the first year in a loamy sand (6.2% C), but decreased in the second year. Greater inhibitory or stimulatory effects were observed in the loamy sand; less distinct results were obtained for a loam (2.8% C). The number of viable soil bacteria was found to be higher in the loamy sand and was postulated to be a result of the higher levels of nitrogen and carbon. Due to the variable plating techniques used by the authors and the differences in precipitation on the experimental fields, the effects of trifluralin on the soil bacteria are difficult to elucidate from this study.

Treflan™ has been found to lower the total counts of the bacteria, fungi, and actinomycetes, but to initially stimulate the counts of *Azotobacter*, nitrogen fixing clostridia, and ammonifiers (Makani *et al.* 1979).

Helmeczi *et al.* (1978) found no significant effects on either the total numbers or the aerobic and cellulolytic bacteria, with field applications of Olitref™ (trifluralin).

In a preliminary study by Horowitz *et al.* (1974), trifluralin applications of 0.67 and 1.34 kg ha^{-1} were found not to affect the numbers of ammonium oxidizers (namely *Nitrosomonas*) and the nitrite oxidizers (namely *Nitrobacter*). Tolkachev and Solyanova (1979) observed no effect on the heterotrophic or nitrifying bacteria over a 7 y period with a yearly application of 2 kg ha^{-1} of commercial trifluralin. No effect was observed on the nitrifying population (*Nitrobacter* sp.) in a clay loam or loam planted to wheat and treated with 1.0 kg ha^{-1} trifluralin (Olson *et al.* 1984).

In field soils treated with 1 or 5 kg ha^{-1} trifluralin, Solbakken *et al.* (1982) found a 24% increase in the Gram-negative non-sporeformers and a 30% decrease in the Gram-variable Coryneform/Arthrobacter group. With pure cultures, Olson *et al.* (1984) found that none of the 18 bacteria studied were affected by 16 $\mu g\ g^{-1}$ Treflan™ EC. Only *Agromyces ramosus*, *B. megaterium*, and a strain of *R. trifolii* were affected by the next higher concentration tested, 400 $\mu g\ g^{-1}$. Affifi and Dowidar (1978) found that trifluralin inhibited the growth and oxygen uptake of *R. leguminosarum* isolated from the roots of peas.

Heinonen-Tanski *et al.* (1982) studied the effect of Illoxan™ (diclofop-methyl) on 25 *Rhizobium* strains isolated from 18 soils. At 100 mg kg^{-1}, Illoxan™ had no effect on the growth of any *Rhizobium* strains cultivated on nutrient agar supplemented with 1% (w/v) beef extract and 5% (w/v) peptone.

Cullimore and McCann (1977) used soil cores to examine the effect of one and 100 $\mu g\ g^{-1}$ trifluralin applications to a loam on 31 genera of algae. *Chlamydomonas*, *Chlorococcum*, *Hormidium*, *Palmella*, *Scytonema*, and *Ulothrix* were the algal genera most sensitive to trifluralin. *Phytoconis*, *Spongiochloris*, *Stichoccocus*, and *Tolypothrix* were more tolerant and *Chlorella* was stimulated by trifluralin. In a 7 y field trial, Tolkachev and Solyanova (1979) found that 2 kg ha^{-1} commercial trifluralin had no effect on the algal population.

Lewis *et al.* (1978) studied the effects of various commercial herbicide formulations and mixtures on soil respiration in a loamy sand (1.2% O.M.) and silty clay loam (3.4% O.M.). They showed that 0.6 kg

ha^{-1} of trifluralin and a mixture of 0.3 kg ha^{-1} trifluralin with 1.1 kg ha^{-1} EPTC, 0.3 kg ha^{-1} diuron, 0.3 kg ha^{-1} linuron, 0.8 kg ha^{-1} dinoseb, or 0.1 kg ha^{-1} metribuzin had no effect on carbon dioxide evolution over a 32 day period. The degradation of ground alfalfa tissue, as measured by CO_2 evolution, was not affected by trifluralin or trifluralin-herbicide mixtures, even when applied at concentrations 100 times the recommended field rates. The addition of 20-20-20 N-P-K fertilizer also did not affect CO_2 evolution. Other workers have also found that carbon dioxide evolution was not affected by Treflan™ (Olson *et al.* 1984) or trifluralin (Rodriguez-Kabana *et al.* 1969).

On the other hand, Davies and Marsh (1977) found that 100 $\mu g\ g^{-1}$ of trifluralin stimulated carbon dioxide evolution in a sandy clay loam (3.9% O.C.) for the first 20 weeks after application; no effect was observed in a sandy loam (1.5% O.C.). A 4 kg ha^{-1} application of Treflan™ (24% a.i.) to an alluvial meadow soil, cropped with soybeans, was found to stimulate respiration for the first 30-60 d (Bakalivanov *et al.* 1979). The use of mineral fertilizer decreased the effect of Treflan™.

In contrast, Kondratenko *et al.* (1981) found that formulated trifluralin, applied at recommended field rates, increased carbon dioxide evolution for only the first day after application. Carbon dioxide evolution was then inhibited and maximum inhibition occurred 7 d after treatment. Stojanovic *et al.* (1972) found that 11,227 kg ha^{-1} of trifluralin or formulated trifluralin inhibited carbon dioxide evolution ca. 26 and 21% respectively. A mixture of formulated

trifluralin, 2,4,5-T, and malathion increased carbon dioxide evolution by 48%.

A 3 and 15 kg ha^{-1} Treflan™ application to a loam chernozem had no effect on catalase, urease, phosphatase, and invertase activity, but increased the cellulose decomposing population (Gruzdev *et al.* 1973). Lewis *et al.* (1978) found no effect of commercial trifluralin or trifluralin-herbicide mixtures on dehydrogenase activity in 2 soils. In a 7 year study, Tolkachev and Solyanova (1979) found no effect on catalase, and reduced urease and invertase activity with applications of 2 kg ha^{-1} of commercial trifluralin. They also observed a 70% increase in cellulolytic activity. In an 8 week study, Davies and Greaves (1981) found that applications of 3.36 and 13.44 kg ha^{-1} Treflan™ to a sandy loam (4.0% O.C.) had no effect on the activities of dehydrogenase, phosphatase, and urease. Addition of 1% (w/w) finely divided lucerne meal did not affect the observed effects of Treflan™.

Davies and Marsh (1977) found that Treflan™ 480 slightly decreased nitrification for the first 2 weeks in a sandy clay loam (3.9% O.C.), while no effect was observed in a sandy loam (1.5% O.C.). No effect was observed on nitrogen mineralization. Treflan™ at 10 and 100 $\mu g\ g^{-1}$ was found to reduce nitrification by 28 and 42% respectively, in four diverse forest soils (Nakos 1980). Other varied effects on nitrifiers and nitrification by trifluralin are reviewed by Alexander (1978).

Horowitz *et al.* (1974) applied trifluralin at 0.67 and 1.34 kg ha^{-1} twice a year over a 4 year period. Soil samples obtained on three occasions, 5 months after trifluralin application, showed no difference in the ammonium- and nitrate-nitrogen contents. In the autumn of the

third year of the study, the nitrate-nitrogen content of the soils was negatively correlated with the number of weeds in the plot; as the efficiency of weed control increased the nitrate-nitrogen content increased. Carbon, nitrogen, and phosphorous mineralization in 2 soils were not affected by field application rates of commercial trifluralin or trifluralin-herbicide mixtures (Lewis *et al.* 1978).

Application of 6 kg ha^{-1} Olitref™ (trifluralin) has been found to decrease the numbers of aerobic nitrogen fixing bacteria in field soil but increase the numbers in laboratory studies (Helmeczi *et al.* 1978). Kondratenko *et al.* (1981) found that formulated trifluralin had no effect on non-symbiotic nitrogen fixation (C2H2 reduction) when applied at recommended field rates. Rennie and Dubetz (1984) studied the effect of trifluralin on the acetylene reducing activity (ARA), i.e., nitrogen fixing potential, of *R. japonicum* inoculates in an irrigated loam. They found that 1.0 kg ha^{-1} trifluralin stimulated ARA in one year, but little effect was observed at 2.0 kg ha^{-1}. In the following year, no effect was observed with a 0.8 kg ha^{-1} application. The stimulation of ARA was not caused by an increase in the numbers of nodule but rather by physiological changes.

Lewis *et al.* (1978) found that field application rates of commercial trifluralin or trifluralin-herbicide mixtures increased sulfur oxidation by ca. 62-192 % in a silty clay loam and a loamy sand.

Little work has been done on the cytological and biochemical effects of herbicides on soil microorganisms. The toxic action of trifluralin is manifested as aberrant mitosis (Hacskaylo and Amato 1968, Lignowski and Scott 1971) due to the reduction in the number or

the absence of microtubules (Bartels and Hilton 1973). Hess and Bayer (1977) reported that trifluralin bound to the flagellar tubulin of the alga, *Chlamydomonas*. The herbicidal activity of diclofop-methyl is due to anti-auxin activity, inhibition of fatty acid biosynthesis, and/or inhibition of photosynthesis (Roberts 1982).

As previously mentioned, the effects of herbicides on denitrification or the denitrifying population has not been extensively studied. No work has been documented on the effects of diclofop-methyl. Trifluralin, at 0.76 or 1.34 kg ha^{-1}, has been found to have no effect on the numbers of denitrifying microorganisms (namely *Pseudomonas*) after 5 mon (Horowitz *et al.* 1974). At recommended field application rates (ca. 1.0 kg ha^{-1}), Treflan™ EC has been found to have no effect on the total denitrifying population in a clay loam and a loam (Olson *et al.* 1984). Recently, Yeomans and Bremner (1985) found that 10 $\mu g\ g^{-1}$ trifluralin had no effect on denitrification in any of the 3 soils studied. At 50 $\mu g\ g^{-1}$, trifluralin enhanced denitrification in a low organic carbon (0.5%) silty clay loam (59% silt, 28% clay), but inhibited nitrite reduction. In a high organic (6.6%) silty clay (49% silt, 41% clay), 50 $\mu g\ g^{-1}$ trifluralin was found to only enhance denitrification.

In conclusion, the effects of trifluralin on microbial populations and processes can vary considerably among researchers, while the effects of diclofop-methyl have not been adequately documented to permit comparisons. The variations in the observed results on the effects of the herbicides are a function of various factors, such as 1) the different methodologies employed, both in field and laboratory

studies, 2) the differences in the physical and chemical characteristics of the soils studied, 3) the environmental differences in field trials, e.g., temperature and rainfall, and 4) the variations in herbicide toxicity to different soil microbial populations. Herbicide experimentation on "natural representatives" of the soil microbial population might dispel some of the disparities occurring in the literature and provide some insight on the influence that different soils have on the interaction of herbicides and soil microorganisms .

INTRODUCTION

Soil fertility is greatly affected by the biochemical transformations resulting from the activity of the soil microbial population. This population is composed of actinomycetes, algae, bacteria, fungi, and viruses. Except for viruses, these organisms regulate soil fertility by acting upon the organic matter (mineralization) and inorganic compounds (immobilization) in the soil. Since soil fertility is important for the maintenance of high crop yields on agricultural land and for species succession on non-agricultural land (forest, tundra, etc.), it is of paramount importance to identify any activities which may increase or decrease the fertility parameters of the soil.

Pesticides are routinely used to increase yields on agricultural land and to control pests in non-argicultural situations. On agricultural land, especially in western Canada, herbicides are the most heavily applied pesticides (McEwan and Stephenson 1979). The methods of treatment, either pre-plant incorporation or post-emergence application, make herbicides potentially more likely to exert effects on the microbial population, and therefore, it is important to determine what effects they have on the soil microbial population and/or microbial processes.

Under anaerobic conditions, both dissimilatory and assimilatory reduction of nitrate can occur. Assimilatory nitrate reduction is classically defined as the reduction of nitrate to ammonia for biosynthetic purposes, and occurs under low availability of ammonium or reduced nitrogenous organic metabolites. Dissimilatory nitrate reduction occurs as an alternate form of respiration; in the absence of

oxygen nitrate is used as a terminal electron acceptor for respiratory electron transport. The process of reduction of nitrogen oxides to dinitrogen is generally termed denitrification, and the form of nitrogen used or produced is primarily dependent upon the bacterial species.

Denitrification has been found to account for a loss of 0 to 70% of the fertilizer nitrogen applied to agricultural soils (Firestone 1982) and is therefore an important factor in the depletion of available soil nitrogen. Various compounds have been found to inhibit denitrification, e.g., acetylene, azide, nitrapyrin, and pesticides (Knowles 1982). Since herbicides are commonly applied to or may end up in agricultural soils, it is of importance to determine if they can stimulate or retard denitrification. Stimulation of denitrification would result in rapid losses of applied fertilizer nitrogen, with subsequent increases in crop production costs, and increases in the quantity of nitrous oxide evolved into the atmosphere, with possible damage to the stratospheric ozone layer. On the other hand, the inhibition of denitrification in areas with high nitrate levels could increase the nitrate and nitrite released into ground water supplies. Nitrites can be toxic to plants, animals, and humans, or may combine with other chemicals to form toxic substances (Sunita and Gupta 1984).

Little work has been done on the effects of herbicides on denitrification, probably reflecting the difficulty in studying the denitrification process (Firestone 1982, Goring and Laskowski 1982). This study was initiated to examine the effect of selected herbicides on denitrification in various soils. When dealing with soil systems, a

large number of variables can affect the denitrification process (Firestone 1982) and under natural conditions it may be difficult to standardize these variables. A laboratory study was therefore undertaken to elucidate the effect of herbicides on the denitrification process. Since the effect of a herbicide in a non-soil microbial culture can differ from that obtained in a soil (Anderson 1978), the study examined the effects of the herbicides in several different soils. Denitrification was measured by following nitrate, nitrite, and nitrous oxide concentrations at regular intervals in an anaerobic soil system.

The herbicides examined in this study were HoeGrass™ and Treflan™. It has been found that the commercial formulation can elicit a different response than that produced by the technical product (Stojanovic *et al.* 1972, Wright and Forey 1972, Heinonen-Tanski *et al.* 1982). For example, in a loamy soil, Percich and Lockwood (1978) found that Aatrex-80 WP caused an early stimulation of microbial populations, which was not observed with atrazine treatment. By examining the effects of both the technical and commercial product, it may be determined whether the herbicide or the non-herbicidal "inert" material(s) affect the denitrification process. Therefore, this study examined the effects of HoeGrass™ and Treflan™ and their respective technical products diclofop-methyl and trifluralin.

HoeGrass™ and Treflan™ are applied at rates of approximately 1 kg ha^{-1}. In this study, the effects on denitrification of 10 and 100 μg active ingredient (a.i.) g^{-1} soil was examined, rates not uncommon in studies reported by other investigators. These higher rates were

chosen to simulate areas of increased herbicide concentrations in the soil, which can be a result of herbicide misuse, e.g., spillage, improper application, washing out of containers, etc., or the re-application of environmentally resistant herbicides. Increased herbicide concentrations can also be found around the adsorption sites of clays and organic matter (Anderson 1978) and the surface of carrier particles in the granular formulation (Olson *et al.* 1984). By examining high herbicide concentrations, it is possible to eliminate from further study compounds that are inactive and, under agricultural rates, unlikely to affect the soil microflora.

Although a laboratory investigation tends to negate the effects of environmental factors, such as variations in pH, temperature, volatilization, and crop roots, various other variables, such as previous pesticide applications and storage of the soils, had to be considered to avoid spurious effects. Previous soil exposure to a herbicide or to a related compound has been found to affect the degradation rate of a herbicide and the microbial populations and/or activities. In Saskatchewan soils, repeated applications of 2,4-D amine and ester formulation have resulted in higher populations of 2,4-D-degrading microorganisms (Cullimore 1981). Soils exhibiting rapid EPTC (*S*-ethyl dipropyl-thiocarbamate) breakdown, as a result of previous EPTC applications, have been found to degrade butylate and vernolate more rapidly (Obrigawitch *et al.* 1983). Under anaerobic conditions, trifluralin has been found to be degraded by various methods (Cripps and Roberts 1978), namely abiotic (Probst *et al.* 1967), microbial metabolic (Carter and Camper 1975) or co-metabolic pathways

(Jacobson *et al.* 1980). Repeated exposure to trifluralin has been found to have no effect on trifluralin degradation (Spain and Veld 1983) but can increase cellulolytic activity (Tolkachev and Solyanova 1979).

In most of the older literature, and even in some recent publications, investigators have used, for experimentation, soils which had been air dried, frozen, or stored, i.e., stressed. Air-drying the soil results in a drastic reduction in the microbial population (Bottner 1985) and enzyme activities (Speir and Ross 1981). Most importantly, it probably results in a reduction of microbial diversity (Salonius 1983). It is not uncommon for soil processes to be based upon synergistic or symbiotic relationships (Aftring *et al.* 1981, Lewis *et al.* 1984) and the reduction in microbial diversity may have produced some of the apparent discrepancies between laboratory and field studies (Malkomes and Whler 1983).

At 1.2% moisture content, soil bacterial and fungal populations have been observed to decline over a 28 day period (Sparling and Cheshire 1979). In contrast to the fungi and bacteria, the yeasts became more capable of recolonizing the soil as the length of storage time increased. The addition of fresh soil (1%) did not restore the soils to their original microbial compositions. Older microbial cells, which are in a dormant or sporulated stage, survive the drying treatment better than younger cells (Soulides and Allison 1961). Reactivation of stressed soil by growing plants in the soil prior to use has been successful at partially restoring microbial activities,

but the type of plant grown in the soil has been found to influence the magnitude of the microbial response (Laanio *et al.* 1973).

Letey *et al.* (1980) stored soils for 7 days at 1.6% (air-dried), 12% and 100% (saturated) water contents previous to using the soils to study the amount of nitrogen evolved during denitrification. They found that the amount of nitrogen evolved was highest in the air-dried soil and lowest in the moist soil. They attributed the lower evolved nitrogen by the moist preincubated soil to depletion of available carbon during preincubation. Alternatively, the higher nitrogen evolution by the air-dried soils may have been due to the increased nutrients provided by the dead microbial cells. The drying-rewetting treatment also increases the rate of decay of native organic matter by 12-30% (Sorensen 1974). Various other authors have documented the effects of storage on microbial populations (Nelson and Parkinson 1978, Ross *et al.* 1980, Tu 1982). Sieving field moist (ca. 30-80%) soil through a 2 mm mesh has also been found to increase net mineral nitrogen and extractable inorganic P contents of the soil compared to that found in intact cores (Ross *et al.* 1985). It is generally recommended that soil be used immediately for experimentation; alternatively, that it can be stored moist at 4°C for a short period of time (Bartlett and James 1980, Salonius 1983). However, the temperature of storage can affect the biomass parameter studied. Carbon dioxide and mineral nitrogen production and ATP content can be differentially affected by different storage temperatures over a 28 d period (Ross *et al.* 1980).

One of the most difficult aspects of soil studies is obtaining a representative soil sample. Local differences exist in the physical and chemical characteristics of the soil. Although these vertical and lateral differences in the soil microbial composition can be minimized by mixing the soil sample before experimentation, wide variations in the soil microbial population can still exist (Alexander 1977). Bacteria prefer to colonize soil aggregates; whereas, fungi are associated largely with extraneous organic material (Greaves and Malkomes 1980). The native soil bacteria are firmly attached to the soil components and are difficult to detach by blending, sonic treatment, or low-speed centrifugal washing (Balkwill *et al.* 1977). In contrast, a laboratory culture of *Arthrobacter globoformis* grown in a soil solution, was found to be easily separated from the soil particles (Balkwill and Casida 1979).

Since this study encompassed several years and several different soils, the soil microbial variability was minimized by utilizing a stock microbial culture prepared from the fresh soils used in this study. By using a stock microbial culture to inoculate the soil, it is was thought possible to minimize the effects of the different microbial populations on denitrification and thus obtain a more accurate measure of the influence herbicides have on the denitrification process in the different soils (Chandra *et al.* 1960, Malkomes and Wohler 1983). Due to the possibility that previous soil pesticide treatment(s) could have had an effect on the microbial population, soils were chosen which had no known history of pesticide applications.

In summary, the purpose of this study was to examine the effects of the commercial herbicides HoeGrass™ and Treflan™, currently used in cereal and oilseed production on the Canadian prairies, and their respective technical products diclofop-methyl and trifluralin, on denitrification in selected representative soils. To minimize the environmental effects, the experiment was performed under defined laboratory conditions. To minimize any microbial differences between the soils, a stock microbial culture was used to inoculated the soils studied just prior to the commencement of each experiment. Denitrification was observed by following the disappearance of nitrate, the appearance and disappearance of nitrite, and the appearance of nitrous oxide. In this manner, it was thought possible to elucidate the effects of the formulated and technical herbicides in soils of varying physical and chemical characteristics. To simulate increased carbohydrate levels encountered near plant roots and to reduce the effects of insufficient soil carbon, the experiment was repeated with the addition of 100 μg glucose g^{-1}.

MATERIALS AND METHODS

I. Soils

Bulk soil samples from the top 20 cm of 6 soils were obtained in May 1984 from agricultural areas in southern Manitoba. These soils had no recent history of pesticide applications. All the soils were air dried for 1 week, then sieved through a 2 mm sieve, and stored in 1.7-L glass jars at ambient room temperature (ca. 24°C).

Except for the water capacity, the pH, the nitrate and sulfate concentrations, and the glucose equivalent, the physical and chemical characteristics listed in Tables 2 and 3 were determined by the Manitoba Provincial Soil Testing (MPST) Laboratory. All concentrations quoted are based on air-dried soil. The electrolytic conductivities of all the soils were 0.020 S m^{-1}. Only soils P3 and P2 were used for the denitrification studies.

Table 2: Physical characteristics of soils.

Soil	Association, Texture	Classification	% Sand	% Silt	% Clay	Water Capacity (mL g^{-1})
P1	Almasippi, sand	Gleyed Rego Black	89	5	6	0.35
P3	Almasippi, sand	Gleyed Rego Black	89	5	6	0.38
BA	Almasippi, sand	Gleyed Rego Black	83	7	10	0.31
P2	Elm River, loam	Cumulic Regosol	44	37	19	0.37
B1	Newdale, clay loam	Orthic Black	34	39	27	0.44
B2	Newdale, clay loam	Orthic Black	37	33	30	0.43
T1	Red River, clay	Gleyed Rego Black	17	28	55	0.45

Table 3: Chemical characteristics of soils.

Soil	pH	% O.M	Glc Equiv. ($\mu g\ g^{-1}$)	NO_3-N ($\mu g\ g^{-1}$)	P ($\mu g\ g^{-1}$)	K ($\mu g\ g^{-1}$)	SO_4-S ($\mu g\ g^{-1}$)
P1	7.9	2.3	-	1.4	2.8	30	2.3
P3	7.2	3.5	16.0	111.3	6.0	190	3.4
BA	7.7	2.6	-	63.0	16.6	115	1.9
P2	7.9	2.5	14.2	28.2	4.0	295	2.9
B1	7.5	7.2	-	84.0	23.2	532	6.7+*
B2	7.6	9.5	-	73.6	60+*	700+*	6.7+*
T1	7.9	7.2	-	117.1	58.8	365	3.0

* Maximum levels measurable by analysis methods.

II. Methods of Analysis

All reagents used in the analyses were certified A.C.S. grade and, unless specified, were obtained from Fisher Scientific Company (Winnipeg, Manitoba). Bacto-peptone, yeast extract, and nutrient broth were obtained from Difco Laboratories (Detroit, MI, U.S.A.). Solvents were high performance liquid chromatography (HPLC) grade, distilled in glass, and were obtained from Caledon Laboratories (Georgetown, Ontario). Glass distilled water was used for all preparations and in all situations requiring water. For the preparation of the mobile phases, the solvents and water were filtered with a 0.45 μm Millipore filter and degassed. Standard nitrate and nitrite solutions were prepared monthly and stored at approximately 4°C. Experimental gases were obtained from Welders Supply Co. Ltd. (Winnipeg, Manitoba).

A stock solution containing 200 μg a.i. mL^{-1} of diclofop-methyl (97.9% methyl 2-[4-(2,4-dichlorophenoxy)phenoxy]propionate, Hoechst) or trifluralin (97.1% α, α, α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine, Eli Lilly & Co) was prepared by dissolving the technical product in 10 mL acetone and diluting to 100 mL with distilled water. Since trifluralin tended to precipitate out of the colloidal solution upon storage, both the trifluralin and diclofop-methyl standards were stored in the dark at ca. 24°C and discarded after the second day. A 1900 μg a.i. mL^{-1} HoeGrass™ (190 g L^{-1}, Manitoba Cooperatives Ltd.) and a 545 μg a.i. mL^{-1} Treflan™ EC (545 g L^{-1}, Manitoba Cooperatives Ltd) stock solution was prepared by diluting the liquid formulation with

distilled water. The stock solutions were stored at 4°C for no longer than 2 weeks.

Water soluble soil phosphorus (Table 3) was extracted with 0.5 mol L^{-1} sodium bicarbonate, pH 8.5, and analyzed by the ascorbic-acid molybdate method employing a Technicon Auto Analyzer (Murphy and Riley 1962). Exchangeable potassium (Table 3) was extracted from 2.5 g of soil by shaking with 25 mL of 1 mol L^{-1} ammonium acetate, pH 7.0, for 1 h. The soil solution was filter through a #1 Whatman filter and analyzed by flame photometry using 0.22% lithium nitrate as an internal standard. The organic matter (O.M.) (Table 3) was determined by the Walkley-Black method as described by Allison (1965).

The water capacity (Table 2) represents the maximum water holding capacity of the soils and was determined by noting the amount of water required to saturate 10 g of soil in a glass funnel (Smith and Tiedje 1979).

In order to facilitate comparison of the initial soil pH (Table 3) to the pH at the end of the reduction experiments, the initial pH was measured in a stirred soil solution containing 10 g soil and 20 mL distilled water. The final pH ranged from 7.2-8.0, with the control soils having a slightly lower pH.

The glucose equivalent or soil "glucose carbon" (Table 3) represents the extractable glucose-C obtained by boiling the soil in 0.01 mol L^{-1} $CaCl_2$ for 1 h (Stanford *et al.* 1975). For this method, 5 g of soil was transferred into a 50 mL graduated centrifuge tube (conical bottom) with 25 mL 0.01 mol L^{-1} $CaCl_2$. To minimize water loss, an air condenser, consisting of a rubber stopper with an attached 30 cm long

glass tube, was inserted into the centrifuge tube. The tubes were then placed in a 100°C water bath for 30 min. After the tubes had cooled, the water lost was replaced. The tubes were then centrifuged at approximately 4000 g for 15 min and the supernatant analyzed for glucose. Glucose analysis was performed by the phenol-H_2SO_4 method (Herbert *et al.* 1971). One milliliter of the supernatant was mixed with 1.0 mL 10% phenol, the solution was agitated on a vortex mixer, and 5.0 mL of concentrated H_2SO_4 was rapidly added. The mixture was allowed to develop color for 30 min and read at 485 nm.

Sulfate-sulfur (Table 3) was extracted from 10 g of soil with 0.001 mol L^{-1} $CaCl_2$. The soil solution was shaken for 30 min and filtered through a #42 Whatman filter paper. The extract was analyzed by the MPST Lab with the Technicon Auto Analyzer, using a modified methylthymol blue method (Lazrus *et al.* 1966).

Various methods were examined to determine the optimum method available for soil nitrate analysis, both in the absence and presence of added glucose. A nitrate standard was used to prepare aqueous samples for the determination of the precision and accuracy of the nitrate methods. A nitrate standard was also used to prepare soils of varying nitrate concentration to determine the efficiency of the analysis methods for soil nitrate. The nitrate analysis methods examined were the 1) brucine sulfanilic acid, 2) hydrazine sulfate, 3) phenoldisulfonic acid, and 4) HPLC methods. An outline of the methodology employed for these methods, and the results obtained, are presented in Appendix 1. The HPLC method (Kuchnicki *et al.* 1985) was ultimately chosen as being the most appropriate for this study. The

development of the HPLC method is outlined in Appendix 2. Nitrate analyses using the HPLC, hydrazine sulfate and phenoldisulfonic acid methods were also compared in six soils (Kuchnicki and Webster 1986) (Appendix 3).

HPLC analysis of nitrate and nitrite was performed using a Waters Scientific (Mississauga, Ont.) Model M45 pump, Model U6K injector, and Model 441 UV detector. The detector was modified with the optional kit to allow detection at 214 nm. A 4 mm I.D. x 250 mm 10 μm LiChrosorb RP-18 column (Merck) was operated at ambient temperature (ca. 24°C). The nitrate peak areas were monitored with a CSI 38 digital integrator (Columbia Scientific Industries) and the nitrite peak heights were measured with an Fisher Recordall S5000 strip chart recorder. The mobile phase was 1:1 methanol-water acidified to pH 3.0 with H_2SO_4. To calibrate the detector and to monitor column performance, standard nitrate and nitrite solutions were routinely analyzed.

When deterioration of nitrite peaks was evidenced, the column was cleaned according to the procedure outlined in the Waters column care manual (#CU84588). The cleaning procedure involved washing the column successively with 100 mL of methanol, methylene chloride, and n-heptane. The column was then again washed with methylene chloride followed by methanol. The flow rate for the cleaning procedure was 2.5 mL min^{-1}.

III. Microbial Culture Preparation

To maximize the variety of microorganisms available, a stock microbial culture was prepared for use in the reduction assays. A soil inoculum was first prepared by diluting 20 g of soil to 100 mL with 0.1% peptone water. The peptone water was prepared by dissolving 1 g Bacto-peptone in 1000 mL distilled water and autoclaving 500 mL portions in 2 L Erlenmeyer flasks for 30 min. The soil used was a fresh moist mixture of soils P1, BA, P2, B1, B2, and T1 (Table 1). The soil dilution was shaken for 30 min and 5 mL of the soil suspension was added to 50 mL of sterile medium, composed of either nutrient broth, soil extract (S.E.) (Wollum 1982), S.E. + 0.5% yeast extract, S.E. + 0.5% peptone, or S.E. + 0.1% glucose. The medium was incubated at 24°C on a reciprocating shaker at 166 strokes min^{-1} (6 cm stroke length). After 48 h, the medium was centrifuged at 4000 g for 30 min and the pellet washed twice with 75 mL of 0.1% peptone water. The final pellets from the various media were combined and diluted to 200 mL with 0.1% peptone water. Ten millilitres of this soil/microbial suspension was then dispensed into test tubes and freeze-dried. The dried product was sealed and stored in a freezer. Before use, the dried product was reconstituted with 10 mL of 0.1% peptone water and 1.5 mL of this suspension was used to inoculate each 20 g portion of soil used for the reduction studies.

IV. Incubation Technique

The reduction assays were performed on 20 g portions of air-dried soil in 250 mL screw top Erlenmeyer flasks. The herbicides were first added to the soil from the stock solutions for a final concentration of 10 or 100 μg a.i. g^{-1} soil. The liquid was then incorporated into the soil by shaking the flask. The final concentration of acetone in the incubation flasks was < 2.5% of the total liquid volume and preliminary studies showed that this concentration of acetone had no effect on anaerobic nitrate and nitrite reduction in Soil P2 or P3 (Appendix IV). Three samples were run simultaneously; a control (no herbicide), and herbicide treatments at the rate of 10 μg g^{-1} and 100 μg g^{-1}. At least 3 replications were provided for each herbicide trial.

In order for both soils to have equivalent nitrate concentrations, 14 mL of a stock 100 μg NO_3-N mL^{-1} solution was added to Soil P2 to bring the NO_3-N concentration up to approximately 100 μg NO_3-N g^{-1}. A 1.5 mL stock microbial culture was added to the flask. To simulate increased carbohydrate levels encountered near plant roots, the experiment was also repeated with the addition of 1 mL of a stock solution containing 2 mg glucose mL^{-1}. These soils were designated as P2+Glc and P3+Glc. A final soil/water ratio of 1:2 was achieved by the addition of distilled water. This ratio was required to facilitate the sampling of the soil solution.

The initial levels of nitrate and nitrate were then determined by shaking the sample for 15 min on a reciprocating shaker at 166 strokes min^{-1} (6 cm stroke length). After shaking, 0.2 mL of the soil solution was removed, filtered with a 0.45 μm filter (MSI) and 1 μL of the

filtrate was injected into the HPLC for nitrate and nitrite analysis. The flask was sealed with an open top screw cap containing a telfon backed silicone septum. The septum enabled the withdrawal of a sample of the soil solution or the flask atmosphere without oxygen contamination of the incubation flask. The flasks were vacuum evacuated for 10 min and flushed with prepurified helium (99.996%) containing $\leq$ 3 ppm oxygen. This degassing operation was performed three times in succession. The slightly increased pressure within the flask was relieved by submerging the flask in water and inserting a needle through the silicone liner.

Since the acetylene inhibition technique was to be used as a measure of denitrification (Balderston *et al.* 1976, Yoshinari and Knowles 1976), 20 mL of purified C_2H_2 (99.6%) was added to each flask with a gas-tight syringe (Hamilton). Acetylene was found to have no effect on anaerobic nitrate and nitrite reduction in either Soil P2 or P3 (Appendix IV). Preliminary measurements for residual oxygen with a helium ionization detector (HID) confirmed that the degassing technique and acetylene additions resulted in oxygen concentrations within the flask of < 1% ambient. The flasks were then incubated at 24°C on a reciprocating shaker at 166 strokes min^{-1} (6 cm stroke length). Periodically, 0.4 mL of the soil solution was removed with a 10 mL syringe for nitrate and nitrite analysis.

Immediately after sampling the soil solution, a 20 μL gas sample was removed with a gas-tight syringe for N_2O analysis. The pressure within the flask was monitored with a modified Warburg respirometer. The modification consisted of the adhesion of a 22 G needle to the flask

connector end of the respirometer. The needle was used to penetrate the silicone septum of the sample flask. The loss in headspace volume, due to pressure measurements, was considered in the calculation of N_2O concentrations. The approximate pressure ranges encountered during analysis were 1.086 to 1.026 atm. A sample of the derivation of the concentration of nitrous oxide in the incubation flask is outlined in Appendix V.

V. Gas Analysis

A 20 μL sample of the flask headspace was removed with a gas-tight syringe (Hamilton) for gas analysis. N_2O was determined with a Varian 2400 gas chromatograph equipped with a tritium foil electron capture detector (ECD). Detector temperature was 200°C. The range was 10-10 and the attenuation was 16. Two 0.3 cm O.D. x 360 cm 80-100 mesh Porapak Q columns were run in parallel. Flow from the desired column was directed into the detector with a gas-tight switching valve. By switching in the alternate column after two injections, it was possible to avoid a large slow-moving peak and thus reduce the waiting time between sample injections. Prepurified nitrogen (99.998%) was used as the carrier gas with a flow rate through each column of 30 mL min^{-1}. Column temperature was 50°C. Peaks heights were measured in mm on a Fisher Recordall 5000. A nitrous oxide standard curve was prepared before each day's analyses (Appendix V).

Nitrogen and oxygen were measured with a Beckman GC 72-5 gas chromatograph equipped with a helium ionization detector (HID) operated at 140°C with a polarizing voltage of 200 V. Prepurified helium was used as the detector gas at a flow rate of 75 mL min^{-1}. Separation of the gases was effected with a 0.3 cm O.D. x 270 cm 60-80 mesh molecular sieve 5A column at ambient temperature (ca. 24°C). Prepurified helium at a flow rate of 30 mL min^{-1} was used as the carrier gas. Peak heights were measured on a Fisher Recordall 5000.

VI. Statistical Analysis

The nitrate and nitrite concentrations for all the treatments were normalized to an assumed initial soil nitrate concentration of 100 μg NO_3-N g^{-1}. To determine the maximum rates of nitrate and nitrite reduction, the data obtained was subjected to linear regression analysis (Zar 1974). Within treatments, the reduction rates, i.e., the linear portion of the curves which produced the maximum rate, were compared. The equality of the regression slopes, i.e., reduction rates, was tested by analysis of covariance (P = 0.05) where

$$F = \frac{\frac{(SS_c - SS_p)}{k - 1}}{\frac{SS_p}{DF_p}}$$

and SSc = "Common regression" residual sum of squares

$$= \Sigma y^2_c - \frac{(\Sigma xy_c)^2}{\Sigma x^2_c}$$

SSp = "Pooled regression" residual sum of squares

$$= \sum_{i=1}^{k} SS_i$$

DFp = Degrees of freedom of "pooled regression"

$$= \sum_{i=1}^{k} n_i - 2k$$

k = number of regression lines compared.

A common curve was then obtained for the statistically equivalent curves. For all cases, the coefficient of determination (r^2), for the linear portion of the nitrate or nitrite reduction curves, was

> 0.93900. The slopes for the different treatments were then compared by analysis of covariance for significance at the P = 0.05 level, in a similar manner as the replicates. If the hypothesis that the three population regression lines had the same slopes was rejected, multiple comparisons of the slopes were performed. The test statistic was

$$q = \frac{b_A - b_B}{SE}$$

where A and B refer to the two lines and

$$SE = \sqrt{\frac{(s^2_{xy})_p}{2}\left(\frac{1}{(\Sigma x^2)_A} + \frac{1}{(\Sigma x^2)_B}\right)}$$

If the slopes were significantly equal, the elevations were tested by

$$F = \frac{\frac{(SS_t - SS_c)}{k - 1}}{\frac{SS_c}{DF_p}}$$

where SS_t = "Total regression" residual sum of squares

$$= \Sigma\, y^2{}_t - \frac{(\Sigma\, xy_t)^2}{\Sigma\, x^2{}_t}$$

If the hypothesis that the three population regression lines had the same elevation was rejected, multiple comparisons of the elevations was performed using the test statistic

$$q = \frac{(Y_A - Y_B) - b_c(X_A - X_B)}{SE}$$

and

$$SE = \sqrt{(s^2_{yx})_p\left(\frac{1}{n_A} + \frac{1}{n_B} + \frac{(X_A - X_B)^2}{(\Sigma x^2)_A + (\Sigma x^2)_B}\right)}$$

The disappearance time of nitrate or nitrite was obtained by extrapolating the linear portion of the reduction curve to the abscissa.

For ease of use, the previously outlined equations were utilized in a spreadsheet, Microsoft Excel™, and its use is illustrated in Appendix VI for the statistical analysis of the control, 10 μg g^{-1}, and 100 μg g^{-1} diclofop-methyl treatments.

RESULTS

AND

DISCUSSIONS

I. General Observations

The initial mixed order reaction rate of nitrate reduction appears to have be influenced by a combination of factors, most importantly, the concentration of oxygen and numbers of denitrifiers (Figure 1). Under steady state denitrification conditions, the system was anaerobic and an optimum concentration of denitrifiers was present, resulting in a constant nitrate reduction rate (Cho and Sakdinan 1978). Since the soil solution was well shaken, diffusion was assumed to be non-limiting and nitrate reduction was zero-order. Under these well shaken conditions, the maximum nitrate reduction rate probably more closely reflected the actual rate than it would have in an unshaken system where diffusion of nitrate to the microbial reaction site may have been the most important limiting factor (Phillips *et al.* 1978, Reddy *et al.* 1978). Rigorous statistical analysis revealed a linear portion to the nitrate reduction curves. As reduction proceeded, nitrate concentrations declined to a level where the reaction rate was affected, resulting in a concentration dependent reaction rate similar to a first-order reaction.

As the nitrate concentration decreased, the nitrite concentration increased, resulting in a typical exponential curve for nitrite formation. Owing to time constraints, it was usually impossible to measure initial nitrite concentrations; however, some information on nitrite formation was obtained in the trifluralin/Treflan™ study. Since in dissimilatory nitrate reduction, nitrate is used as a terminal electron acceptor, it could be assumed that the maximum rate of nitrate disappearance would be equal to the maximum rate of nitrite appearance. However, rates for nitrite formation were considerably less than for

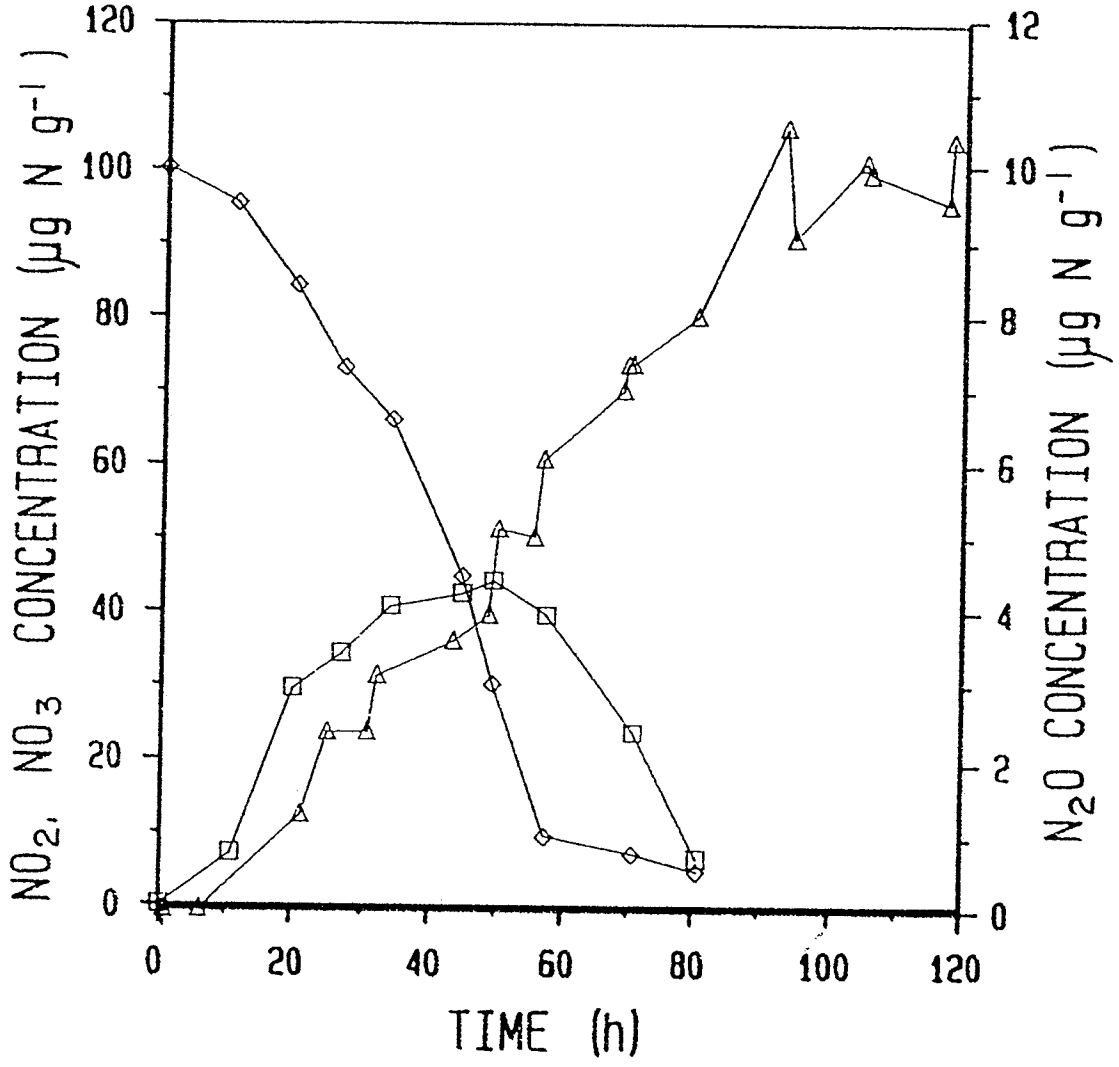

Figure 1: Nitrate (◊————◊), nitrite (□————□), and nitrous oxide (△————△) (right side axis) concentrations for the control in Soil P3. Data presented for one of several replications.

nitrate disappearance (Table 4) and may have been due to the assimilation of a portion of the NO_3-N and/or the reduction of some nitrite to nitric oxide. The maximum nitrite concentration occurs when almost all of the nitrate has been depleted.

Nitrous oxide concentrations were first detected approximately 12 to 20 h after initiation of the experiment (Figure 1). Initial nitrous oxide formation appeared to be approximately linear. Since the acetylene inhibition technique was used, it was assumed that the total concentration of N_2O-N evolved would equal the initial concentration of NO_3-N (Ryden *et al.* 1979, Vinther *et al.* 1982). However, the maximum N_2O-N concentrations obtained were never more than 15% of the initial NO_3-N concentrations. The nitrous oxide production curve leveled out shortly after nitrite disappeared, followed by a slight decrease in the the nitrous oxide level. Decreases in nitrous oxide levels have been found with 1% acetylene after 120 h of incubation (Yeomans and Beauchamp 1978). Under anaerobic conditions 0.1% acetylene is sufficient to inhibit the reduction of nitrous oxide for at least 4 d (Keeney *et al.* 1985); however, under a 100% carbon dioxide atmosphere, a 5% greater loss of nitrous oxide was observed after 4 d and a 36% greater loss after 7 d. The concentration of acetylene used in these assays, 10%, was assumed to be sufficient to inhibit nitrous oxide reduction during the experiment (Yoshinari and Knowles 1977, Yoshinari *et al.* 1977, Smith *et al.* 1978, Ryden *et al.* 1979).

Acetylene inhibition of nitrous oxide reduction is known to be alleviated by sulfide (Tam and Knowles 1979, Evans *et al.* 1985). Evans *et al.* (1985) found that with 1 kPa acetylene, the combination of carbon

Table 4. Comparison of nitrate reduction and nitrite evolution rates for the control samples.

Soil	NO3 Reduction† (μg NO_3-N g^{-1} h^{-1})	NO2 Evolution‡ (μg NO_2-N g^{-1} h^{-1})	% Δ*
P2	9.1	2.8	-69.2
P2+Glc	20.4	10.0	-51.0
P3	2.4	2.3	-4.2
P3+Glc	17.4	10.8	-37.9

† mean of 3 replications
‡ mean of 2 replications
* % difference from nitrate rate.

dioxide and sulfide caused greater alleviation than by sulfide alone. In a non-glucose amended soil with 52 μg S g^{-1} added, nitrous oxide reduction commenced at 75 h and was completed by 150 h. The authors stated that alleviation could be achieved with ca. 7.5 μg S g^{-1}. The sulfate content of the two soils used in this study was < 3.4 μg SO_4 g^{-1} and the equivalent sulfide concentration would probably not be sufficient to affect nitrous oxide reduction. The short incubation times and the higher level of acetylene used (10%) should also have precluded any initial interferences from sulfide.

Some difficulty was experienced with the tritium electron capture detection system. At reproducible injection volumes, the detector appeared to become quickly saturated to nitrous oxide (Figure 2). The standard curves were also poorly reproducible from day to day. Figure 2 illustrates the best response obtained; it was not uncommon to obtain a 50% difference in the response between two successive injections of 160 pmoles. To avoid any possibility that the inconsistencies in peak height were caused by the adsorption of nitrous oxide to the stainless steel tubing of the chromatographic column, the column was preconditioned before use with three 10 μL samples of nitrous oxide (Delwiche and Rolston 1976).

The amount of nitrogen evolved as nitrous oxide and dinitrogen has been found to account for 88-100% of the NO_3-N lost, and the amount of NO_3-N lost has been found to correlate highly with the amount of nitrogen evolved as nitrous oxide and dinitrogen (Burford and Bremner 1975, Vinther 1982). On the other hand, Tiedje (1982) stated that with the MPN method, denitrifiers generally accounted for only 30-80% of the

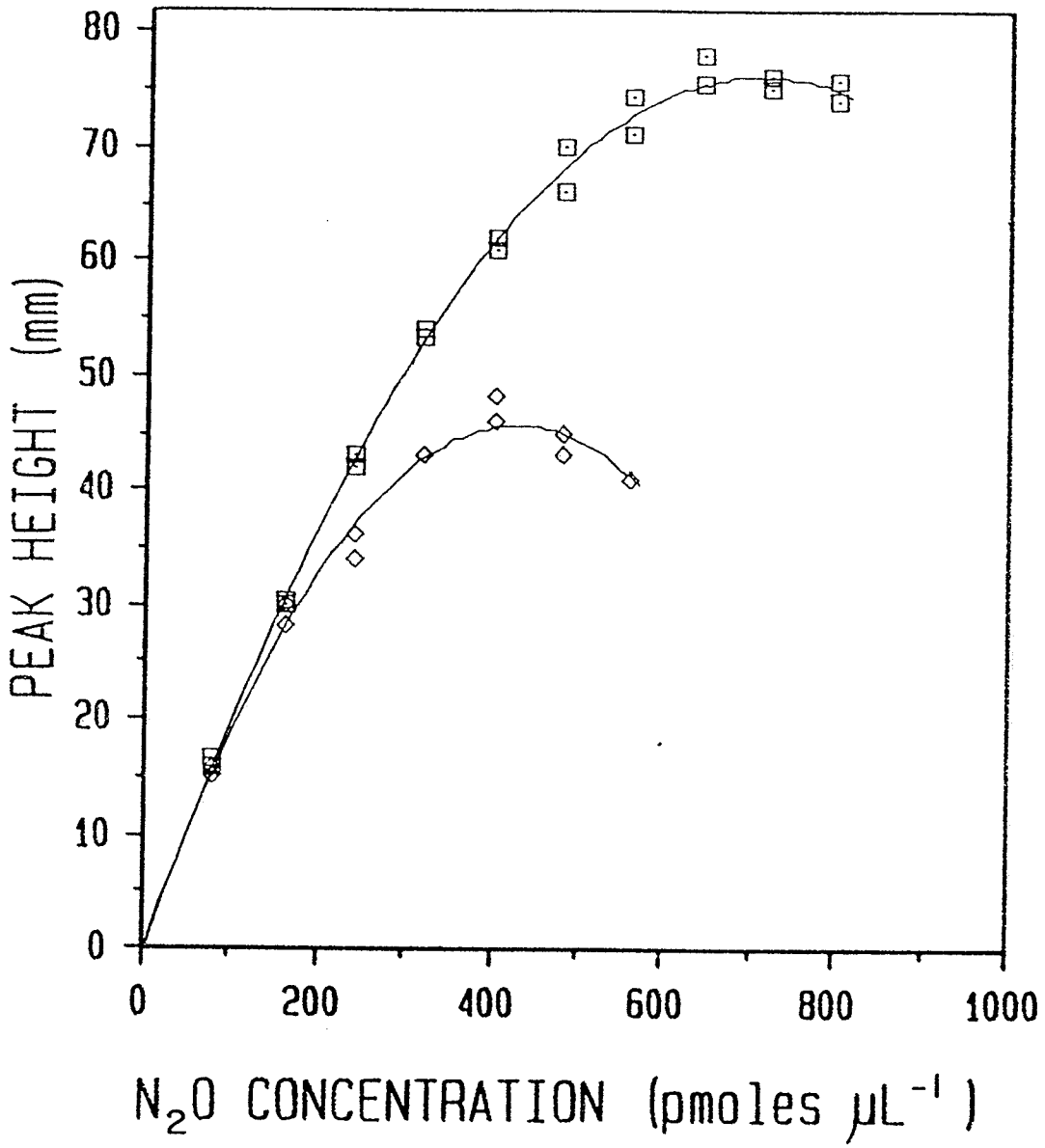

Figure 2: Nitrous oxide standard curves produced on consecutive days (□,◊) using a tritium foil ECD-GC. Two consecutive analyses were performed at each concentration indicated.

nitrate removed. It would therefore be presumptuous to assume that in this experiment the available nitrate was eventually reduced to nitrous oxide. Owing to the difficulty in obtaining reliable nitrous oxide concentrations, the nitrous oxide data were ignored in the following discussions. Only the anaerobic disappearance of nitrate and the appearance and disappearance nitrite were considered, i.e., anaerobic transformations of nitrate and nitrite, rather than denitrification *per se*.

II. Effects of Diclofop-Methyl and HoeGrass™ on Anaerobic Nitrate and Nitrite Reduction

A. Soils P2 and P2+Glc

Nitrate reduction curves for soils treated with diclofop-methyl and the commercial formulation HoeGrass™ were not substantially different from the control soils (Figure 3). Nitrite concentrations reached a peak at approximately the time that nitrate concentrations became minimum and these times did not differ for the various treatments. However, maximum nitrite concentrations obtained were approximately 37% lower for the 100 $\mu g\ g^{-1}$ treatments. In contrast, Grant and Payne (1982) found that 100 $\mu g\ mL^{-1}$ 2,2-dichloropropionic acid (dalapon) treatments produced higher nitrite levels and longer nitrite persistence times than the controls when cultures of *P. perfectomarinus* were grown in a 20% artificial seawater medium.

Through active transport, microorganisms can concentrate nutrients within the cell. Since the nitrite assay measures only extracellular nitrite concentrations, the difference in the soil nitrite concentrations at elevated diclofop-methyl levels may reflect a differential ability to concentrate greater amounts of nitrite intracellularly. Alternatively, the apparent concentration of nitrite is dependent on both the rate at which nitrate is reduced to nitrite and the rate at which nitrite is reduced to nitric oxide. Since the rate of nitrate reduction was not affected by the 100 $\mu g\ g^{-1}$ treatments (Table 5), the rate of nitrite reduction may be affected. The reduction of nitrite can be simply illustrated by the Michaelis-Menton theory of

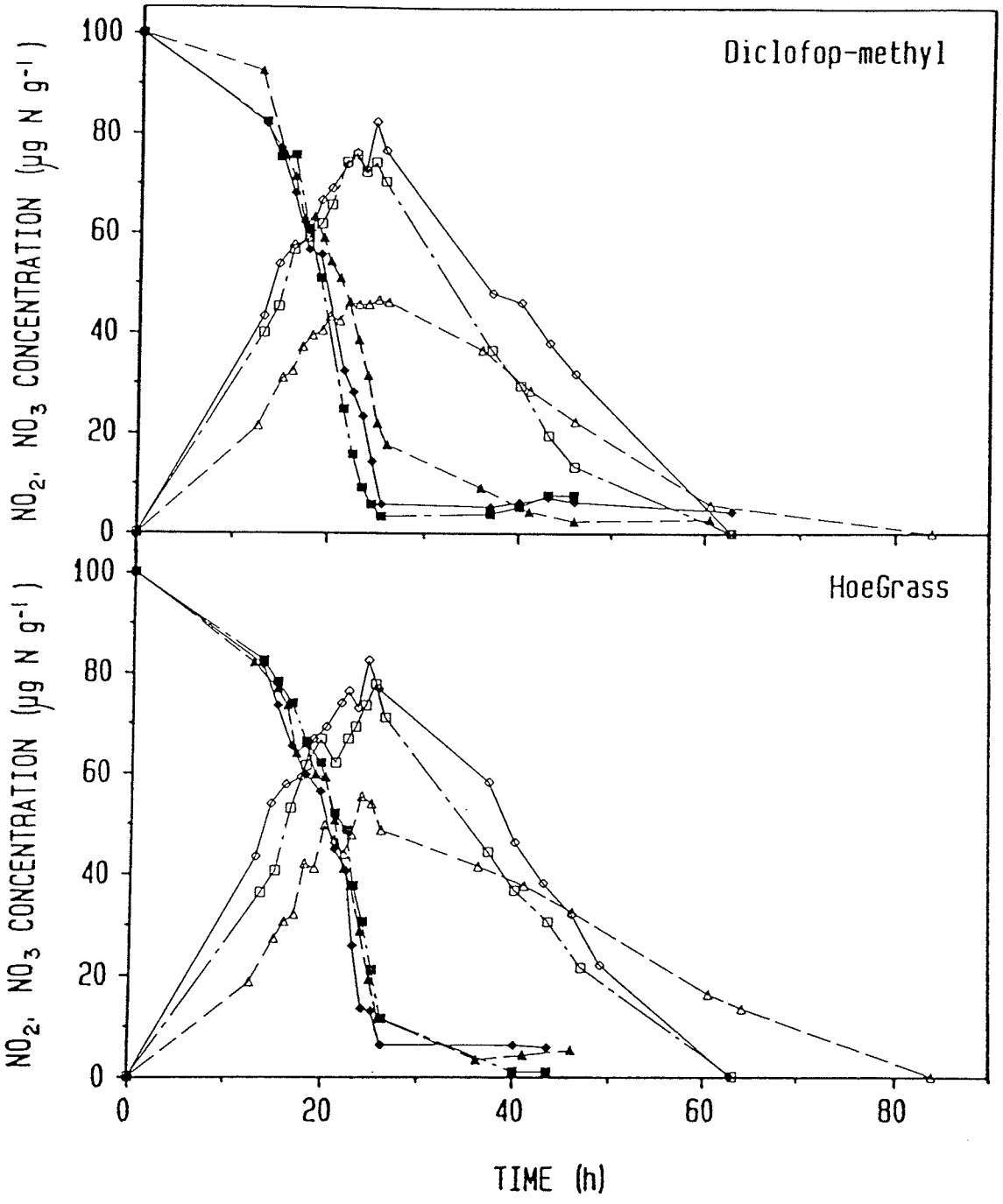

Figure 3: The effect of diclofop-methyl and HoeGrass™ on anaerobic nitrate (closed symbols) and nitrite (open symbols) reduction in Soil P2. Control (◊————◊), 10 µg g^{-1} (□———□), 100 µg g^{-1} (△———△). Data presented for one of several replications.

Table 5: Rates of nitrate reduction ($\mu g\ NO_3\text{-}N\ g^{-1}\ h^{-1}$) as affected by diclofop-methyl and HoeGrass™. Values not followed by the same letter are significantly different at P = 0.05 level.

P2	Control(6)	10 $\mu g\ g^{-1}$(3)	100 $\mu g\ g^{-1}$(3)
Diclofop-Me	7.3 ± 0.8†a	8.5 ± 0.6 b	7.4 ± 1.2 a
HoeGrass™	7.3 ± 0.8 a	7.2 ± 1.1 a	7.5 ± 0.6 a
P2+Glc			
Diclofop-Me	20.1 ± 1.8 cd	19.6 ± 2.8 cd	18.6 ± 1.9 c
HoeGrass™	20.1 ± 1.8 cd	22.1 ± 3.1 d	21.3 ± 2.2 d
P3			
Diclofop-Me	I:††1.1 ± 0.1(4)e	1.0 ± 0.1(1)e	1.2 ± 0.1(2)e
	F: 2.4 ± 0.5 f	3.1 ± 0.2(4)g	2.3 ± 0.3 fh
HoeGrass™	I: 1.1 ± 0.1(4)e	1.1 ± 0.1(2)e	1.0 ± 0.1(2)e
	F: 2.4 ± 0.5 f	2.5 ± 0.6 f	1.9 ± 0.6 h
P3+Glc			
Diclofop-Me	I: 9.3 ± 1.1 j	7.7 ± 0.6 k	5.7 ± 0.9 m
	F: 17.1 ± 2.2 np	21.4 ± 2.6 q	19.5 ± 1.5 pq
HoeGrass™	I: 9.3 ± 1.1 j	8.7 ± 0.5 j-	6.0 ± 0.4 m
	F: 17.1 ± 2.2 np	17.7 ± 1.9 n	14.8 ± 1.0 r

(1) one analysis and mean of (2) two replicates, (3) three replicates, (4) four replicates, and (6) replicates.
† Average ± confidence interval at 95% level.
†† "I" and "F" denote initial and final rates for biphasic reduction.

$$E + (NO_2) \longrightarrow E(NO_2) \longrightarrow E + NO$$

where E is the enzyme responsible. It may be possible that the higher herbicide treatment level enhanced the reduction of nitrite to nitrous oxide, i.e., the overall process, or resulted in a larger amount of the enzyme-substrate complex ($E(NO_2)$); i.e., the herbicide may have increased the rate of formation, k_1, or decreased the rate of dissolution, k_2, of the enzyme-nitrite complex.

The maximum nitrite concentrations obtained for the controls, in relation to the initial nitrate concentrations, were also considerably higher than those obtained by most investigators (Bollag and Henninger 1976, Cho and Sakdinan 1978, Sorensen 1978, Grant and Payne 1982, McKenney *et al.* 1982); however, some investigators have found nitrite concentrations as high as 50-85% of the initial nitrate concentration (Volz and Starr 1977, Komatsu *et al.* 1978). The diversity of procedures found in denitrification experiments and ion analysis prevents easy explanation of this discrepancy. It appears that factors such as the agitation of the incubation flask, the initial nitrate or oxygen concentration, the rate of nitrate reduction, and the use of column or incubation flask apparatus have no bearing on the maximum nitrite levels obtained. The time interval between sampling and analysis may be important in determining the concentration of nitrite obtained. It was observed that if the filtered samples were refrigerated and analyzed the following day, they were occasionally found to contain up to 50% less nitrite. Since extraction and analysis were not performed under strictly aseptic conditions, both chemical and/or biological transformations of nitrite may have occurred. The

potentiality of nitrite loss thus necessitated the analysis of the filtered samples immediately after sampling.

In Soil P2, except for the increase in the maximum rate of nitrate reduction by the 10 $\mu g\ g^{-1}$ diclofop-methyl treatment, the other herbicide treated soils did not differ significantly from the control soils (Table 5). The nitrate reduction rate of 7.3 $\mu g\ NO_3\text{-}N\ g^{-1}\ h^{-1}$ contrasts sharply with the best rate of 0.124 $\mu g\ NO_3\text{-}N\ g^{-1}\ h^{-1}$ obtained by Stanford *et al.* (1975), in a soil containing an extracted "glucose-C" concentration of 164 $\mu g\ g^{-1}$. These authors believed their results reflected both diffusion and nitrate reduction in an unshaken soil. The higher reduction rate in Soil P2, which contains a lower extractable carbon content, is probably due to the soil having been shaken, thus removing the effects of nitrate diffusion to the microbial site.

The times at which nitrate disappeared in Soil P2 differed significantly between all treatments (Table 6), even though the reduction rates were equivalent (Table 5). The 100 $\mu g\ g^{-1}$ treatments consistently produced a 1 to 2 h increase in nitrate disappearance times. This difference in disappearance times for samples with equivalent reduction rates implies that the initiation time for nitrate reduction may have been affected by the presence of diclofop-methyl, possibly due to its effect on the biosynthetic pathways of microbial growth and/or nitrate reductase synthesis.

Nitrite reduction rates in Soil P2 (Table 7) did not differ significantly for any of the 10 $\mu g\ g^{-1}$ treatments but were significantly reduced for the 100 $\mu g\ g^{-1}$ treatments, with HoeGrass™

Table 6. Times for complete nitrate disappearance (h) as calculated from best fit regression line. Values not followed by the same letter are significantly different at P = 0.05 level.

P2		Time (h) of nitrate disappearance for Control[6]	10 $\mu g\ g^{-1}$[3]	100 $\mu g\ g^{-1}$[3]
Diclofop-Me		26.5 ± 3.6†a	24.0 ± 3.6 b	28.1 ± 4.2 c
HoeGrass™		26.6 ± 3.8 a	28.7 ± 5.7 d	27.8 ± 4.9 e
P2+Glc				
Diclofop-Me		15.8 ± 2.2 f	16.2 ± 1.9 g	16.4 ± 2.0 g
HoeGrass™		15.6 ± 2.0 f	15.4 ± 1.9 f	16.6 ± 1.9 h
P3				
Diclofop-Me	I:††	97.6 ± 13.1[4]ij	97.2 ± 13.8[1]ik	97.8 ±14.5[2]jk
	F:	62.2 ± 15.9 mn	52.6 ± 9.9[4]p	62.8 ± 16.2 m
HoeGrass™	I:	97.6 ± 13.1[4]ij	93.9 ± 7.1[2]q	100.0 ± 18.0[2]r
	F:	62.9 ± 15.8 mn	60.6 ± 15.1 n	76.2 ± 14.2 s
P3+Glc				
Diclofop-Me	I:	24.9 ± 7.3 t	25.9 ± 7.9 u	24.7 ± 12.0 v
	F:	22.5 ± 1.7 w	22.5 ± 1.3 x	25.2 ± 1.0 y
HoeGrass™	I:	24.9 ± 7.3 t	25.7 ± 6.0 t	25.4 ± 11.1 z
	F:	22.5 ± 1.7 w	22.6 ± 1.7 w	25.0 ± 1.8 aa

(1) one analysis and mean of (2) two replicates, (3) three replicates, (4) four replicates, and (6) replicates.

† Average ± confidence interval at 95% level.

†† "I" and "F" denote initial and final rates for biphasic reduction.

Table 7. Rates of nitrite reduction ($\mu g\ NO_2^-\text{-}N\ g^{-1}\ h^{-1}$) as affected by diclofop-methyl and HoeGrass™. Values not followed by the same letter are significantly different at P = 0.05 level.

P2	Control(6)	10 $\mu g\ g^{-1}$(3)	100 $\mu g\ g^{-1}$(3)
Diclofop-Me	2.34 ± 0.58†a	2.82 ± 0.20 a	1.25 ± 0.03 b
HoeGrass™	2.34 ± 0.58 a	2.39 ± 0.12 a	0.89 ± 0.17 c
P2+Glc			
Diclofop-Me	3.28 ± 1.00 de	2.49 ± 0.21 d	2.58 ± 0.08 d
HoeGrass™	3.28 ± 1.00 de	2.67 ± 0.22 e	2.41 ± 0.92 e
P3			
Diclofop-Me	1.41 ± 0.19 f	1.35 ± 0.17(4)f	0.86 ± 0.03 g
HoeGrass™	1.41 ± 0.19 f	1.45 ± 0.32 f	0.94 ± 0.17 g
P3+Glc			
Diclofop-Me	2.27 ± 0.22 h	2.29 ± 0.19 h	1.71 ± 0.18 i
HoeGrass™	2.27 ± 0.22 h	2.43 ± 0.20 h	1.27 ± 0.06 j

Mean of (3) three replicates, (4) four replicates, and (6) replicates.
† Average ± confidence interval at 95% level.

being more effective at reducing the rate. The nitrite disappearance times were not affected by the 10 $\mu g\ g^{-1}$ treatments (Table 8). The non-significant 3.7 h decrease in the time of nitrite disappearance observed for the 10 $\mu g\ g^{-1}$ treatment is probably a function of the 2.5 h decrease in the nitrate disappearance time (Table 6), rather than an actual decrease in the nitrite disappearance time.

The reduced nitrite rates for the 100 $\mu g\ g^{-1}$ treatments resulted in large increases in the time of nitrite disappearance; however, it should be noted that the confidence intervals for the nitrite disappearance times were very large, viz., ca. 19-43%. The large confidence intervals may partially have been a result of the inability to exactly define the initiation time of the assays, the flask to flask variations in oxygen concentrations, and/or the larger error encountered in soil nitrite determinations. As in this study, Komatsu *et al.* (1978) also found that even though the reduction curve characteristics, i.e., the rates of nitrate and nitrite reduction, remained the same, the time-course of replicates differed slightly.

With the addition of an energy source (Soil P2+Glc), maximum rates of nitrate and nitrite reduction were initiated earlier and maximum nitrite concentrations were approximately equivalent for all the treatments (Figure 4). In contrast to Soil P2, the time at which the nitrite maximum appeared was increased for the 100 $\mu g\ g^{-1}$ treatments, with a greater increase noted for the HoeGrass™ treatment. Although the nitrate reduction rates appeared to decrease with increasing diclofop-methyl concentrations, the rates were not statistically different (Table 5). However, the time of nitrate disappearance was

Table 8: Times for complete nitrite disappearance (h) as calculated from best fit regression line. Values not followed by the same letter are significantly different at the P = 0.05 level.

P2	Time (h) of nitrite disappearance for Control[6]	10 μg g^{-1}[3]	100 μg g^{-1}[3]
Diclofop-Me	55.8 ± 14.2↑a	52.1 ± 14.1 a	64.4 ± 16.6 b
HoeGrass™	56.4 ± 15.0 c	56.2 ± 15.3 c	81.5 ± 30.8 d
P2+Glc			
Diclofop-Me	37.4 ± 19.1 e	37.1 ± 14.9 e	38.0 ± 14.4 f
HoeGrass™	37.4 ± 19.1 e	37.3 ± 15.0 e	43.2 ± 14.3 g
P3			
Diclofop-Me	85.8 ± 16.1 h	81.8 ± 18.0[4] i	114.0 ± 38.0 j
HoeGrass™	85.1 ± 16.1 h	85.8 ± 17.3 h	135.1 ± 30.4 k
P3+Glc			
Diclofop-Me	54.0 ± 24.6 m	52.6 ± 22.7 m	60.7 ± 25.9 n
HoeGrass™	53.2 ± 23.8 m	56.5 ± 2.10 p	85.9 ± 37.0 q

Mean of [3] three replicates, [4] four replicates, and [6] replicates.
↑ Average ± confidence interval at 95% level.

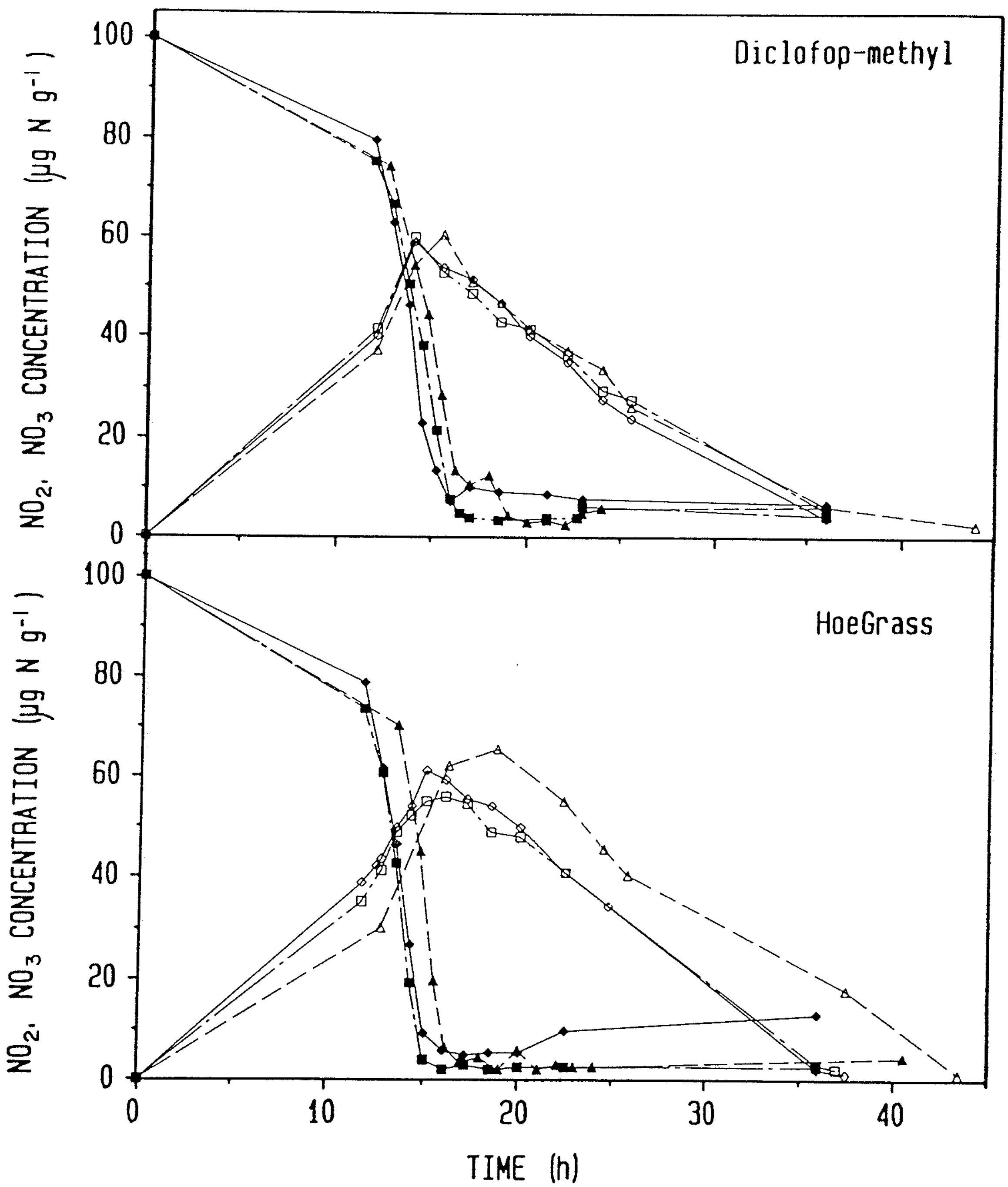

Figure 4: The effect of diclofop-methyl and HoeGrass™ on anaerobic nitrate (closed symbols) and nitrite (open symbols) reduction in soil amended with 100 $\mu g\ g^{-1}$ glucose (Soil P2+Glc). Control (◇———◇), 10 $\mu g\ g^{-1}$ (□— — —□), 100 $\mu g\ g^{-1}$ (△— — — —△). Data presented for one of several replications.

significantly increased by both diclofop-methyl treatments and by the 100 μg g^{-1} HoeGrass™ treatment (Table 6). In the glucose amended soil, nitrite reduction rates appeared to be affected by the diclofop methyl and HoeGrass™ treatments, but these differences were not statistically significant (Table 7). However, the nitrite disappearance times were significantly increased for the 100 μg g^{-1} treatments, with HoeGrass™ producing the greater increase (Table 8).

Under the prevailing experimental conditions, it appears that in soils P2 and P2+Glc, only the 10 μg g^{-1} diclofop-methyl treatment had any significant effect on nitrate reduction, increasing the rate ca. 1.3 μg NO_3-N g^{-1} h^{-1} and producing a corresponding 2.5 h decrease in the disappearance time. The 100 μg g^{-1} treatments consistently increased the nitrate disappearance time without a concomitant decrease in the rate. Cervelli and Rolston (1983) found that atrazine applied at 3 μg g^{-1} also stimulated the rate of nitrate reduction in a soil column.

Under aerobic conditions, the ester bond of diclofop-methyl undergoes rapid hydrolysis to diclofop; up to 17% of the diclofop-methyl has been found to hydrolyze within 15 min of its application to soil (Martens 1978) and the amount of hydrolysis is dependent upon the soil type (Smith 1977). Under aerobic or anaerobic conditions, 86-90% is degraded within two days (Klingman and Ashton 1982, Karanth *et al.* 1984). Under anaerobic conditions diclofop has been found to have a half-life of approximately 150 d in a sandy loam (62% sand, 32% silt, 0.95% OM) (Martens 1978). In the non-glucose amended soils, it may be possible that at the low concentration,

diclofop-methyl and/or diclofop are either serving as an energy source for the denitrifiers or stimulating nitrate reduction. However, the low anaerobic biological degradation probably precludes its use as an energy source. At the high concentration, the time of initiation of nitrate reduction is affected, producing increases in disappearance times. An increase in the disappearance time, without a corresponding decrease in the reduction rate, implies partial inhibition either of a metabolic process(es) or of nitrate reductase synthesis. In the non-glucose amended soil the rate of nitrite reduction is affected by the higher herbicide treatment, resulting in a large increase in the disappearance time. In this case, enzyme function also appears to be affected, which in turn affects the time of nitrite disappearance. Bollag and Henninger (1976) also reported a temporary accumulation of nitrite for 100 $\mu g\ g^{-1}$ 2,4-D treatments.

B. Soils P3 and P3+Glc

Since soil P3 had a slightly higher amount of carbohydrate available for reductions (Table 3), it was expected that nitrate and nitrite reduction would be initiated sooner; however, this was not the case. Maximum nitrate reduction for Soil P3 was initiated at a later time (Figure 5) than in Soil P2 (Figure 3). Maximum nitrite levels, about 45 $\mu g\ NO_2\text{-}N\ g^{-1}$, were slightly lower than obtained for the P2+Glc samples (Figure 4).

Regression analysis of the nitrate curves revealed that some of the curves were biphasic. Of the replications examined for each treatment, from a minimum of 1 of 4, to a maximum of 4 of 6 of the

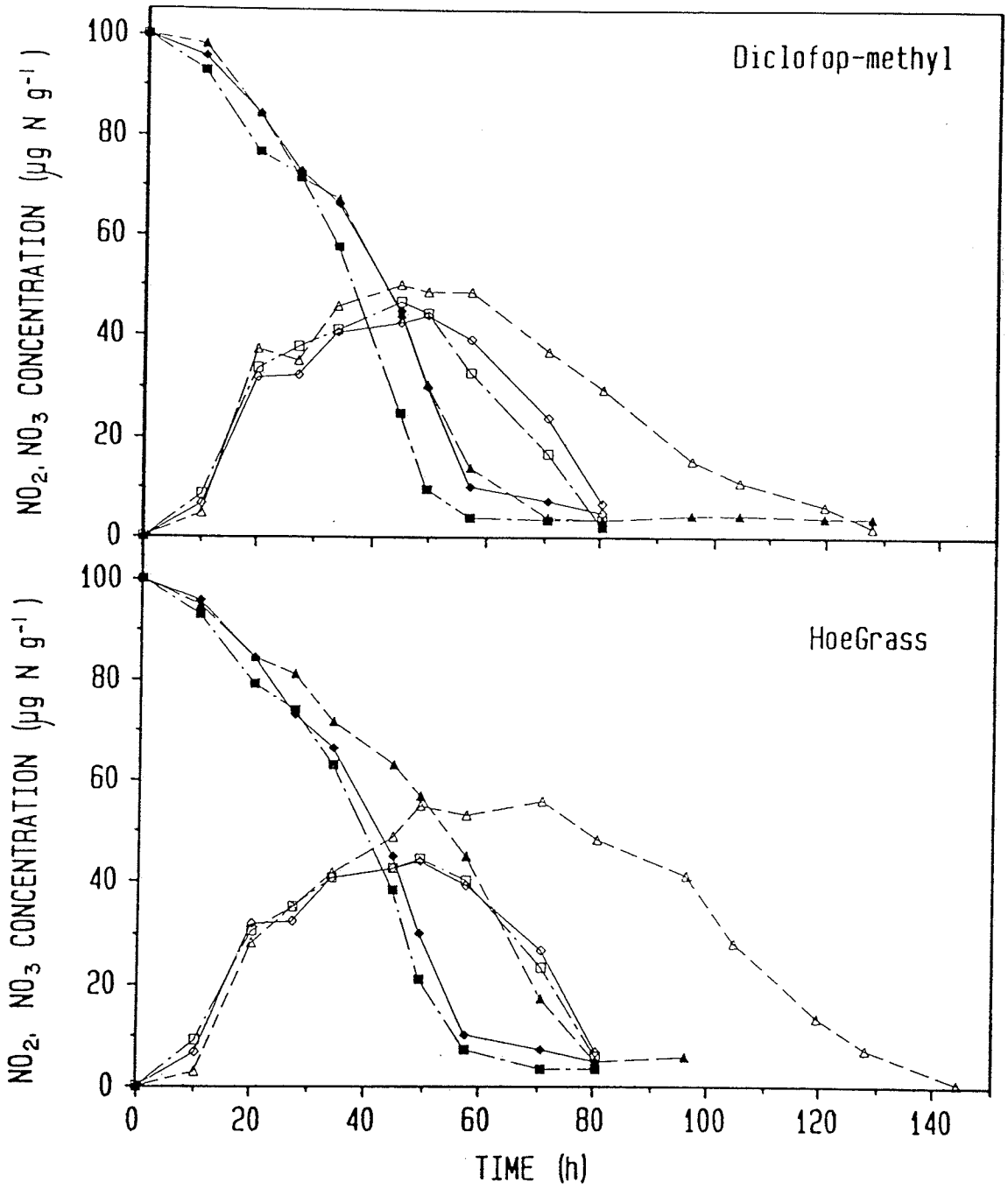

Figure 5: The effect of diclofop-methyl and HoeGrass™ on anaerobic nitrate (closed symbols) and nitrite (open symbols) reduction in Soil P3. Control (◊————◊), 10 µg g^{-1} (□————□), 100 µg g^{-1} (△————△). Data presented for one of several replications.

nitrate reduction curves were biphasic. When biphasic reduction occurred, the initial phase began at approximately 10.5 h and the final phase at about 34.0 h. The biphasic nature of the nitrate reduction curves may be an indication of microbial succession. Since a mixed microbial culture was used, it was possible that a growth factor was affecting the predominating microorganism(s), and thus limiting the observed maximum nitrate reduction rates.

Smith and Parsons (1985) speculated that the persistent denitrifying activity of their dried soils was due to the existence of two distinct denitrifier populations, one slow growing and one fast growing. Smith and Tiedje (1979) consistently observed a biphasic pattern for nitrous oxide evolution. They speculated that as the soil became anaerobic, oxygen inhibition of the native denitrifying enzymes was removed producing the initial linear phase. Oxygen removal derepressed enzyme synthesis such that within 4-8 h after anoxia the cell attained maximum denitrification capacity, resulting in the second linear region of nitrous oxide production. It may be possible that this phenomenon also occurred for nitrate reduction.

The initial nitrate reduction rates were approximately 1/2 the final nitrate rate (Table 5). There were no significant differences in the initial nitrate reduction rates between the treatments, resulting in an average reduction rate of 1.1 ± 0.1 μg NO_3-N g^{-1} h^{-1}. The final reduction rates showed more variation but were statistically equivalent for all the treatment replications, irrespective of the occurrence of the initial reduction. The 10 μg g^{-1} diclofop-methyl treatment had a significantly higher rate, 3.1 ± 0.2 μg NO_3-N g^{-1} h^{-1}, and the 100 μg

g^{-1} HoeGrass™ treatment rate was significantly lower, 1.9 ± 0.61 $\mu g\ g^{-1}\ h^{-1}$.

The approximate 30% differences in the final nitrate reduction rates for the 10 $\mu g\ g^{-1}$ diclofop-methyl and 100 $\mu g\ g^{-1}$ HoeGrass™ treatments resulted in a 10 h decrease and 14 h increase respectively, in the time of nitrate disappearance (Table 6). Unfortunately, the large confidence intervals for this soil, up to 25.8%, places doubt on the validity of the statistical comparisons. The 10 $\mu g\ g^{-1}$ treatments appeared to consistently decrease the time of nitrate disappearance, although this appeared to be significant only for the final diclofop-methyl time, a 10 h decrease, and the initial HoeGrass™ time, a 4 h decrease.

Nitrite reduction rates in Soil P3 were approximately 1/2 the rates observed in Soil P2 (Table 7). The rates observed in Soil P3 for the 100 $\mu g\ g^{-1}$ treatments of diclofop-methyl or HoeGras™ were significantly lower. The lowered rate resulted in large increases in the time of nitrate disappearance (Table 8). Although the slightly lowered reduction rate for the 10 $\mu g\ g^{-1}$ diclofop-methyl treatment was not significantly lower, the nitrite disappeared 4 h earlier. This reduction in the nitrite disappearance time probably reflects the 10 h decrease in nitrate disappearance (Table 6, Figure 5) rather than any decrease in the times of nitrite disappearance.

With the addition of glucose to this soil (P3+Glc), the most noticeable effects were 1) the maximum concentration of nitrite increased almost 2 fold and 2) all the replications of each treatment exhibited a distinct biphasic pattern of nitrate reduction (Figure 6).

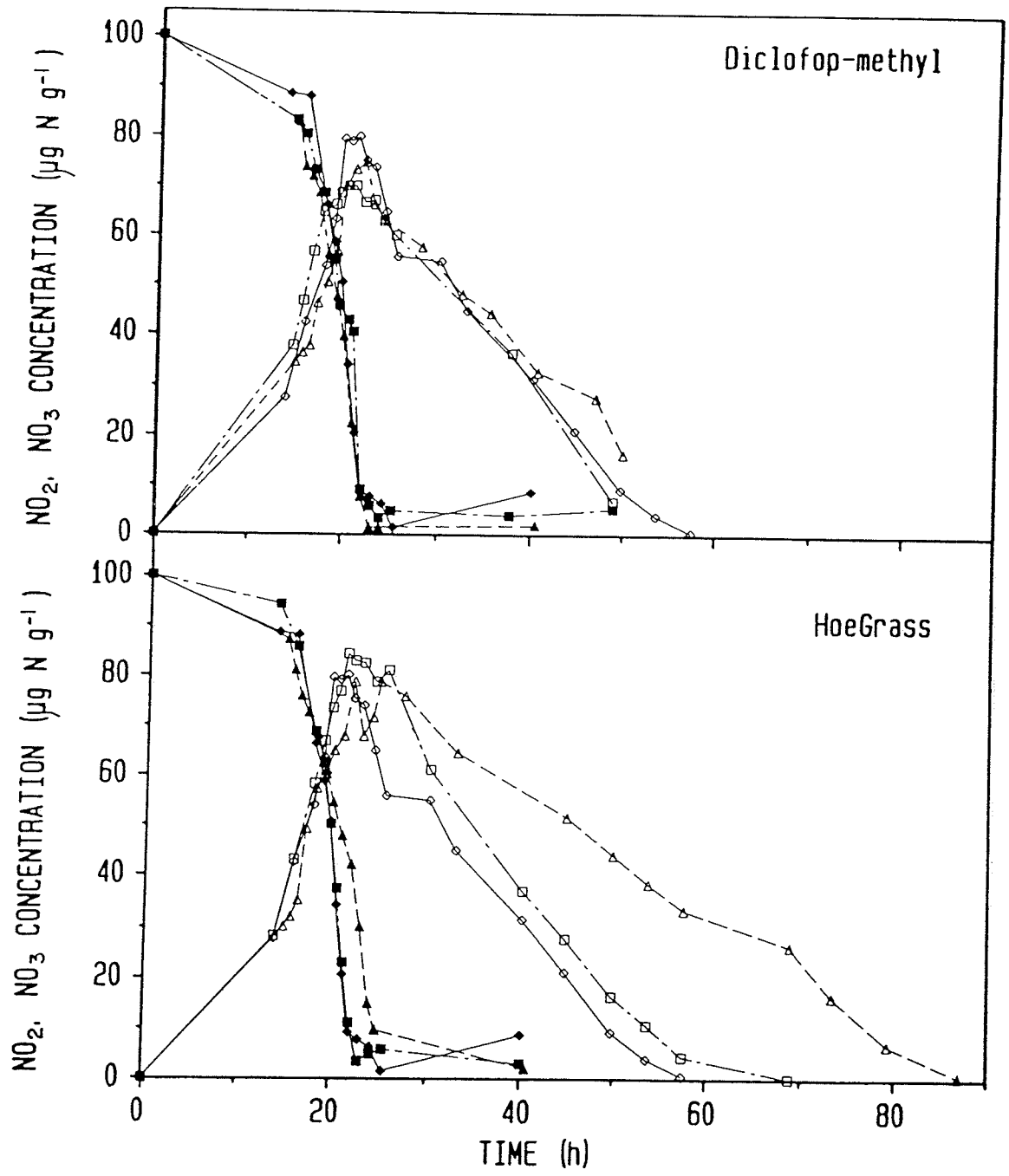

Figure 6: The effect of diclofop-methyl and HoeGrass™ on anaerobic nitrate (closed symbols) and nitrite (open symbols) reduction in soil amended with 100 $\mu g\ g^{-1}$ glucose (Soil P3+Glc). Control (◊————◊), 10 $\mu g\ g^{-1}$ (□— — —□), 100 $\mu g\ g^{-1}$ (△— — — —△). Data presented for one of several replications.

The initial reduction began at approximately 17.0 h and the final reduction at approximately 20.5 h. The increase in an available energy source would appear to be the determining factor in the appearance of the initial reduction. The nitrate reduction rates increased dramatically, almost to levels observed in P2+Glc (Table 5). Except for the 100 $\mu g\ g^{-1}$ diclofop-methyl treatment, the initial and final reduction rates for Soil P3+Glc increased about 6.2-8.8 fold over those for Soil P3, but the difference between the initial and final reduction rates of Soil P3+Glc remained approximately 2 fold.

The initial nitrate reduction rate decreased with increasing concentrations of diclofop-methyl or HoeGrass™. As for Soil P3, the final nitrate reduction rate of the 10 $\mu g\ g^{-1}$ diclofop-methyl treatment was significantly higher than the control and the 100 $\mu g\ g^{-1}$ HoeGrass™ treatment was significantly lower. Fewer apparent differences were noted for the final disappearance times (Table 6) and only the 100 $\mu g\ g^{-1}$ treatments were appreciably different, revealing an approximate 3 h increase.

Nitrite reduction rates in Soil P3+Glc were 1.4 to 2.0 times greater than in soil P3 and were again only significantly different for the 100 $\mu g\ g^{-1}$ treatments (Table 7). Except for the 100 $\mu g\ g^{-1}$ diclofop-methyl treatment, nitrite disappearance times (Table 8) decreased approximately 37% from those in Soil P3; a decrease of 33% was observed in Soil P2+Glc. The disappearance time for the 100 $\mu g\ g^{-1}$ HoeGrass™ treatments was again greater than for the 100 $\mu g\ g^{-1}$ diclofop-methyl treatment.

C. Comparisons Between Soils

As the readily decomposable C source in a soil increases, the rate of nitrate reduction is expected to increase (Burford and Bremner 1975, Stanford *et al.* 1975). Although Soil P3 has a slightly higher % O.M. and concentration of soil "glucose-C" than Soil P2 (Table 3), the rate of nitrate reduction was 68% less than in Soil P2. It would appear that nitrate reduction in Soil P3 is limited by the concentration of a nutrient other than glucose. Stanford *et al.* (1975) found that their sandy soils had very low contents of soil "glucose-C" (24-51 $\mu g\ g^{-1}$) and were poorly correlated with either zero- or first-order reaction kinetics. They postulated that sandy soils may have developed a "deficiency of certain nutrients". In our soils with 100 $\mu g\ g^{-1}$ glucose added, the difference between the nitrate reduction rates of soils P2+Glc and P3+Glc was reduced to 15%. The addition of glucose almost completely alleviated the retardation of nitrate reduction. It appears that the explanation of a nutrient deficiency may not completely account for the differences in nitrate reduction between these two soils. It is possible that the different chemical and/or physical characteristics of the soils enhanced different populations of denitrifiers (Malkomes and Wohler 1983) and that with the addition of glucose, the populations stimulated by the glucose became similar. The stimulation of different populations, over the time course of the assay, could also account for the biphasic reduction rates obtained for Soil P3.

Except for the 10 $\mu g\ g^{-1}$ diclofop-methyl treatment, the nitrate reduction rates of soils P2 and P2+Glc were not significantly affected

by the herbicide treatment (Table 5). On the other hand, the nitrate reduction rates for soils P3 and P3+Glc were sporadically affected by the treatment. The 10 $\mu g\ g^{-1}$ diclofop-methyl treatment in soils P2, P3, and P3+Glc significantly increased the final nitrate reduction rate, generally with a resultant decrease in the disappearance time, and was possibly due to the stimulation of nitrate reductase synthesis and/or function. Except for the diclofop-methyl treatment in Soil P3, the 100 $\mu g\ g^{-1}$ treatments increased nitrate disappearance times, generally without decreases in the reduction rate.

At 85% field capacity in a sandy loam (85% sand, 3.2% O.M.), Smith (1977) found that 68% of the diclofop-methyl, applied at the rate of 2 $\mu g\ g^{-1}$, disappeared within 24 h; whereas, only 40% disappeared in silty clay (32% sand, 38% silt, 11.5% O.M.). Martens (1978) found that, under aerobic or anaerobic conditions, diclofop degraded more slowly in a sandy loam (62.4% sand, 1.0% O.C.) vs. a loamy sand (79.4% sand, 3.2% O.C.). In the presence of 4-chloro-*o*-toluidine, Bollag and Kurek (1980) found a higher concentration of nitrate remaining and of nitrite accumulating with increasing concentrations of sand. The inhibitory effect of 4-chloro-*o*-toluidine was attributed to there being less organic matter available to bind the aniline.

In this study it was assumed that the reduced clay content of Soil P3 might result in less herbicide being complexed by the clay, thus, leaving more herbicide free to affect nitrate and nitrite reduction. A comparison of the final nitrate reduction rates (Table 5) reveals a greater probability of inhibition in the sand (P3), but only at the higher level of Hoegrass™ tested. The percent increase in the nitrate

disappearance time with the 100 $\mu g\ g^{-1}$ treatments was also greater in both P3 and P3+Glc. On the other hand, an increase in the carbon status, i.e., the addition of glucose, did not appear to mitigate or intensify the effect of the herbicide. Consequently, nitrate reduction rates and disappearance times would appear to correlate with the clay content of the two soils. In contrast to the nitrate reduction rates, the herbicide decreased nitrite reduction rates more in the loam (P2) than the sand (P3). With the addition of glucose to both soils, the sand (P3+Glc) had the greater decreased rate as compared to the loam (P3+Glc). The effect of the herbicide on nitrite reductions does not appear to correlate with either the clay or the organic matter content of the soil. A greater selection of soils in future studies may provide more conclusive evidence of this phenomenon.

Diclofop-methyl and HoeGrass™ generally had little effect on nitrate reduction rates at the levels tested. The lower diclofop-methyl level tested, 10 $\mu g\ g^{-1}$, increased the nitrate reduction rates in both soil P2 and P3 and the higher HoeGrass™ level tested, 100 $\mu g\ g^{-1}$, decreased the rate in soils P3 and P3+Glc. Nitrite reduction rates were consistently reduced at the higher level tested, 100 $\mu g\ g^{-1}$. HoeGrass™ appeared to have a greater effect than diclofop-methyl at reducing nitrate or nitrite reduction, thus demonstrating the need to study both the formulated and technical product.

III. Effects of Trifluralin and Treflan™ on Anaerobic Nitrate and Nitrite Reduction

A. Soils P2 and P2+Glc

The nitrate and nitrite reduction curves for the controls and the herbicide treated soils typified the expected trends (Figure 1 vs. Figure 7). The maximum nitrite concentration appeared at approximately the disappearance time of nitrate. Maximum nitrite concentrations for all treatments were approximately 60 $\mu g\ NO_2\text{-}N\ g^{-1}$, but the maxima appeared approximately 12 h later for the 100 $\mu g\ g^{-1}$ trifluralin treatment.

Regression analysis of the linear portions of the nitrate reduction curves revealed no significant difference for any of the treatments, except the 100 $\mu g\ g^{-1}$ trifluralin treatment (Table 9). The average nitrate reduction rate of all the significantly similar treatments was $9.0 \pm 0.4\ \mu g\ NO_3\text{-}N\ g^{-1}\ h^{-1}$. Regression analysis also revealed that the nitrate reduction curves could probably be regarded as having two linear regions. However, it was difficult to ascertain initial linearity objectively; 3 of the 6 control curves generated appeared to have this initial linear region while 1 or 2 of the herbicide treated soils exhibited a possible initial linear phase. The initial rate for the control was $3.7 \pm 0.4\ \mu g\ NO_3\text{-}N\ g^{-1}\ h^{-1}$. Only the final nitrate reduction rates are given in Table 9.

Significant increases in the disappearance times were found for the 10 $\mu g\ g^{-1}$ trifluralin, and the 100 $\mu g\ g^{-1}$ trifluralin and Treflan™ treatments (Table 10). A 2.9 and 9.3 h increase in disappearance times was obtained by the 10 and 100 $\mu g\ g^{-1}$ trifluralin treatments

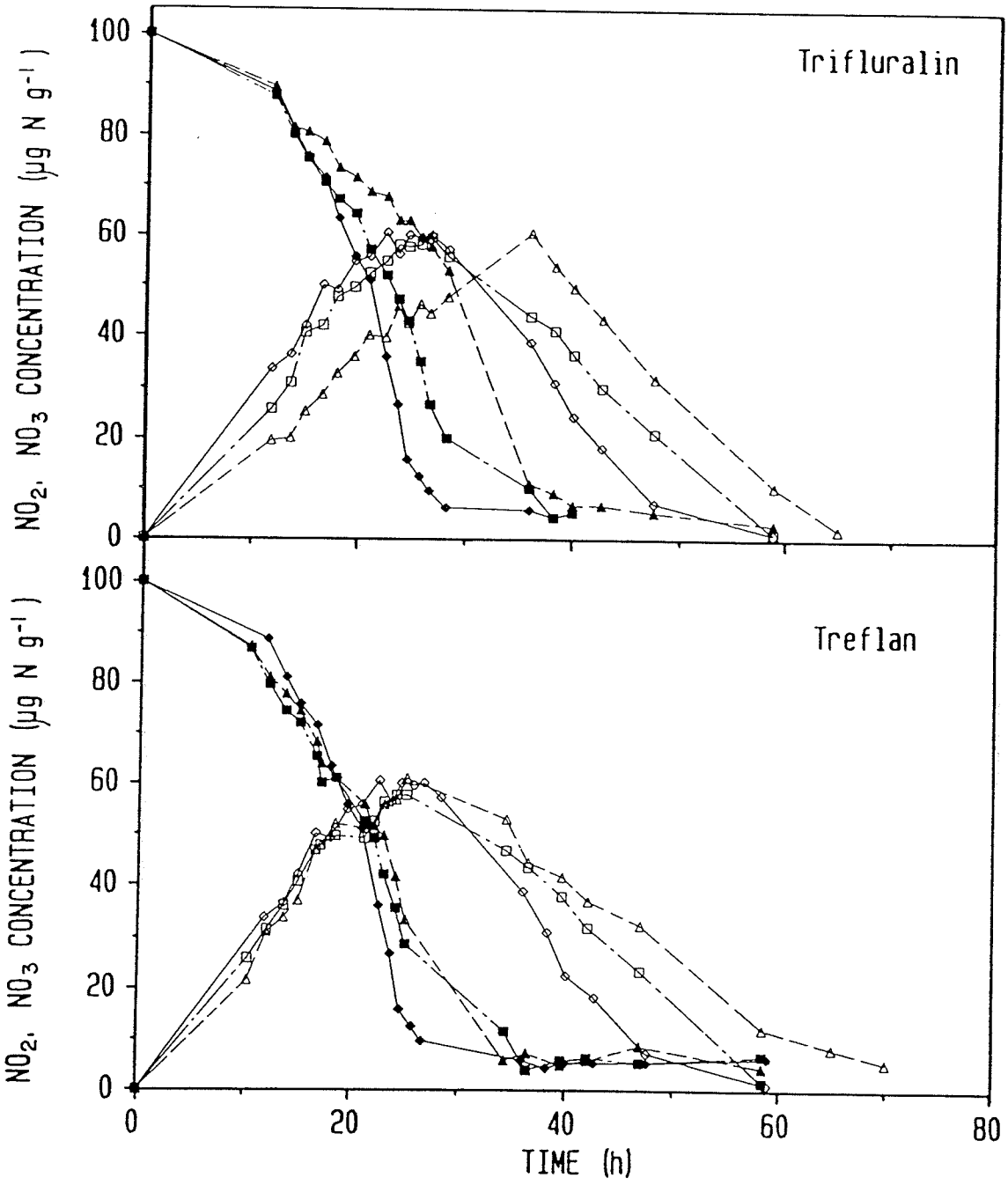

Figure 7: The effect of trifluralin and Treflan™ on anaerobic nitrate (closed symbols) and nitrite (open symbols) reduction in Soil P2. Control (◊————◊), 10 µg g^{-1} (□——□), 100 µg g^{-1} (△——△). Data presented for one of several replications.

Table 9: Rates of nitrate reduction ($\mu g\ NO_3\text{-}N\ g^{-1}\ h^{-1}$) as affected by the presence of trifluralin and Treflan™. Values not followed by the same letter are significantly different at the P = 0.05 level.

P2	Control(6)	10 $\mu g\ g^{-1}$(3)	100 $\mu g\ g^{-1}$(3)
Trifluralin	9.1 ± 0.7†a	8.5 ± 0.5 a	5.6 ± 0.2 b
Treflan™	9.1 ± 0.7 a	8.5 ± 1.2 a	8.3 ± 1.4 a
P2+Glc			
Trifluralin	20.4 ± 2.8 c	23.8 ± 2.2 c	23.2 ± 2.4 c
Treflan™	20.4 ± 2.4 c	22.7 ± 1.5 c	21.3 ± 3.7 c
P3			
Trifluralin I:	††1.1 ± 0.1 d	1.5 ± 0.1 d	1.4 ± 0.1 d
F:	2.4 ± 0.2 e	2.6 ± 0.5 e	2.9 ± 0.3 e
Treflan™ I:	1.1 ± 0.1 d	1.5 ± 0.3 d	1.3 ± 0.1 d
F:	2.4 ± 0.2 e	2.7 ± 0.1 e	2.9 ± 0.8 e
P3+Glc			
Trifluralin I:	3.3 ± 0.5 f	3.5 ± 0.7 f	3.0 ± 0.5 f
F:	17.4 ± 2.3 g	15.2 ± 1.7 g	17.5 ± 3.0 g
Treflan™ I:	3.3 ± 0.5 h	4.5 ± 1.1 i	1.9 ± 0.5 j
F:	17.4 ± 2.3 g	18.9 ± 3.2 g	18.7 ± 2.3 g

Mean of (3) three and (6) six replicates.
† Average ± confidence interval at 95% level.
†† "I" and "F" denote initial and final rates for biphasic reduction.

Table 10: Times for complete nitrate disappearance (h) as calculated from best fit regression line. Values not followed by the same letter are significantly different at the P = 0.05 level.

P2	Time (h) of nitrate disappearance for Control(6)	10 μg g^{-1}(3)	100 μg g^{-1}(3)
Trifluralin	26.4 ± 4.0†a	29.3 ± 4.1 b	35.7 ± 3.7 c
Treflan™	26.5 ± 4.0 a	26.4 ± 5.1 a	28.5 ± 4.9 d
P2+Glc			
Trifluralin	15.9 ± 1.6 e	15.9 ± 1.7 e	16.6 ± 1.5 f
Treflan™	15.9 ± 1.6 e	18.2 ± 1.2 g	17.4 ± 1.1 f
P3			
Trifluralin	I:††96.2 ± 68.2 h	76.4 ± 52.4 i	78.7 ± 70.4 i
	F: 61.1 ± 14.5 j	62.7 ± 10.4 j	64.2 ± 11.7 k
Treflan™	I: 96.2 ± 68.2 h	78.8 ± 57.7 i	78.8 ± 54.9 i
	F: 60.9 ± 14.4 jm	62.8 ± 10.1 j	59.6 ± 15.6 m
P3+Glc			
Trifluralin	I: 39.6 ± 26.5 n	39.1 ± 27.2 n	41.1 ± 27.2 p
	F: 23.3 ± 1.6 q	22.7 ± 1.7 r	24.1 ± 2.1 q
Treflan™	I: 39.1 ± 25.8 mn	27.6 ± 17.1 s	72.8 ± 50.3 t
	F: 23.1 ± 1.4 q	21.9 ± 1.6 r	24.1 ± 1.9 q

Mean of (3) three and (6) replicates.
† Average ± confidence interval at 95% level.
†† "I" and "F" denote initial and final rates for biphasic reduction.

respectively. Treflan™ had less effect than trifluralin on nitrate reduction rates or disappearance times.

Nitrite reduction rates were significantly lower for both treatments of trifluralin or Treflan™ (Table 11), and the Treflan™ treatments decreased the rate more than the trifluralin treatments. The decrease in nitrite reduction rates resulted in large increases in the nitrite disappearance times (Table 12). The disappearance times increased with increasing herbicide concentration. Taking into account the increases in the nitrate disappearance times, the 10 $\mu g\ g^{-1}$ treatments produced an increase in the nitrite disappearance times of 11 and 34% for the trifluralin and Treflan™ treatments respectively. An increase of 10 and 36% for trifluralin and Treflan™ respectively was also noted for the 100 $\mu g\ g^{-1}$ treatments. It appears that the lower herbicide concentration is sufficient to retard nitrite reduction and further increases in the herbicide concentration are not effective.

With the addition of glucose (P2+Glc), nitrate and nitrite reduction were initiated earlier (Figure 8). Maximum nitrite concentrations were slightly higher, 65-70 $\mu g\ NO_2\text{-}N\ g^{-1}$, and with increasing herbicide concentrations, there was slight increase in the time at which the maximum appeared. Nitrate reduction rates increased more than 20-fold and there was little, if any difference between the treatments (Table 9). The average rate for all the treatments was 21.3 $\pm$ 1.9 $\mu g\ NO_3\text{-}N\ g^{-1}\ h^{-1}$. Nitrate disappeared approximately 31-50% faster than in Soil P2. The disappearance times were significantly increased for the 10 $\mu g\ g^{-1}$ Treflan™ and both 100 $\mu g\ g^{-1}$ treatments (Table 10). The nitrite reduction rates were not affected by any of the herbicide

Table 11. Rates of nitrite reduction ($\mu g\ NO_2\text{-}N\ g^{-1}\ h^{-1}$) as affected by the presence of trifluralin and Treflan™. Values not followed by the same letter are significantly different at the P = 0.05 level.

P2	Control(6)	10 $\mu g\ g^{-1}$(3)	100 $\mu g\ g^{-1}$(3)
Trifluralin	3.44 ± 0.32↑a	2.49 ± 0.31 b	2.36 ± 0.13 b
Treflan™	3.44 ± 0.32 a	1.94 ± 0.55 c	1.57 ± 0.33 c
P2+Glc			
Trifluralin	4.03 ± 0.70 d	3.64 ± 0.50 d	3.73 ± 0.44 d
Treflan™	4.03 ± 0.70 d	3.67 ± 0.71 d	3.12 ± 0.27 d
P3			
Trifluralin	1.36 ± 0.31 eg	1.03 ± 0.10 f	1.18 ± 0.31 ef
Treflan™	1.36 ± 0.31 g	1.25 ± 0.13 g	1.57 ± 0.14 g
P3+Glc			
Trifluralin	2.68 ± 0.18 hij	2.61 ± 0.33 h	2.14 ± 0.40 i
Treflan™	2.68 ± 0.18 hij	2.54 ± 0.37 j	1.40 ± 0.09 k

Mean of (3) three and (6) replicates.
↑ Average ± confidence interval at 95% level.

Table 12. Times for complete nitrite disappearance (h) as calculated from best fit regression line. Values not followed by the same letter are significantly different at P = 0.05 level.

P2	Time (h) of nitrite disappearance for Control(6)	10 $\mu g\ g^{-1}$(3)	100 $\mu g\ g^{-1}$(3)
Trifluralin	47.0 ± 9.1†a	55.2 ± 15.3 b	60.8 ± 18.8 c
Treflan™	47.0 ± 9.1 a	63.0 ± 28.3 d	65.9 ± 24.0 e
P2+Glc			
Trifluralin	31.9 ± 15.1 f	32.5 ± 13.2 f	33.9 ± 14.3 g
Treflan™	32.7 ± 16.2 f	32.9 ± 15.0 f	35.2 ± 16.4 h
P3			
Trifluralin	122.5 ± 17.8 i	132.1 ± 36.8 j	153.2 ± 24.9 j
Treflan™	121.5 ± 14.0 i	131.0 ± 23.7 m	147.1 ± 21.2 n
P3+Glc			
Trifluralin	55.7 ± 24.4 p	55.3 ± 21.8 p	67.3 ± 35.3 q
Treflan™	56.0 ± 22.3 p	56.1 ± 23.3 p	93.6 ± 44.5 r

Mean of (3) three and (6) replicates.
† Average ± confidence interval at 95% level.

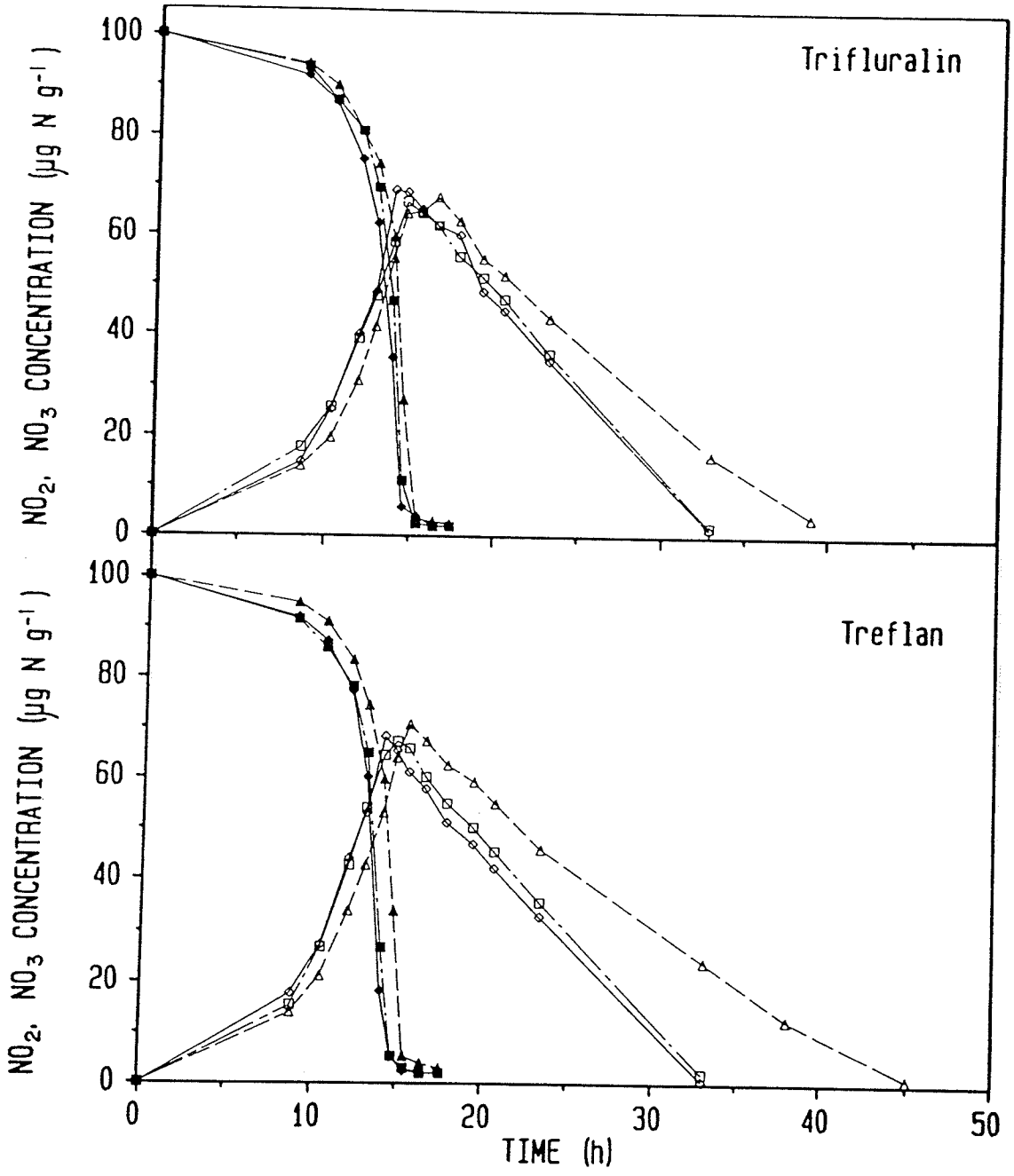

Figure 8: The effect of trifluralin and Treflan™ on anaerobic nitrate (closed symbols) and nitrite (open symbols) reduction in soil amended with 100 $\mu g\ g^{-1}$ glucose (Soil P2+Glc). Control (◊———◊), 10 $\mu g\ g^{-1}$ (□– – –□), 100 $\mu g\ g^{-1}$ (△– – –△). Data presented for one of several replications.

treatments (Table 11). The nitrite disappearance times were slightly increased, ca. 4%, by both 100 $\mu g\ g^{-1}$ treatments (Table 12). The nitrite reduction rates increased approximately 20% over those of Soil P2; the disappearance times of the controls decreased approximately 30%.

Trifluralin is strongly adsorbed to and inactivated by organic matter in the soil (Grover 1974, Horowitz *et al.* 1974, Weber *et al.* 1974, Harrison and Weber 1975) and thus removed from the effects of degradation (Grover 1974, Wheeler *et al.* 1979, Zeyer and Kearney 1983). Yeomans and Bremner (1985) incubated 3 soils under a helium atmosphere and found that after 8 days, 10 $\mu g\ g^{-1}$ trifluralin had no effect in any of the soils. In a low organic carbon (0.5% O.C.) silty clay loam (13% sand, 28% clay), 50 $\mu g\ g^{-1}$ trifluralin enhanced denitrification, i.e., nitrogen evolution was increased, but inhibited nitrite reduction. In a high organic (6.6%) silty clay (10% sand, 41% clay), 50 $\mu g\ g^{-1}$ trifluralin was found to only enhance denitrification. In this study, the addition of glucose to Soil P2, i.e., increasing in the carbon status of the soil, appeared to alleviate the effect of the herbicides on anaerobic reductions, especially nitrite reduction. However, Yeomans and Bremner (1985) also observed no effect on nitrite levels or denitrification in a clay loam (31% sand, 30% clay) with 3.3% O.C. It may be possible that the observed effects on nitrite reduction are moderated by the soluble fraction of soil organic carbon rather than the total soil organic carbon. Nitrate reduction has been found to correlate better with the extractable glucose carbon (Stanford *et al.* 1975) and this may also be the case for nitrite. Nitrogen and nitrous oxide evolution were also found to be highly correlated with

water-soluble organic carbon (Burford and Bremner 1975). Bromacil, chlorotoluron, diquat, diuron, glyphosate, and simazine have been found to preferentially bind to the water-soluble soil organic matter (Madhun *et al.* 1986).

With regard to the observed effects of herbicides on denitrification, Yeomans and Bremner (1984) found that "the conclusions reached were not significantly affected by the time of sampling". In the present study this was not the case. An examination of the reduction curves for nitrate and nitrite (Figure 7) reveals that at certain times the treatments do have differential effects. For example, the average difference between the nitrite concentrations of the control and 10 $\mu g\ g^{-1}$ diclofop-methyl treatment in Soil P2 at 35.75 h is 13.8% while at 42.50 h the difference is 66%. The time of sampling may thus determine the perceived affect of a herbicide.

B. Soils P3 and P3+Glc

In Soil P3, all the nitrate reduction curves exhibited biphasic behavior (Figure 9). The maximum nitrite concentrations obtained were approximately 65 $\mu g\ NO_2\text{-}N\ g^{-1}$ and increased levels were observed for the 100 $\mu g\ g^{-1}$ trifluralin, and the 10 and 100 $\mu g\ g^{-1}$ Treflan™ treatments. The 100 $\mu g\ g^{-1}$ Treflan™ treatment produced an approximate 30% increase in the maximum nitrite concentration. The time at which the maximum appeared was slightly increased by the herbicide treatments.

Both the initial and final nitrate reduction rates were not

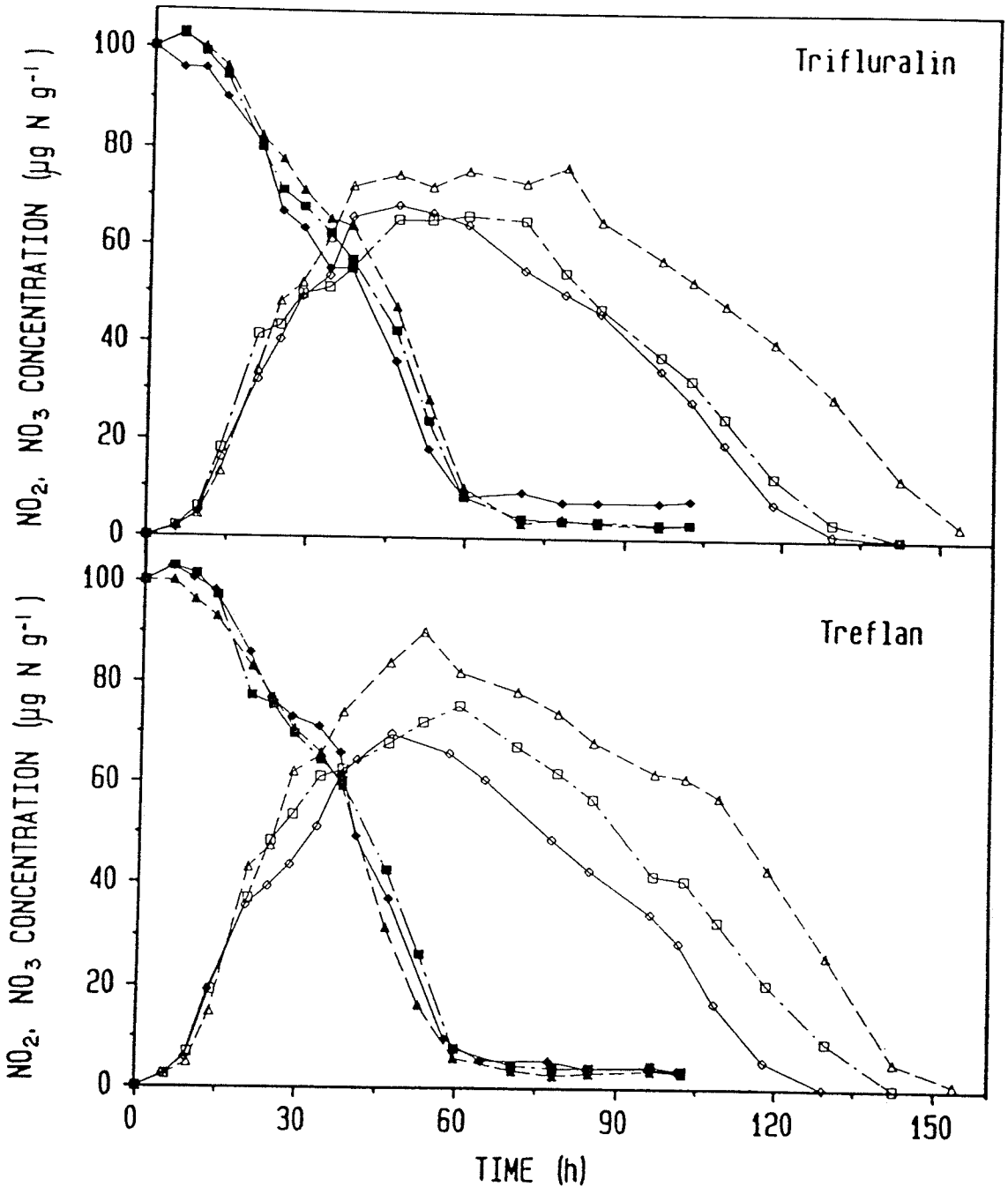

Figure 9: The effect of trifluralin and Treflan™ on anaerobic nitrate (closed symbols) and nitrite (open symbols) reduction in Soil P3. Control (◊————◊), 10 µg g^{-1} (□——— —□), 100 µg g^{-1} (Δ——— —Δ). Data presented for one of several replications.

significantly different for any of the treatments (Table 9), but the 100 $\mu g\ g^{-1}$ trifluralin treatment significantly increased the final disappearance time by approximately 3 h (Table 10). The rates of nitrite reduction were sporadically decreased by the trifluralin treatments, with the 10 $\mu g\ g^{-1}$ treatment being significantly more inhibitory than the 100 $\mu g\ g^{-1}$ (Table 11). Disappearance times increased substantially with increasing herbicide concentration (Table 12) and there was little difference between equivalent treatments of trifluralin and Treflan™.

With the addition of glucose (P3+Glc) there was a dramatic increase in the maximum nitrite concentrations observed, but fewer differences in the maximas between the treatments (Figure 10); the time of appearance of the 100 $\mu g\ g^{-1}$ maxima was slightly increased. The control nitrite maxima increased approximately 50% over those in the non-glucose amended soil.

The initial nitrate reduction rates for the Treflan™ treatments varied sporadically (Table 9). There were no significant differences between the final reduction rates for any of the trifluralin or Treflan™ treatments. The average of the final rates, 17.5 ± 1.1 $\mu g\ NO_3\text{-}N\ g^{-1}\ h^{-1}$, was approximately 7 times that obtained in Soil P3. Both the 10 $\mu g\ g^{-1}$ trifluralin and Treflan™ treatments significantly reduced the time of nitrate disappearance by approximately 0.6 h (Table 10), an effect not observed in Soil P3. The addition of glucose also lowered the error associated with the disappearance times.

Nitrite reduction rates varied slightly for the trifluralin treatments and were only significantly reduced by the 100 $\mu g\ g^{-1}$

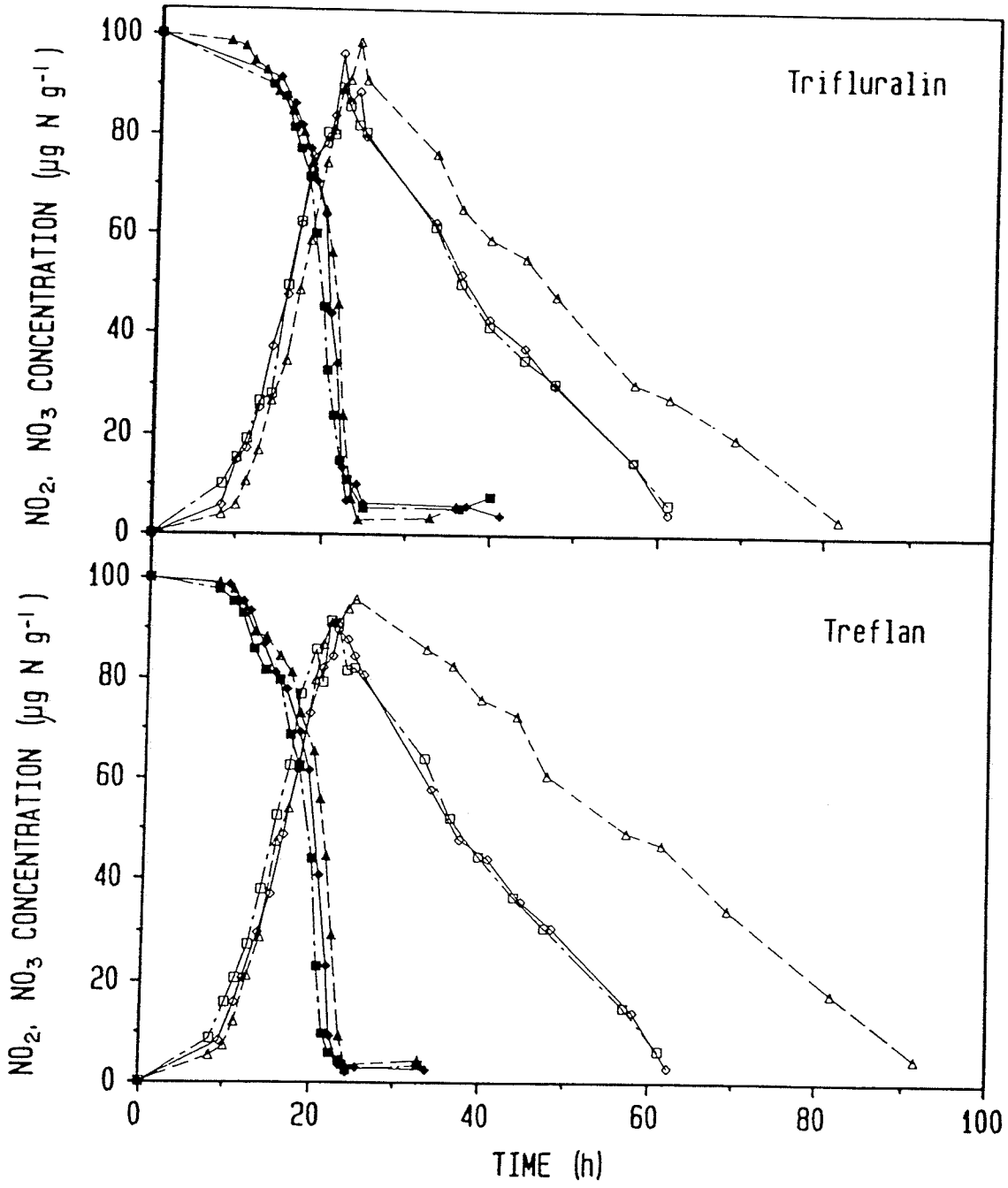

Figure 10: The effect of trifluralin and Treflan™ on anaerobic nitrate (closed symbols) and nitrite (open symbols) reduction in soil amended with 100 $\mu g\ g^{-1}$ glucose (Soil P3+Glc). Control (◊————◊), 10 $\mu g\ g^{-1}$ (□— — —□), 100 $\mu g\ g^{-1}$ (△— — — —△). Data presented for one of several replications.

Treflan™ treatment (Table 11). Both the 100 $\mu g\ g^{-1}$ trifluralin and Treflan™ treatments produced an increase in the nitrite disappearance times, with the Treflan™ treatment being more effective (Table 12). Except for the 67% increase in Soil P3+Glc by the 100 $\mu g\ g^{-1}$ Treflan™ treatment, the 100 $\mu g\ g^{-1}$ trifluralin and Treflan™ treatments increased the nitrite disappearance times by approximately 21% in both Soil P3 and P3+Glc.

3. Comparisons Between Soils

Some experimentation with trifluralin and Treflan™ treatments of Soil P3 was first initiated in the spring of 1985. The same analysis was performed in early 1986 and was found to produce statistically different results. The soil nitrate level of the dried and sieved stored soil had risen from approximately 90.6 ± 1.7 $\mu g\ NO_3\text{-}N\ g^{-1}$ to 111.3 ± 2.0 $\mu g\ NO_3\text{-}N\ g^{-1}$. Nitrate and nitrite reduction was found to proceed at lower rates in 1985 and the maximum levels of nitrite obtained were generally 5-10% lower than in 1986. These initial analyses (1985) were not included in the reduction rate or disappearance time calculations. It would appear that within the "normal" time-frame of the experiment, e.g., 2 months, good reproducibility is attained; however, over an extended period of time, the reproducibility between the initial and final analysis is poor. As previously stated in the introduction, soil storage can affect the parameters studied. In this instance the soil was stored air-dried in glass containers. Air-drying soil has been shown to increase the denitrification rate (Letey *et al.* 1980) and increase the level of biomass nitrogen (Ross *et al.* 1980).

Ross *et al.* (1980) also found that 1 of 4 soils showed an approximate 40% increase in the mineralizable nitrogen content between the 28th and 56th day of storage at 25°C.

Nitrate reduction rates were 3.5 times greater in Soil P2 than Soil P3 and nitrate disappeared from Soil P2 in approximately 1/2 the time. In view of the higher % O.M. and glucose equivalent content of Soil P3, the increase in rates is a contradiction of classical thinking; i.e., nitrate reduction increases with increasing carbon supply. It is more probable that the difference in carbon levels between the two soils is not significant and that the reductions are limited by another nutrient(s), reversing the expected trend of increased nitrate reduction with increased carbon content (Stanford *et al.* 1975).

In Soil P3, the maximum nitrite concentrations attained were greater than in Soil P2 and were especially noticeable in the glucose amended soils, P3+Glc (Figure 10) vs. P2+Glc (Figure 8). The higher amounts of nitrite appearing in Soil P3 may be related to the slower nitrate reduction rate in this soil, although inferentially, slower nitrate reduction rates should result in lower nitrite levels.

With the exception of the 100 $\mu g\ g^{-1}$ trifluralin treatment in Soil P2, the final nitrate reduction rates in both soils were not affected by any of the herbicide treatments. The 10 $\mu g\ g^{-1}$ treatments had no effect or increased the final nitrate disappearance times in Soil P2 and Soil P2+Glc, had no effect in Soil P3, and decreased the times in Soil P3+Glc. The 100 $\mu g\ g^{-1}$ treatments generally increased the final disappearance times in all soils except Soil P3+Glc, where no effects were observed. Except for the 9.3 h (35%) increase by 100 $\mu g\ g^{-1}$

trifluralin in Soil P2, any increases in the final disappearance times were less than 14% of the control.

Trifluralin is known to be strongly adsorbed and inactivated by the soil organic matter (Grover 1974, Horowitz *et al.* 1974, Weber *et al.* 1974, Harrison and Weber 1975, Solbakken *et al.* 1982). Since organic matter is required for optimum nitrate reduction, it is questionable how the higher organic matter soil (P3) is affected less by the herbicide if the herbicide is adsorbed to the organic matter. Utilization of the organic matter during reduction should expose the microorganism to higher levels of the herbicide. This would imply that trifluralin is relatively non-toxic to soil microorganisms at the levels examined. Growth studies of 18 pure bacterial cultures has revealed little effect by 400 $\mu g\ g^{-1}$ of Treflan EC and no effect by 16 $\mu g\ g^{-1}$ (Olson *et al.* 1984). At 100 $\mu g\ mL^{-1}$ of trifluralin, Jacobson *et al.* (1980) found that under discontinuous anaerobiosis none of the ^{14}C label entered the nucleoside pool of sewage microflora, indicating that trifluralin was co-metabolized. Co-metabolism of a herbicide may have less of an effect on the microbial anabolic and catabolic pathways (Perry 1979).

Nitrite reduction rates were approximately 2.5 times less in Soil P3 than Soil P2. Nitrite reduction rates were significantly reduced in Soil P2 by all trifluralin and Treflan™ treatments and in Soil P3+Glc by the 100 $\mu g\ g^{-1}$ Treflan™ treatment. In the non-glucose amended soils, nitrite disappearance times increased in the presence of the herbicides and increased with increasing herbicide concentrations in Soil P2. With the addition of glucose, only the 100 $\mu g\ g^{-1}$ treatments were effective at increasing the disappearance times. With exceptions, the higher the

soil carbon content, the less of a difference was observed in the disappearance times.

There appeared to be little effect of trifluralin/Treflan™ on nitrate reduction rates and sporadic but substantial effects on nitrite rates. The disappearance times of nitrate and, to a greater extent nitrite, were affected, especially at the higher level. Assuming equivalent microbial populations, it would appear that the herbicide does not affect nitrate reductase but can effect nitrite reductase. The retardation of the reduction process, as shown by the increase in disappearance times, probably resulted from the inhibition of another biosynthetic process. As outlined in the Historical, the varied responses of the microbial population to this herbicide nullify an attempt to elucidate the actual cause of the retardation of nitrite reduction without other growth indicators, such as CO_2 evolution, CFU, and enzyme activities. It is, however, relatively apparent that the soils may differentially influence the effect of this herbicide on anaerobic reductions, with the addition of organic carbon to the soil reducing the perceived effect.

IV. Comparisons Between Herbicides of Effects on Anaerobic Nitrate and Nitrite Reductions

Statistical analyses of the set of control replicates from the diclofop-methyl/HoeGrass™ work and the trifluralin/Treflan™ work were performed separately. The average nitrate reduction rates for the control replicates of the same soil were in good agreement between the two sets of experiments, e.g., the nitrate reduction rates of Soil P3 for the diclofop-methyl/HoeGrass™ experiment (Table 5) and for the trifluralin/Treflan™ experiment (Table 9). Only the nitrate reduction rates of Soil P2 were considerably different and may have been due to the length of time between the two sets of experiments. Twelve months elapsed between the first and last set of assays; the effect of diclofop-methyl/HoeGrass™ in Soil P2 was the first phenomenon examined, while the effect of trifluralin/Treflan™ was the last. The difference between the controls of Soil P2 may be due to the effects of storage on either the soil or the stock microbial culture. If this was the case, experiments performed over considerable time intervals could produce inconsistent results, no matter how consistent the experimental method. An indepth study of the microbial populations of the stock culture or the chemical characteristics of the stored soils might provide some insight into this phenomenon.

In both the diclofop-methyl/HoeGrass™ and the trifluralin/Treflan™ work, the nitrate reduction rates were sporadically and minimally affected (Tables 5 and 9). The 10 $\mu g\ g^{-1}$ diclofop-methyl treatments increased the nitrate reduction rates in Soil P2, P3, and P3+Glc; the 100 $\mu g\ g^{-1}$ Hoegrass™ treatments decreased the rates in Soil P3 and

P3+Glc; and the 100 $\mu g\ g^{-1}$ trifluralin treatment decreased the rate in Soil P2.

The final nitrate disappearance times for the controls of the two experiments were also in close agreement (Tables 6 and 10). Disappearance times for the herbicide treatments of the two experiments revealed no general trends. However, it is significant to note that the confidence intervals for the final nitrate disappearance times obtained for Soil P3 were unusually high in both experiments, from 16.1-26.1%, and this large variation may mask any significant trends. Diclofop-methyl and HoeGrass™ had less of an effect on the disappearance times in Soil P2 and P2+Glc, while trifluralin and Treflan™ had less of an effect in Soil P3 and P3+Glc. Little is known about the actual interaction of the soil and diclofop-methyl as it affects the microbial population, and from this study, it is difficult to definitively state whether the clay or organic matter content was the more important factor in moderating the effects. On the other hand, the effects of trifluralin on the nitrate disappearance times appeared to be moderated in the higher % O.M. soil or in the soils amended with glucose. In view of the limited effects of the herbicides on nitrate reduction rates, it appears that nitrate reductase is only slightly affected by these herbicides. Further work with other herbicides, purified nitrate reductase, and biosynthetic pathways will clarify the nature of the effects on nitrate disappearance.

A close examination of the nitrite control values from the diclofop-methyl/HoeGrass™ and trifluralin/Treflan™ work revealed a greater difference between the reduction rates of nitrite than that

experienced for nitrate. The greatest differences between the controls were observed in 1) Soil P2, the first (diclofop-methyl) and last (trifluralin) experiments done, and 2) Soil P2+Glc, the second (diclofop-methyl) and second last (trifluralin) experiments done (Tables 7 and 11). The differences in the rates of the controls were approximately 5-10 times greater than for P3 and P3+Glc. The confidence intervals for the nitrite reduction rates were also proportionally larger than that experienced for nitrate reduction. It would appear that nitrite is more sensitive to the experimental conditions and/or the soil properties. As hypothesized for nitrate reduction, the lengthy storage period may have affected the observed nitrite reduction rate. Changes in temperature of 5°C have been found to greatly affect the nitrite reduction curve (McKenney *et al.* 1984). It was found that the nitrite reduction rate decreased with decreasing temperature in both soils, but the maximum concentration of nitrite formed increased with increasing temperature in one soil and decreased in the other. All assays in this study were performed at a minimum temperature of 24°C or the prevailing room temperature; any increases in temperature, caused by fluctuating room temperatures, could have affected the time course of the replicates and be reflected as errors in the disappearance times.

The nitrite reduction rates were not affected by the 10 $\mu g\ g^{-1}$ treatments of diclofop-methyl and HoeGrass™ in all soils (Table 7). Trifluralin and Treflan™ at 10 $\mu g\ g^{-1}$ sporadically affected nitrite rates in the non-glucose amended soils (Table 11). At 100 $\mu g\ g^{-1}$, diclofop-methyl and HoeGrass™ treatments decreased the reduction rates

in all soils except P2+Glc, while trifluralin and Treflan™ treatments again sporadically affected the rates. The disappearance times were also minimally affected by the 10 $\mu g\ g^{-1}$ diclofop-methyl and HoeGrass™ treatments (Table 8), compared to trifluralin and Treflan™ treatments (Table 12); however, trifluralin and Treflan™ were not effective in the glucose amended soils. At 100 $\mu g\ g^{-1}$, diclofop-methyl and Hoegrass™ treatments had a greater effect at increasing the disappearance times than trifluralin and Treflan™, except for the Treflan™ treatment in Soil P3+Glc.

An examination of the nitrate and nitrite reduction rates and disappearance times revealed that in most cases the effects of the herbicides were moderated by the addition of glucose. The decrease in the effect of the herbicide with glucose addition to the soil may be due to the binding of the herbicide to the glucose molecule, to the stimulation of a different population, or to the increase in the metabolic rate of the microbial cells. On the other hand, Trevors (1982) found that pentachlorophenol inhibition of the electron transport system activity was increased with the addition of 1% glucose. Pure culture studies with diclofop-methyl and trifluralin could help to elucidate the apparent effect of glucose.

In general, diclofop-methyl (HoeGrass™) had a greater effect on the anaerobic reductions than trifluralin (Treflan™). On initial examination, the observed lesser effect of trifluralin could be attributed to the differences in solubility between trifluralin and diclofop-methyl; trifluralin is less soluble in water than diclofop-methyl (Table 13). Based only on the herbicide solubilities,

Table 13: Solubilities of diclofop-methyl and trifluralin in water.

Diclofop-methyl (mg L-1)	Trifluralin (mg L-1)	Reference
-	24.0 @ 27°C	Spencer (1973)
-	0.3	Helling (1976)
0.3 @ 22°C	<1.0 @ 27°C	Worthing (1979)
-	0.05	Weber *et al.* (1980)
3.0 @ 22°C	4.0 @ 20°C	Spencer (1982)
50.0 @ 22°C	<1.0 @ 27°C	Hartley & Kidd (1983)

the more soluble herbicide, diclofop-methyl, would be presumed to be degraded more rapidly (Alexander 1973) or in this case, have a greater effect on the reduction processes. As previously mentioned, under aerobic conditions trifluralin binds strongly to the soil organic matter, is relatively immobile, and has a half-life of approximately 28 d (Grover 1974, Weed and Weber 1974, Golab *et al.* 1979). Diclofop-methyl rapidly degrades to diclofop, which undergoes strong binding or complexing with the soil and has a half-life of 150 d under anaerobic conditions (Smith 1977, Martens 1978). No $^{14}CO_2$ was evolved from ^{14}C-labelled diclofop-methyl after 25 wk of anaerobic incubation (Martens 1978). Bollag and Nash (1974) studied the effect of 11 urea herbicides on nitrous oxide and carbon dioxide production in a liquid medium. The most soluble herbicides, monuron, metobromuron, and fenuron (230 mg L^{-1} to 2.85 g L^{-1}, Hartley & Kidd 1983), were found to have no effect on nitrous oxide or carbon dioxide production at concentrations up to 200 g mL^{-1}. The six less soluble herbicides (18-105 mg L^{-1}) were found to inhibit nitrous oxide production and stimulate carbon dioxide production at 50 g mL^{-1}. The only exceptions were metoxuron (678 mg L^{-1}) which inhibited nitrous oxide production at 100 and 200 g mL^{-1} and chloroxuron (4.0 mg L^{-1}) which was only inhibitory at 100 g mL^{-1}. Herbicide toxicity appeared to be inversely related to the solubility of the herbicides.

Herbicide-microbe interactions are a function of various interrelated variables, such as physico-chemical properties, toxicity of the herbicide, species composition of the population, and interspecies interactions. Trifluralin has been found to be relatively

non-toxic to most soil microorganisms; of the 18 bacterial strains studied in aerobic pure cultures, 2 of 2 denitrifiers and 10 of 12 nitrate reducers were not affected by 400 $\mu g\ g^{-1}$ of Treflan™ EC after 2-3 d (Olson et al. 1984). No studies on the toxicity of diclofop-methyl to microbial isolates are available.

A factor which should also be considered is that the native soil microorganisms are firmly attached to the soil components (Balkwill et al. 1977). The organic particles, which may represent as little as 15% of the colonizable surface area, can be populated by 60% (by mass) of the soil microorganisms; mineral particles are only minimally colonized (Scow 1982). The close proximity of the organically associated microorganisms and the organically bound herbicide may be of more importance than the amount of herbicide in solution.

The present study indicated that at 10 and 100 $\mu g\ g^{-1}$, diclofop-methyl and HoeGrass™ could stimulate or inhibit the nitrate reduction rate, whereas trifluralin and Treflan™ generally had no effect. Nitrate disappearance times were affected more by diclofop-methyl or HoeGrass™ and the inhibition was greatest at 100 $\mu g\ g^{-1}$ in Soil P3 and P3+Glc. Nitrite reduction was not affected by diclofop-methyl or HoeGrass™ at 10 $\mu g\ g^{-1}$; whereas, trifluralin or Treflan™ generally inhibited nitrite reduction in the non-glucose amended soils. At the 100 $\mu g\ g^{-1}$ level, all the herbicides inhibited nitrite reduction in Soil P2 but not in Soil P2+Glc. Diclofop-methyl and HoeGrass™ were inhibitory in Soil P3 and P3+Glc. The nitrite disappearance times were increased by 10 $\mu g\ g^{-1}$ trifluralin or Treflan™ in both non-glucose amended soils; sporadic smaller increases or

decreases occurred for diclofop-methyl and HoeGrass™. At 100 $\mu g\ g^{-1}$, the herbicides increased the nitrite disappearance times in all soils, but diclofop-methyl and HoeGrass™ generally increased the times to a greater extent. Overall it appears that the addition of glucose to both soils generally mitigates the effects of the herbicides on nitrate and nitrite reduction rates and times. No conclusions can be drawn on the effects of the other soil constituents until a wider selection of soils is studied.

CONCLUSIONS

For both the diclofop-methyl/HoeGrass™ and trifluralin/Treflan™ work, the nitrate reduction rates ranged from a low of 1.0 ± 0.1 μg NO_3-N g^{-1} h^{-1} in Soil P3 to a high of 23.8 ± 2.2 μg NO_3-N g^{-1} h^{-1} in Soil P2+Glc. Nitrate reduction was also biphasic in Soil P3, with the initial rate approximately 1/2 the final rate. Nitrite reduction rates ranged from 0.86 ± 0.03 μg NO_2-N g^{-1} h^{-1} in Soil P3 to 4.03 ± 0.70 μg NO_3-N g^{-1} h^{-1} in Soil P2+Glc.

This study showed that the technical and formulated herbicide could have different effects on the rate of nitrate reduction. For example, at 10 μg g^{-1} diclofop-methyl enhanced nitrate reduction in 3 of 4 soils, whereas HoeGrass™ had no effect. At 100 μg g^{-1} HoeGrass™ was inhibitory in Soil P3 and P3+Glc and diclofop-methyl had little effect. The nitrate disappearance times were generally not affected by the 10 μg g^{-1} HoeGrass™ treatments. An increase in the disappearance times was observed for almost all the 100 μg g^{-1} treatments, with Soil P3 and P3+Glc experiencing the greater increases in the time. Nitrite reduction rates were not affected by diclofop-methyl or HoeGrass™ at 10 μg g^{-1} in all soils or in Soil P2+Glc at 100 μg g^{-1}. Substantial decreases in the nitrite reduction rates were observed in soils P3 and P3+Glc by the 100 μg g^{-1} treatments and there was a difference between the diclofop-methyl and HoeGrass™ treatments in soils P2 and P3+Glc. The disappearance times were slightly increased by some 10 μg g^{-1} treatments and by almost all 100 μg g^{-1} treatments. The greater increases were observed in Soil P3 vs. Soil P2, the non-amended soils vs. the glucose amended soils, and by the HoeGrass™ treatments.

Except for the 100 $\mu g\ g^{-1}$ trifluralin treatment in Soil P2, trifluralin and Treflan™ treatments had no effect on the rate of nitrate reduction in all soils. The nitrate disappearance times were generally not affected by the 10 and 100 $\mu g\ g^{-1}$ treatments in soils P3 and P3+Glc, but increased in soils P2 and P2+Glc. There was little difference in the nitrate disappearance times between trifluralin and Treflan™. In Soil P2, concentrations as low as 10 $\mu g\ g^{-1}$ trifluralin or Treflan™ generally had an inhibitory effect on the rate of nitrite reduction, and concentrations as high as 100 $\mu g\ g^{-1}$ did not further enhance this effect; the other soils were sporadically affected. The nitrite disappearance times were increased by the 10 $\mu g\ g^{-1}$ herbicide treatments in the non-glucose amended soils. At 100 $\mu g\ g^{-1}$, the nitrite disappearance times were increased, especially in Soil P3 and P3+Glc. Treflan™ treatment produced either equivalent or larger increases in the disappearance times than obtained with trifluralin treatment.

Generally the effects of the herbicides on nitrate and nitrite reduction rates and times were moderated by the addition of glucose to the soil and the effect was most noticeable for trifluralin and Treflan™. All the herbicides had little effect on nitrate reduction rates and trifluralin or Treflan™ had less of an effect than diclofop-methyl or HoeGrass™. Nitrite reduction rates were usually affected by the 100 $\mu g\ g^{-1}$ diclofop-methyl or HoeGrass™ treatments. The herbicides generally increased the nitrate and nitrite disappearance times at the 100 $\mu g\ g^{-1}$ treatment level. In all soils

the herbicides affected the nitrate reduction rates and disappearance times to a lesser extent than the nitrite reduction rates and times.

This study showed that even though the anaerobic reduction rate may not be affected by a herbicide, the times at which nitrate or nitrite disappears can be affected. Since anaerobic reduction of inorganic ions depends on other microbial processes, e.g., the overall microbial metabolic rate, availability of energy sources, membrane transport, enzyme synthesis, and electron transport, it is possible for a herbicide to inhibit or retard a variety of these processes without affecting the reductases. In this manner the time at which the inorganic ion disappears can be affected. The addition of glucose moderates the effect of the herbicides, possibly due to the increase in metabolic activity of an identical population or to the selection of an alternate population differentially affected by the herbicide.

It is difficult to determine the importance of the observed effects to actual agricultural situations. This study was done under ideal conditions to eliminate the effects of the environment on anaerobic reductions and is a good preliminary indicator of potential herbicide effects. The study can provide an indication of 1) whether the technical product or the formulation additive(s) is producing the observed effect, 2) whether the herbicide, rather than the environment, is effective at eliciting a microbial response, and 3) the minimum concentration that will probably produce a response to the herbicide in the field. In this manner, the study provides a simple and relatively fast technique to elucidate whether a herbicide has the potential to produce an effect on anaerobic nitrogen transformations under field conditions.

REFERENCES

Afifi, A.F. and A.E. Dowidar. 1978. Trifluralin effect on *Psium-Rhizobium* relationship. Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, II Asteilung 133: 394-399 (Soils Fert. Abst. 43: 7801).

Aftring, R.P., B.E. Chalker, and B.F. Taylor. 1981. Degradation of phthalic acids by denitrifying, mixed cultures of bacteria. Appl. Environ. Microbiol. 41: 1177-1183.

Alexander, M. 1973. Nonbiodegradable and other recalcitrant molecules. Biotechnol. Bioeng. 15: 611-647.

Alexander, M. 1977. Introduction to soil microbiology. J. Wiley & Sons, N.Y. 467 pp.

Alexander, M. 1982. Most probable number method for microbial populations. In Methods of Soil Analysis. Part 2. A.L. Page (ed.), Am. Soc. of Agron., Inc., Madison, WI. Agronomy 9: 815-820.

Allison, L.E. 1965. Organic carbon. In Methods of Soil Analysis. Part 2. C.A. Black (ed.), Am. Soc. of Agron., Inc., Madison, WI. Agronomy 9: 1367-1378.

Anderson, J.R. 1978. Pesticide effects on non-target soil microorganisms. In Pesticide Microbiology. J.R. Hill and S.J.L. Wright (ed.), Academic Press Inc. (London) Ltd. pp. 313-533.

Anderson, W.P. 1983. Weed Science: Principles. 2nd Edition. West Publishing Co., St. Paul, Mn. 655 pp.

Bailey, L.D. and E.G. Beauchamp. 1973. Effects of temperature on nitrate and nitrite reduction, nitrogenous gas production, and redox potential in a saturated soil. Can. J. Soil Sci. 53: 213-218.

Bakalivanov, D., A. Peneva, and M. Lalova. 1979. Interaction of the herbicides Sencor and Treflan with the microflora of a chernozem smolnitsa soil. Pochvoznanie i Akrokhimiya 14: 89-95. (Soils Fert. Abst. 43: 7343).

Baker, A.S. 1967. Colorimetric determination of nitrate in soil and plant extracts with brucine. J. Ag. Food Chem. 15: 802-806.

Balderston, W.L., B. Sherr, and W.J. Payne. 1976. Blockage by acetylene of nitrous oxide reduction in *Pseudomonas perfectomarinus*. Appl. Environ. Microbiol. 31: 504-508.

Balkwill, D.L. and L.E. Casida, Jr. 1979. Attachment to autoclaved soil of bacterial cell from pure cultures of soil isolates. Appl. Environ. Microbiol. 37: 1031-1037.

Balkwill, D.L., T.E. Rucinsky, and L.E. Casida, Jr. 1977. Release of microorganisms from soil with respect to transmission electron microscopy viewing and plate counts. Antonie van Leeuwenhoek J. Microbiol. Serol. 43: 73-87.

Bartels, P.G. and J.L. Hilton. 1973. Comparison of trifluralin, oryzalin, pronamide, propham, and colchicine treatments on microtubules. Pest. Biochem. Physiol. 3: 462-472.

Bartlett, R. and B. James. 1980. Studying dried, stored soil samples - some pitfalls. Soil Sci. Soc. Am. J. 44: 721-724.

Bollag, J.-M. and N.M. Henninger. 1976. Influence of pesticides on denitrification in soil and with an isolated bacterium. J. Environ. Qual. 5: 15-18.

Bollag, J.-M. and E.J. Kurek. 1980. Nitrite and nitrous oxide accumulation during denitrification in the presence of pesticide derivatives. Appl. Environ. Microbiol. 39: 845-849.

Bollag, J.-M. and C.L. Nash. 1974. Effect of chemical structure of phenylureas and anilines on the denitrification process. Bull. Environ. Contam. Toxicol. 12: 241-248.

Bottner, P. 1985. Response of microbial biomass to alternate moist and dry conditions in a soil incubated with ^{14}C- and ^{15}N-labelled plant material. Soil Biol. Biochem. 17: 329-337.

Breazeale, F.W. and N.D. Camper. 1970. Bacterial, fungal and actinomycete populations in soils receiving applications of 2,4-dichlorophenoxyacetic acid and trifluralin. Appl. Environ. Microbiol. 19: 379-380.

Bremner, J.M. 1965. Inorganic forms of nitrogen. In Methods of Soil Analysis. Part 2. C.A. Black (ed.), Am. Soc. of Agron., Inc., Madison, WI. Agronomy 9: 1179-1237.

Buchholz, A.E., C.I. Verplough, and J.L. Smith. 1982. A method for the simultaneous measurement of less than a part-per-million of chloride, nitrate, and sulfate in aqueous samples by nonsuppressed ion chromatography. J. Chromatogr. Sci. 20: 499-501.

Burford, J.R. and J.M. Bremner. 1975. Relationships between the denitrification capacities of soils and total, water-soluble and readily decomposable soil organic matter. Soil Biol. Biochem. 7: 389-394.

Carter Jr., G.E. and N.D. Camper. 1973. Soil microbiol studies with trifluralin. In Proc. 26th Annu. Meeting Southern Weed Sci. Soc., Clemson Univ., S.C. Weed Abstr. 24: 538.

Carter Jr., G.E. and N.D. Camper. 1975. Soil enrichment studies with trifluralin. Weed Sci. 23: 71-74.

Chandra, P., W.R. Furtick, and W.B. Bollen. 1960. The effect of four herbicides on microorganisms in nine Oregon soils. Weeds 8: 589-598.

Cervelli, S. and D.E. Rolston. 1983. Influence of atrazine on denitrification in soil columns. J. Environ. Qual. 12: 482-486.

Cho, C.M. and L. Sakdinan. 1978. Mass spectrometric investigation on denitrification. Can. J. Soil Sci. 58: 443-457.

Cripps, R.E. and T.R. Roberts. 1978. Microbial degradation of herbicides. In Pesticide Microbiology. J.R. Hill and S.J.L. Wright (ed.), Academic Press Inc. (London) Ltd. pp. 669-730.

Cullimore, D.R. 1971. Interaction between herbicides and soil microorganisms. Residue Rev. 35: 65-80.

Cullimore, D.R. 1981. The enumeration of 2,4-D degraders in Saskatchewan soils. Weed Sci. 29: 65-80.

Cullimore, D.R. and A.E. McCann. 1977. Influence of four herbicides on the algal flora of a prairie soil. Plant Soil 46: 499-510.

Davidson, E.A., M.K. Strand, and L.F. Galloway. 1985. Evaluation of the most probable number method for enumerating denitrifying bacteria. Soil Sci. Soc. Am. J. 49: 642-645.

Davies, H.A. and M.P. Greaves. 1981. Effect of some herbicides on soil enzyme activities. Weed Res. 21: 205-209.

Davies, H.A. and J.A.P. Marsh. 1977. The effect of herbicides on respiration and transformation of nitrogen in two soils. II. Dalapon, pyrazone, and trifluralin. Weed Res. 17: 373-378.

Delwiche, C.C. and D.E. Rolston. 1976. Measurement of small nitrous oxide concentrations by gas chromatography. Soil Sci. Soc. Am. J. 40: 324-327.

Domsch, K.H., G. Jagnow, and T.-H. Anderson. 1983. An ecological concept for the assessment of the side effects of agrochemicals on soil microorganisms. Residue Rev. 86: 65-105.

Evans, D.G, E. Beauchamp, and J.T. Trevors. 1985. Sulfide alleviation of the acetylene inhibition of nitrous oxide reduction in soil. Appl. Environ. Microbiol. 49: 217-220.

Firestone, M.K. 1982. Biological denitrification. In Nitrogen in Agricultural Soils. F.J. Stevenson (ed.), Am. Soc. of Agron., Inc., Madison, WI. Agronomy 22: 289-326.

Gamble, T.N., M.R. Betlach, and J.M. Tiedje. 1977. Numerically dominant denitrifying bacteria from world soils. Appl. Environ. Microbiol. 33: 926-939.

Gerritse, R.G. 1979. Rapid simultaneous determination of nitrate and nitrite by high-performance liquid chromatography. J. Chromatogr. 171: 527-529.

Golab, T., W.A. Althaus, and H.L. Wooten. 1979. Fate of [^{14}C]Trifluralin in Soil. J. Agric. Food Chem. 27: 163-179.

Goring, C.A.I. and D.A. Laskowski. 1982. The effects of pesticides on nitrogen transformations in soils. In Nitrogen in Agricultural Soils. F.J. Stevenson (ed.), Am. Soc. of Agron., Inc., Madison, WI. Agronomy 22: 689-720.

Grant, M.A. and W.J. Payne. 1982. Effects of pesticides on denitrifying activity in salt marsh sediments. J. Environ. Qual. 11: 369-372.

Greaves, M.P. and H.P. Malkomes. 1980. Effects on soil microflora. In Interactions Between Herbicides and the Soil. R.J. Hance (ed), Academic Press Inc., London. pp 223-253.

Grover, R. 1974. Adsorption and desorption of trifluralin, triallate, and diallate by various adsorbents. Weed Sci. 22: 405-408.

Gruzdev, G.S., A.F. Mozgovoi, and I.V. Kuz'mina. 1973. Effect of treflan on biological activity of soil under sunflower. Izv. timiryazev. sel.'-khoz. Akad. 4:136-141 (Soils Fert. Abstr. 37: 250).

Hacskaylo, J. and V.A. Amato. 1968. Effect of trifluralin on roots of corn and cotton. Weed Sci. 16: 513-515.

Haddad, P.R. and A.L. Heckenberg. 1982. High-performance liquid chromatography of inorganic and organic ions using low-capacity ion-exchange columns with indirect refractive index detection. J. Chromatogr. 252: 177-184.

Harrison, G.W. and J.B. Weber. 1975. Comparative phytotoxicities of five herbicides in ten North Carolina Soils. Proc. 28th Am. South. Weed Sci. Soc. pp. 283-291.

Hart, L.T. and A.D. Larson. 1966. Effect of 2,4-dichlorophenoxyacetic acid on different metabolic types of bacteria. Bull. Environ. Contam. Toxicol. 1: 108-120.

Hartley, D. and H. Kidd. 1983. The agrochemicals handbook. Royal Society of Chemistry, England.

Heinonen-Tanski, H, G. Oros, and M. Kecskeses. 1982. The effect of soil pesticides on the growth of red clover rhizobia. Acta Agric. Scand. 32: 283-288.

Helling, C.S. 1976. Dinitroaniline herbicides in soils. J. Environ. Qual. 5: 1-15.

Helmeczi, B., M. Bessenyei, and M. Nagy. 1978. Effects of dinitroaniline-type herbicides on several physiological groups of soil-bacteria. Weed Abstr. 29: 605.

Henninger, N.M. and J.-M. Bollag. 1976. Effect of chemicals used as nitrification inhibitors on the denitrification process. Can. J. Soil Sci. 22: 668-672.

Herbert, D., P.J. Phipps, and R.R. Strange. 1971. Chemical analysis of microbial cells. In Methods in Microbiology. J.R. Norris and D.W. Ribbons (ed.), Academic Press, N.Y. pp. 272-274.

Hess, F.D. and D.E. Bayer. 1977. Binding of herbicide trifluralin to *Chlamydomonas* flagellar tubulin. J. Cell Sci. 24: 361-360.

Horowitz, M., T. Blumenfeld, G. Herzlinger, and H. Hulin. 1974. Effects of repeated applications of ten soil-active herbicides on weed population, residue accumulation and nitrification. Weed Res. 14: 213-220.

Jacobson, S.N., N.L. O'Hara, and M. Alexander. 1980. Evidence for cometabolism in sewage. Appl. Environ. Microbiol. 40: 917-921.

Kaiser, P., J.J. Pochon, and R. Cassini. 1970. Influence of triazine herbicides on soil microorganisms. Residue Rev. 32: 211-233.

Kamphake, L.J., S.A. Hannah, and J.M. Cohen. 1967. Automated analysis for nitrate by hydrazine reduction. Water Resour. Res. 1: 205-216.

Karanth, N.G.K., J.P.E. Anderson, and K.H. Domsch. 1984. Degradation of the herbicide diclofop-methyl in soil and influence of pesticide mixtures on its persistence. J. Biosci. 6: 829-837.

Keeney, D.R. and D.W. Nelson, 1982. Nitrogen - Inorganic forms. In Methods of Soil Analysis. Part 2. A.L. Page (ed.), Am. Soc. of Agron., Inc., Madison, WI. Agronomy 9: 643-698.

Keeney, D.R., K.L. Sahrawat, and S.S. Adams. 1985. Carbon dioxide concentration in soil: Effects on nitrification, denitrification and associated nitrous oxide production. Soil Biol. Biochem. 17: 571-573.

Klingman, G.C. and F.M. Ashton. 1982. Weed Science: Principles and Practices. 2nd Edition. J. Wiley & Sons, N.Y. 449 pp.

Knowles, R. 1982. Denitrification. Microbiol. Rev. 46: 43-70.

Kok, S.U., K.A. Buckle, and M. Wootton. 1983. Determination of nitrate and nitrite in water using high-performance liquid chromatography. J. Chromatogr. 260: 189-192.

Komatsu, Y., M. Takagi, and M. Yamaguchi. 1978. Participation of iron in denitrification in waterlogged soil. Soil Biol. Biochem. 10: 21-26.

Kondratenko, W.I. A.V. Voevodin, and S.S. Islamov. 1981. Changes in soil microflora under the influence of herbicides. Weed Abstr. 31: 1246.

Kuchnicki, T.C., L.P. Sarna and G.R.B. Webster. 1985. Determination of nitrate, nitrite, and phosphate at 214 nm by reverse phase HPLC. J. Liq. Chromatogr. 8: 1593-1609.

Kuchnicki, T.C. and G.R.B. Webster. 1986. A comparison of HPLC analysis of nitrate in soils with the phenoldisulfonic acid and hydrazine sulfate methods. Can. J. Soil Sci. 66: 151-157.

Kuryndina, T.I. 1965. The effect of herbicides on the microflora of orchard soil. Sb. nauch. Rab. Inst. Sadov. I.V. Michurina II: 113-116 (Weed Abstr. 17: 1938).

Kuzyakina, T.I. 1971. Changes in the microflora populations of a derono-podzolic soil with the use of different rates of simazine. Dokl. TSKhA 172: 142-145 (Weed Abstr. 24: 820).

Laanio, T.L., P.C. Kearney, and D.D. Kaufman. 1973. Microbial metabolism of dinatramine. Pestic. Biochem. Physiol. 3: 271-277.

Lal, R, and D.M. Saxena. 1980. Cytological and biochemical effect of pesticides on microorganisms. Residue Rev. 73: 49-85.

Lazrus, A.J., J.C. Hill, and J.P. Hodge. 1966. A new colorimetric microdetermination of sulphate ion. Automation in Anal. Chem., Technicon Symposium 1865, Mediad 1966. pp 291-293.

Letey, J., A. Hadas, N. Valoras, and D.D. Focht. 1980. Effect of preincubation treatments on the ratio of N_2O/N_2 evolution. J. Environ. Qual. 9: 232-235.

Leuenberger, U., R. Gauch, K. Reider, and E. Baumgartner. 1980. Determination of nitrate and bromide in foodstuffs by high-performance liquid chromatography. J. Chromatogr. 202: 461-468.

Lewis, D.L., R.E. Hodson, and L.F. Freeman III. 1984. Effects of microbial community interactions on transformation rates of xenobiotic chemicals. Appl. Environ. Microbiol. 48: 561-565.

Lewis, J.A., G.C. Papavizas, and T.S. Hora. 1978. Effect of some herbicides on microbial activity in soil. Soil Biol. Biochem. 10: 137-141.

Lignowski, E.M. and E.G. Scott. 1971. Trifluralin and root growth. Pl. Cell Physiol. 12: 701-708.

Madhun, Y.A., J.L. Young, and V.H. Freed. 1986. Binding of herbicides by water-soluble organic materials from soil. J. Environ. Qual. 15: 64-68.

Makani, A.A.M., M. Abdel-Nasser, and A.A. Abdel-Moneim. 1979. Effect of some pesticides on certain microorganisms, contribution to soil fertility. Zentralbl. Bakteriol. Parastenkd. Ikfektionskr. Hyg., II. 134: 5-12.

Malkomes, H.-P. and B. Wohler. 1983. Testing and evaluating some methods to investigate side effects of environmental chemicals on soil microorganisms. Ecotox. Environm. Saftey 7: 284-289.

Marsh, J.A.P. and H.A. Davies. 1981. Effects of dichlorprop and mecoprop on respiration and transformation of nitrogen in two soils. Bull. Environ. Contam. Toxicol. 26: 108-115.

Martens, R. 1978. Degradation of the herbicide [14C]-diclofop-methyl in soil under different conditions. Pestic. Sci. 9: 127-134.

McCann, A.E. and D.R. Cullimore. 1979. Influence of pesticides on the soil algal flora. Residue Rev. 72: 1-31.

McElhannon, W.S. and H.A. Mills. 1981. Suppression of denitrification with nitrapyrin. HortScience 16: 530-531.

McElhannon, W.S., H.A. Mills, and P.B. Bush. 1984. Simazine and atrazine - Suppression of denitrification. HortScience 19: 218-219.

McEwan, F.L. and G.R. Stephenson. 1979. The Use and Significance of Pesticides in the Environment. J. Wiley and Sons, N.Y., 538 pp.

McKenney, D.J., G.P. Johnson, and W.I. Findlay. 1984. Effect of temperature on consecutive denitrification reactions in Brookston clay and Fox sandy loam. Appl. Environ. Microbiol. 47: 919-926.

McKenney, D.J., K.F. Shuttleworth, J.R. Vriesacker, and W.I. Findlay. 1982. Production and loss of nitric oxide from denitrification in anaerobic Brookston clay. Appl. Environ. Microbiol. 43: 534-541.

Mills, H.A. 1984. A procedure for rapidly evaluating the effect of chemicals on denitrification. Commun. Soil Sci. Plant Anal. 15: 1007-1016.

Mills, H.A. and W.S. McElhannon. 1984. Terrazole suppression of denitrification. HortScience 19: 54-55.

Molnar, I., H. Knauer, and S. Wilk. 1980. High-performance liquid chromatography of ions. J. Chromatogr. 201: 225-240.

Mordaleva, L.G., I.I. Murzinova, and A.V. Kotlyarova. 1981. On the effect of herbicides on biological activity of compact chernozem soil. Agrokhimiya 12: 99-102 (Weed Abstr. 32: 224).

Murphy, J. and J.P. Riley. 1962. A modified single solution method for determination of phosphate in natural waters. Anal. Chim. Acta 27: 31-36.

Nakos, G. 1980. Effects of herbicides used in forestry on soil nitrification. Soil Biol. Biochem. 12: 517-519.

Nelson, D.W. and L.E. Sommers. 1982. Total carbon, organic carbon, and organic matter. In Methods of Soil Analysis. Part 2. A.L. Page (ed.), Am. Soc. of Agron., Inc., Madison, WI. Agronomy 9: 539-579.

Nelson, L.M. and D. Parkinson. 1978. Effect of freezing and thawing on survival of three bacterial isolates from an artic soil. Can. J. Soil Sci. 24: 1468-1474.

Obrigawitch, T., A.R. Martin, and F.W. Roeth. 1983. Degradation of thiocarbamate herbicides in soils exhibiting rapid EPTC breakdown. Weed Sci. 31: 187-192.

Olson, B.M., R.B. McKercher, and J.J. Germida. 1984. Microbial populations in trifluralin-treated soil. Plant Soil 76: 379-387.

Osterloh, J. and D. Goldfield. 1984. Determination of nitrate and nitrite ions in human plasma by ion exchange-high performance liquid chromatography. J. Liq. Chromatogr. 7: 752-763.

Parr, J.F. 1974. Effects of pesticides on microorganisms in soil and water. In Pesticides in Soil and Water. W.D. Guenzi (ed.), SSSA Inc., Madison, WS. pp 315-340.

Parr, J.F. and S. Smith. 1973. Degradation of trifluralin under laboratory conditions and soil anaerobiosis. Soil Sci. 115: 55-63.

Percich, J.A. and J.L. Lockwood. 1978. Interaction of atrazine with soil microorganisms: Population changes and accumulation. Can. J. Soil Sci. 24: 1145-1152.

Perry, J.J. 1979. Microbial cooxidations involving hydrocarbons. Microbiol. Rev. 43: 59-72.

Peshakov, G., E. Raikov, and D. Tsvetanov. 1969. Investigating the effect of various herbicides on the soil microflora and on ammonification and nitrification in the soil. Pochv. Agrokhim. 4: 89-94 (Weed Abstr. 20: 1856).

Phillips, R.E., K.R. Reddy, and W.H. Patrick. 1978. The role of nitrate diffusion in determining the order and rate of denitrification in flooded soil: II. Theoretical analysis and interpretation. Soil Sci. Soc. Am. J. 42: 272-278.

Probst, G.W., T. Golab, R.J. Herberg, F.J. Holzer, S.J. Parka, C. Van der Schans, and J.B. Tepe. 1967. Fate of trifluralin in soils and plants. J. Ag. Food Chem. 15: 592-599.

Rankov, E. and E. Elenkov. 1970. The effect of Treflan on soil microflora. Pochv. Agrokhim. 5: 127-136 (Soils Fert. Abstr. 34: 405).

Reddy, K.R., W.H. Patrick, and R.E. Phillips. 1978. The role of nitrate diffusion in determining the order of denitrification in flooded soil: I. Experimental results. Soil Sci. Soc. Am. J. 42: 268-272.

Rennie, R.J. and S. Dubetz. 1984. Effect of fungicides and herbicides on nodulation and N_2 fixation in soybean fields lacking indigenous *Rhizobium japonicum*. Agron. J. 76: 451-454.

Roberts, H.A. 1982. Weed Control Handbook: Principles. Blackwell Scientific Publications, London. 533 pp.

Rodriguez-Kabana, R. and E.A. Curl. 1980. Nontarget effects of pesticides on soilborne pathogens and disease. Ann Rev. Phytopathol. 18: 311-332.

Rodriguez-Kabana, R., E.A. Curl, and H.H. Funderburk. 1969. Effect of trifluralin on growth of *Sclerotium rolfsii* in liquid culture and soil. Phytopathol. 59: 228-232.

Rolston, D.E. and S. Cervelli. 1980. Denitrification as affected by irrigation frequency and applied herbicides. In Agrochem. Res.-Biota Interactions Soil Aq. Eco., pp 189-199 (cited in Yeomans and Bremner 1985).

Ross, D.J., K.R. Tate, A. Cairns, and K.F. Meyrick. 1980. Influence of storage on soil microbial biomass estimated by three biochemical procedures. Soil Biol. Biochem. 12: 369-374.

Ross, D.J., T.W. Speir, K.R. Tate, and V.A. Orchard. 1985. Effects of sieving on estimations of microbial biomass, and carbon and nitrogen mineralization, in soil under pasture. Aust. J. Soil Res. 23: 319-324.

Rudzinski, W.E., D. Bennett, and V. Garcia. 1982. Retention of ionized solutes in reversed-phase high performance liquid chromatography. J. Liq. Chromatogr. 5: 1295-1312.

Ryden, J.C., L.J. Lund, and D.D. Focht. 1979. Direct measurement of denitrification loss from soils: I. Laboratory evaluation of acetylene inhibition of nitrous oxide reduction. Soil Sci. Soc. Am. J. 43: 104-110.

Salonius, P.O. 1983. Effects of air drying on the respiration of forest soil microbial populations. Soil Biol. Biochem. 15: 199-203.

Scow, K.M. 1982. Rate of biodegradation. In Handbook of chemical property estimation methods. W.J. Lyman, W.F. Reehl, and D.H. Rosenblatt (ed.), McGraw Inc., N.Y. pp. 9.1-9.85.

Sethunathan, N. 1970. Foliar sprays of growth regulators and rhizosphere effect in *Cajanus cajan* Millsp. II. Qualitative changes in the rhizosphere and certain metabolic changes in the plant. Plant Soil 33: 71-80.

Small, H., T.S. Stevens, and W.C. Bauman. 1975. Novel ion exchange chromatographic method using conductimetric detection. Anal. Chem. 47: 1801-1809.

Smith, A.E. 1977. Degradation of the herbicide dichlorfop-methyl in prairie soils. J. Agric. Food Chem. 25: 893-898.

Smith, A.E. 1979 Transformation of [14C]diclofop-methyl in small field plots. J. Agric. Food Chem. 27: 1145-1148.

Smith, A.E. 1982. Herbicides and the soil environment in Canada. Can. J. Soil Sci. 62: 433-460.

Smith, M.S., M.K. Firestone, and J.M. Tiedje. 1978. The acetylene inhibition method for short-term measurement of soil denitrification and its evaluation using nitrogen-13. Soil Sci. Soc. Am. J. 42: 611-615.

Smith, M.S. and L.L. Parsons. 1985. Persistence of denitrifying enzyme activity in dried soils. Appl. Environ. Microbiol. 49: 316-320.

Smith, M.S. and J.M. Tiedje. 1979. Phases of denitrification following oxygen depletion in soil. Soil Biol. Biochem. 11: 261-267.

Solbakken, E., H. Hole, O. Lode, and T.A. Pedersen. 1982. Trifluralin persistence under two different soil and climatic conditions. Weed Res. 22: 319-328.

Sorensen, L.H. 1974. Rate of decomposition of organic matter in soil as influenced by repeated air drying-rewetting and repeated additions of organic material. Soil Biol. Biochem. 6: 287-292.

Sorensen, J. 1978. Capacity for denitrification and reduction of nitrate to ammonia in a coastal marine sediment. Appl. Environ. Microbiol. 35: 301-305.

Soulides, S.A. and F.E. Allison. 1961. Effect of drying and freezing soils on carbon dioxide production, available mineral nutrients, aggregation, and bacterial population. Soil Sci. 91: 291-298.

Southwick, L.M. and G.H. Willis. 1979. Interaction of trifluralin with soil components under flooded anaerobic conditions. In Abstracts of the 178th National Meeting of the American Chemical Society (Soils Fert. Abst. 44: 3255).

Spain, J.C. and P.A. Van Veld. 1983. Adaptation of natural microbial communities to degradation of xenobiotic compounds: Effects of concentration, exposure time, inoculum, and chemical structure. Appl. Environ. Microbiol. 45: 428-435.

Sparling, G.P. and W.V. Cheshire. 1979. Effects of soil drying and storage on subsequent microbial growth. Soil Biol. Biochem. 11: 317-319.

Speir, T.W. and D.J. Ross. 1981. A comparison of the effects of air-drying and acetone dehydration on soil enzyme activities. Soil Biol. Biochem. 13: 225-229.

Spencer, E.Y. 1973. Guide to the Chemicals Used in Crop Production. 6th Edition, Agriculture Canada Publication #1093, 542 pp.

Spencer, E.Y. 1982. Guide to the Chemicals Used in Crop Production. 6th Edition, Agriculture Canada Publication #1093, 595 pp.

Stanford, G., R.A. Vander Pol, and S. Dzienia. 1975. Denitrification rates in relation to total and extractable soil carbon. Soil Sci. Soc. Am. Proc. 39: 284-289.

Stojanovic, B.J., M.V. Kennedy, and F.L. Shuman, Jr. 1972. Edaphic aspects of the disposal of unused pesticides, pesticide wastes, and pesticide containers. J. Environ. Qual. 1: 54-62.

Sunita, S. and V.K. Gupta. 1984. Extractive photometric determination of nitrite in polluted water in ppm level using p-nitroaniline and guaiacol. Intern. J. Environ. Anal. Chem. 19: 11-18.

Tabatabai, M.A. and W.A. Dick. 1983. Simultaneous determination of nitrate, chloride, sulfate, and phosphate in natural waters by ion chromatography. J. Environ. Qual. 12: 209-213.

Tam, T.Y. and R. Knowles. 1979. Effects of sulfide and acetylene on nitrous oxide reduction by soil and *Pseudomonas aeruginosa*. Can. J. Microbiol. 25: 1133-1138.

Thayer, J.R. and T.C. Huffaker. 1980. Determination of nitrate and nitrite by high-pressure liquid chromatography: Comparison with other methods for nitrate determination. Anal. Chem. 102: 110-119.

Tiedje, J.M. 1982. Denitrification. In Methods of Soil Analysis. Part 2. A.L. Page (ed.), Am. Soc. of Agron., Inc., Madison, WI. Agronomy 9: 1011-1026.

Tolkachev and Solyanova. 1979. The changes in the soil microflora with the repeated year-by-year use of herbicides in cotton crops. Byulleten' Vsesoyuznogo Nauchno-Issledovatevskogo Instituta sel'Skokhozyaaist-vennoi Mikrobiologgii. 32: 83-85 (Soils Fert. Abstr. 43: 998).

Torstensson, L. 1974. Effects of MCPA, 2,4,5-T, linuron, and simazine on some functional groups of soil microorganisms. Swedish J. Agric. Res. 4: 151-169.

Trevors, J.T. 1982. Effect of pentachlorophenol on electron transport system activity in soil. Bull. Environ. Contam. Toxicol. 29: 727-730.

Tu, C.M. 1982. Physical treatment and reinoculation of soil: Effects on microorganisms and enzyme activities. Soil Biol. Biochem. 14: 57-61.

Tu, C.M. and J.R.W. Miles. 1976. Interactions between insecticides and soil microbes. Residue Rev. 64: 17-65.

Tulabaev, B.D. 1972. The effect of different rates of herbicides on the microflora of a serozem-meadow soil under cotton crops. Khimiya sel'Khoz. 10: 776-780 (Weed Abstr. 22: 1911).

Tyagny-Ryadno, M.G. 1967. Effect of herbicides on the microflora and agrochemical properties of the soil. Trudy Kamenets-podolsk. sel'Kohz. Inst. 9: 43-48 (Weed Abstr. 18: 1440).

Van der Houwen, O.A.G.J., J.A.A. van der Linden, and A.W.M. Indemans. 1982. Evaluation of the use of lithium nitrate as a test substance for the determination of the hold-up time on a reversed-phase packing. J. Liq. Chromatogr. 5: 2321-2341.

Van Os, M.J., J. Slaninà, C.L. De Ligny, W.E. Hammers, and J. Agterdenbos. 1982. Determination of traces of inorganic anions by means of high-performance liquid chromatography of Zipax-SAX columns. Anal. Chimica Acta 144: 73-82.

Vilsmeier, K. 1984. Determination of dicyandiamide, nitrite and nitrate in soil extracts by high pressure liquid chromatography. Z. Pflanzenernaehr. Bodenk. 147: 264-268.

Vinther, F.P., H.G. Memon, and V. Jensen. 1982. Populations of denitrifying bacteria in agricultural soils under continuous barley cultivation. Pedobiologia 24: 319-328.

Volz, M.G. and J.L. Starr. 1977. Nitrate dissimilation and population dynamics of denitrifying bacteria during short term continuous flow. Soil Sci. Soc. Am. J. 41: 891-896.

Wainwright, M. 1978. A review of the effects of pesticides on microbial activity in soils. J. Soil Sci. 29: 287-298.

Weber, J.B., P.J. Shea, and H.J. Strek. 1980. An evaluation of nonpoint sources of pesticide pollution runoff. In Environmental Impact of Nonpoint Source Pollution. M.R. Overlash and J.M. Davidson (ed.), Ann Arbor Science Publishers Inc., Ann Arbor, Mich. pp 69-98.

Weber, J.B., S.B. Weed, and T.W. Waldrep. 1974. Effect of soil constituents on herbicide activity in modified-soil field plots. Weed Sci. 22: 454-459.

Weed, S.B. and J.B. Weber. 1974. Pesticide-organic matter interactions. In Pesticides in soil and water. W.D. Guenzi (ed.), Soil Sci. Soc. Am., Inc., Madison, WI. pp 39-66.

Weeraratna, C.S. 1980. Effect of dalapon-sodium on nitrification and denitrification in a tropical loam soil. Weed Res. 20: 291-293.

Wheeler, W.B., G.D. Stratton, R.R. Twilley, Li-Tse Ou, D.A. Carlson, and J. M. Davidson. 1979. Trifluralin degradation and binding in soil. J. Agric. Food Chem. 27: 702-706.

Wollum II, A.G. 1982. Cultural methods for soil microorganisms. In Methods of Soil Analysis. Part 2. A.L. Page, R.H. Miller, D.R. Keeney (ed.), Am. Soc. of Agron., Inc., Madison, WI. Agronomy 22: 781-802.

Wright, S.J.L. and A. Forey. 1972. Metabolism of the herbicide barban by a soil *Penicillium*. Soil Biol. Biochem. 4: 207-213.

Worthing, C.R. 1979. The Pesticide Manual. 6th Edition. PCPC Publications, England. 655 pp.

Yeomans, J.C. and E.G. Beauchamp. 1978. Limited inhibition of nitrous oxide reduction in soil in the presence of acetylene. Soil Biol. Biochem. 10: 517-519.

Yeomans, J.C. and J.M. Bremner. 1985. Denitrification in soil: Effects of herbicides. Soil Biol. Biochem. 17: 447-452.

Yoshinari, T. and R. Knowles. 1976. Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. Biochem. Biophys. Res. Commun. 69: 705-710.

Yoshinari, T. and R. Knowles. 1977. Acetylene inhibition of nitrous oxide reduction and measurement of denitrification and nitrogen fixation in soil. Soil Biol. Biochem. 9: 177-183.

Yoshinari, T., R. Hynes, and R. Knowles. 1977. Acetylene inhibition of nitrous oxide reduction and measurement of denitrification and nitrogen fixation in soil. Soil Biol. Biochem. 9: 177-183.

Zayed, S.M.A.D., I.Y. Mostafa, H.S.H. Attaby, and Y.M. Adam. 1982. The use of radiorespirometry for evaluating the effect of trifluralin on the microbiological activity of *Fusarium oxysporum* and *Trichoderma viridae*. Isotope Radiation Res. 14: 117-121 (Soils Fert. Abstr. 47: 1597).

Zar, J.H. 1974. Statistical Analysis. Prentice Hall Inc., N.J. 614 pp.

Zeyer, J. and P.C. Kearney. 1983. Microbial dealkylation of trifluralin in pure culture. Pest. Biochem. Physiol. 20: 10-18.

APPENDICES

I. Nitrate Analysis Methodology

Numerous methods have been developed for the analysis of nitrate concentrations in soil, e.g., the brucine, hydrazine sulfate, phenoldisulfonic acid, and cadmium reduction methods (Keeney and Nelson 1982). In this study, the precision and accuracy of the brucine, hydrazine sulfate, and phenoldisulfonic acid (PDS) methods, and their suitability for use in the reduction assays, were compared. The precision of the methods was determined by comparing nitrate standard curves prepared by each method. A preliminary assessment of the accuracy of the methods was determined by recovery, from Soil P1 (Tables 1 and 2), of various concentrations of added nitrate.

Standard curves for the brucine method (Baker 1967) were prepared by adding 0.4 mL of 5 M NaCl to a 2.0 mL aqueous nitrate sample and cooling the mixture in an ice-water bath. Then 2.0 mL of 80% H_2SO_4 was added and the solution was immediately mixed using a vortex mixer. After cooling the solution, 0.1 mL of brucine-sulfanilic acid solution was added. The brucine-sulfanilic acid solution was prepared by dissolving 1.0 g of brucine sulfate and 0.1 g of sulfanilic acid in 70 mL of hot distilled water and then slowly adding 3 mL concentrated H_2SO_4. The mixture was cooled and made up to 100 mL with distilled water. After the addition of the brucine-sulfanilic acid solution, the nitrate sample was heated in a 100°C water bath for 20 min., then cooled to room temperature, and the absorbance read at 420 nm against a reagent blank in a Bausch and Lomb (B&L) Spectronic 710, using a 10 mm cuvette.

For the modified hydrazine sulfate method (Kamphake *et al.* 1967), 0.6 mL 1 M NaOH, 3.4 mL 0.42 mM anhydrous cupric sulfate, 1.6 mL 4.7 mM hydrazine sulfate, and 1.6 mL color reagent were respectively added to an aqueous nitrate sample. The sample was mixed after the addition of each reagent. The color reagent contained 0.2% *n*-1-napthylethylene diamine-dihydrochloride and 4% sulfanilamide in 10% v/v phosphoric acid. The nitrate-reagent mixture was made up to 10 mL with distilled water, allowed to stand for 5 min, and read at 540 nm in a B&L Spectronic 710. In the automated method (MPST Lab), the nitrate-reagent mixture is heated to 53°C before the addition of the color reagent. Nitrate standard curves were therefore also prepared from samples which were heated to 55°C, prior to the addition of the color reagent.

The modified phenoldisulfonic acid (PDS) (Bremner 1965) requires that the nitrate sample be free of water. The aqueous nitrate sample was therefore first oven-dried at 95°C. Then 2 mL of phenoldisulfonic acid solution was added and the mixture was allowed to stand for 15 min. The phenoldisulfonic acid solution was made by mixing 450 mL concentrated H_2SO_4, 70 mL 90% (ca.) phenol, and 225 mL fuming H_2SO_4 in a Kjeldhal flask and heating in boiling water for 2 h (prepared by the technician, V. Huzel). The nitrate-acid mixture was then washed with distilled water into a 50 mL volumetric flask, titrated with concentrated NH_4OH until a yellow color developed, and made up to 50 mL with distilled water. The mixture was cooled and read at 415 nm with a B&L Spectronic 710.

Since the anaerobic nitrate reduction study was also to be performed with the addition of 100 μg glc g^{-1} to the soils, the PDS method was evaluated for its accuracy in determining the concentration of nitrate in soil samples amended with 100 μg glc g^{-1}. Glucose was found to interfere with PDS nitrate analysis by decreasing the concentration of nitrate recovered from the samples. Various pre-assay modifications were attempted to decrease the glucose interference. These modifications included: 1) removal of the glucose from aqueous samples by utilizing Sep-Pak cartridges, charcoal, 30% hydrogen peroxide, or ion exchange chromatography, and 2) drying at lower temperatures.

Both silica (#51910) and C_{18} (#51900) Sep-Pak cartridges (Waters Associates Inc., Mississauga, Ont.) were examined for their effectiveness at removing the glucose from a 5 mL sample (the amount used for the PDS assay) containing 100 mg glc L^{-1}. For the charcoal modification, 1-3 g of charcoal was added in increasing 1 g volumes to a 5 mL aqueous glucose sample (100 mg glc L^{-1}). The sample was agitated with a vortex mixer for 1 min, filtered through #1 Whatman filter paper, and the solution was analyzed for glucose. The hydrogen peroxide modification consisted of the addition of 35% hydrogen peroxide to the dried nitrate-glucose sample. The mixture was allowed to stand for 48 h before re-drying and analyzing for nitrate and glucose concentrations. For all the modifications, the amount of glucose was determined by the phenol-H_2SO_4 method (Herbert *et al.* 1971).

The anion exchange chromatography modification was performed using a Bio-Rad AG 1-X8 20-50 mesh analytical grade resin (Bio-Rad Laboratories, Mississauga, Ont.). AG 1-X8 ion exchange resin is a strongly basic resin with quaternary ammonium functional groups attached to a divinylbenzene copolymer lattice. The chromatography columns were prepared by placing a distilled water slurry, containing 3.0 g of beads, into a 0.5 x 37 cm glass column. The anion column was then washed with 3 volumes of water. From the relative selectivity table (Bio-Rad catalogue (1984) p 14), citrate and acetate were determined to be the most suitable of the available mobile phases. Various concentrations, volumes, and combinations of potassium acetate and sodium citrate were evaluated. Flow through the anion column was effected through the use of a peristaltic pump (Harvard Apparatus Co., Rover, MA). A 10 mL sample containing 100 mg NO_3-N L^{-1} and 100 mg glc L^{-1} was used to determine the effectiveness of the eluants.

The efficiency of the analysis methods in determining the concentrations of soil nitrate in Soil P1 was examined by preparing soil of varying nitrate concentrations. Ten grams of soil was placed in a 250 mL Erlenmeyer flask and nitrate was added to the soil for a final concentration of 10, 20, 40, 60, or 80 μg NO_3-N g^{-1}, from a 100 mg NO_3-N L^{-1} (KNO_3) stock solution. The total amount of liquid in the flask was made up with distilled water to 10 mL. The soil solution was then shaken for 30 min on a reciprocating shaker at 130 strokes min^{-1} (6 cm stroke length) and placed in a 90°C oven for 24 h, to evaporate the water.

For the brucine method, the nitrate in the prepared soil was extracted with 10 mL 2 M KCl or a silver sulfate-copper sulfate solution for 30 min on a reciprocating shaker. The silver sulfate-copper sulfate solution was prepared by mixing 20 mL of 1 mol L^{-1} copper sulfate and 100 mL of 0.6% silver sulfate and diluting to 1 L with distilled water. For the KCl-extracted soils, the brucine method of nitrate analysis was modified so that 0.4 mL of distilled water was added to the soil extract instead of the 0.4 mL 5 M NaCl.

The automated hydrazine sulfate method necessitated extraction of the nitrate from the prepared soil by shaking the soil with 0.5 mol L^{-1} sodium bicarbonate, pH 8.0, + 1 g of activated charcoal for 30 min. The soil extract was filtered through #30 Whatman filter paper and analyzed with a Technicon Auto Analyzer, based on a modification of the automated colorimetric procedure of Kamphake *et al.* (1967).

For the PDS method, the nitrate in the prepared soil was extracted with 25 mL of the silver sulfate-copper sulfate solution. The soil and extracting solution were shaken for 30 min, during which time 0.16 g of calcium hydroxide and 0.50 g of magnesium carbonate were also added. After filtration, the nitrate in the soil extract was determined by a modified PDS method (Bremner 1965). For this procedure, 5.0 mL of the soil extract was pipetted into a 50 mL beaker and evaporated to dryness at 95°C. The concentration of the nitrate was determined with a B&L Spectronic 710 at 415 nm, using a 10 mm light path.

Nitrate standard curves produced by the brucine method were reproducible and the curves produced at various times were highly correlated; the correlation coefficient (r) was > 0.99520 for the

linear portions of the standard curves (Figure 11). The curves were linear from approximately 0.02-0.10 $\mu g\ mL^{-1}$. Not unexpectedly, it was inadvertently found that inadequate mixing of the sample tended to decrease the absorbance values and to produce non-linear responses at concentrations > 0.05 $\mu g\ mL^{-1}$. Soil extracted with 2 M KCl resulted in slightly higher accuracy than extraction with the silver sulfate-copper sulfate solution; however, even extraction with KCl resulted in poor nitrate recovery values (Figure 12). Baker (1967) found that chloride, at the levels used for extraction, interfered with the rate of color development, and color fading increased with increasing chloride concentrations. Organic matter, nitrite, and strong oxidizing and reducing agents are also known to interfere with the test (Keeney and Nelson 1982).

The manual hydrazine sulfate method was found to result in larger variations in the standard curve values, with $r > 0.95434$ for the linear portions of the curve (Figure 13). Response was linear from approximately 0.05-0.20 $\mu g\ mL^{-1}$. When the sample was heated at 55°C before addition of the color reagent, a logarithmic standard curve was produced, but the precision increased slightly. Owing to the poorer precision of the manual hydrazine sulfate method, the analysis of soil nitrate was not attempted.

Standard curves produced by the PDS method were well correlated ($r > 0.99939$) (Figure 14), although it is recommended that the analysis be bracketed by known standards. The curves were linear from 0.2-2.0 $\mu g\ mL^{-1}$. Extraction and analysis of nitrate from Soil P1 revealed essentially 100% recovery of the added nitrate (Figure 15). As a

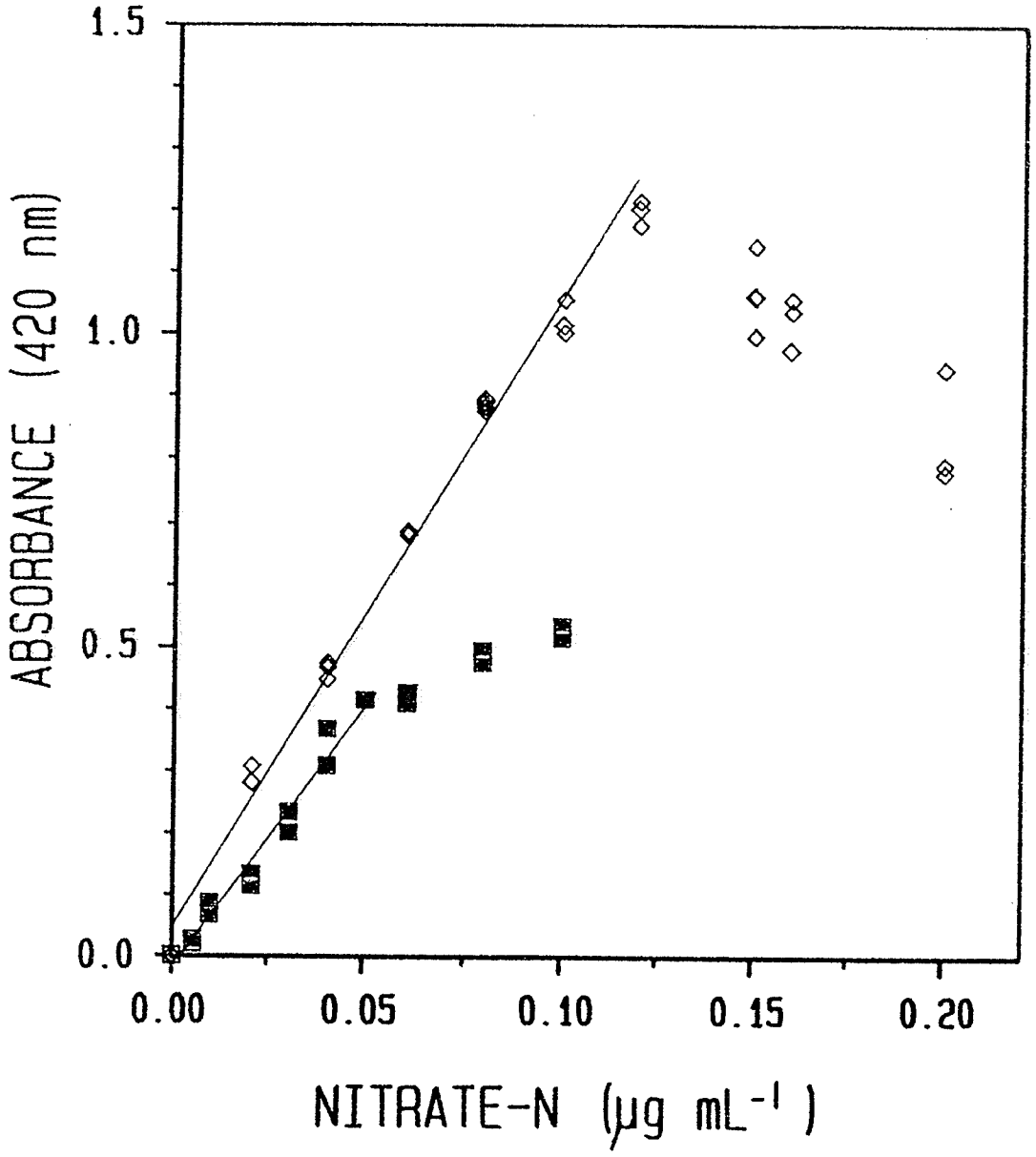

Figure 11: Brucine standard curves. The replicates, 3 for ◊ and 2 for ■, were done on consecutive days. Analysis points indicated as ■ were not mixed before reagent addition.

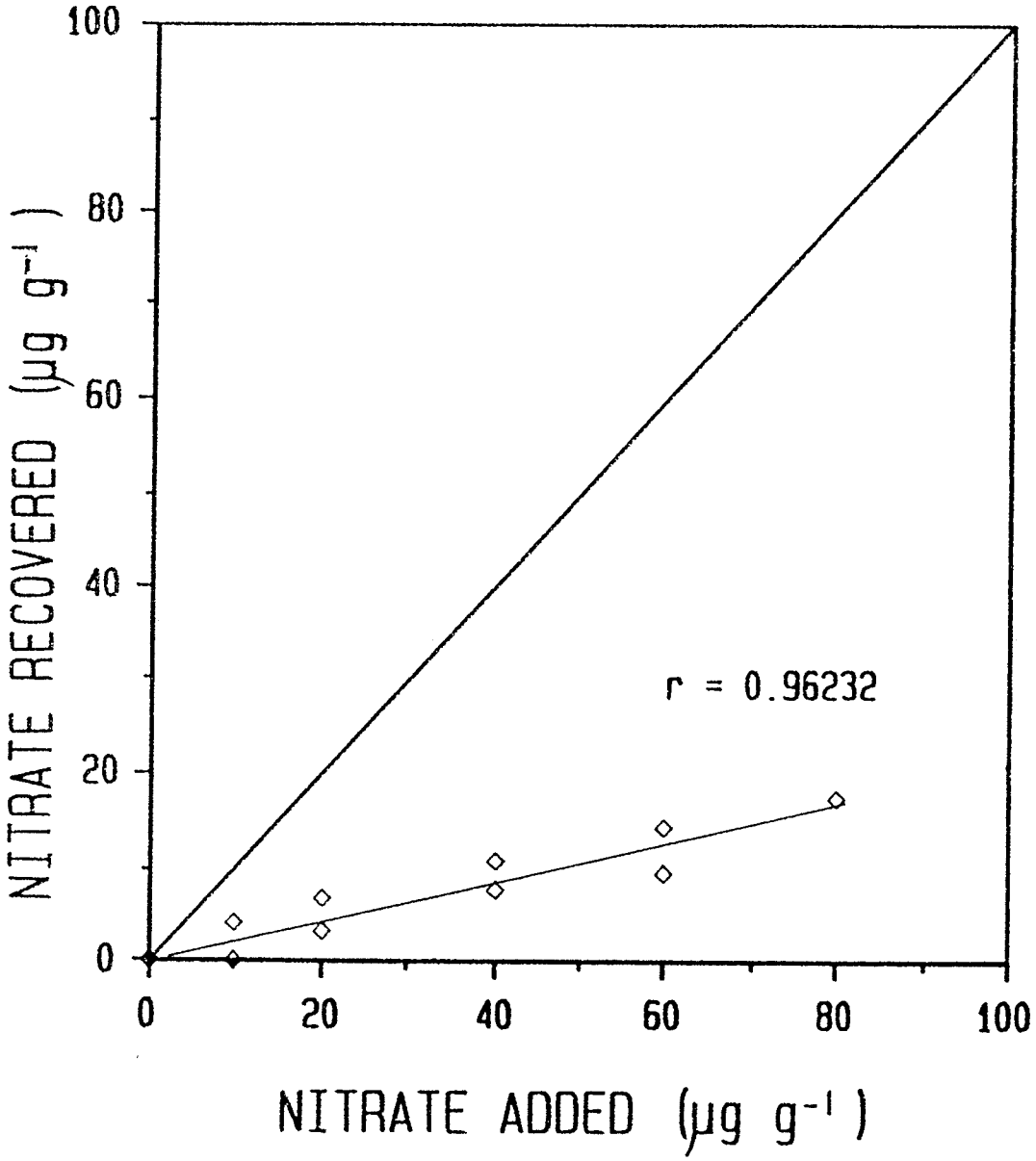

Figure 12: Analysis of nitrate added to Soil P1 by the brucine method. Two analyses for each indicated concentration were performed on successive days. The 100% recovery curve is indicated as "_____".

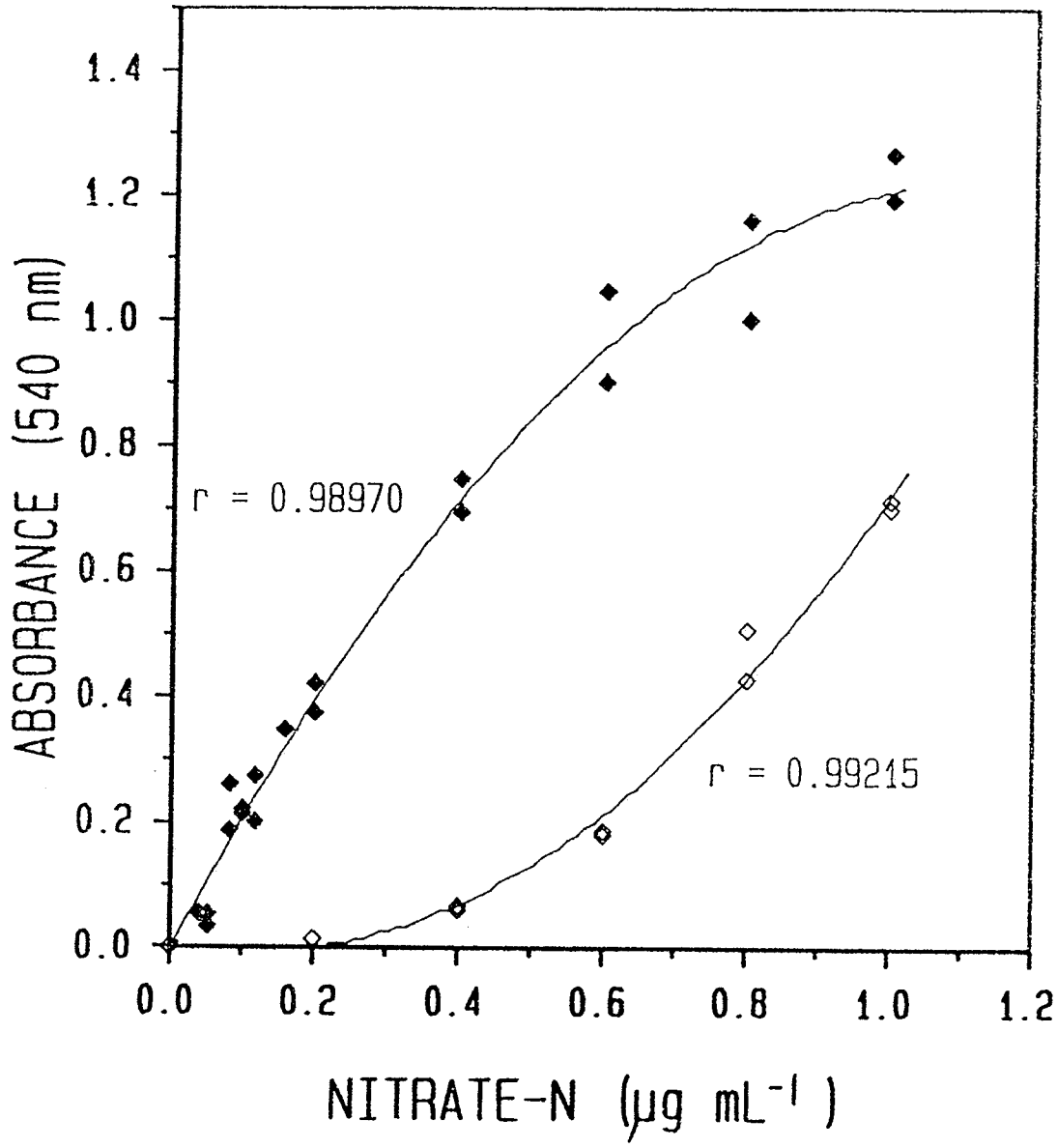

Figure 13: Hydrazine sulfate standard curves. Each indicated concentration (◊—or—♦) represents the average of two replicates performed on successive days. For the standard curve indicated as ♦———♦, the samples were heated to 55°C prior to color reagent addition.

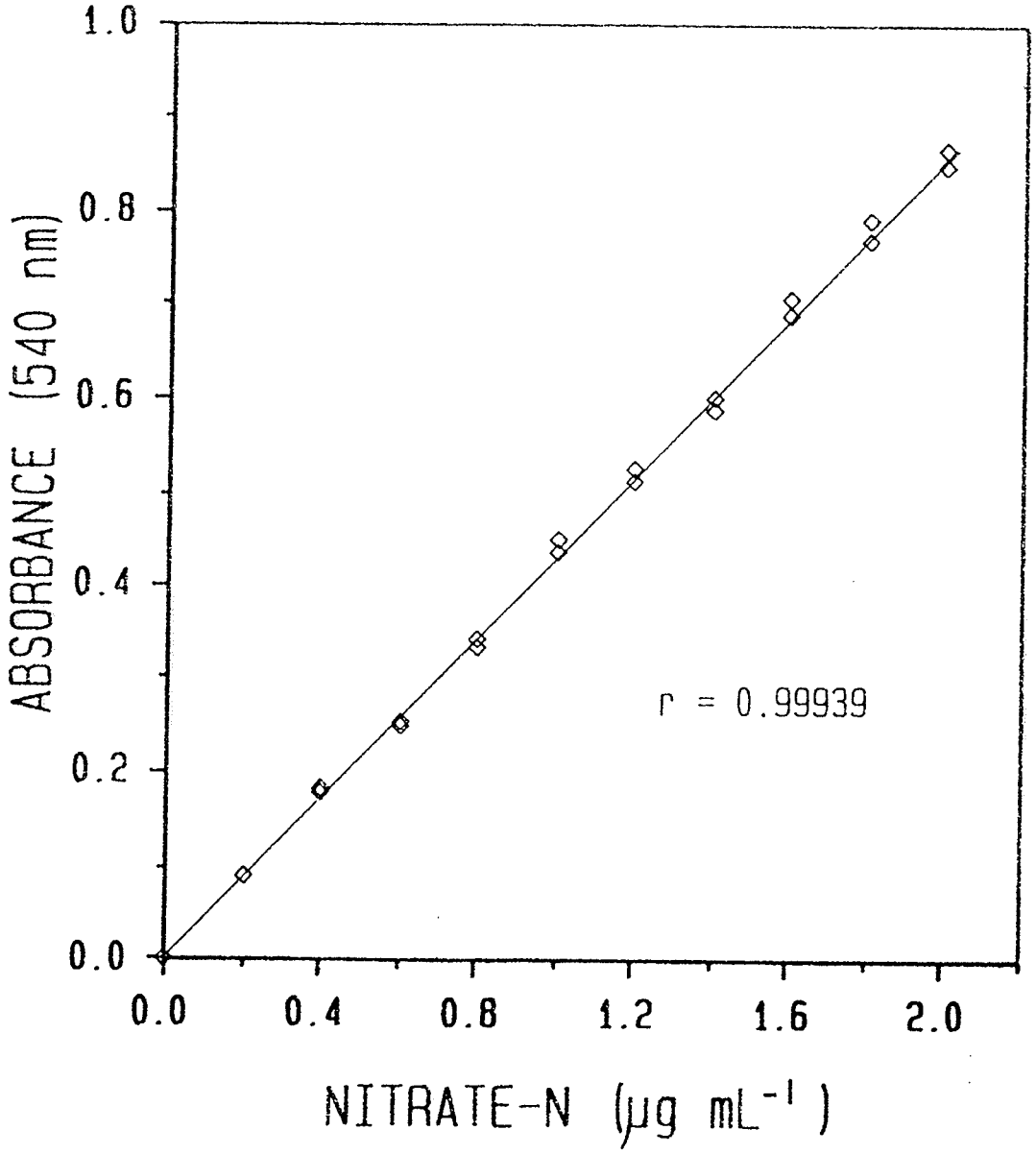

Figure 14: Phenoldisulfonic acid standard curves. Two analyses for each indicated concentration were performed on successive days.

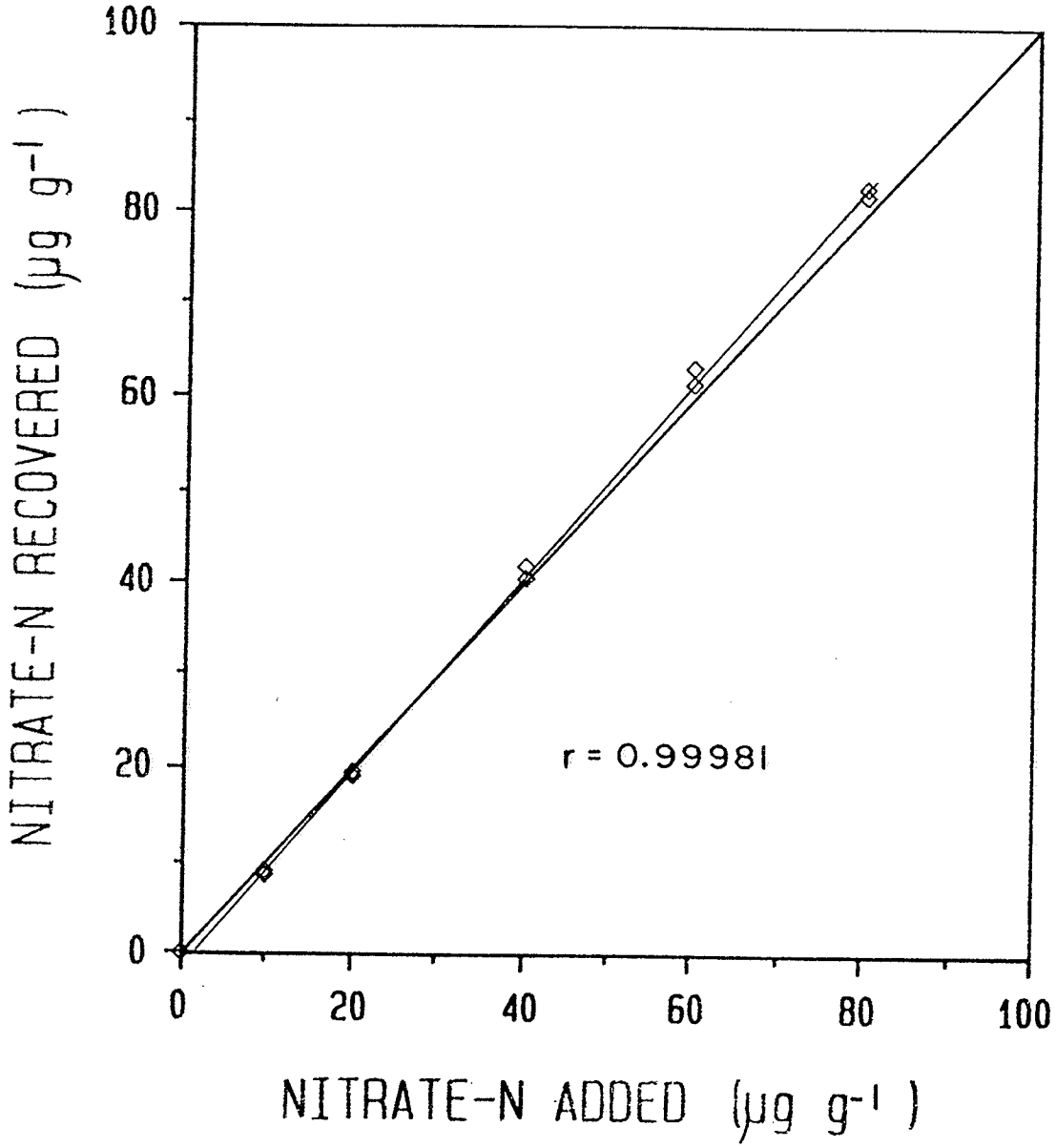

Figure 15: Nitrate analysis of Soil P1 by the phenoldisulfonic acid method. Two analyses for each indicated concentration were performed on successive days. The 100% recovery curve is indicated as "_____".

result of the high recoveries of the added nitrate by the PDS method, this method was originally chosen for nitrate analysis in the nitrate reduction study.

Organic matter is known to interfere with the PDS method (Keeney and Nelson 1982). Since glucose was to be added to the soils for the reduction assays, the nitrate recovery experiment was repeated with the addition of 100 νg glc g^{-1} to Soil P1. At the temperature originally used for drying the extraction, 95°C, it was found that glucose interfered with nitrate analysis (Figure 16). For glucose concentrations < 150 μg g^{-1}, the amount of glucose recovered from the soil was found to be greater than the amounts added (Figure 17) and the difference between that added and recovered, increased as the amount of glucose added decreased. Nitrate concentrations from 20-80 μg g^{-1} had no effect on the recoveries of glucose.

Various methods were employed to remove the glucose interference of nitrate analysis. The Sep-Pak C_{18} cartridge was found to remove very little glucose from the 5 mL sample and the silica cartridge was not effective at removing any glucose. For the charcoal modification, it was found that as the amount of charcoal increased, the concentration of glucose remaining decreased, however, even with a 3 g charcoal addition, only 1/2 of the added glucose was removed. Hydrogen peroxide is often used to oxidize organic matter in soils (Nelson and Sommers 1982), but hydrogen peroxide was found unsuitable because of the violent reaction of phenoldisulfonic acid with the dried hydrogen peroxide treated sample.

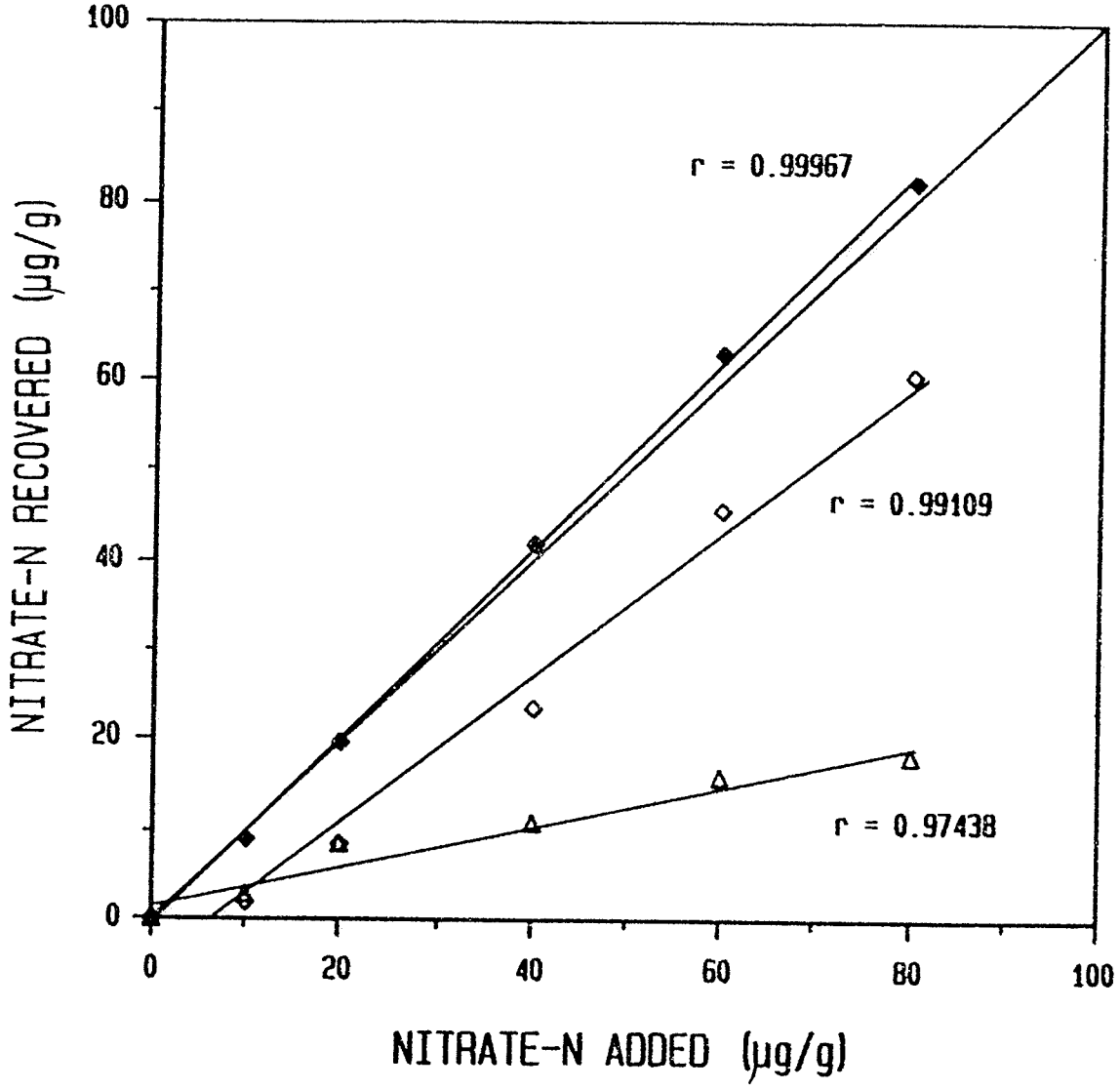

Figure 16: Nitrate analysis of Soil P1 amended with glucose by the phenoldisulfonic acid method. Two analyses for each indicated concentration were performed on successive days. The 100% recovery curve is indicated as "_____". Soil extract was dried at 60°C (♦——♦), 80°C (◊——◊), and 95°C (△——△).

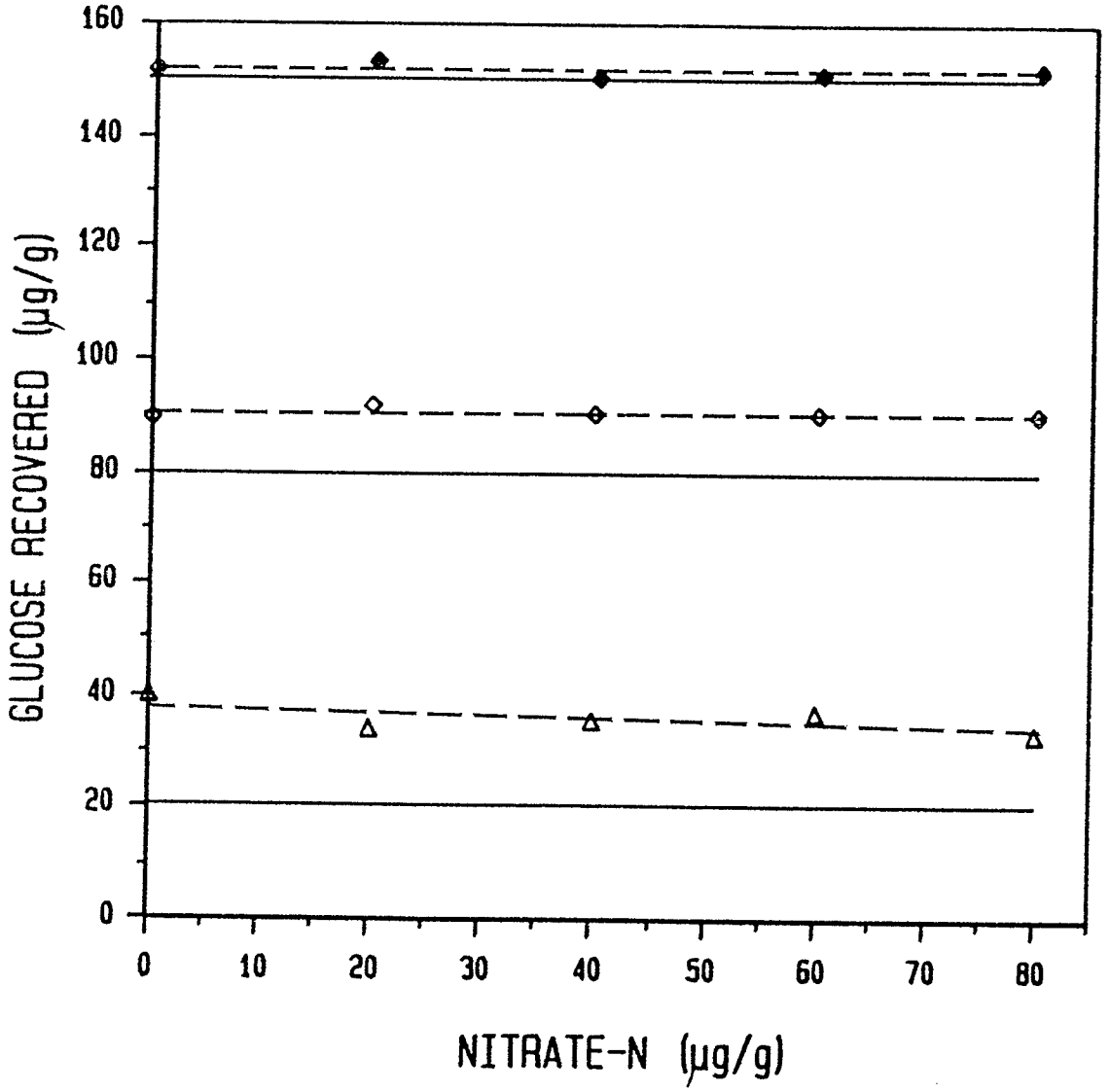

Figure 17: Recovery of 20 (△———△), 80 (◇———◇), and 150 (♦———♦) $\mu g\ g^{-1}$ of glucose added to Soil P1. Each data point represents the mean of two analyses performed on successive days. The 100% recovery of added glucose is indicated as "_____".

Ion exchange chromatography proved to be the most promising of the modifications, although it was the most tedious. Of the various combinations attempted, only two were found suitable. The 10 mL glucose-nitrate sample was added and eluted with 70 mL water and then either 100 mL 0.1 M potassium citrate followed by 100 mL 1.0 M potassium acetate or 60 mL 1.0 M potassium citrate followed by 60 mL 1.0 M potassium acetate. With both 0.1 M and 1.0 M potassium citrate, approximately 15% of the total glucose eluted with the initial water fractions. With 0.1 M potassium citrate, less than 0.36 μg glc mL^{-1} was recovered in any of the citrate or acetate fractions, for a total glucose recovery of 2.33 μg mL^{-1} (Figure 18). Nitrate eluted in a wide band, for total a recovery of 127.3 μg mL^{-1} . The 1.0 M potassium citrate eluted the majority of the glucose concurrent with small concentrations of nitrate (Figure 18), for a total glucose recovery of 60.7 μg mL^{-1}. Approximately 10 μg mL^{-1} of nitrate came off with the 1.0 M potassium citrate. The majority of the nitrate, 83.8 μg mL^{-1} was eluted with the 1.0 M potassium citrate.

Concurrent with the ion exchange method, an experiment was performed to study the effect of the evaporation temperature on nitrate recoveries by the PDS method. It was found that by evaporating the sample at a lower temperature, it was possible to remove the interference from glucose in Soil P1. As the evaporation temperature decreased from 95° to 80° to 60°C, the amount of nitrate recovered increased to approximately 100% (Figure 16). A decrease in temperature also resulted in an increase in the correlation coefficient (r). None of these nitrate analysis methods and PDS modifications were employed

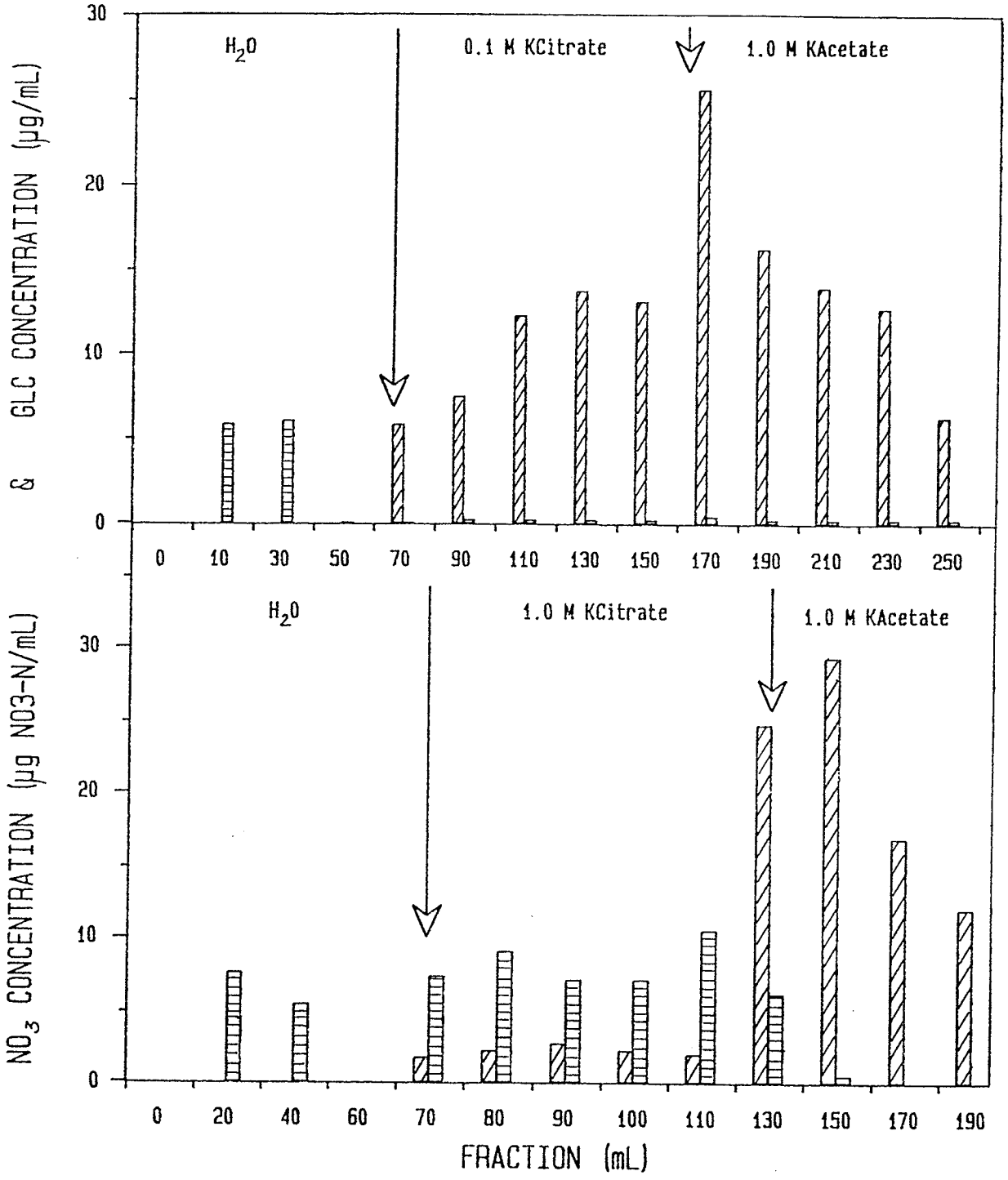

Figure 18: The separation of a mixture of 100 μg NO_3-N mL^{-1} and 100 μg glc mL^{-1} with a Bio-Rad AG 1-X8 anion exchange column using 0.1 M potassium citrate or 1.0 M potassium citrate and 1.0 M potassium acetate. ▤ glucose, ▨ nitrate.

in the nitrate reduction study. An alternate method, HPLC, was found to be more suitable for the analysis of nitrate and to give consistent recoveries in various soils. The development of the HPLC nitrate analysis method is outlined in Appendix II and a comparison of the HPLC method with the PDS and hydrazine sulfate methods is outlined in Appendix III.

II. HPLC Analysis of Inorganic Ions

Numerous methods have been developed for the analysis of nitrate concentrations in soil, e.g., the phenoldisulfonic acid, brucine, and cadmium reduction methods (Keeney and Nelson 1982). In recent years high performance liquid chromatography (HPLC) has become popular for nitrate analysis and has been used to determine nitrate levels in food (Leuenberger *et al.* 1980), human plasma (Osterloh and Goldfield 1984), water (Kok *et al.* 1983), sewage sludge (Gerritse 1979), and soil (Vilsmeier 1984). The analyses are usually based upon the principles of ion chromatography HPLC, first reported by Small *et al.* (1975); both an analytical and suppressor column were used and the concentration of the ions was measured with a conductivity detector. Ion chromatography coupled with a conductivity detector has been used by numerous investigators (Buchholz *et al.* 1982, Van Os *et al.* 1982, Tabatabai and Dick 1983). Typically, nitrate is eluted within 5 to 6 min, with a detection limit of 0.03-0.1 mg L^{-1}. Haddad and Heckenberg (1982) used a low-capacity ion exchange column with an indirect refractive index detector. Retention times of 4.58 min for nitrite and 5.63 min for nitrate were obtained, with detection limits of 0.08 and 0.12 mg L^{-1} respectively.

Recently, reverse phase (RP) column packings have been used for the separation of inorganic ions. Molnar *et al.* (1980) used a LiChrosorb RP-18 column with a mobile phase of 0.002 M tetrabutylammonium hydroxide plus 0.05 M phosphate buffer (pH 6.7) to separate various anions. Kok *et al.* (1983) used a Radial-Pak C_{18} μBondapak column, with a PIC A UV grade reagent as the mobile phase, to

separate nitrate and nitrite in water. Vilsmeier (1984) utilized a RP-C_{18} Spheri-5D column, with a mobile phase of 1 mol L^{-1} tetrabutylammonium hydrogen sulfate and 25 mmol L^{-1} NaH_2PO_4 (pH 6.7), to separate nitrate, nitrite, and dicyandiamid. The nitrate was extracted from soil with water or $CaCl_2$.

In this study, various mobile phases were examined for their suitability for use in RP-HPLC for the detection and quantitation of nitrate and nitrite. The mobile phases examined were: distilled water, distilled water acidified with HCl, H_2SO_4, or acetic acid, potassium hydrogen phthalate buffer (pH 3.0), acetonitrile, methanol, isopropanol, and a paired ion chromatographic (PIC) reagent.

HPLC analysis was performed using a Waters Scientific (Mississauga, Ont.) Model M45 pump, Model U6K injector, and Model 441 UV detector. The detector was modified with the optional kit to allow detection at 214 nm. A 4 mm I.D. x 250 mm 10 μm LiChrosorb RP-18 column (Merck) was operated at ambient temperature (ca. 24°C). The nitrate peak areas were monitored with a CSI 38 digital integrator (Columbia Scientific Industries) and the peak heights of the ions were monitored with an Omniscribe strip chart recorder.

Low UV PIC A (tetrabutylammonium hydrogen sulfate) (Waters Scientific, Mississauga, Ont.) was prepared by dissolving the contents of the vial in one litre of distilled water. The potassium hydrogen phthalate buffer ($KHC_8H_4O_4$) was made by adding 22.4 mL 0.1 M HCl to 50 mL 0.1 M potassium hydrogen phthalate.

Distilled water, at pH > 5.5 and flow rates of 0.5 to 1.0 ml min^{-1}, was ineffective at separating nitrate and nitrite. As the pH

was lowered to 3.0 with H_2SO_4, the separation between nitrate and nitrite increased, with nitrate eluting first (Figure 19a). The choice of the acidifying agent was important in determining the elution characteristics; acidification to pH 3.0 with HCl (Figure 19b) resulted in slightly longer retention of nitrate and nitrite, reduced sensitivity, and a noisier baseline. However, in all cases, phosphate was found to have the same retention time as nitrate.

Potassium hydrogen phthalate buffer, pH 3.0, was effective at separating nitrate and nitrite, but peak shape deteriorated (Figure 19c). Nitrate, nitrite, and phosphate can be more effectively separated with 4 mM potassium hydrogen phthalate, pH 4.0, using a Vydac anion column (Haddad and Heckenberg 1982). Although nitrate and nitrite have been shown to be separated by 45-50 mM phosphate buffer, pH 3.0, using a Partisil-10 SAX (strong anion exchange) column (Thayer and Huffaker 1980), under these experimental conditions, 5 mM potassium dihydrogen phosphate, pH 3.2, was not effective at separating phosphate and nitrate. In contrast to the separation of nitrate, nitrite, and phosphate possible with Dionex low-capacity anion resin using 3.0 mM $NaHCO_3$ + 2.4 mM Na_2CO_3 (Tabatabai and Dick 1983), the use of 0.1 M $NaHCO_3$, pH 7.6, produced poor separation with the LiChrosorb RP-18 column.

Isopropanol-water mixtures were found to result in a dramatic reduction in nitrate sensitivity. However, a 3:1 isopropanol-water mixture was effective a slightly separating phosphate from nitrate (Figure 19d).

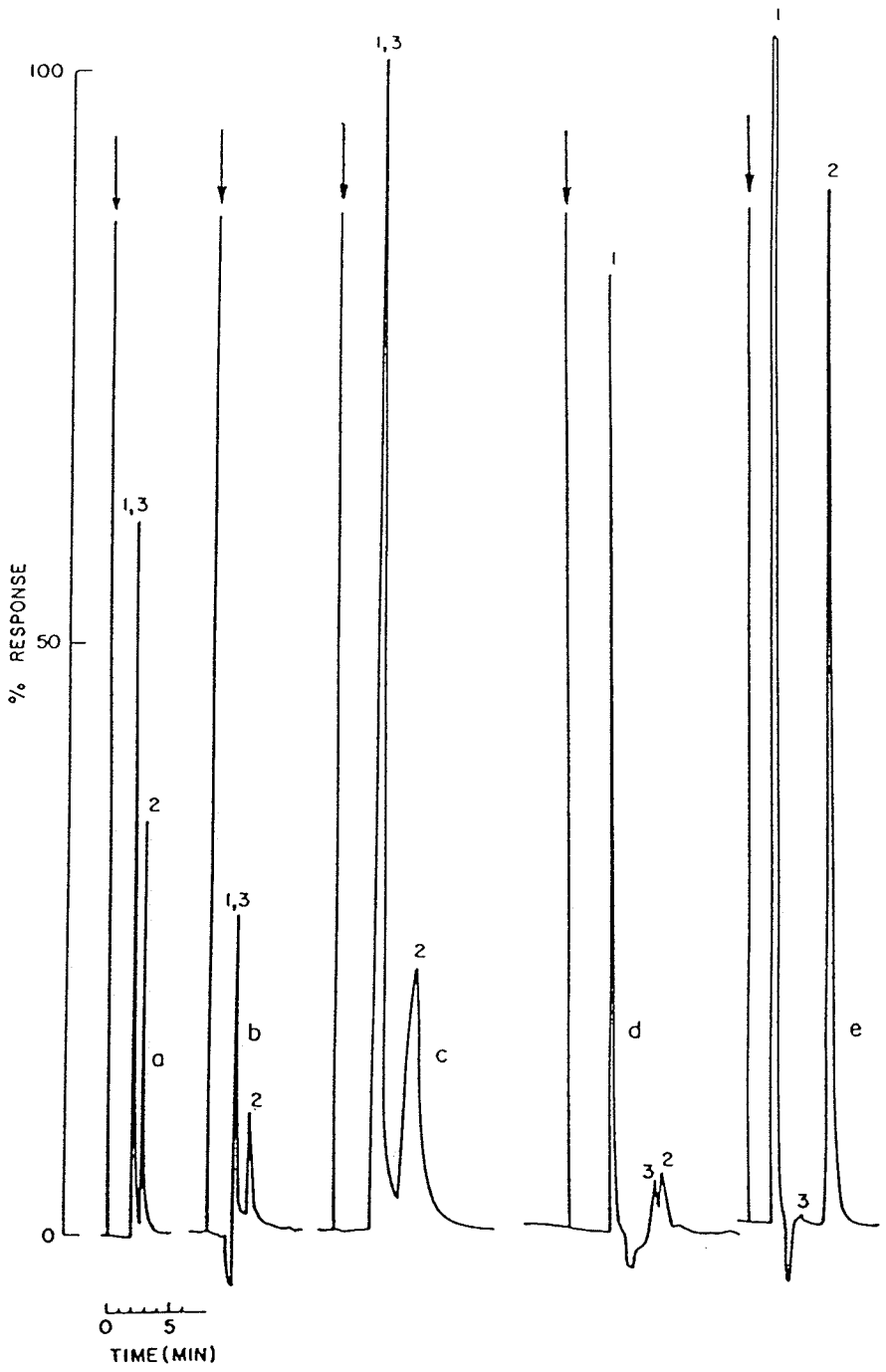

Figure 19: HPLC chromatograms of a 25 mg L^{-1} nitrate, 25 mg L^{-1} nitrite, and 400 mg L^{-1} phosphate sample with mobile phases of a) water acidified to pH 3.0 with H_2SO_4, b) water acidified to pH 3.0 with HCl, c) potassium hydrogen phthalate buffer pH 3.0, d) 3:1 isopropanol/water acidified to pH 3.0 with H_2SO_4, and e) 1:1 methanol/water acidified to pH 3.0 with H_2SO_4. Peak: 1 = nitrate, 2 = nitrite, and 3 = phosphate. Chart speed 0.1 in min^{-1}.

A 1:1 mixture of methanol-water, pH 2.9, was found to result in the best separation of phosphate from nitrate (Figure 19e). Phosphate does not absorb appreciably at 214 nm and the high concentration of phosphate needed to elicit a detector response indicates that phosphate would be essentially non-detectable at concentration normally found in our soils, i.e., < 100 $\mu g\ g^{-1}$ (Table 3). A concentration of 2000 mg L^{-1} was required to confidently determine the retention time for phosphate. At this high concentration, it was found that column performance deteriorated with phosphate use. The deterioration was manifested by a broadening of the nitrate peak width and, more significantly, by excessive noise at maximum absorbance, making peak height determination difficult. Column efficiency was restored by eluting the column with 100 mL methanol, 100 mL methylene chloride, 100 mL *n*-heptane and then methylene chloride and methanol again. The column was then washed with 1000 mL of distilled water. Flow rates were approximately 2.5 mL min^{-1}. Successive analyses of 2 mg L^{-1} nitrate, 100 mg L^{-1} nitrite, and 400 mg L^{-1} phosphate were also found to eventually result in temporary deterioration of the nitrite peak. However, it appears that the levels of phosphate normally found in our soils did not appreciably interfere with nitrate and nitrite analysis.

The separation of phosphate from nitrate was enhanced by the use of methanol in the mobile phase and may be due to a moderating effect of the methanol on the column (Rudzinski *et al.* 1982). The methanol may interact with the bonded phase allowing the ionic species to interact with the bonded alcohol. Rudzinski *et al.* (1982) found that a Partisil 5-ODS column, with 1:3 methanol-water as the mobile phase,

resulted in essentially "unretained" nitrate. Van der Houwen *et al.* (1982) speculated that very polar uncharged substances could interact with residual silanol groups and an increase in an organic modifier would increase this interaction. No retention was speculated for charged substances unless a hydrophobic portion was present. For this study, it was speculated that the addition of an alcohol with a larger carbon number would have a more modifying effect.

Various proportions of methanol-water-isopropanol were tried to optimize the retention times and sensitivities of the three ions. It was found that a 1:1:1 ratio resulted in adequate separation of nitrate, nitrite, and phosphate (Figure 20). As the ratio of methanol declined, the retention time of phosphate decreased and approached that of nitrate. Conversely, as the isopropanol ratio increased, the phosphate retention increased.

Low UV PIC A (PIC A) was effective at separating nitrate and nitrite (Figure 21). In contrast to the other mobile phases studied, PIC A eluted nitrite first. Interestingly, phosphate concentrations of up to 2000 mg L^{-1} were not detectable, possibly due to poor separation of phosphate. Table 14 shows the retention times of nitrate, nitrite, and phosphate for PIC A and 1:1 methanol-water.

An absorbance of 0.001 was obtained for 0.05 mg L^{-1} nitrate and 2.5 mg L^{-1} nitrite, utilizing a mobile phase of 1:1 methanol-water, pH 3.0. With PIC A, approximately 1.0 mg L^{-1} nitrate and 0.6 mg L^{-1} nitrite were required to produce absorbances of 0.001. Using the same type of column with a PIC A mobile phase and UV detection, Kok *et al.* (1983) calculated the detection limit to be 1.0 mg L^{-1} for both

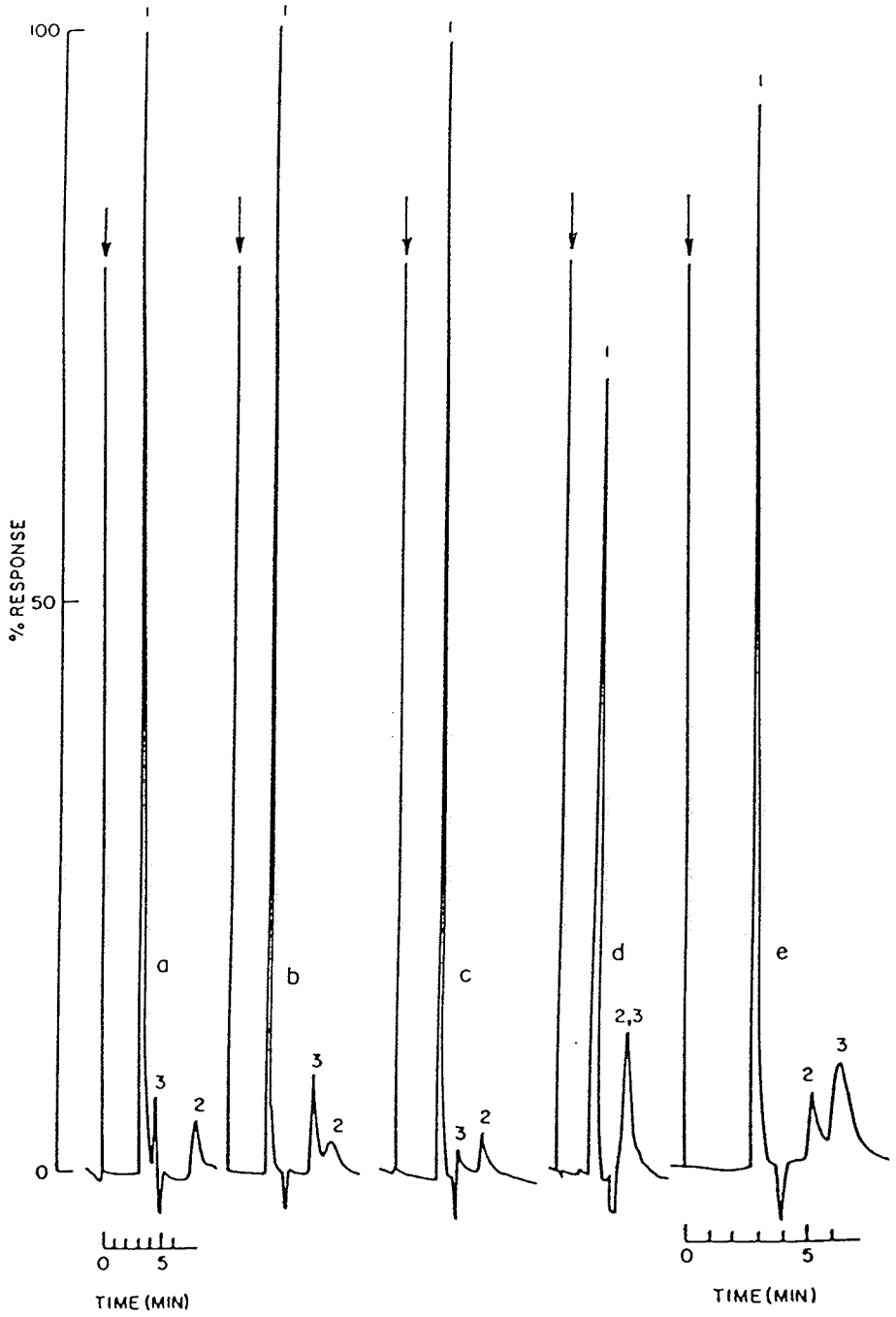

Figure 20: HPLC chromatograms of a 25 mg L^{-1} nitrate, 25 mg L^{-1} nitrite, and 400 mg L^{-1} phosphate sample with ratios of a) 2:1:1, b) 1:1:1, c) 1:14.1:1, d) 0.86:1:1, and e) 0.67:1:1 of methanol/water/ isopropanol acidified to pH 3.0 with H2SO4. Peak: 1 = nitrate, 2 = nitrite, and 3 = phosphate. Chart speed was 0.1 in min^{-1} except for 'e', which was 0.2 in min^{-1}.

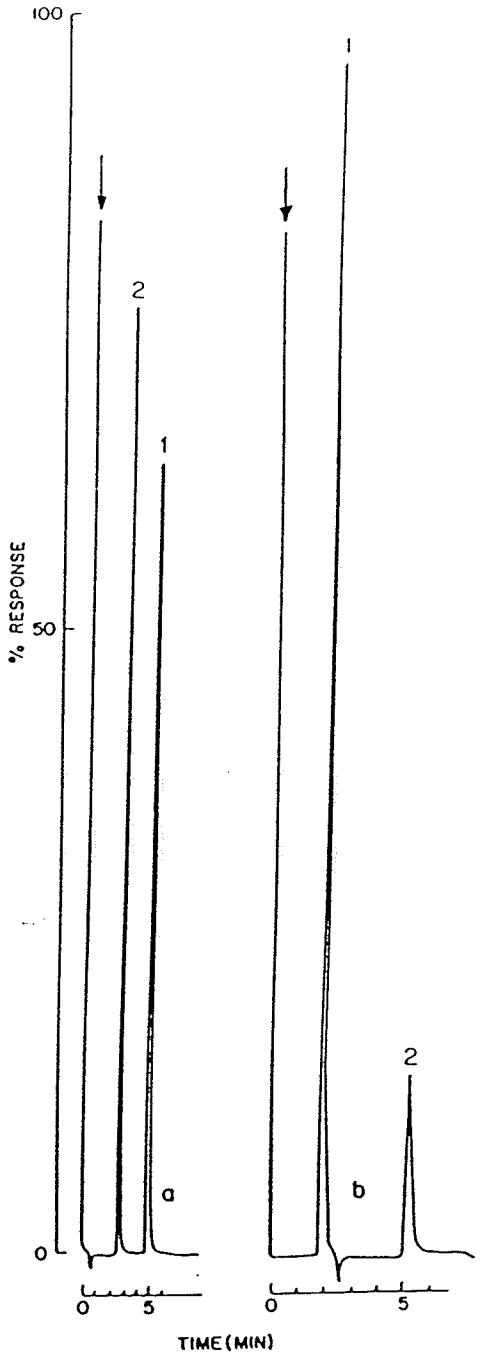

Figure 21: Comparison of chromatograms produced by a 50 mg L^{-1} nitrate, 50 mg L^{-1} nitrite, and 400 mg L^{-1} phosphate sample with a) Low UV PIC A and b) 1:1 methanol/water acidified to pH 3.0 with H_2SO_4. Flow rates were 2.5 mL min^{-1} for PIC A and 1 mL min^{-1} for methanol/water. Peak: 1 = nitrate, 2 = nitrite, and 3 = phosphate. Chart speed was 0.1 in min^{-1} for 'a' and 0.2 in min^{-1} for 'b'.

Table 14: Retention times (min) of nitrate, nitrite, and phosphate with 1:1 methanol-water and Low UV PIC A.

Mobile Phase	Retention Time (min) for Nitrate	Nitrite	Phosphate
1:1 MeOH-H_2O	1.62	4.90	2.57
Low UV PIC A	5.08	2.92	n.d.

n.d. - not detectable

nitrate and nitrite. The nitrate absorbances with 1:1 methanol-water tended to exhibit more variability. At the 95% confidence level, 5 injections of 1 mg NO_3-N L^{-1} produced absorbances varying by 10.0% and at 100 mg NO_3-N L^{-1}, by 1.7%. With PIC A, the absorbances varied by < 1.2% at all concentrations. The nitrite absorbance values for 2.6-400 mg NO_2-N L^{-1} varied within 3.6% for both 1:1 methanol-water and PIC A.

The nitrate standard curve produced with 1:1 methanol-water was linear from 0.1 to 80 mg NO_3-N L^{-1} with r = 0.99769 (Figure 22), while the nitrite standard curve was linear from 2.5 to 400 mg NO_2-N L^{-1} with r = 0.99934 (Figure 23). Nitrate concentrations > 80 mg NO_3-N L^{-1} resulted in non-linear responses. Nitrite concentrations > 400 mg NO_2-N L^{-1} were not analyzed. With PIC A, the nitrate standard curve was linear from 0.75 to 100 mg NO_3-N L^{-1} with r = 0.999125 (Figure 22) and the nitrite curve was linear from 0.75 to 175 mg NO_2-N L^{-1} with r = 0.98942 (Figure 23). The 1:1 methanol-water system provided an approximate 10 times increase in sensitivity over that obtained with PIC A. In contrast, with PIC A sensitivity to nitrite was doubled. The 1:1 methanol-water system gave better overall sensitivity than the PIC A reagent, although at a reduction in confidence. The 1:1 methanol-water mixture is less expensive and works at a lower flow rate, viz., 1.0 vs 2.5 mL min^{-1} for PIC A, resulting in an overall savings in mobile phase expense.

The results indicate that 1:1 methanol-water is an effective eluant for the analysis of nitrate and nitrite by RP-HPLC. The use of 1:1 methanol-water at a flow rate of 1.0 ml min^{-1} would result in a

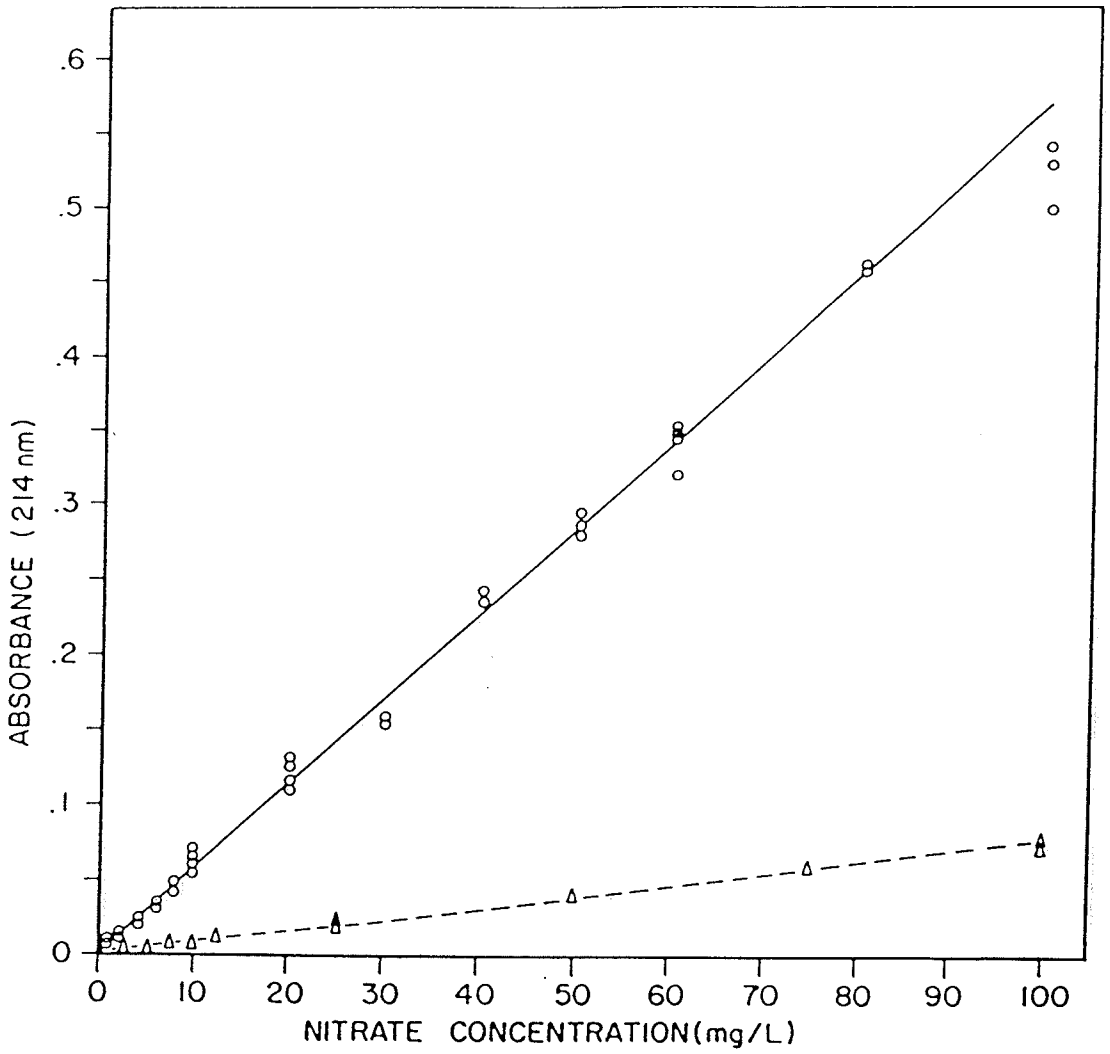

Figure 22: Standard curves for nitrate generated with 1:1 methanol-water (o———o) and Low UV PIC A (Δ-----Δ) mobile phases. A minimum of 3 analyses for the 1:1 methanol/water and 2 for the PIC A were performed at each indicated concentration. The 100 mg L^{-1} analyses for 1:1 methanol/water were not included in the statistical analysis.

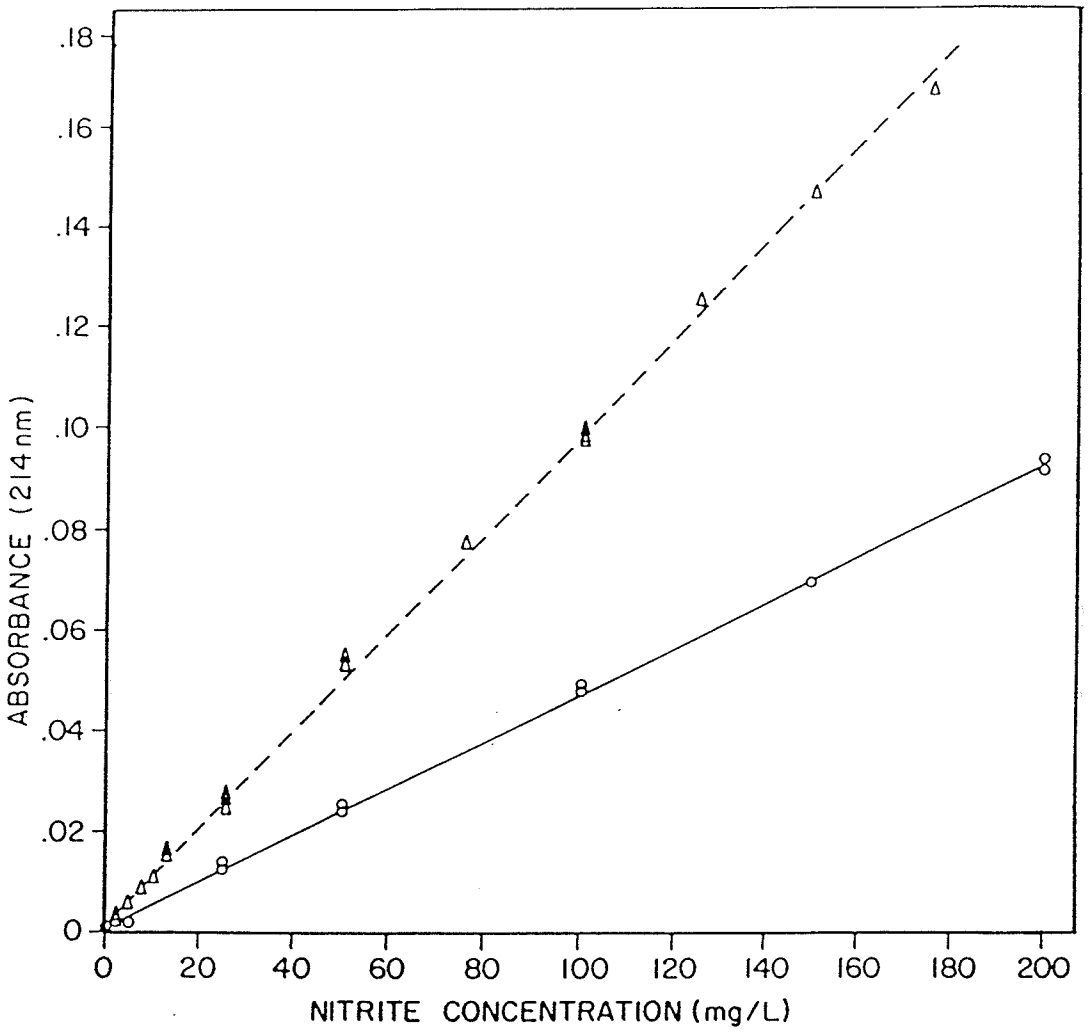

Figure 23: Standard curves for nitrite generated with 1:1 methanol/water (o———o) and Low UV PIC A (Δ----Δ) mobile phases. Each concentration assayed represents a minimum of 2 analyses.

substantial savings in the cost of the mobile phase without deterioration in HPLC performance. For phosphate analysis, 1:1:1 methanol-water-isopropanol, pH 3.0, can be used, although detection at another wavelength would provide more sensitivity.

III. Comparison of Nitrate Analysis Methods

The efficiency of the HPLC nitrate analysis method (Kuchnicki *et al.* 1985) was compared to the phenoldisulfonic acid (PDS) (Bremner 1965) and the hydrazine sulfate (Kamphake *et al.* 1967) methods in 6 soils of varying physical and chemical properties. The PDS and hydrazine sulfate methods were chosen since the PDS method has been intensely used by the Soil Science Department (University of Manitoba) and the automated hydrazine sulfate method is used by the MPST Laboratory. The PDS method also produced the most reliable standard curves and was the most accurate for the extraction and analysis of Soil P1 (Appendix I). The feasibility of using the soil extractants a) water, b) silver sulfate-copper sulfate, used for the PDS method, and c) sodium bicarbonate, used for the hydrazine sulfate method, as extractants for the HPLC analysis method was also examined.

The collection, storage, and chemical analyses of the 6 soils used in this study is outlined in the Materials and Methods section. The physical and chemical characteristics of the soils are given in Tables 2 and 3.

Soil of varying nitrate concentrations was produced by adding 2.0 g (HPLC method), 2.5 g (hydrazine sulfate method) or 5.0 g (PDS method) of soil to a 250 mL Erlenmeyer flask. Nitrate was added to the soil at concentrations of 5, 10, 20, 40, 60, 80, and 100 μg NO_3-N g^{-1} from a 50 mg NO_3-N L^{-1} (KNO_3) stock solution. The total amount of liquid in the flask was made up with distilled water to 5.0 mL (10.0 mL for the PDS method). The soil solution was then shaken at 24°C on a reciprocating

shaker at 130 strokes min^{-1} (6 cm stroke length) for 30 min and placed in a 90°C oven for 24 h, to evaporate the water.

For HPLC analysis, 6.0 mL of water or 1:1 methanol-water, pH 3.0 (methanol-water), was then added to the flask to extract the nitrate. After shaking for 45 min, the soil solution was filtered through #1 Whatman filter paper and 1.0 mL of the filtrate was passed through a 0.45 μm disposable syringe filter (Micron Separations Inc., N.Y.). Normally, less than 8 μL of this filtrate was needed for injection into the HPLC for analysis at 214 nm. Standard nitrate solutions were also analyzed each day to calibrate the detector.

For the automated hydrazine sulfate method, the soil was extracted with 0.5 mol L^{-1} sodium bicarbonate, pH 8.5, for 30 min. The extract was filtered and analyzed with a Technicon Auto Analyzer, based on a modification of the automated colorimetric procedure of Kamphake *et al.* (1967).

For the PDS method, the soil was extracted with 25 mL of a silver sulfate-copper sulfate solution. This solution was prepared by mixing 20 mL of 1 mol L^{-1} copper sulfate and 100 mL of 0.6% silver sulfate and diluting to 1 L with distilled water. The soil and extracting solution were shaken for 30 min, during which time 0.16 g of calcium hydroxide and 0.50 g of magnesium carbonate were also added. After filtration, the nitrate in this extract was then determined by a modified PDS method (Bremner 1965). For this procedure, 5.0 mL of the soil extract was pipetted into a 50 mL beaker and evaporated to dryness at 60°C. The concentration of the nitrate was determined with a B&L Spectronic 710 at 415 nm, using a 10 mm light path.

At a detector sensitivity sufficient to produce maximum nitrate peaks, soil samples extracted with water and analyzed with methanol-water showed only one major peak, nitrate (Figure 24a). The other smaller peaks were due to chloride, phosphate, and other unknown compounds. At 10 times the detector sensitivity, the minor peaks were shown to interfere minimally with the nitrate peak (Figure 24b). The methanol-water extracts were less colored than the water extracts. Chromatograms of the methanol-water extracts revealed slightly decreased peak heights of the minor peaks, probably due to less extraction of organic and inorganic substances.

To determine if any of the compounds eluted at the same time as nitrate, the water and methanol-water extracts of the soils were analyzed with Low UV PIC A as the mobile phase. In all cases only the nitrate produced a major peak. Limited analysis with PIC A showed, except for decreased sensitivity, little difference in nitrate response between methanol-water or water as the extractant.The silver sulfate-copper sulfate and sodium bicarbonate extractions were also analyzed by HPLC.

The silver sulfate-copper sulfate extracts were relatively colorless, and on analysis revealed only the nitrate peak. Sodium bicarbonate extractions gave the poorest chromatograms due to contaminants in the sodium bicarbonate extracting solution used by the MPST Laboratory.

When analyzed by HPLC, both the silver sulfate-copper sulfate (Figure 25a) and the sodium bicarbonate (Figure 25b) extracts showed no correlation with any of the soil physical and chemical characteristics

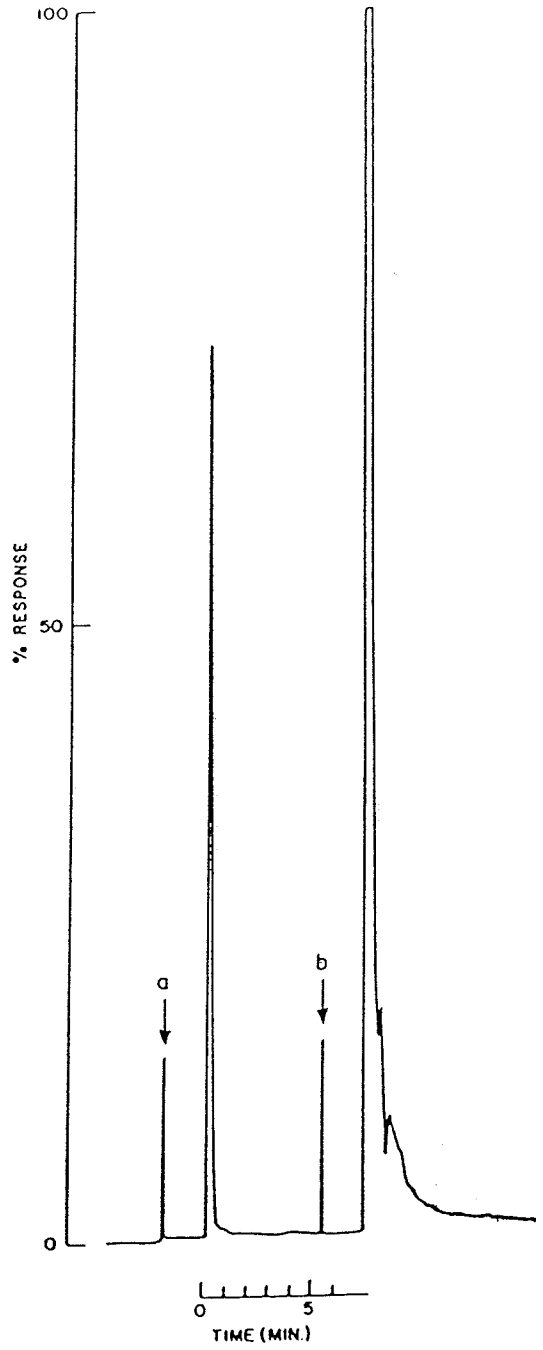

Figure 24: Representative HPLC chromatograms of Soil T1 extracted with water, pH 5.5. Chart speed was 0.2 in min^{-1}. The arrow indicates the point of sample injection. Absorbance units full scale were a) 0.5 and b) 0.02. The absorbance of the nitrate sample was 0.358.

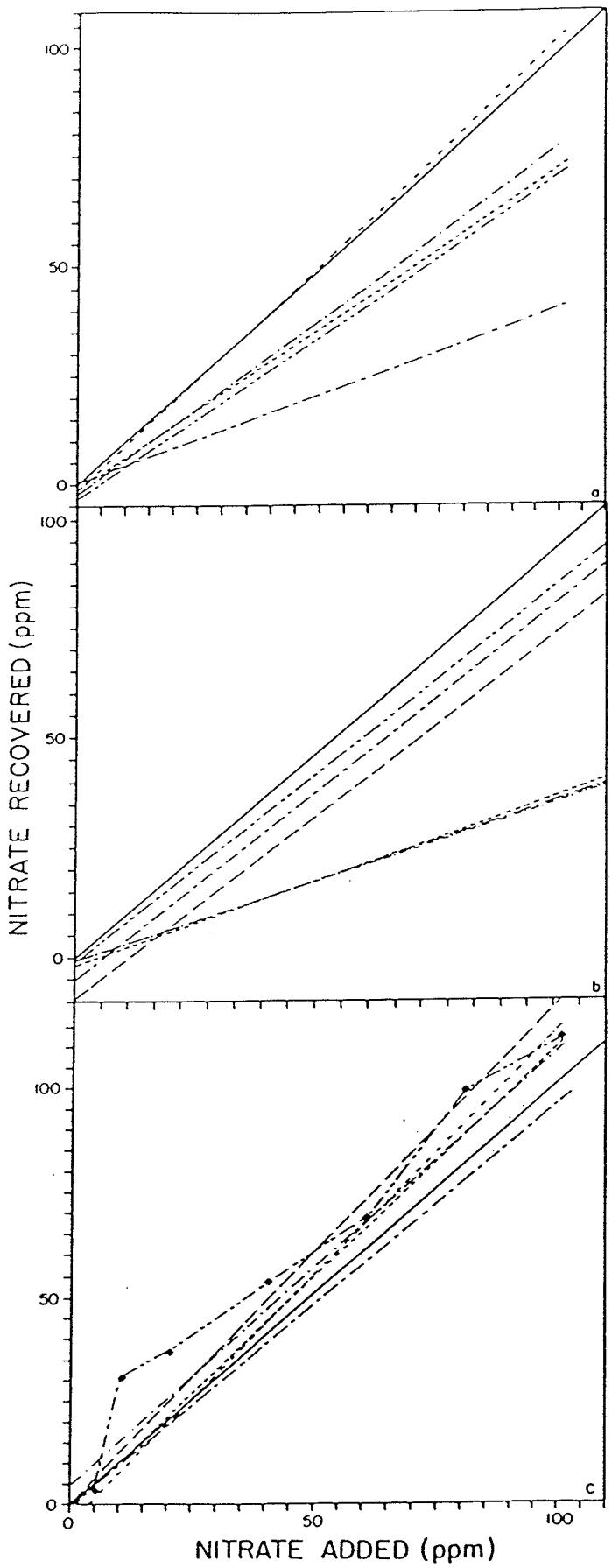

Figure 25: Efficiency of HPLC analysis with soil extractants a) sodium bicarbonate, pH 8.5, b) silver sulfate-copper sulfate, and c) 1:1 methanol/water, pH 3.0. Regression curves are averages of two replicates with $r \geq 0.98241$. P1 (— — —), BA (-·-·-), P2 (— — —), B1 (-----), B2 (—·—·—), T1 (— — —), 100% recovery (————).

(Tables 2 and 3). Sodium bicarbonate extraction efficiencies ranged from 35 to 95%; those for silver sulfate-copper sulfate ranged from 40 to 105%. The bicarbonate extractions frequently yielded negative recovery values at the lowest nitrate additions and were especially serious for soils P1 and P2 (Figure 25b), where where the concentration recovered at the 5 and 10 μg NO_3-N g^{-1} additions ranged from -1.1 to -5.0 μg NO_3-N g^{-1}.

The methanol-water extractions (Figure 25c) generally resulted in nitrate recoveries greater than 100% and in the case of soil T1, as high as 125%. For soil T1 the response was initially non-linear. The higher response for the methanol-water extractions is puzzling. The use of PIC A showed no other major co-extractives. A co-extractive would also be extracted at a constant concentration and would thus be expected to produce a linear regression curve parallel to the 100% recovery line. A more in-depth analysis of the physical and chemical properties of the soils may provide some insight to this phenomenon.

PDS analysis of the six soils revealed the expected trend of organic matter interference (Keeney and Nelson 1982). The higher organic content soils, T1, B2, and B1, had lower nitrate recoveries (Figure 26a). The 95% confidence intervals (Table 15) were usually greater for PDS analysis. BA and P1 had recoveries greater than 100%.

The hydrazine sulfate method was more precise than the PDS method; however, at low nitrate additions, negative values were frequently obtained for nitrate recoveries (Figure 26b). Recoveries ranged from 86.7 to 93.6% with an average recovery for the six soils of 90.2 ± 1.3

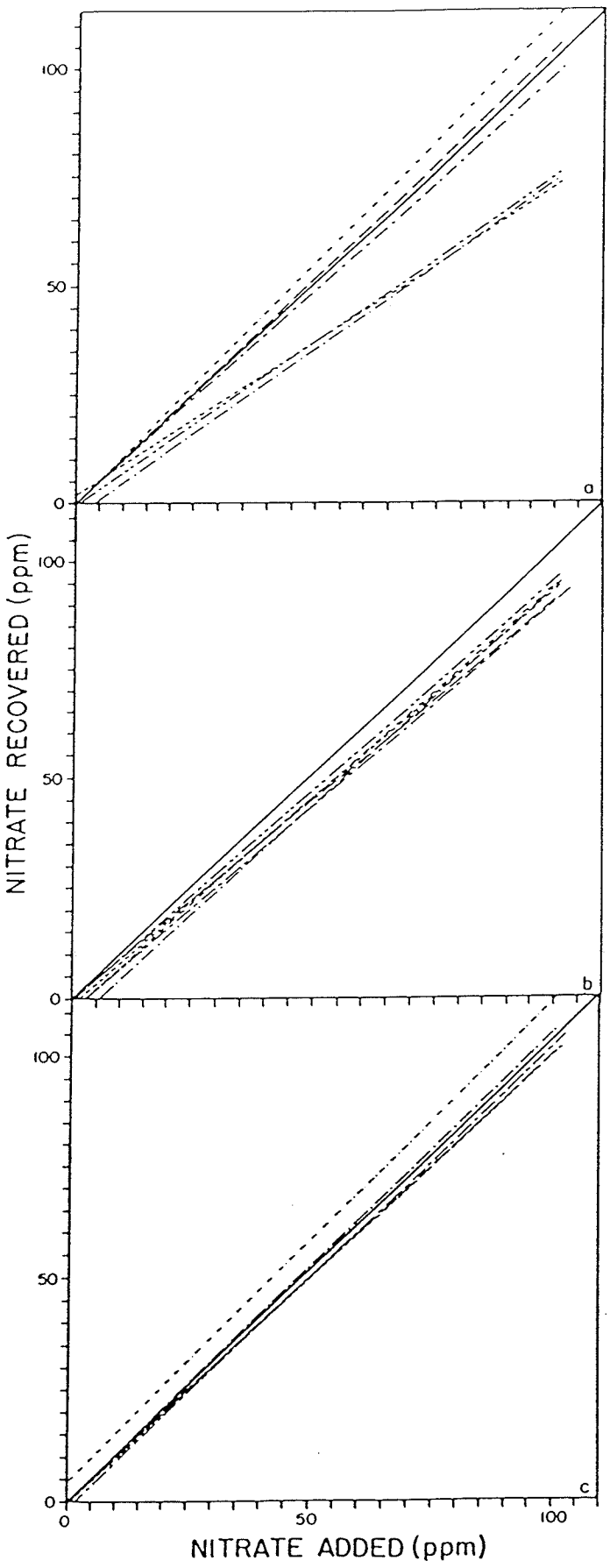

Figure 26: Efficiency of nitrate analysis methods a) phenoldisulfonic acid, b) hydrazine sulfate, and c) HPLC with water extraction. Regression curves are averages of two replicates with $r \geq 0.98627$. P1 (— — —), BA (-·-·-), P2 (— — — —), B1 (- - - -), B2 (—·—·—), T1 (— — — —), 100% recovery (————)..

(P = 0.05) (Table 15). Although the soils with higher organic matter (Table 3) exhibited slightly more variation, statistical analysis revealed that the physical and chemical characteristics of the soils used in this study had no effect upon recoveries of added nitrate.

Excluding the analysis of BA soil, the HPLC method gave equivalent precision with and was more accurate than the hydrazine sulfate method (Figure 26c, Table 15). Recoveries ranged from 97.4 to 105.8% with an average of 100.6 ± 1.2 (P = 0.05). Recoveries were not affected by the chemical and physical properties of the soils. The variation between analysis of the various soils can be attributed more to the variation within the soil samples than to experimental errors. The cause of the 112.0% recovery obtained with BA soil is unknown. The position of the linear regression curve (Figure 26c) suggests a co-extractive. However, analysis with PIC A as the mobile phase revealed no peak which could account for the approximate 13% increase in recovery. If an interfering compound is present, it is not separated from the nitrate or is not detectable with the PIC A system.

The HPLC method compares favorably with or is better than the hydrazine sulfate and PDS methods of nitrate analysis (Table 15). The HPLC method enables a smaller sample size than either of the other methods. However the minimum sample size is limited by the statistics of analysis; as the sample size decreases the sampling error becomes greater. HPLC analysis also allows on-going nitrate analysis of an aqueous soil sample with little perturbation of the system, proving helpful in studying soil solution reactions.

Table 15: Comparison of three methods for the recovery of 100 μg NO_3-N g^{-1} added to six Manitoba soils and the correlation coefficient (r) of recovery of added nitrate for the six concentrations studied.

	Method					
	HPLC		Hydrazine Sulfate		PDS	
SOIL	% recovery†	r	% recovery†	r	% recovery†	r
T1	105.8±3.0	0.99745	93.6±6.0	0.99325	73.5±4.1	0.99453
B2	100.8±3.0	0.99526	91.6±4.4	0.99634	72.8±7.3	0.98627
B1	97.6±1.2	0.99948	90.8±2.8	0.99838	71.4±1.6	0.99906
P2	100.8±2.0	0.99811	87.9±1.9	0.99920	96.7±1.4	0.99964
P1	97.5±2.2	0.99906	88.0±2.4	0.99870	102.9±1.0	0.99988
BA	112.0±3.1	0.99515	89.2±2.1	0.99906	109.0±5.3	0.99592
Average	100.6±1.2*	0.99652*	90.2±1.3	0.99635		

† ±95% confidence interval, mean of 2 replicates except BA-HPLC method which is mean of 3 replicates

* Excluding soil BA

The results indicate that water is an efficient extractant of soil nitrate at agriculturally important levels, provided the soil particle does not disperse as a result of deflocculation. The use of 1:1 methanol-water, pH 3.0, is an effective eluant for the analysis of nitrate by RP-HPLC.

IV. Effects of Acetone and Acetylene on Anaerobic Nitrate and Nitrite Reduction

Denitrification can be affected by numerous compounds (Knowles 1982). The low solubilities of the technical products in water (Table 13) necessitated that the stock solutions of the technical products be made up in 10% acetone. Speir and Ross (1981) found that acetone dehydration of soil (2.5:1 acetone/water) reduced the activites of invertase, amylase, cellulase, xylanase, protease, and phosphatase, but not urease and sulfatase. Acetylene is used to inhibit the reduction of nitrous oxide to dinititrogen and is also known to reduce cell metabolic activity at 3.0 kPa (Balderston *et al.* 1976). Preliminary trials therefore were undertaken to determine the effects of both acetone and acetylene on nitrate and nitrite reduction.

The nitrate and reduction assays were performed as outlined in the Materials and Methods section. Two assays were run simultaneously, a control flask and a treatment flask. The control flask had no acetylene or acetone added. To the treatment flask was added 20 mL of acetylene or 10 mL of a 10% acetone-water solution. There were 3 replicates for each treatment.

An addition of 10% acetylene to the flask had no statistical effect ($P = 0.05$) on nitrate and nitrite reduction rates or disappearance times in either Soil P2 or P3 (Tables 16 and 17 and Figure 27). The presence of 2.5% acetone also had no statistical effect on nitrate and nitrite reduction rates or disappearance times in either soil (Table 16 and 17 and Figure 28).

Table 16: The effect of 10% acetylene and 2.5% acetone on nitrate and nitrite reduction in Soil P2 and P3. Values not followed by the same letter are significantly different at P = 0.05 level.

	Nitrate Reduction Rates ($\mu g\ NO_3\text{-}N\ g^{-1}\ h^{-1}$)			
Soil	Control	10% Acetylene	Control	2.5% Acetone
P2	8.2±1.4 a	8.0±1.7 a	7.9±1.1 ab	7.5±1.4 ab
P3	2.4±0.8 c	2.5±0.9 c	2.9±1.2 c	2.7±1.0 c

	Nitrite Reduction Rates ($\mu g\ NO_2\text{-}N\ g^{-1}\ h^{-1}$)			
Soil	Control	10% Acetylene	Control	2.5% Acetone
P2	2.56±0.61 d	2.49±0.67 d	2.80±0.50 d	2.79±0.59 d
P3	1.32±0.37 e	1.47±0.40 e	1.24±0.27 f	1.27±0.24 ef

Table 17: The effect of 10% acetylene and 2.5% acetone on nitrate and nitrite disappearance times in Soil P2 and P3. Values not followed by the same letter are significantly different at P = 0.05 level.

	Nitrate Disappearance Times (h)			
Soil	Control	10% Acetylene	Control	2.5% Acetone
P2	26.6±5.4 a	26.9±5.6 a	28.1±6.2 b	28.1±8.1 b
P3	61.8±18.4 c	61.4±19.0 c	62.5±16.2 c	62.0±14.1 c

	Nitrite Disappearance Times (h)			
Soil	Control	10% Acetylene	Control	2.5% Acetone
P2	54.6±16.8 d	53.8±17.0 d	50.5±26.1 e	49.8±24.3 e
P3	91.2±19.8 f	89.6±20.1 f	88.6±18.8 g	89.1±18.9 f

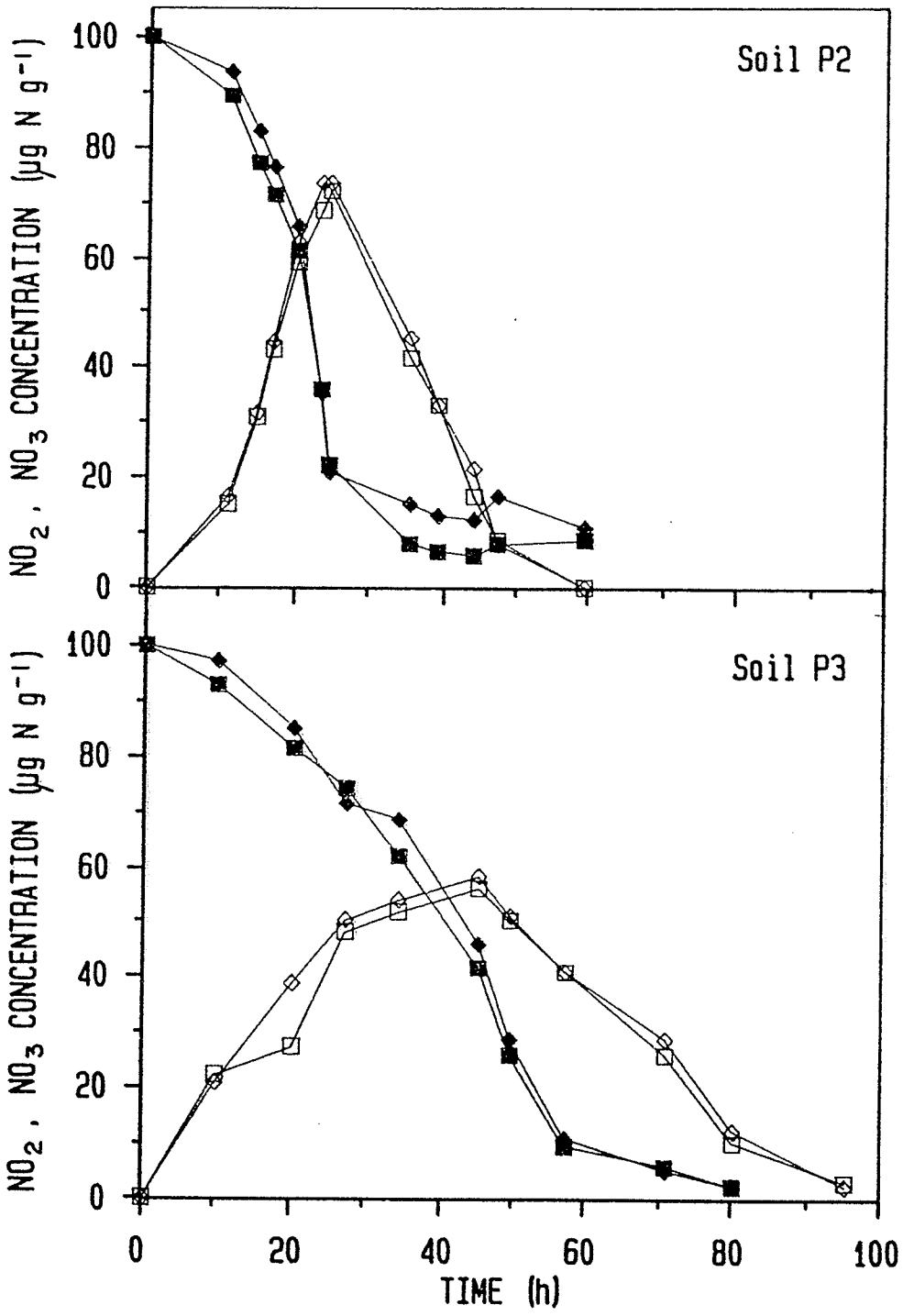

Figure 27: The effect of 10% acetylene on anerobic nitrate (closed symbols) and nitrite (open symbols) reduction in Soil P2 and P3. Control ◊———◊, 10% acetylene treatment □———□.

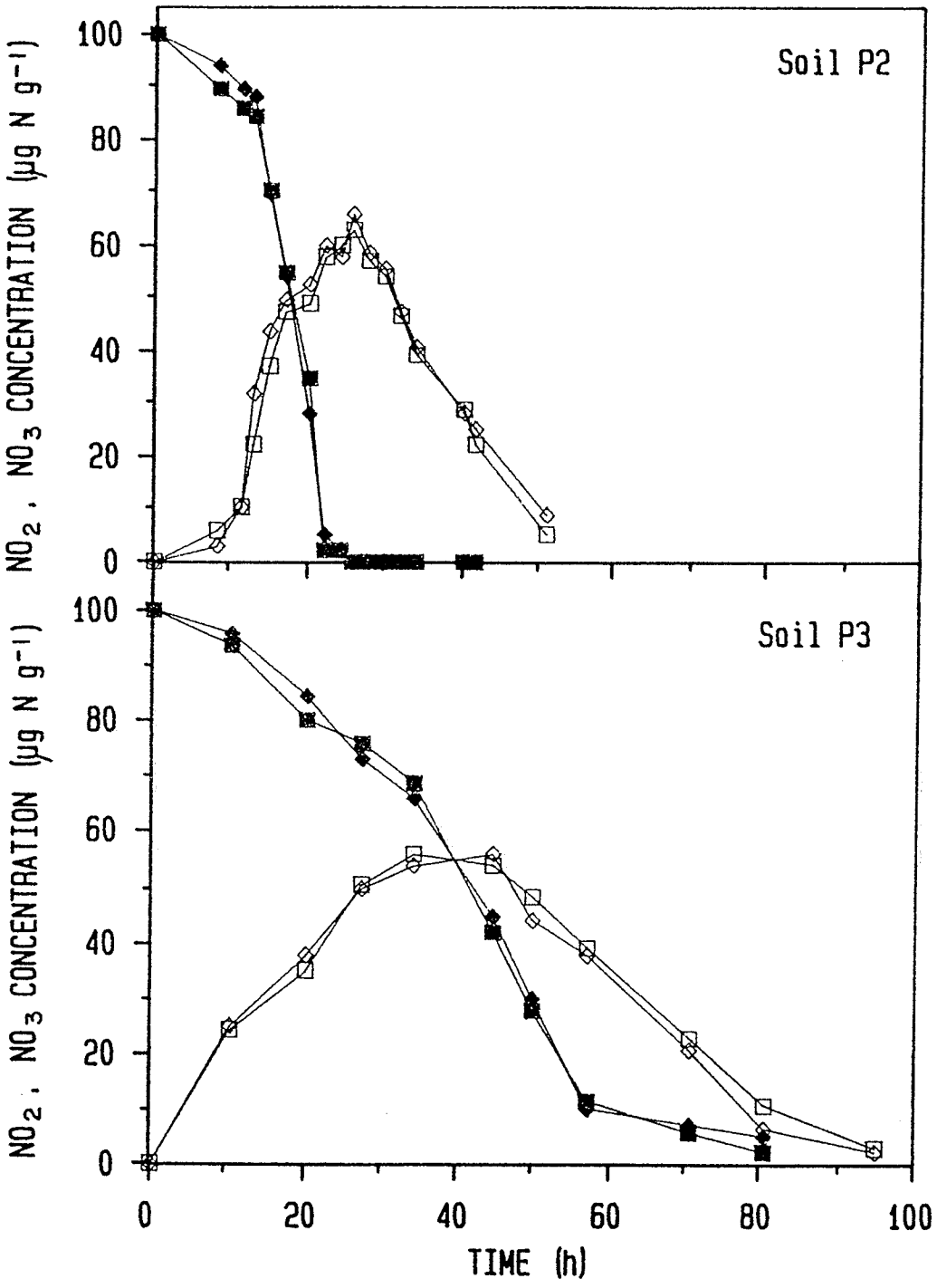

Figure 28: The effect of 2.5% acetone on anerobic nitrate (closed symbols) and nitrite (open symbols) reduction in Soil P2 and P3. Control ◊———◊, 10% acetylene treatment □———□.

There is also a possiblity that acetone can interact synergistically or antagonistically with a pesticide (Burell and Corke 1980); however, due to the insolubility of trifluralin in water, the recommended procedure of testing for pesticide-solvent interactions could not be undertaken (Stratton *et al.* 1982).

REFERENCES

Burrell, R.E. and C.T. Corke. 1980. Interactions of the solvent acetone with the fungicides benomyl and captan in fungal assays. Bull. Contam. Toxicol. 25: 554-559.

Stratton, G.W., R.E. Burrell, C.T. Corke. 1982. Technique for identifying and minimizing solvent-pesticide interactions in bioassays. Arch. Environm. Contam. Toxicol. 11: 437-445.

V. Nitrous Oxide Concentration Derivations

A. VAPOR PHASE:

1. STANDARD:

A 0.2 mL sample of N_2O was diluted to 1024.5 mL with He.

22414 mL is occupied by 1 mole of gas

Thus, standard is: $0.2/22414*1 = 8.923$ E-06 moles
$= 8.92$ μmoles @ STP

The temperature of the assay was 24°C (ambient), thus

$$n_1 T_1 = n_2 T_2$$

$$(8.922)(273) = n(297)$$

$$n = 8.20 \ \mu\text{mole}$$

Standard flask was 1024.5 mL, thus have total of

$$8.20/1024.5 = 0.0080 \ \mu\text{moles mL}^{-1}$$

$$= 8.01 \text{ pmoles } N_2O \ \mu\text{L}^{-1}$$

Samples from 10 to 100 μL of standard were injected into GC and peak heights were noted. The amount injected was plotted vs. the peak height, e.g., a 20 μL injection of the standard contains 0.16 nmoles of N_2O, and produced a peak height of 30 mm.

A quadratic equation is obtained where y = peak height in mm and x = amount N_2O in nmoles.

$$y = -153.015x^2 + 215.351x - 0.328$$

$r^2 = 0.999$
$p < 0.0001$
S.E. = 0.962
C.V. = 2.412

2. SAMPLE:

At 20.6 h the control was sampled and a 20 μL gas sample produced a peak height of 16.5 mm.

Using the above formula the amount of N_2O in the sample is:

$$16.5 = -153.015x^2 + 215.351x - 0.328$$

$$0 = -153.015x^2 + 215.351x - 16.838$$

Using the formula: $x = \frac{b \pm (b^2-4ac)}{2a}$

$$x = \frac{-215.351 \pm (215.3512 - 4*153.015*16.838)}{2*(-153.015)}$$

$$x = 0.083 \text{ nmoles}$$

Thus the concentration of N_2O is

$$(0.083/20) = 0.0041521 \text{ nmoles } \mu L^{-1}$$
$$= 0.00415 \text{ } \mu\text{moles mL}^{-1}$$

The pressure was measured with a mercury differential manometer and registered 3.65 cm. Thus, the pressure within the incubation flask is

$$76.00 + 3.65 = 79.65 \text{ cm Hg}$$

At 24°C the water vapor pressure is 2.2377 cm Hg. Thus the adjusted pressure is

$$79.65 - 2.2377 = 77.41 \text{ cm Hg}$$

The concentration of N_2O at incubation pressures is

$$[N2O] = \frac{0.00415 * 77.41}{76}$$

$$= 0.00423 \text{ } \mu\text{moles mL}^{-1}$$

A decrease in flask pressure indicates a loss of N_2O from the incubation flask. This decrease can be calculated as:

(Previous Pr.-Present Pr.)/76 * N2O Conc.

$$((78.06-77.41)/76.00) * 0.00423 = 0.00004 \text{ } \mu\text{moles mL}^{-1}$$

Thus the total concentration of N_2O at time 20.6 h is

$$0.00423 + .00004 = 0.00427 \text{ } \mu\text{moles mL}^{-1}$$

The incubation flask had a total volume of 260.0 mL, but 20.0 g of Soil P3 displaces 8.5 mL of water. Volume of water added to flask is 40.0 mL. Thus total vapor phase volume is

$$(260.0-40.0-8.5) = 211.5 \text{ mL}$$

The total amount of N_2O-N in the vapor phase of the incubation flask is:

$$0.00427*211.5 = 0.903 \text{ } \mu\text{moles}$$

Since 20.0 g of soil was used in the study, the concentration in the vapour phase is

$$0.903/20.0 = 0.0452 \text{ } \mu\text{moles } N_2O \text{ g}^{-1}$$

or $0.0452 * 28 = 1.26$ $\mu g\ N_2O\text{-}N\ g^{-1}$ soil

B. AQUEOUS PHASE:

Ostwald absorption coefficient for N_2O in distilled water is 0.614 mL mL^{-1} @ STP

$pV = nRT$ where p = partial pressure of N_2O

$$p = \frac{(0.0000009 \text{ moles})(0.08206 \text{ atm-L } ^\circ\text{mole}^{-1})(297)}{(0.2115 \text{ L})}$$

$p = 1.04$ E-04 atm

$$\text{Conc. in solution} = \frac{k}{p}$$

At the partial pressure of N_2O, the amount in solution is

$$\frac{0.614}{1} = k = \frac{x}{1.04 \text{ E-04}}$$

$x = 6.39$ E-05 ml N_2O mL^{-1} H_2O

$= 63.8$ nL mL^{-1}

Since have a total of 40.0 mL of water and all (and only) the water is available for absorption, total amount of N_2O in the water is

$63.8 * 40.0 = 255$ nL

Since 22.414 nL of N_2O-N weights 28.0 ng, 255 nL weights:

$$\frac{(255)(28.0)}{22.414} = 319 \text{ ng}$$

or

$$= \frac{0.32}{20.0}$$

$= 0.02$ $\mu g\ g^{-1}$

Thus the total concentration of N_2O-N is

Vapour phase + aqueous phase = 1.26 + 0.02

$= 1.28$ $\mu g\ g^{-1}$

NOTE: Since the concentration of N_2O-N in the aqueous phase was small, $\approx$ 1.6%, this calculation was omitted in the determination of the total concentration of N_2O-N.

VI. Statistical Derivations

A sample of the data for the control, 10 μg g^{-1} and 100 μg g^{-1} diclofop-methyl (DM) treatments used for multiple linear regression analysis is shown in Table 18. The "Σx^2" represents the sum of the squares of the deviation from the treatment mean $\bar{X}$. The "Tot Σx^2" represents the sum of the squares of the deviation from the "Total" regression mean $\bar{X}$, i.e., the mean of the sum of the control, 10 μg g^{-1}, and 100 μg g^{-1} treatments. The appropriate means are found in Table 19.

Table 19 illustrates the values generated for testing the difference between three regression functions, i.e., testing the difference between the reduction rates or the elevations. In this sample, since the calculated F for the slope ($F_{(slope)}$), 0.13, was less than that obtained for the F value from the F-distribution table (3.29) (Zar 1974); the slopes (nitrate reduction rates) were equivalent. If the slopes had differed, Table 20 illustrates the procedure used to determine which slopes were equivalent. The Newman-Keuls multiple range test was used to test for differences between the 10 and 100 μg g^{-1} treatments, while the Dunnett's test was used to compare the control with the other treatments.

In contrast, the elevations differed in this analysis, $F_{(elevation)} > F_{0.05(1),2,37}$. In this case, multiple comparisons are made to determine between which elevations the differences occurred (Table 21). Again the Newman-Keuls and Dunnett's tests were used where appropriate. All the elevations were determined to be different, $q > q_{0.05,33,2}$.

The table also illustrates the intercept values at both the ordinate and abscissa, and their respective coefficients of variation (C.V.)

Table 19: A sample of the raw data and calculated deviations from the means for the linear portions of nitrate reduction curves.

TIME	CONTROL	Σx^2	Tot. Σx^2	Σxy	Tot. Σxy	Σy^2	Tot. Σy^2
18.75	55.7	15.82	15.83	-117.87	-78.22	878.31	386.45
21.50	32.2	1.51	1.51	-7.53	4.72	37.65	14.76
22.50	28.2	0.05	0.05	-0.49	1.80	4.56	61.49
23.50	23.4	0.60	0.59	-2.06	-9.74	7.09	159.81
24.50	14.1	3.14	3.14	-21.21	-38.86	143.13	481.44
25.50	5.9	7.69	7.68	-55.91	-83.52	406.57	908.52
18.75	56.2	15.82	15.83	-119.86	-80.21	908.20	406.36
21.50	32.2	1.51	1.51	-7.53	4.72	37.65	14.76
23.50	19.5	0.60	0.59	-5.07	-12.75	43.08	273.63
24.50	13.5	3.14	3.14	-22.27	-39.92	157.84	508.13
25.50	5.8	7.69	7.68	-56.19	-83.79	410.61	914.56
Σ 250.00	286.70	57.56	57.56	-415.98	-415.78	3034.73	4129.90
TIME	**D. M. 10**						
19.50	61.9	13.01	10.43	-75.80	-83.50	441.60	668.65
21.00	52.0	4.44	2.99	-23.42	-27.59	123.53	254.67
22.25	48.6	0.73	0.23	-6.61	-6.02	59.51	157.71
23.25	37.6	0.02	0.27	-0.47	0.81	10.80	2.43
24.25	30.2	1.31	2.31	-12.21	-8.88	114.18	34.13
25.25	20.9	4.59	6.35	-42.83	-38.17	399.43	229.27
26.25	11.2	9.88	12.40	-93.30	-87.46	881.24	617.11
19.50	68.3	13.01	10.43	-98.89	-104.17	751.54	1040.60
21.00	57.6	4.44	2.99	-35.22	-37.28	279.37	464.76
22.25	53.9	0.73	0.23	-11.16	-8.56	169.37	318.92
23.25	42.6	0.02	0.27	0.24	3.42	2.94	43.01
24.25	36.6	1.31	2.31	-4.90	0.85	18.37	0.31
25.25	30.3	4.59	6.35	-22.68	-14.47	112.06	32.97
26.25	20.7	9.88	12.40	-63.44	-54.02	407.46	235.37
Σ 323.50	572.40	67.96	69.96	-490.68	-465.05	3771.40	4099.90
TIME	**D.M. 100**						
20.00	59.1	9.00	7.45	-71.44	-62.93	567.12	531.69
21.00	50.5	4.00	2.99	-30.43	-25.00	231.47	209.04
22.00	41.0	1.00	0.53	-5.71	-3.62	32.65	24.59
23.00	37.4	0.00	0.07	0.00	0.37	4.47	1.85
24.00	28.4	1.00	1.62	-6.89	-9.71	47.41	58.40
25.00	18.9	4.00	5.16	-32.77	-38.93	268.49	293.84
26.00	11.3	9.00	10.70	-71.96	-80.93	575.31	612.15
20.00	57.1	9.00	7.45	-65.44	-57.47	475.86	443.45
21.00	48.0	4.00	2.99	-25.43	-20.68	161.65	143.00
22.00	41.0	1.00	0.53	-5.71	-3.62	32.65	24.59
23.00	39.0	0.00	0.07	0.00	0.80	13.80	8.75
24.00	27.4	1.00	1.62	-7.89	-10.98	62.18	74.68
25.00	22.9	4.00	5.16	-24.77	-29.84	153.41	172.70
26.00	12.0	9.00	10.70	-69.86	-78.64	542.22	578.00
Σ 322.00	494.00	56.00	57.03	-418.30	-421.17	3168.72	3176.72

Table 19: Calculations used for testing the difference between three regression functions.

	REGRESSION LINE			POOLED	COMMON	TOTAL
	Control	10 μg g-1	100 μg g-1	REG.	REG.	REG.
Σx^2	57.56	67.96	56.00		181.52	57.03
Σxy	-415.98	-490.68	-418.30		-1324.96	-421.17
Σy^2	3034.73	3771.40	31168.72		9974.84	3176.72
n	11	14	14			39
b	-7.23	-7.22	-7.47	-7.31	-7.30	-7.39
Res SS	28.26	228.87	44.17	301.29	303.64	66.24
Res DF	9	12	12	33	33	37
Res Mean Sq	3.14	19.07	3.68	9.13	9.20	1.79
X	22.73	23.11	23.00			22.73
Y	26.06	40.89	35.29			36.04
t	2.26	2.18	2.18		2.04	2.01
C.V.	0.49	1.14	0.55		0.45	1.04

$k = 3$

$F_{(slope)} = 0.13$ $\qquad$ $F_{(elevation)} = -12.90$

$F_{.05(1),2,33} = 3.29$ $\qquad$ $F_{.05(1),2,37} = 3.26$

Table 20: Calculations used for multiple comparisons among slopes.

k = 2

Comparison between Control and 10 $\mu g\ g^{-1}$:

S.E.	=	0.383
v	=	33
Calculated q	=	0.020
q.05,v,2	=	2.879
Common slope	=	-7.22
C.V.	=	0.56

Comparison between 10 and 100 $\mu g\ g^{-1}$:

S.E.	=	0.386
v	=	33
Calculated q	=	-0.648
q.05,v,2	=	0.879
Common slope	=	-7.33
C.V.	=	0.56

Comparison between Control and 100 $\mu g\ g^{-1}$:

S.E.	=	0.420
v	=	33
Calculated q	=	-0.577
q.05,v,2	=	2.879
Common slope	=	-7.35
C.V.	=	0.59

Table 21: Calculations for multiple comparisons among elevations.

	Control/10	10/100	Control/100
b_c	-7.30	-7.30	-7.30
a	200.75	206.36	197.56
q	-14.40	7.90	3.89

$q_{(0.05,33,2)} = 2.879$

	Intercept	C.V.
Control	192.0	30.2
10	209.5	29.2
100	203.2	32.0
At y = 0 :		
Control	26.3	3.6
10	28.7	5.7
100	27.8	4.9