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SHORT-TERM MEASUREMENT OF SOIL DENITRIFICATION
USING C_2H_2 INHIBITION:
RESPOSNSE TO ANAEROBIOSIS AND THE
EFFECT OF THE RHIZOSPHERE

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SHORT-TERM MEASUREMENT OF SOIL DENITRIFICATION USING C_2H_2 INHIBITION: RESPONSE TO ANAEROBIOSIS AND THE EFFECT OF THE RHIZOSPHERE

By

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ABSTRACT

SHORT-TERM MEASUREMENT OF SOIL DENITRIFICATION
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Methodological limitations make it difficult or impossible to measure rates of soil denitrification or ratios of the two denitrification products, N_2O and N_2, under natural conditions. Practical methods of reducing denitrification loss in agricultural practice are not available.

Acetylene inhibits the reduction of N_2O to N_2. This eliminates the methodological problem of high atmospheric N_2 concentrations masking the denitrification products and permits measurement of low denitrification rates during a short-term analysis. The concentration of C_2H_2 required for effective inhibition was shown to increase as soil NO_3^- concentrations decrease. When low concentrations (approximately 0.1 pg/g soil) of NO_3^-, as carrier-free $^{13}NO_3^-$, were added to anaerobic soil slurries 0.1 atm C_2H_2 was required for inhibition. Denitrification rates of anaerobic slurries determined by the C_2H_2 inhibition method were compared to rates determined by a method using the short-lived radioactive isotope of N, ^{13}N. There were no consistent differences between the results with the two methods.

Furthermore, direct ^{13}N measurements of the ratio $N_2O/(N_2O + N_2)$ agreed with indirect measurements using C_2H_2, that is, N_2O production rate by soils without C_2H_2 divided by rates with C_2H_2. It was concluded that C_2H_2 inhibition is a valid, sensitive, and convenient method of measuring denitrification rate. However, the dependence on NO_3^- concentration and the slow diffusion of inhibitor and product gases requires that the method be cautiously used in poorly defined conditions or undisturbed soils.

After the imposition of anaerobiosis on soil slurries, two distinct phases of denitrification rate were observed. Phase I denitrification rate was always linear, was not inhibited by chloramphenicol, was increased slightly or not at all by carbon amendments, and lasted for 1 to 3 hours after the onset of anaerobiosis. Phase I was attributed to the activity of pre-existing denitrifying enzymes in the soil. Results of phase I assays indicated that denitrifying enzymes are present even in well aerated soils. Following phase I, enzyme synthesis was derepressed and denitrification rate increased. Chloramphenicol inhibited this increase. In soils without carbon amendment a second linear phase, phase II, was attained after 4 to 8 hours of anaerobic incubation. The linearity of this phase was attributed to full derepression of denitrifying activity by the indigenous population and to lack of significant growth of denitrifiers. Carbon amendment eliminated or abbreviated the linearity of this phase and the rate continued to increase, apparently due to growth. Phase I but not phase II was increased by decreased aeration state of the soil <u>in situ</u>. Therefore, phase I may be more directly

related to natural denitrification rates.

The effect of roots on denitrification was studied with C_2H_2 inhibition methods. Anaerobic assays of soil slurries indicated that a greater supply of carbon increases the potential for denitrification in the rhizosphere. This was observed with greenhouse and fresh field soils. A split-plate experiment suggested that denitrifying activity decreases rapidly in the first few mm away from the root. A specific enrichment of denitrifiers relative to aerobes was observed in planted soils. Soils with and without intact plants were also assayed for denitrification rate. These soils were water-saturated and in an aerobic atmosphere, thus approximating natural denitrifying conditions. When soil NO_3^- concentrations were high the results conformed to the prevailing view that denitrification is enhanced in the rhizosphere. Yet, at low NO_3^- concentrations the denitrification rate was significantly lower in planted soils than in unplanted. This is believed to be the result of competition for NO_3^- between plant uptake and denitrifiers. High NO_3^- concentrations caused a significant increase in $N_2O/(N_2O + N_2)$ in these soils.

To Susan and Hannah

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I am indebted to Dr. James Tiedje, chairman of my guidance committee, for establishing an excellent environment in which to conduct research and for allowing me to carry out this research independently.

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CHAPTER I

INTRODUCTION: DIRECTING SOIL MICROBIOLOGY RESEARCH TOWARD MINIMIZING DENITRIFICATION LOSSES

Biological Abstracts cites 112 publications in 1976-77 related to denitrification. This intense activity has succeeded in defining the important parameters for denitrification, for example: aeration state, supply of electron donor, and supply of electron acceptor, and it has begun to reveal the biochemistry of denitrification. Yet the rewards of this research have been limited. We have not approached a level of understanding which permits quantitative correlation of denitrification rates with natural conditions. Reliable measurements of the magnitude of denitrification loss and the ratio of the two denitrification products, N_2O and N_2, are not available. Nor has this research provided practical strategies for reducing nitrogen lost through denitrification.

This large allocation of research resources is not unjustified. Enough information is available to indicate that denitrifiers claim a significant fraction of the N applied to agricultural soils. Methodological limitations (to be discussed later) and inherent soil variability have resulted in a large range of values reported for denitrification loss, usually between 15% of applied fertilizer (Allison, 1955) and 70% (Rolston et al., 1976). Hauck has recently reviewed much of this literature (personal communication) and tentatively concluded that the best estimate of average N loss from agricultural soils lies between 20 and 30%.

There are reasons for the great interest in denitrification,

other than the efficient use of fertilizer. Of special concern has been the suggestion that N_2O from fertilizer catalyzes a significant destruction of atmospheric ozone (Johnston, 1972; CAST, 1976) possibly resulting in serious environmental perturbations. It is my opinion that there are no valid estimates of the percentage of denitrification gases released as N_2O under natural conditions, therefore, N_2O flux can only be grossly approximated. Even given a greater understanding of atmospheric chemistry than currently exists (Crutzen, 1976) the magnitude of this hazard cannot presently be determined.

Several recent reviews have examined the denitrification literature in detail. A comprehensive review here would add very little. Instead I will acknowledge pertinent research at the beginning of each chapter and where appropriate. Among the reviews which can be recommended are: Focht (1978) on methodology, Payne (1973) on biochemistry and microbiology, Focht and Verstraete (1977) on biochemical ecology, and a general review by Delwiche and Bryan (1976).

The major objective of this introductory chapter is to relate the direction of my research and the type of denitrification research being conducted elsewhere to denitrification loss in agricultural practice. Many laboratory studies are, perhaps casually, justified by the possibility of increasing the efficiency of nitrogen use. The validity of this justification is rarely examined critically. Is it economically feasible to manipulate agronomic practice to reduce denitrification? What information, which can be obtained by soil microbiologists, is most likely to yield tangible rewards? I will suggest four general approaches to minimizing denitrification, discuss what is known and what needs to be known about soil microbiology for

the development of these approaches, and speculate on their probability of success.

Any pretention of agricultural economics is denied. These discussions are in no way intended to be complete cost analyses; innumerable assumptions and simplifications have been made. I will simply attempt to make suggestive comparisons of relative costs. The production and cost figures have been obtained from personal communication with Dr. M. L. Vitosh and from M.S.U. extension bulletins E-1110, E-802, and E-857.

Two major factors are ignored in the following discussions. First, it is assumed that the environmental cost of increased N_2O production is not significant. Yet, I do not wish to imply that this is necessarily true. Second, it should be emphasized that agronomic practice will ultimately be determined only by controlled field studies under conditions which approximate those of farming. Laboratory studies can merely define the important factors and suggest possible strategies.

Strategies which sacrifice yield.

This is the most general approach to be discussed and includes numerous possibilities. Among these are rotations with low value legumes such as alfalfa which can increase soil organic nitrogen but do not provide the cash return of, for example, continuous corn cropping. In irrigated systems, water input (and soil moisture) could be reduced to less than that necessary for optimum growth.

A simple approach is to reduce the application of nitrogen fertilizer. Although there are undoubtedly cases where more nitrogen is

applied than necessary, the alternatives considered here are to apply nitrogen to approximate maximum yield or most profitable return, or to deliberately sacrifice yield to reduce nitrogen loss. There is good evidence that denitrification rate is related to soil NO_3^- concentrations. Apparent first-order kinetics for denitrification rate and NO_3^- concentration are observed in soils provided that the supply of electron donor is not limiting and NO_3^- concentrations are below about 40 ppm N in solution (Starr and Parlange, 1975; Stanford et al., 1975).

To examine the probability of success with these strategies, compare some costs involved in the production of irrigated corn for grain. This farming system is now fairly common in Michigan; its use is increasing. It was chosen for this and the following discussions because it involves a medium value crop, a relatively high N input, and offers a number of opportunities for controlling N transformations.

A yield of 180 bu/A and a corn price of 2.50 $/bu is assumed. The crop value is then 450$/A. A yield reduction of 5% would cost 22.50 $/A. Approximately 200 lb N/A is recommended for irrigated corn. If all of this were applied as anhydrous ammonia (0.12 $/lb), the cost of N fertilizer would be 24 $/A. More commonly about 2/3 is applied as anhydrous ammonia and 1/3 is applied as N solution (0.24 $/lb) in the irrigation water. In this case fertilizer would cost 32 $/A. It will be assumed that Hauck's estimate of denitrification loss is accurate; approximately 25% of the fertilizer applied is denitrified. The denitrification cost is then about 8 $/A.

It is concluded that if denitrification could be totally eliminated, which seems very unlikely, even a small yield reduction would

cost much more, 22.50$, than would be gained in fertilizer cost, 8 $. For this strategy to become marginally acceptable, N cost must at least triple relative to corn price. In the U.S. agricultural system, this does not seem likely in the near future. The cost of N is only a small part of the total cost of crop production. Fertilizer N, even if used inefficiently, provides a large economic return. Researchers anticipating the application of their denitrification studies to farm operation should be aware of this. The same reasoning applies to another topic currently under extremely active investigation, N_2 fixation.

Specific chemical inhibition of denitrification.

Chemicals which specifically inhibit nitrification have been discovered; these include carbon disulfide and nitrapyrin (N-Serve). N-Serve is still being evaluated, but it appears that it will have some practical application, at least under certain conditions. This suggests that comparable inhibitors of denitrification might exist. (Though it is not clear that direct inhibition of denitrification is preferable to the indirect approach of inhibiting nitrification and reducing the substrate supply for denitrification.)

If such an inhibitor is to be found, it seems most likely to arise from basic research on denitrification. Some of the data already available indicate that a general inhibitor is less likely for denitrification than for nitrification. The taxonomic diversity of denitrifiers is apparently greater than that of nitrifiers. Payne (1973) lists 15 genera of denitrifiers, whereas Nitrosomonas and Nitrobacter are considered to be primarily responsible for soil

nitrification and only Nitrosomonas need be affected to inhibit nitrification. (Schmidt's (1978) recent fluorescent antibody studies suggest, however, that nitrifiers may be considerably more diverse than commonly believed.) More significantly, the biochemistry and control mechanisms of denitrification are apparently more diverse. It has been observed that some denitrifiers require NO_3^- and anaerobiosis to derepress aynthesis of denitrifying enzymes, but others do not require NO_3^- (W. J. Payne, personal communication). Pseudomonas perfectomarinus synthesizes all denitrifying enzymes simultaneously (Payne et al., 1971). However, Micrococcus denitrificans synthesizes first NO_3^-, then NO_2^- reductase (Lam and Nicholas, 1969). NO_2^- reductases of several organisms have been shown to be heme proteins, but not the copper containing NO_2^- reductase of Achromobacter cycloclastes (Iwasaki and Matsubara, 1972).

On the other hand, some degree of biochemical unity, necessary for the function of a general inhibitor, might be expected. For example, a component with an absorption maxima at 573 nm related to the binding of nitric oxide has been observed in all denitrifiers examined (W. J. Payne, personal communication; Rowe et al., 1977). In this laboratory it has been demonstrated that N_2O is a freely diffusible intermediate for essentially all soil denitrifiers (Firestone et al., 1977 and unpublished data), contrary to some earlier assertions that the denitrification pathway is variable (for example; Stefanson, 1972).

If a specific denitrification inhibitor were available, its use in soils might actually have unfavorable results. It is probably not feasible to inhibit the first step in denitrification, NO_3^- reduction

to NO_2^-, because it is also carried out in plant and microbial assimilation of NO_3^-, presumably by similar mechanisms (see Payne, 1972, for a comparison of assimilatory and dissimilatory NO_3^- reduction). If the first unique reactions of denitrification were inhibited, the accumulation of toxic NO or NO_2^- could occur.

If a workable denitrification inhibitor were available, it is reasonable to assume that its cost would be comparable to that of N-Serve, about 3 $/A. The application costs of N-Serve are insignificant since it is suited for injection with anhydrous ammonia. This might not be true of a denitrification inhibitor and application cost might be an additional 1 to 2 $/A. Comparing this to the previously calculated denitrification cost of 8 $/A and assuming no change in yield, at least a 50% inhibition of denitrification would be necessary for even a marginal profit.

It is apparent that any strategy for reducing denitrification must have a very small cost to be profitable. The remaining two strategies to be considered have minimal cost and, in fact, merely involve a refinement of current recommended farm practice. They are also more directly related to the research described in this thesis.

Scheduling nitrogen application to favor plant, rather than denitrifier, utilization of NO_3^-.

It seems obvious that plants and denitrifiers, particularly denitrifiers in the rhizosphere, compete for soil NO_3^-. However, to my knowledge this viewpoint has not been explicitly stated or critically examined. There is evidence (reviewed in Chapter 4) that potential denitrification rates are increased in the rhizosphere and it is

frequently concluded that plants stimulate denitrification (for example; Focht and Verstraete, 1977) by increasing the supply of carbonaceous substrate and so the demand for electron acceptor. Yet merely by removing NO_3^- from soil plants would be expected to reduce denitrification under some conditions. It might be possible to schedule the application of N to favor the plant in this competitive relationship.

To devise such a strategy more information is required about the relative rates of denitrification in rhizosphere and non-rhizosphere soil. The effect of soil conditions and stage of plant growth on the rhizosphere activity and on the rate of plant uptake, and the dependence of denitrification and plant uptake rates on soil NO_3^- concentrations must also be determined. Initial investigations of these relationships are reported in Chapter 4. With further information of this kind it might be possible to predict, for example, that at X days after planting uptake is rapid yet the rhizosphere population has not become very active. At X + Y days plant uptake might begin to decline and the potential rhizosphere activity increase due to exudation and death of root cells. It could then be suggested that N be applied so that maximum NO_3^- concentration occurs at time X and is greatly reduced at time X + Y.

The cost of this strategy is small. In fact, it may be zero since it is currently practiced to some extent. Split applications, applying a portion of the N before planting and a portion after plant emergence, have been found to increase the efficiency of fertilizer use and improve the chances of optimum plant growth. The cost of an additional trip across the field to fertilize is about 2 $/A, if done by the farm operator. On irrigated soils there is no significant

application cost since N solution can be added to the irrigation water. However, N solution is somewhat more expensive than anhydrous ammonia or solid N fertilizer. Although these methods are currently in use, it is doubtful that the schedules have been optimized for maximum plant growth or minimum denitrification. Computer models may eventually be available to schedule irrigation for farmers (Jackson et al, 1977). With sufficient knowledge about the relationship between plant growth and soil nitrogen transformations, scheduling of N applications could be incorporated into this service.

Scheduling irrigation to minimize denitrification.

It has been established that soil moisture, through its effect on soil aeration, is of primary importance in determining denitrification rate (Bremner & Shaw, 1958). Increased denitrification rates have been associated with application of irrigation water and rainfall (Ardakani et al., 1977). This relationship suggests that in irrigated systems soil moisture might be better controlled to reduce denitrification. A reasonably wide range of soil moisture, from about 50 to 100% of field capacity, permits optimum plant growth. Therefore, there is some opportunity to manipulate soil moisture without reducing yields.

The quantitative relationship between soil moisture and denitrification rate has not been determined. A steady-state approach to this question is insufficient; the dynamics of the response to changes in aeration state are also important. How soon after irrigation does denitrification rate begin to increase? How long do denitrifiers and their abilities to denitrify persist under drying conditions?

Our lack of understanding of the effect of soil moisture on denitrification is demonstrated by the following two strategies. They are opposite, yet both are reasonable based on current limited knowledge. Frequent brief irrigation could be scheduled to keep soil moisture at a low, constant level. This might reduce denitrification by keeping the soil continuously well-aerated. Trickle irrigation might be the best way of accomplishing this. On the contrary, increasing the drying period between irrigations could greatly reduce the size and activity of the denitrifying community minimizing the response to the brief period of saturation during irrigation.

It seems likely that these practices would actually increase efficiency of water use and so might decrease costs. An indication of the possible relative expense of altering irrigation practice can be obtained from the fuel and labor cost of 2 $/A inch of irrigation water. (In Michigan, irrigated corn requires about 8 inches of water per year.) Therefore, the cost of refined irrigation management would probably be much less than the denitrification cost of 8 $/A and could yield additional profits from increased efficiency of water use.

Research objectives.

Many of the questions posed in this chapter can be answered by laboratory research. I believe the following research objectives to be consistent with the need for lowcost methods of increasing the efficiency of N fertilizer use.

1. Develop new methods for the study of soil denitrification.

Denitrification methodology has been reviewed in detail by Focht (1978). Current methods, briefly discussed in Chapter 2, are

insensitive or tedious or difficult to apply to undisturbed systems. A large part of my thesis research has been devoted to investigation and development of new methods.

The reported inhibition by acetylene of N_2O reduction to N_2 (Balderston et al., 1976; Yoshinari and Knowles, 1976) was seen to be a potentially useful tool. Very sensitive analytical techniques are available for N_2O measurement. This method also eliminates the problem of detecting low rates of N_2 production against a very high atmospheric background of this gas. Chapter 2 considers the application of C_2H_2 inhibition methodology to soil denitrification studies.

The number of soil denitrifiers is not directly related to soil denitrifying activity primarily because denitrifiers can respire either O_2 or N oxides. Nevertheless, enumeration of soil denitrifiers is frequently of interest. Several currently used methods of enumeration have been evaluated and found to be generally unsatisfactory. Appendix A will present our investigations of possible modifications.

It is difficult or impossible to study the in situ physiology of denitrifiers or any other specific soil microorganism. A possible solution is the use of an antibiotic (rifampicin) resistance marker. This would permit quantitative recovery of the microorganism from soil. Preliminary investigations of this approach are presented in Appendix B.

The use of the radioactive isotope ^{13}N, despite its ten minute half-life, is an exciting new development in denitrification research. My role in the development and use of ^{13}N methodology has been secondary to that of other members of our group (Mary and Richard Firestone and Michael Betlach), and so this subject will be only briefly dis-

cussed in Chapter 2. A complete report of this method is in preparation (Tiedje et al., 1978).

2. Investigate the denitrification response to the depletion of O_2 from soil.

During the investigation of the C_2H_2 inhibition method two linear phases of denitrification rate were observed after the imposition of anaerobiosis. My second major objective was to determine the cause of this pattern and relate it to the response to decreases in soil aeration, i.e., irrigation or rainfall, in nature. It was anticipated that this research might reveal:

A. The absence or presence of denitrifying activity (functional denitrifying enzymes) in dry soils.

B. The mechanisms involved in the denitrification response to reduced aeration. Possible mechanisms include removal of O_2 inhibition, derepression of synthesis of denitrifying enzymes, and growth of denitrifiers.

C. The rate of response to increased moisture.

D. The magnitude of the response.

E. The times and conditions for significant denitrification loss from agricultural soils.

3. Investigate the effect of plants on denitrification.

I have hypothesized that the competition between plants and denitrifiers is of great importance to the fate of soil NO_3^-. Earlier investigations (reviewed briefly in Chapter 4) have not considered this aspect but have apparently sought only to observe a stimulatory

effect of roots on denitrification, sometimes leading to ambiguous results. Chapter 4 describes the application of newly developed methods to the following questions:

A. Can the presence of plants reduce denitrification rate under some conditions?

B. When can a stimulatory effect be expected?

C. If the denitrification rate is increased or reduced, what is the mechanism?

This introductory discussion should not be taken to imply that all denitrification research must be justified by agricultural necessity. Denitrification is a critical reaction in the Earth's nitrogen cycle, and so has relevance well beyond agricultural practice.

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CHAPTER II

THE ACETYLENE INHIBITION METHOD FOR SHORT-TERM MEASUREMENT OF SOIL DENITRIFICATION AND ITS EVALUATION USING ^{15}N

The increased cost of fixed nitrogen and the possibility that soil-evolved N_2O may contribute to atmospheric ozone depletion (McElroy, 1976) have caused renewed interest in the process of denitrification. Despite a considerable mass of research on denitrification, reliable values for rates of N_2 and N_2O production in field soils are lacking. Due to limited sensitivity, previous methods have required extensive amendment of soils and/or long term incubation. Although these methods have elucidated the basic controlling factors, the dynamics of denitrification and the quantitative effects of environmental or management parameters on natural soils are largely unknown.

The inhibition of N_2O reductase by acetylene in pure culture was reported by Yoshinari and Knowles (1976) and by Balderston, et al (1976). It is now widely accepted that N_2O is an obligatory, and probably freely diffusible intermediate in the denitrification pathway (Payne, 1973; St. John and Hollocher, 1977). Therefore, one would expect the quantity of N_2O produced by C_2H_2-inhibited microorganisms to be a direct measure of the total gaseous N produced without inhibition. If N_2O is the sole denitrification product, analysis is greatly simplified since N_2O, unlike N_2, is a minor atmospheric constituent (approximately 300 ppb) and can be assayed by sensitive gas chromatographic detectors. The successful application of this method to soil denitrification studies has been reported by Yoshinari, et al

(1977).

I have used gas chromatography and $^{13}NO_3^-$ to evaluate the acetylene inhibition technique in soils and have identified the conditions and soil types for which this method is valid and for which blockage of N_2O reductase is complete. The radioactive isotope, ^{13}N, provides an extremely sensitive assay, with excellent temporal resolution, which can be used without alteration of native NO_3^- concentration.

MATERIALS AND METHODS

The soils used are described in Table 1. Soils were near field capacity when collected and, without drying, were passed through a 5 mm sieve and stored in sealed plastic bags at 2^{o} C until used. The storage period varied from 1 day to 6 months.

Soil slurries were used in most of the C_2H_2 inhibition assays. This made it easier to amend the soils and simplified interpretation of the results by limiting the effects of diffusion. Soil, usually 75 g fresh weight, was placed in 125 ml erlenmeyer flasks and the desired amendments, with 30 ml of H_2O, were stirred in. All solute additions were on the basis of weight per fresh weight of soil. The aerobic assays were conducted on soils, without added water, in 50 ml centrifuge tubes. The vessels were sealed with a rubber stopper which was pierced by a glass tube capped with a serum stopper. Flasks were made anaerobic by twice evacuating and flushing with He or Ar. Acetylene was added as desired. Slurries were incubated on a rotary shaker at 250 rpm. In all gas chromatography experiments there were at least three replicates for all treatments.

Samples of the headspace ($\leq$ 0.5 ml) were periodically removed

Table 1. Characteristics of soils used.

Series	Texture	Classification	pH	% Organic Matter	Mineralizable C*
Brookston	Loam	Typic argiaquoll	7.6	3.2	152
Carlisle	Muck	Typic metasaprist	6.5	85.0	233
Miami	Sandy loam	Typic hapludalf	6.6	2.7	166
Spinks	Loamy sand	Psammentic hapludalf	6.4	1.5	126

* Mineralizable C is in units of μgC/g soil and was determined by the method of Burford and Bremner (1975).

with a Pressure-lock syringe (precision Sampling Corp., Baton Rouge). In most experiments a Carle model 8515 gas chromatograph (Carle Instruments Inc., Fullerton, Calif.) with a microthermistor detector was used to analyze gases. The sensitivity of this instrument was increased by addition of a 5X/10X operational amplifier. The lower limit of detection was about 100 ppm (v/v). A Porapak Q column (3 mm x 1.8 m) was used to separate CO_2, N_2O, and C_2H_2. A switching valve made it possible to direct the effluent of this column either to the detector or to a Molecular Sieve 5A column (3 mm x 1.8 m) to separate N_2, O_2, and NO. The column temperature was 45°C and the carrier gas was He at a flow of 25 ml/min. Peak areas were determined with a computing integrator.

A Perkin-Elmer model 900 gas chromatograph (Norwalk, Conn.) with a ^{63}Ni electron capture detector (Rasmussen, et al., 1976) operated at 300°C was used for measuring N_2O concentrations from ambient to 100 ppm. Excellent separation of N_2O was achieved by operating the Porapak Q column at ambient temperatures. The carrier gas was 5% CH_4 in Ar with a flow rate of 40 ml/min.

The ^{13}N was generated at the MSU Cyclotron by the reaction $^{16}O(p,\alpha)^{13}N$ using water as a target; the details of the production, characterization and detection of this isotope are described elsewhere (Tiedje, et al., 1978). In these experiments the ^{13}N used was $\geq$ 85% $^{13}NO_3^-$ with minor quantities of $^{13}NH_4^+$ and $^{13}NO_2^-$. In some experiments NH_4^+ was removed prior to use by making the ^{13}N solution alkaline and evacuating to dryness. Approximately 1 mCi of ^{13}N (4 pg NO_3^- + NO_2^-) was added to the flasks containing soil slurries. In certain experiments $^{14}NO_3^-$ carrier was also added. To determine rates

of gas production, a gas flushing system similar in principal to the one of Gersberg et al. (1976) was used. The flask containing a soil slurry and incubated on a magnetic stirrer, was connected to a helium sparging system which continuously flushed gaseous products into the differential trapping system (Tiedje et al. 1978) which separated $^{13}N_2O$ from $^{13}N_2$ to allow quantitation of each gas. Specific activity of the denitrification products was assumed equal to that of the reactants, which was determined by counting a subsample of the ^{13}N solution and by extracting the soil following the incubation and measuring $NO_3^- + NO_2^-$ by Technicon Autoanalyzer standard methods.

In all experiments, the concentration of N_2O in solution was calculated from the measured headspace concentration and denitrification rates were corrected accordingly. In some slurries as much as 1/2 of the total N_2O produced remained in solution. It was verified that published values of the Bunsen absorption coefficient approximated the N_2O solubility in this system by adding N_2O, in the concentration range we normally encountered, to autoclaved soil slurries. The slurries were shaken, allowed to equilibrate, and the quantity of N_2O remaining in the headspace was determined. At 20° C I obtained a coefficient of 0.74 which is slightly higher than the published value of 0.66 for pure water solutions.

Assays for testing completeness of acetylene inhibition:

Three approaches were used to determine the completeness of the C_2H_2 inhibition in soil slurries. Anaerobic slurries of Brookston soil were preincubated for 48 hours with 0.5% added glucose to deplete naturally occurring NO_3^-. After this preincubation period the flask atmosphere was replaced as before. Various quantities of C_2H_2 were

added and allowed to mix for 25 min prior to injection of 0.25 ml of N_2O. N_2O remaining in the headspace was periodically determined.

The second approach evaluated the recovery of added NO_3^--N as N_2O-N. Unamended Brookston soil was preincubated for 48 hours as an anaerobic slurry. Eight ppm NO_3^--N and 0.1% glucose were added after injecting the desired quantities of C_2H_2. The quantity of N_2O present after 24 and 48 hours was determined.

The third and most rigorous test of the effectiveness of C_2H_2 inhibition was conducted with ^{13}N methods. Brookston soil was preincubated anaerobically for 48 hours with 4 ppm NO_3^--N and 0.5% glucose. Preliminary experiments had shown that with this treatment all of the added NO_3^- would be depleted and that N_2O reducing activity would be high. Acetylene was added to the flasks as desired and the contents mixed for 1 to 2 hours. Carrier-free ^{13}N was then added to soil incubated on a rotary shaker. After approximately 15 min a 10 ml gas sample was removed by syringe and injected into the differential ^{13}N gas trapping system to determine $^{13}N_2O$ and $^{13}N_2$ produced. Correction was made for differential solubility of the two gases. To some flasks 2 ppm $^{14}NO_3^-$-N were added before the addition of ^{13}N.

RESULTS

Completeness of acetylene inhibition of N_2O reduction in soils:

Reduction of N_2O added to soil in the presence of varying C_2H_2 concentrations is shown in Table 2. The N_2O concentration slowly decreased even in the flasks with 1 atm C_2H_2. This could be due to slow adsorption reactions rather than biological reduction. Whereas very low concentrations of C_2H_2 did inhibit N_2O reduction, 0.15 atm

Table 2. Reduction of nitrous oxide by soil in the presence of various acetylene concentrations.

pC_2H_2	Incubation time		
	2 hours	7 hours	24 hours
(atm)	μmoles N_2O remaining*		
0	9.9 ± .06† (88)≠	7.6 ± .10 (68)	0 (0)
0.01	10.4 ± .24 (93)	9.7 ± .28 (87)	7.3 ± .12 (65)
0.04	10.6 ± .17 (95)	9.7 ± .16 (87)	9.0 ± .37 (80)
0.15	10.5 ± .07 (94)	10.1 ± .37 (90)	10.1 ± .34 (90)
1.0	10.8 ± .31 (96)	10.5 ± .16 (94)	10.3 ± .34 (92)

* 11.2 μmoles N_2O added to each flask.

† Limits are $\bar{s}_{\bar{x}}$ $t_{.05}$

≠ Values in parentheses are N_2O at indicated time ÷ N_2O at 15 min x 100.

C_2H_2 was required for maximum inhibition.

The stoichiometric conversion of NO_3^- to N_2O in the presence of various concentrations of acetylene is shown in Table 3. The recovery of N_2O was complete for all acetylene concentrations at 24 hours but by 48 hours N_2O was apparently further reduced in flasks containing the lower concentrations of C_2H_2. In this experiment and the previous one, I verified that significant quantities of N_2O were not produced by the NO_3^- depleted soils with 1 atm C_2H_2 but without added NO_3^-.

The effectiveness of the acetylene in inhibiting the reduction of $^{13}N_2O$ to $^{13}N_2$ is reported in Table 4. Because of the high specific activity of $^{13}NO_3^-$, the NO_3^- concentration was extremely low. Under these conditions high acetylene concentrations ($\geq$ 0.15 atm) were required to obtain an effective block. When a small quantity (2 ppm) of $^{14}NO_3^-$-N was added, a dramatic increase in N_2O was noted. This suggests NO_3^- aids the inhibition of N_2O reduction. This interpretation is also supported by the other two experiments since high concentrations of acetylene were needed when NO_3^- concentrations were low but not when NO_3^- was high (first 24 hours of second experiment, (Table 3).

Determination of soil denitrification rates:

Table 5 provides a comparison of denitrification rates measured by the C_2H_2 inhibition method and the ^{13}N method. The experiments necessarily differed in that the soil for the ^{13}N assay was continously stripped of product gases while for the acetylene inhibition assay the flasks were sealed and incubated on a rotary shaker. In spite of these differences the results were reasonably similar for the two methods. Furthermore, the ratios of N_2O/(total gaseous N) determined

Table 3. Recovery of added nitrate as nitrous oxide in the presence of various acetylene concentrations.

pC_2H_2	Incubation time	
	24 hours	48 hours
(atm)	μmoles N_2O	
0	0	0
0.01	23.1 ± 7.0† (108)*	19.7 ± 5.4 (92)
0.04	23.0 ± 6.0 (108)	21.0 ± 9.5 (98)
0.15	20.1 ± 2.5 (94)	19.3 ± 0.2 (90)
1.0	20.6 ± 3.5 (96)	20.4 ± 0.4 (96)

* Percent of added NO_3^--N recovered as N_2O-N; 42.6 μmoles NO_3^- added per flask.

† Limits are $\bar{s}_{\bar{x}}$ $t_{.05}$

Table 4. Effect of various concntrations of acetylene on the ratio of denitrification products as determined by ^{13}N.

pC_2H_2	$^{13}N_2O/(^{13}N_2O + {}^{13}N_2)$
(atm)	
0	0.03
.01	0.26
.05	0.69
.15	0.95
0 + 2 ppm NO_3^--N	0.91
.05 + 2 ppm NO_3^--N	0.96

Table 5. Comparison of denitrification rates and ratios of products determined by ^{13}N and C_2H_2 inhibition methods.

Soil	Denitrification rate		Ratio of products	
	^{13}N	C_2H_2 Inhib.	^{13}N	C_2H_2 Inhib.
	—nmoles gas · g^{-1} · min^{-1}—		——$\delta N_2O/\delta(N_2 + N_2O)$——	
Brookston* (0-1)†	0.51	0.49	0.05	0.04
Brookston* (2-3)	0.70	1.45	0.34	0.26
Carlisle (4-5)	2.15	1.88	0.48	0.52
Spinks (7-9)	0.10	0.20	0.74	0.64

* Brookston soil was amended with 8 ppm $^{14}NO_3^-$-N and 0.03 atm acetylene; others received no added $^{14}NO_3^-$ and 0.1 atm acetylene.

† Hours after onset of anaerobiosis are in parentheses.

by the ^{13}N method were similar to those determined by the acetylene inhibition method. For the latter method the ratio was determined by comparing N_2O produced in the presence of acetylene to N_2O produced by the same soil in the absence of acetylene (Yoshinari, et al. 1977). Both methods indicate that the total denitrification rate of the Brookston soil, as well as the proportion of N_2O, increased soon after the onset of anaerobic conditions. In the sealed vessels with NO_3^- present, the reduction of headspace N_2O does not appear to be significant since the ratio of gaseous products was similar in the sealed and continuous flow (^{13}N) assay systems. It was also observed that when the flasks without C_2H_2 were evacuated and flushed to remove accumulated N_2O, the rate of N_2O production resumed at essentially the same rate (data not shown). Therefore, the rate of release of N_2O was apparently independent of N_2O concentration in the headspace.

During the C_2H_2 inhibition assay of the Brookston soil, we also monitored CO_2 concentration in the headspace. There was no significant difference between the flasks with and without C_2H_2, suggesting that 0.03 atm C_2H_2 had little effect on soil respiration. In other experiments with up to 1 atm acetylene I have observed no significant effect of C_2H_2 on CO_2 evolution within a 12 hour assay period. I have also observed that the rate of N_2O production was not affected by C_2H_2 concentrations up to 1 atm.

The C_2H_2 inhibition method was used to compare denitrification rates of four different soils over a longer time period. The soils were incubated as anaerobic slurries at 18° C with 8 ppm NO_3^--N and 0.04 atm acetylene added. The time course of N_2O production is plotted in Figure 1. Shortly after the onset of anaerobic conditions, the

Figure 1. Nitrous oxide production by 4 C_2H_2 inhibited soils. Points are means of 3 replicates.

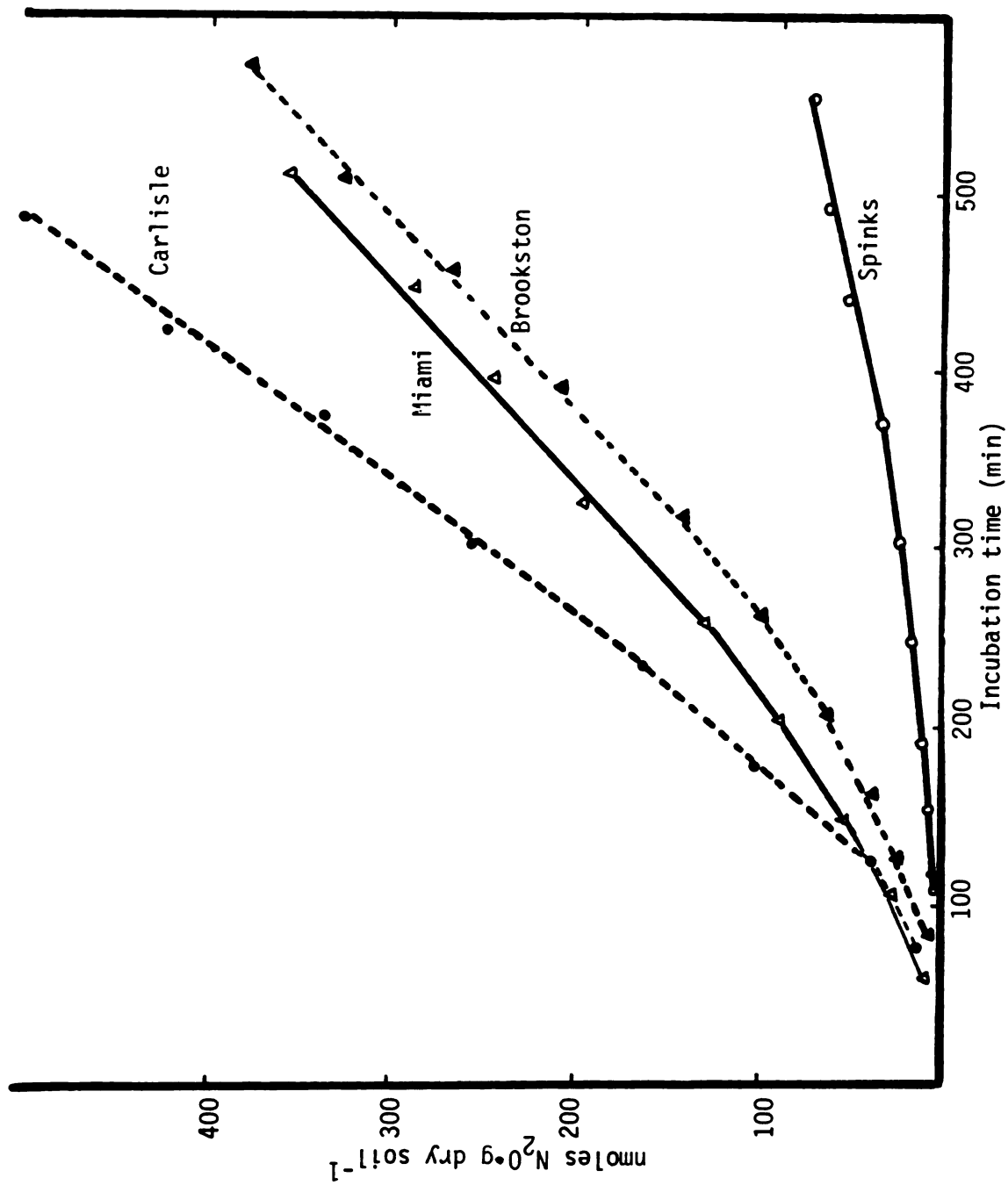
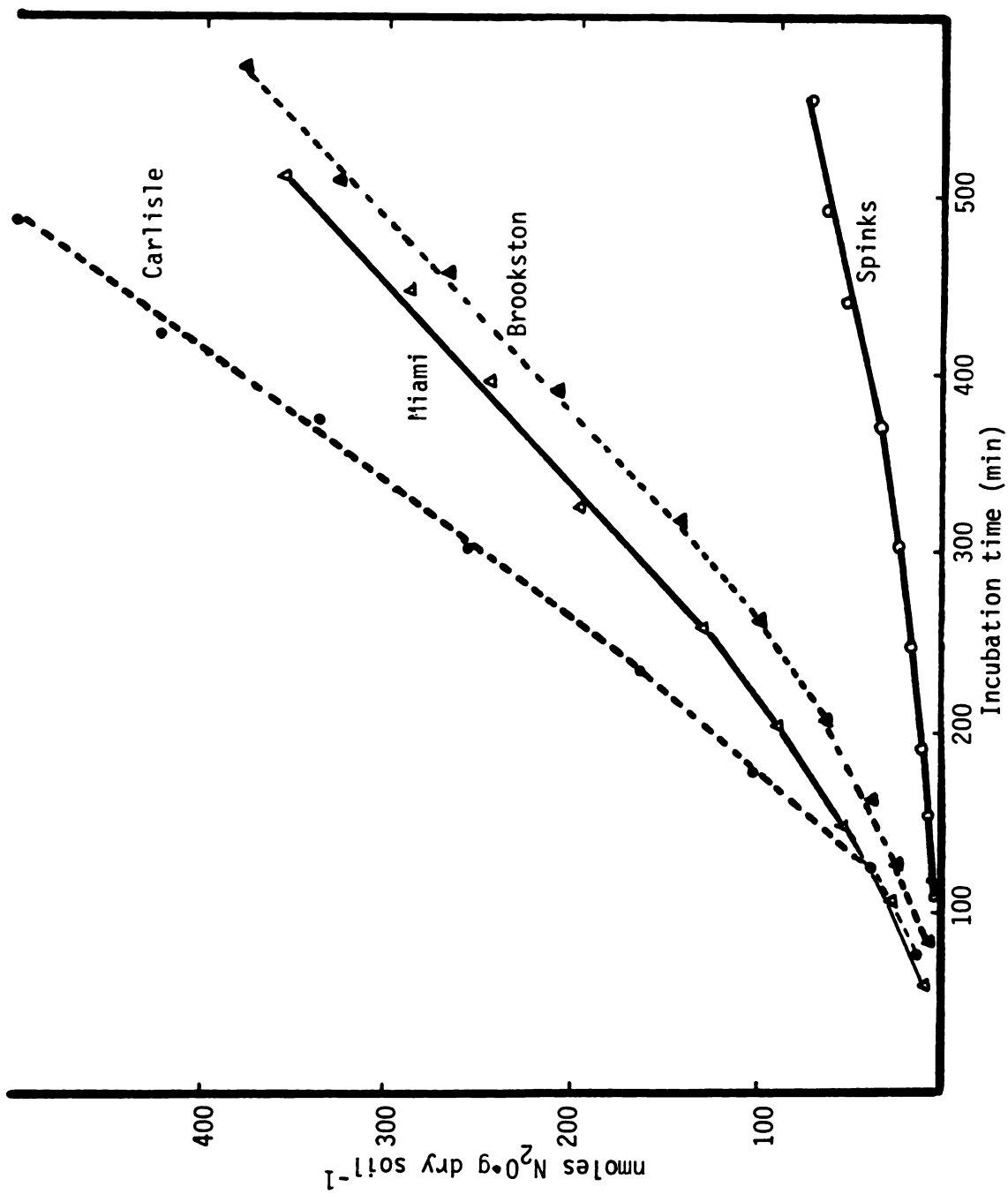

Carlisle
Miami
Brookston
Spinks
100
200
300
400
500
Incubation time (min)
100
200
300
400
nmoles N_2O•g dry soil^{-1}

Figure 1. Nitrous oxide production by 4 C_2H_2 inhibited soils. Points are means of 3 replicates.

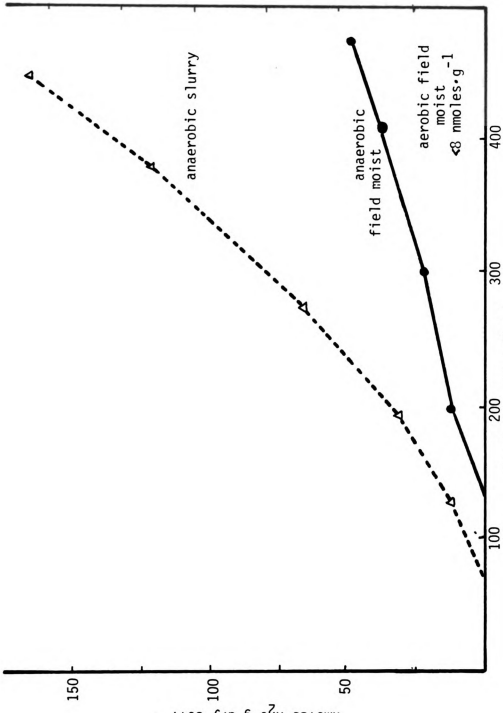

Carlisle
Miami
Brookston
Spinks
100
200
300
400
500
Incubation time (min)
100
200
300
400
nmoles N_2O•g dry soil^{-1}

rates increased for all soils until an approximately constant rate was attained. The soils differed in the time required to reach linearity and also in the ratio of the final linear rate to the first rate observed. The denitrification rates corresponded with mineralizable carbon.

I have found that denitrification rates can easily be measured without carbon or NO_3^- amendments. In some soils denitrification rates were stimulated by these amendments, however in other soils the rates were not increased by either or both additions. These effects have not been thoroughly investigated by me but other work in our laboratory, conducted primarily by Mary Firestone, has demonstrated that in anaerobic soil slurries denitrification rate is independent of NO_3^- concentration between about 10 and 1000 ppm N.

Since the C_2H_2 inhibition method requires no substrate additions, we have been able to apply it to less disturbed soils. Figure 2 allows a comparison of the denitrification rates of an unamended anaerobic slurry, anaerobic soil aggregrates (no water added), and aerobic aggregates. The Miami soil, which had been sieved and kept at 2^oC for 5 months, was used in all cases. The reduced rate in the anaerobic aggregrates without added water suggests that substrate diffusion limited the denitrification rate. The soil had been stored moist (near field capacity) and so contained ample water for biological activity. Production of N_2O could not be detected in the aerobic treatment using the microthermister detector (detection limit 8 nmoles $\cdot$ g^{-1}). However, with the much more sensitive electron capture detector we were able to observe a linear increase in headspace N_2O which continued throughout the experiment (32 hours). This

Figure 2. Comparison of denitrification by the Miami soil under different experimental conditions. All soils were treated with C_2H_2. Points are means of 3 replicates.


anaerobic slurry
anaerobic field moist
aerobic field moist <3 mmoles·g⁻¹
Incubation time (min)
100
200
300
400
50
100
150
moles N2O·g dry soil-1

rate was 2.7×10^{-4} nmoles $\cdot g^{-1} \cdot min^{-1}$ which is about 1000 x less than for the anaerobically incubated soils. Headspace gas analysis after 32 hours indicated that O_2 had not decreased by more than 4%.

DISCUSSION

I believe that the C_2H_2 inhibition method will be extremely valuable in denitrification research since it allows determination of denitrification rates in unamended soils and within a short time period, thus minimizing changes due to the assay environment. My purpose was to evaluate this method by comparison with an independant method and to determine the assay conditions for acceptable results. I confirmed the finding of Yoshinari et al. (1977) that acetylene blocks the reduction of N_2O by the indigenous soil microflora. However, I found that this inhibition was enhanced in the presence of nitrate. Thus, when soil nitrate concentrations were low, e.g. less than a few ppm, higher acetylene concentrations were needed to obtain a suitable degree of inhibition. My findings indicate that 0.1 atm of acetylene should be adequate for low nitrate samples (or samples where the nitrate would be exhausted before the assay is terminated).

The marked effect of nitrate on N_2O reduction also has important implications for the question of what causes N_2O production in soils. As shown in Table 4, as little as 2 ppm NO_3^--N caused a shift from only 3% N_2O to 91% N_2O. This finding and further experiments in our laboratory conducted by Mary Firestone (unpublished results) indicate that NO_3^- concentration is of primary importance in determining the percentage of denitrification gases released as N_2O.

The absence of a detectable effect of C_2H_2 on soil respiration

suggests that the inhibitor does not have confounding short-term influences on soil processes which could, directly or indirectly, alter denitrification rates. Acetylene is a biologically active compound, however, (for example; Brouzes and Knowles, 1971) and it may be necessary to adjust the concentration used to the system being studied.

The similarity of rates measured by the ^{13}N and the acetylene inhibition methods was very encouraging. It is particularly interesting that comparison of rate of N_2O production by inhibited and uninhibited soils provided a measurement of $N_2O/(N_2O + N_2)$ which correlated well with $^{13}N_2O/(^{13}N_2O + ^{13}N_2)$. This approach is apparently valid for the assay conditions described here, however caution is advised in its application to other systems.

The acetylene inhibition method has several advantages over previously used methods. The problem of lack of sensitivity due to the high atmospheric concentration of N_2 is eliminated. When coupled with electron capture detector analysis of N_2O, it is extremely sensitive. A significant advantage is that substrate additions and long term incubations are not required and only generally available equipment is necessary.

Since C_2H_2 is water soluble and, as a gas, is readily diffusable in a porous matrix, the method should be applicable to undisturbed systems--soil columns or cores, or perhaps field studies. I have made preliminary attempts to conduct C_2H_2 inhibition assays on soil cores with mixed results, probably because diffusion of product and/or inhibitor was limiting for our chamber design. Other potential problems are the effect of nitrate concentration on the inhibition and the oxidation of C_2H_2 which can occur under aerobic conditions. Though I

feel the method still holds promise for work with undisturbed soils, simple designs may not suffice and any procedure developed will have to be thoroughly tested to verify its reliability.

I observed that denitrification rates increased within a few hours after the imposition of anaerobic conditions. Soil assays without added electron donor generally yielded bilinear plots of accumulated N_2O vs. time (Figure 1). The rapid temporal changes in denitrifying activity which occur after the imposition of anaerobiosis in the laboratory should be analogous to the processes which occur when a soil or a portion of the microsites becomes anaerobic in nature. Therefore, these temporal patterns have been examined in detail. The results of these studies are presented in the next chapter.

The strong effect of O_2 on the indigenous denitrifying enzymes was indicated by the 1000 fold difference in denitrification rates of soil aggregrates under aerobic vs. anaerobic atmospheres. The aerobic rate was equivalent to 2 kg · ha furrow slice^{-1} · 100 days^{-1}. Denitrification obviously proceeds at a very slow rate in well aerated soils. However, whenever oxygen becomes limiting at a microsite, the indigenous denitrifying enzymes should immediately begin reducing nitrate. Because of this strong O_2 effect significant amounts of nitrate could be rapidly lost from soils following irrigation or rainfall. This scenario would predict that denitrification occurs in periodic bursts, in response to changes in O_2 status, against a background of very slow, yet continuous denitrification.

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CHAPTER III

PHASES OF DENITRIFICATION FOLLOWING OXYGEN DEPLETION IN SOIL

It is generally believed that denitrification occurs in agricultural soils predominantly under saturated or near-saturated conditions (Focht and Verstraete, 1977). Soil denitrification rates have been shown to increase with added water or reduced aeration (Bremner and Shaw, 1958; Ardakani et al., 1977; Pilot and Patrick, 1972). This is consistent with known physiology of denitrifiers; O_2 both inhibits denitrifying enzyme activity and represses synthesis of new denitrifying enzymes (Payne, 1973). There are reports suggesting biological production of nitrogen gases in well-aerated soils (Starr et al., 1974; Broadbent and Clark, 1965; Bremner & Blackmer, 1978), though the significance of nitrogen loss under these conditions has not been determined. The close relationship between aeration state and denitrification rate indicates that it is important to characterize the short-term soil response to reduced aeration. Until now, methodological limitations have precluded studying the dynamics of the soil response, that is, how rapidly and to what extent denitrification rates change following the onset of anaerobic or partially anaerobic conditions. Nor has the biological component of this response, the short-term reaction of soil denitrifiers to imposed anaerobiosis, been examined. In this chapter I address these questions which are important to the basic understanding, and to the potential control and prediction of denitrification.

MATERIALS AND METHODS

Soils used were Brookston loam, Spinks loamy sand, Miami sandy loam, and Carlisle muck; their characteristics have been presented previously (chapter 2). The maximum water holding capacity, as determined by saturating soil in a filter paper funnel, was 43 ml/100 g for the Brookston and 36 ml/100 g for the Spinks and Miami. After collection the soils were stored at 2 to 4 C without drying. However, for many experiments fresh samples were collected from the same sites immediately before experimentation. All soils were at or below field capacity when collected.

The validity of the acetylene inhibition method for measuring denitrification rates has been established previously (chapter 2). The methods used here were similar. Briefly: soil to be assayed was made into a slurry and incubated anaerobically in closed flasks. Acetylene (0.1 atm) was added to inhibit the reduction of N_2O to N_2. Nitrate was also added to the soil suspensions, 10 ppm NO_3^--N (soil fresh wt basis) for short-term assays and repeated additions of 200 ppm for longer experiments. Rapid attainment of anaerobiosis for phase I assays was insured by magnetically stirring the suspensions while evacuating and flushing the flasks; incubation was under a He atmosphere. There were at least 3 replicates of all treatments.

To determine denitrification rates in aerobic atmospheres, soils were passed through a 4 mm sieve to break up large clods and remove debris. This treatment had little effect on smaller, stable aggregates. Thirty five g of soil was gently shaken with 0.1 atm C_2H_2 in

a stoppered 50 ml centrifuge tube, then compacted by gently tapping the tube.

The concentrations of N_2O were measured by gas chromatography using ^{63}Ni electron capture and microthermistor detectors as previously described.

When chloramphenicol (Calbiochem; La Jolla, Cal.) was used to inhibit protein synthesis, it was added to the suspension at 0.27 mg/g soil. These experiments were done with a mixture of 50 g washed silica sand and 5 g Brookston soil as well as the Brookston soil alone. The sand mixture was incubated anaerobically with succinate and NO_3^- several days before the experiment to develop denitrifying populations.

Pure cultures of denitrifiers were grown aerobically to log phase in nutrient broth (Difco; Detroit, Mi.). They were harvested on 0.45 μ Millipore filters and washed with three volumes of phosphate buffer (pH 7.0, 0.05 M). The filters with the cells were placed in flasks of nitrate broth (Difco). The flasks were then made anaerobic and assayed in the presence of acetylene in the same manner as the soil suspensions. Some cultures were also assayed in nitrate broth with 0.5 g/ℓ Na thioglycollate, a reducing agent. The flasks with thioglycollate were evacuated and flushed with He 16 hours before innoculation. The denitrifiers had been isolated from soils and were fully characterized during earlier work in this laboratory (T. N. Gamble, The Commonality of Numerically Dominant Denitrifier Strains Isolated from Various Habitats, M.S. Thesis, 1976). They were: Pseudomonas fluorescens biotype II 72, P. fluorescens biotype IV 206, P. stutzeri 224, P. aureofaciens 59, and Flavobacterium sp. 175.

Numbers refer to our strain designation.

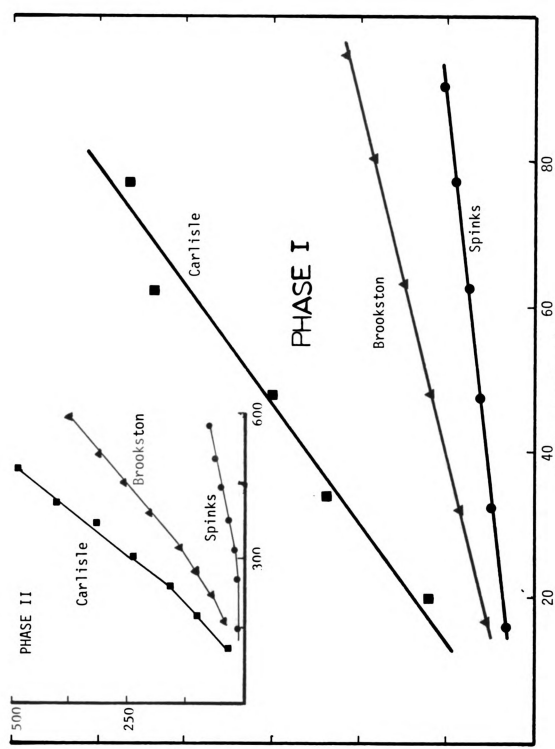
Most probable numbers of denitrifiers were determined with a microtiter system (Rowe et al., 1977). Disappearance of nitrate and nitrite from nitrate broth was determined with diphenylamine.

RESULTS

Two linear phases of denitrification were observed following the imposition of anaerobiosis on aerobic soil (Figure 3). Acetylene was used in these experiments so N_2O production actually represents total denitrification. Phase I lasted from approximately 15 min, the time for N_2O to reach easily measured concentrations, to between 1 and 3 hours. Previous work had shown that this initial period is followed by an increase in rate until a second linear phase, phase II, is established. Some phase II data from this earlier work is included in Figure 3 to demonstrate the biphasic nature of denitrification rates. The duration of both phases was quite variable among soils. However, the pattern was observed consistently with both fresh and stored (moist) soil samples.

In some experiments attempts were made to eliminate O_2 more rapidly and completely. These included deaerating the solution and flask before adding the soil, varying the duration of evacuating and flushing the flasks, and agitating the suspensions while evacuating. These procedures did not alter the bilinear pattern indicating that residual O_2 or delayed establishment of equilibrium was not responsible for the observed phases.

Figure 3. Two linear phases of denitrification in 3 C_2H_2 inhibited soils. Points are the means of 3 replicates.


PHASE I
Carlisle
Brookston
Spinks
Incubation time (min)
20
40
60
80
20
40
60
nmoles N2O•g dry soil-1
PHASE II
Carlisle
Brookston
Spinks
600
300
250
500

Autoclaved soils do not evolve N_2O at a significant rate.

The effect of chloramphenicol on denitrification rate is illustrated in Figure 4. This compound is an inhibitor of protein synthesis but would not be expected to reduce the activity of enzymes already present. Phase I rate was not reduced, but very slightly increased by chloramphenicol. This small increase (which has been consistently observed) could be due to diversion of carbon supply from protein synthesis to respiratory processes. Of greater significance is the effect on the shift from phase I to phase II. Phase II rates were markedly reduced by chloramphenicol. Inactivation of chloramphenicol by soil binding and microbial decomposition would be expected in soil, so this experiment was also conducted with silica sand mixed with a small quantity of soil. In this case the increased rate from phase I to phase II was almost totally eliminated (Figure 4). Succinate (1%) was added to the sand, allowing growth and thus accounting for the lack of a distinct linear phase II in the absence of chloramphenicol. These results indicate that the rate increase following phase I is a result of the synthesis of denitrifying enzymes and suggest that phase I, but not phase II, is a measure of the activity of pre-existing denitrifying enzymes.

This conclusion is supported by the effect of glucose additions to soil. Though the effect was quite dependent on the soil sample, the results in Table 6 are representative of the stimulatory pattern. Phase I rates were not increased at all in some soils and only slightly increased in others. Phase I remained linear in glucose-amended soils. Phase II denitrification was often but not always stimulated by glucose. The second linear phase was always of reduced duration

Figure 4. Effect of chloramphenicol on denitrification rates in a soil and a sand. Points are the means of 3 replicates. Numbers in parentheses are rates (nmoles $N_2O \cdot g$ dry $soil^{-1} \cdot min^{-1}$) with 95% confidence limits.

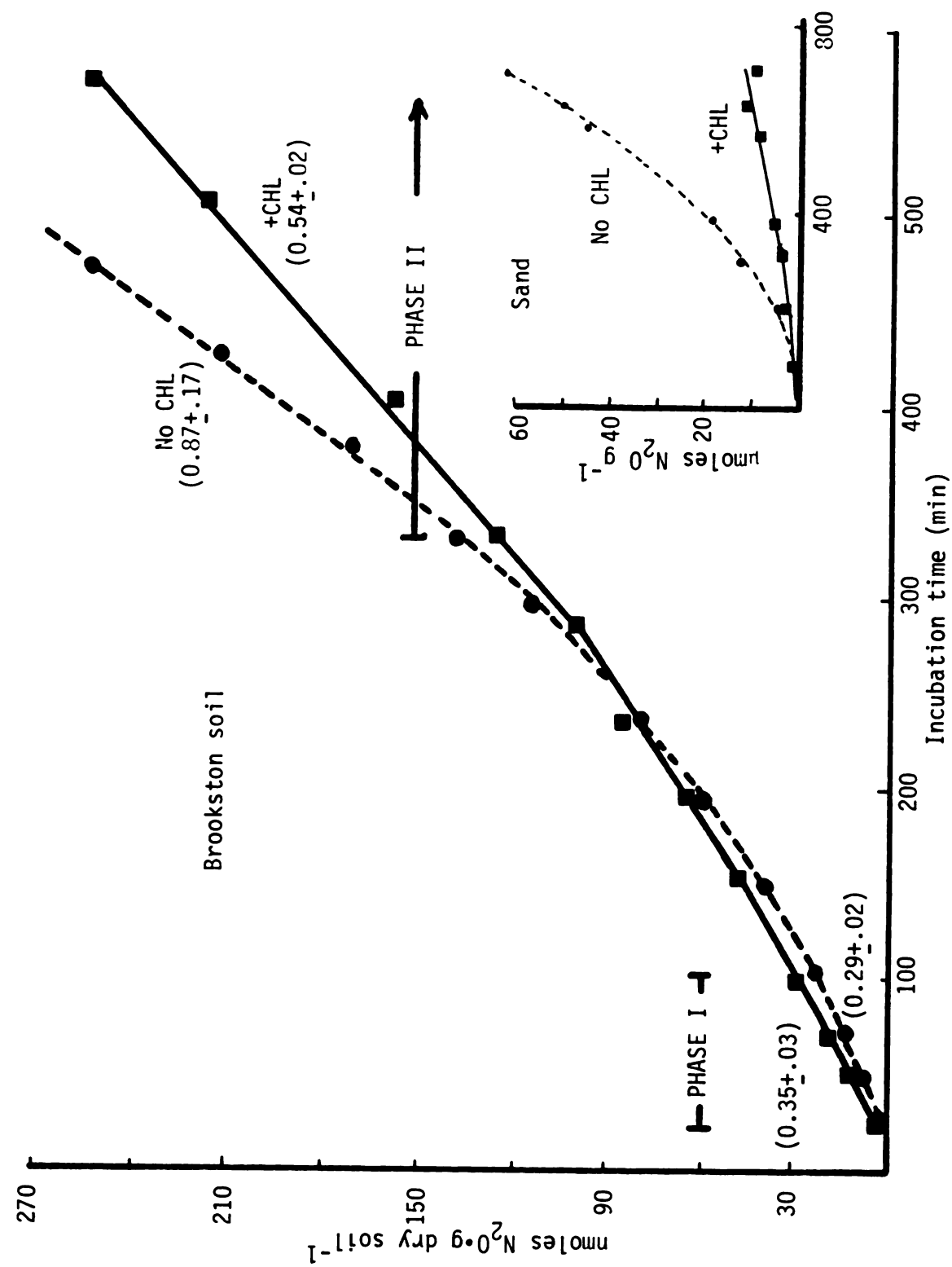

nmoles N_2O•g dry soil^{-1}
270
210
150
90
30
100
200
300
400
500
Incubation time (min)
Brookston soil
No CHL
(0.87+_.17)
+CHL
(0.54+_.02)
PHASE II
PHASE I
(0.35+_.03)
(0.29+_.02)
Sand
No CHL
+CHL
μmoles N_2O g^{-1}
60
40
20
400
800

Figure 4. Effect of chloramphenicol on denitrification rates in a soil and a sand. Points are the means of 3 replicates. Numbers in parentheses are rates (nmoles $N_2O \cdot g$ dry $soil^{-1} \cdot min^{-1}$) with 95% confidence limits.

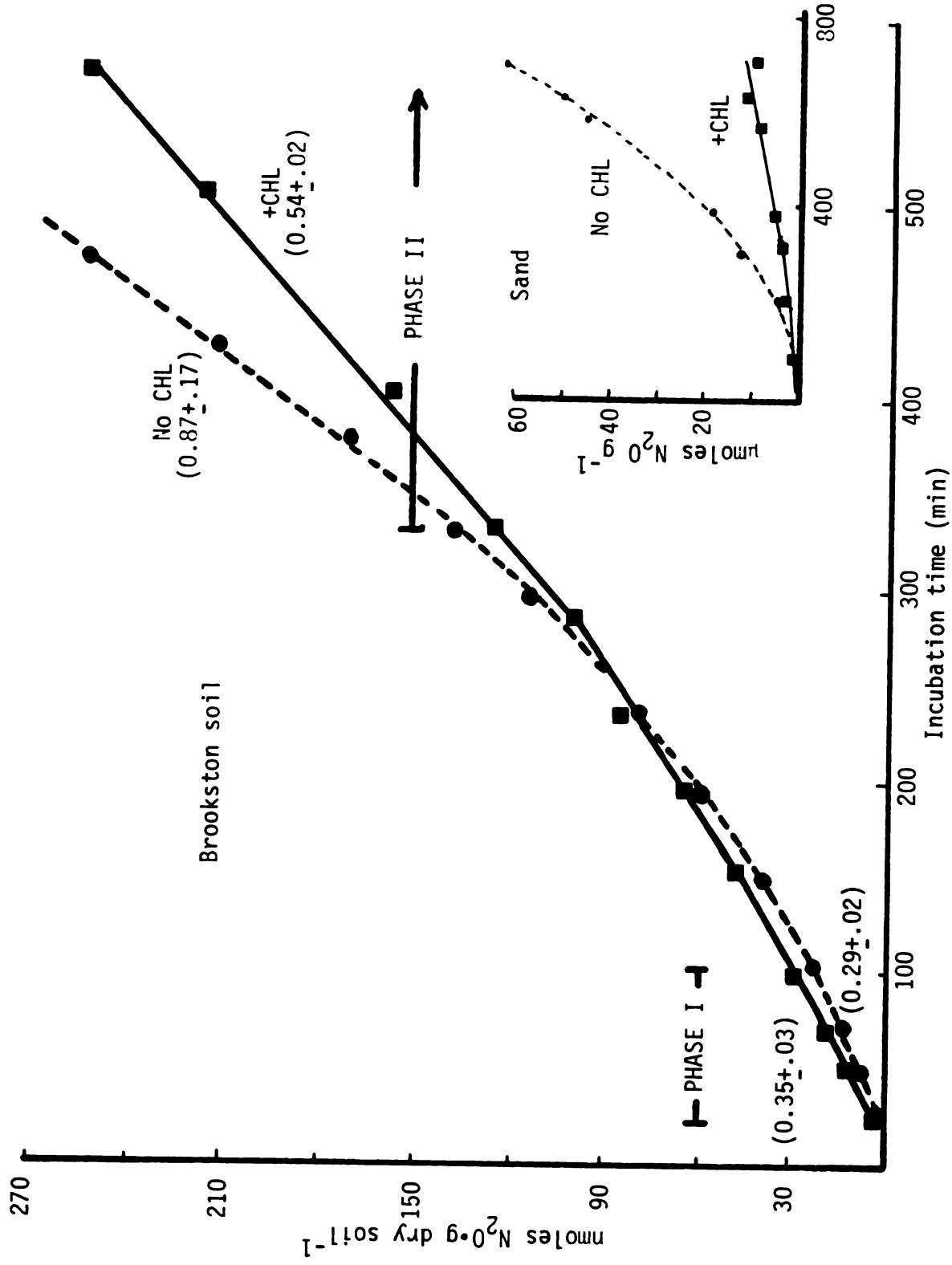

nmoles N2O•g dry soil-1
270
210
150
90
30
100
200
300
400
500
Incubation time (min)
Brookston soil
No CHL
(0.87+_.17)
+CHL
(0.54+_.02)
PHASE II
PHASE I
(0.35+_.03)
(0.29+_.02)
Sand
No CHL
+CHL
60
40
20
umoles N2O g-1
300
400
500

Table 6. Effect of glucose additions on denitrification rate of Brookston soil.

Time of anaerobic incubation	Denitrification rate	
	No glucose	Glucose added
hours	---nmoles $N_2O \cdot g$ dry $soil^{-1} \cdot min^{-1}$----	
0 to 1½ (Phase I)	0.21 ± .03*	0.25 ± .03†
6 to 8	0.56 ± .09	0.56 ± .08≠
24 to 27	0.67 ± .06	2.82 ± .16≠
72 to 75	0.60 ± .07	1.43 ± .09 ≠

* $s_b t_{.05}$

† 0.15% glucose added

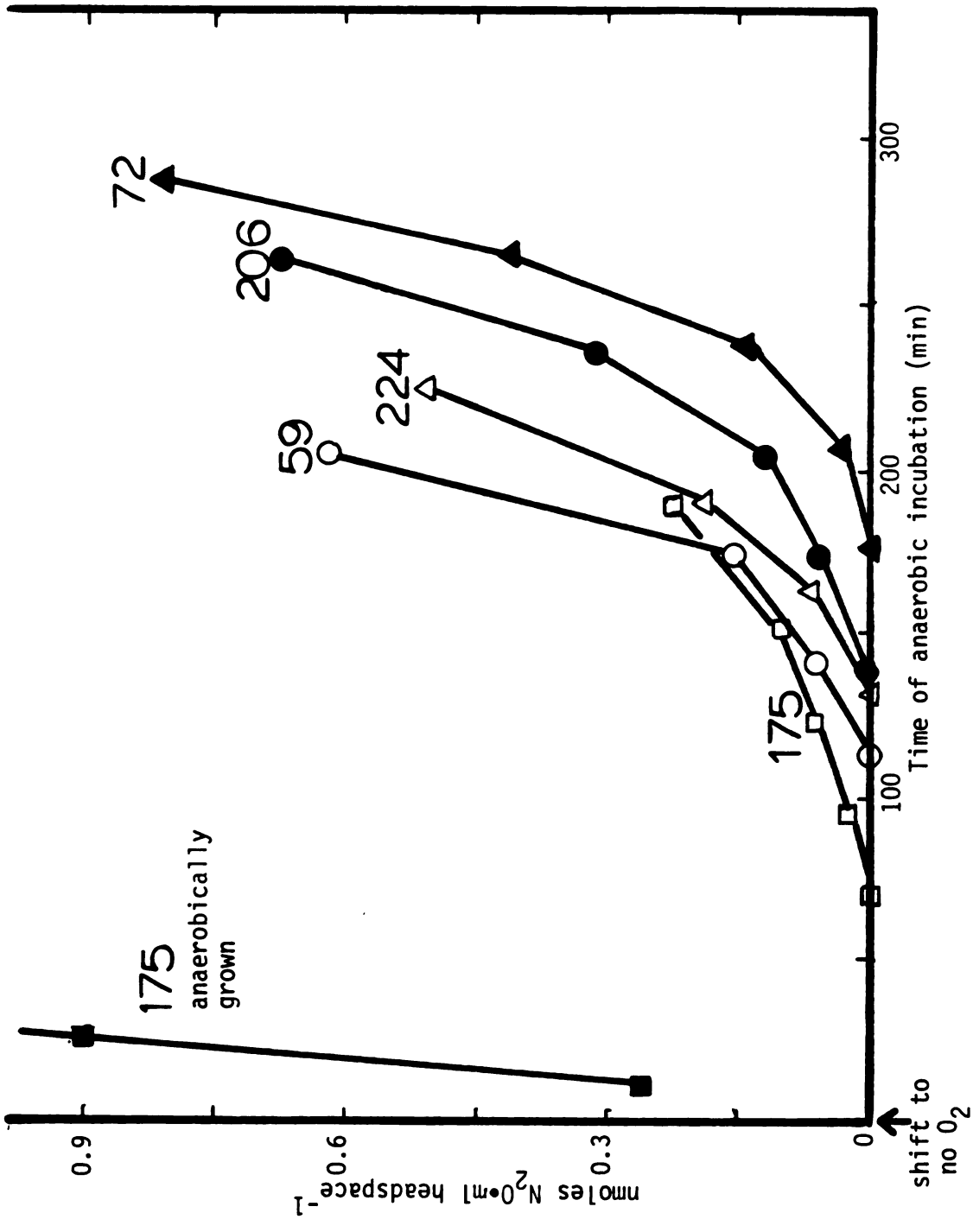
≠ 0.1% glucose added

and was followed or completely replaced by a logarithmic increase in rate when glucose was added. Without a carbon amendment, the denitrification rate remained essentially constant, i.e., phase II persisted, for up to 3 days. It is concluded that during phase I the enzymatic capacity to denitrify is a more important limitation than the supply of electron donor, but that enzyme synthesis and the potential for growth during continued incubation increases the demand for electron donor, making this the more important limiting factor. The data also suggest that a significant increase in number of denitrifers does not occur in anaerobic soils unless an energy supply is added.

To determine the role of microbial growth in the denitrification rate changes, denitrifiers were enumerated by an MPN procedure after various periods of anaerobic incubation. Increasing numbers could be detected only in soils with added glucose. However, the methods used are not precise enough to detect increases of less than an order of magnitude.

Pure cultures of denitrifiers shifted from aerobic to anaerobic conditions exhibited a distinct lag phase before beginning to denitrify (Figure 5). Nitrous oxide first appeared between 95 and 205 min after the onset of anaerobiosis; most of the organisms required about 2 hours to produce detectable N_2O. Flavobacterium sp. 175 grown anaerobically in NO_3^- broth, harvested and assayed in the same manner as the aerobic cultures began producing N_2O immediately. The reducing agent, thioglycollate, did not decrease lag times indicating that neither residual O_2 nor a high initial E_h were delaying denitrification. The lag times correspond quite well with the time of shift from phase I to phase II in anaerobic soils and further support the

Figure 5. Derepression of denitrifying enzyme synthesis by pure cultures of 5 strains of denitrifiers. Denitrifiers were grown either aerobically or in one case anaerobically, harvested, and then incubated anaerobically with NO_3^-.


72
206
224
59
175
175
anaerobically
grown
0.9
0.6
0.3
0
nmoles N_2O•ml headspace^{-1}
100
200
300
Time of anaerobic incubation (min)
shift to
no O_2

conclusion that the phase I rate is unaffected by de novo enzyme synthesis.

Since phase I is a measure of the activity of in situ denitrifying enzymes it should respond to altered soil aeration in the field. This was demonstrated by sampling the Miami soil, planted to corn, immediately before and after 4 hours of irrigation. Within 1 hour of sampling the soils were made anaerobic and phase I and II rates were determined. Phase I rate of the sample taken after irrigation was approximately twice that of the sample taken before (Figure 6). However, there was no difference in phase II rates. This comparison serves as a control to show that there were not significant chemical or physical differences between the two samples which could account for the difference in the phase I rates. The increase can be attributed to the partial derepression of denitrifying enzyme synthesis caused by reduced soil aeration during irrigation. It is interesting that a significant amount of in situ activity was detected even in the sample taken prior to irrigation. This soil was at 16% water content and so may be considered reasonably well aerated.

The relationship between phase I rate and soil aeration was further established by adding various amounts of water to 50 g Spinks soil in 125 ml flasks. The flasks were left open for 16 hours before phase I rates were determined. Denitrifying activity was present in all the treatments but was greatly increased at water contents above 25% (Table 7).

Nitrous oxide evolution from soils in an aerobic atmosphere, with 0.1 atm C_2H_2 added could also be observed (Figure 7). Miami soil at

Figure 6. Phase I and phase II denitrification by Miami soil sampled before and after irrigation. Points are the means of 3 replicates. Numbers in parentheses are rates (nmoles $N_2O \cdot g$ dry $soil^{-1} \cdot min^{-1}$) with 95% confidence limits.

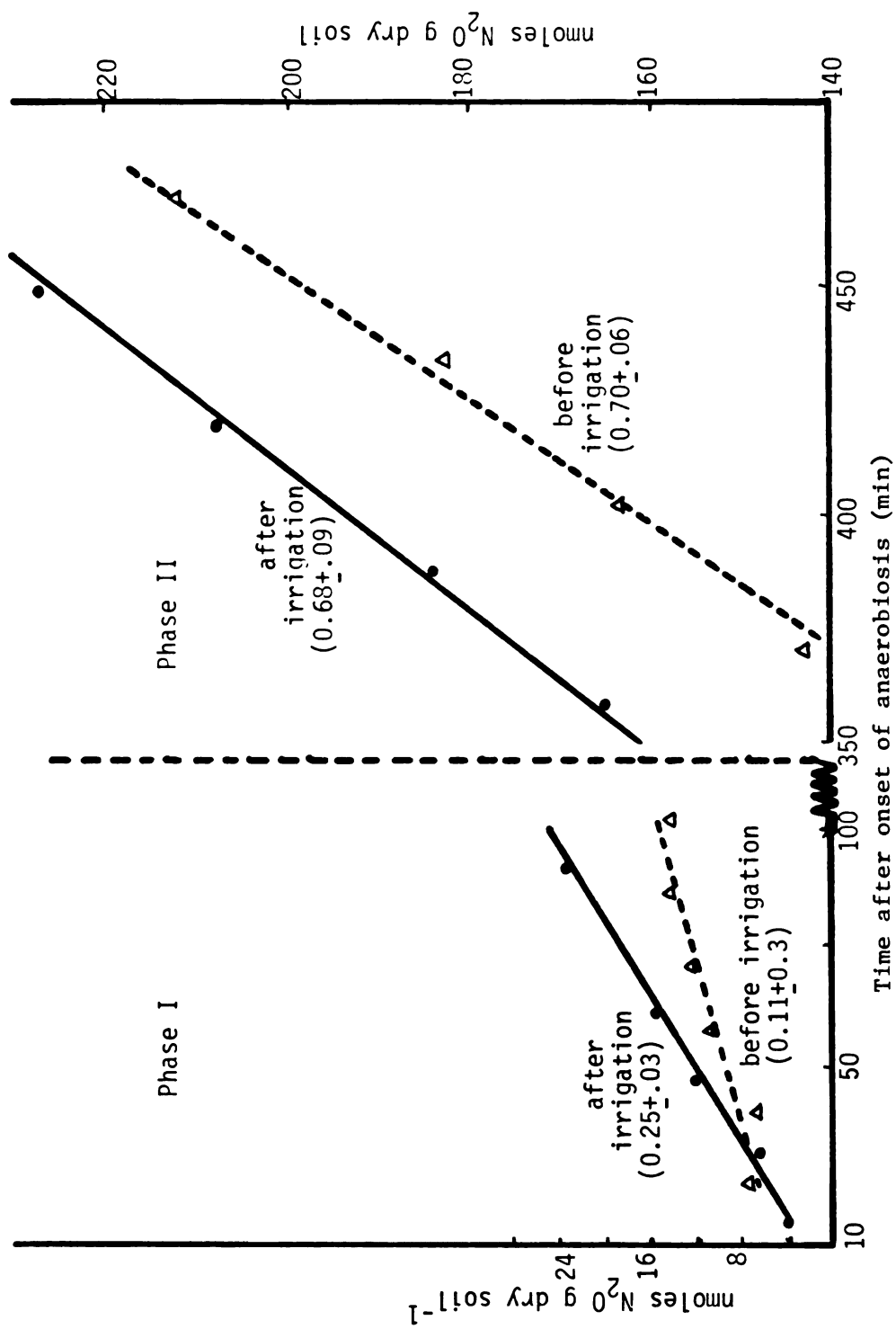
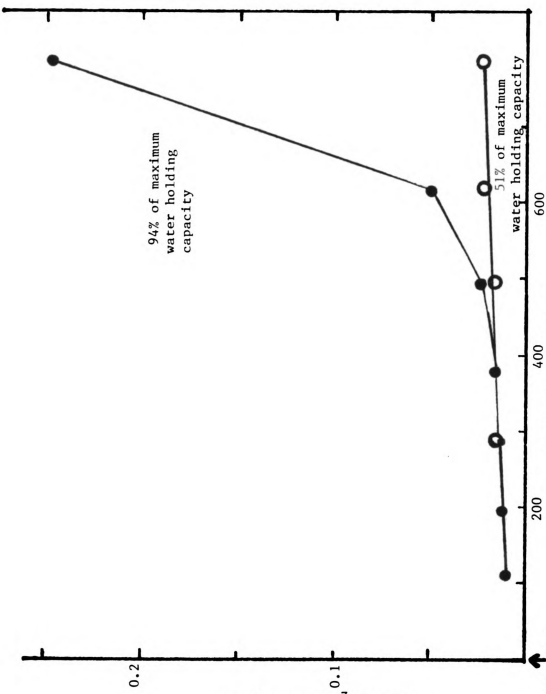

nmoles N_2O g dry soil^{-1}
8
16
24
10
50
100
350
400
450
Time after onset of anaerobiosis (min)
Phase I
after irrigation (0.25+.03)
before irrigation (0.11+0.3)
Phase II
after irrigation (0.68+.09)
before irrigation (0.70+.06)
140
160
180
200
220
nmoles N_2O g dry soil

Table 7. Phase I denitrification rate of Spinks loamy sand preincubated aerobically at varying water contents.

Percent gravimetric water content	Denitrification rate
	nmoles $N_2O \cdot g$ dry $soil^{-1} \cdot min^{-1}$
6	0.06 ± .02*
16	0.05 ± .01
27	0.10 ± .02
37	0.16 ± .06
58	0.38 ± .09

* $\pm s_b t_{.05}$

Figure 7. Evolution of N_2O by C_2H_2-inhibited Miami soil in an aerobic atmosphere. Water was added to half of the samples at the beginning of the incubation. There were 5 replicates per treatment.


nmoles N_2O·ml headspace^{-1}
0.2
0.1
H_2O added
200
400
600
Incubation time (min)
94% of maximum water holding capacity
51% of maximum water holding capacity

51% of maximum water holding capacity and the same soil adjusted to 94% of maximum water holding capacity (approximately saturation) just prior to the assay were compared for pattern of N_2O evolution. A very low rate of N_2O evolution, 1.1×10^{-14} moles $N_2O \cdot$ ml headspace$^{-1} \cdot min^{-1}$, was observed from the drier soil and initially from the moistened soil. Approximately six hours after wetting, the rate began to increase rapidly. Similar results were observed with several other soil samples. The lag period ranged from 4 to 7 hours. The O_2/N_2 ratio in the headspace, as determined by gas chromatographic analysis was reduced by less than 5% during these experiments.

DISCUSSION

It is concluded that the following sequence of events occurs when a soil or soil microsite becomes anaerobic: first, the O_2 inhibition of native denitrifying enzymes is removed, which results in an initial linear phase of denitrification (phase I). After a lag period of at least one hour newly synthesized denitrifying enzymes become functional and the rate increases (if permitted by substrate supply). At 4 to 8 hours all denitrifiers are fully derepressed so each cell has attained its maximum capacity to denitrify (phase II). At this point denitrification rate can increase only by an increase in number of cells; significant growth will occur only when the supply of electron donor is large. These events and their relationship to the phases observed are summarized in Table 8. It should be noted that phases IIa and IIb were referred to as phases II and III in an earlier report (Tiedje et al., 1978). This designation has been changed to avoid the

Table 8. Generalized description of the phases of soil denitrification after the imposition of anaerobic conditions.

Phase	Generalized time interval (hours)		Characteristics of rate	Explanation
	Start	End		
I	1/4	1-3	Constant	Activity of pre-existing enzymes
Transition	1-3	4-8	Increasing	Active derepression of enzyme synthesis
IIa (low available carbon)	4-8	Indefinite	Constant	Indigenous community fully derepressed, no significant growth
IIb (high available carbon)	4-8	Indefinite	Increasing	Growth of denitrifiers

implication that these are always distinct phases.

The evidence for this explanation of the phases can be summarized as follows: first, various methods of rapidly removing O_2 had no effect, indicating that residual O_2 could not account for the lower initial rate. Chloramphenicol reduced phase II denitrification rates but not phase I, suggesting that the increase was due to enzyme synthesis. The small initial stimulation by glucose relative to subsequent stimulation demonstrates that the capacity to utilize electron donor increases after phase I. Pure cultures of soil denitrifiers exhibited a lag time consistent with the duration of phase I before demonstrating derepression of denitrifying enzymes. Payne and Riley (1969) observed a slightly shorter lag time, 40 min, with the marine denitrifier, Pseudomonas perfectomarinus. This could be due to a difference in the physiology of this organism or might be related to a difference in habitat; it would be interesting to compare the phase pattern of soils with that of sediments, which are more or less continuously anaerobic.

Phase I and phase II rates measure completely different factors in soil denitrification. Phase II rate corresponds to what is generally called denitrification potential in the literature (for ex.; Balasubramanian and Kanehiro, 1976). My results indicate that this rate is a function of the number of soil denitrifiers and the limitations imposed on them by the energy and electron acceptor supply, pH, and temperature. All of these factors might be altered by imposition of the assay conditions. I have shown that addition of an energy source has a major effect on the rate and the pattern in phase II. It is also probable that moistening of a completely dry soil has

effects similar to adding an energy source. Denitrification potential has been shown to correlate very well with mineralizable carbon (Bremner and Shaw, 1958). In fact, a measurement of mineralizable C probably provides about as much information about soil denitrification as does denitrification potential. Phase I, in contrast to phase II or denitrification potential, is sensitive to the aeration state of the native soil. Phase I is essentially an enzyme assay and reflects the immediate biological effect of changes in soil moisture and aeration--extremely important factors for soil denitrification. Therefore, phase I rate appears to provide more information about in situ denitrifying activity than does denitrification potential and should be a more useful approach to the study of soil denitrification.

The importance of derepression of denitrifying enzyme synthesis in nature, as well as in laboratory incubations, is indicated by the increase in phase I rate of soil during field irrigation. Also when water was added to aerobic soils a pattern was observed similar to that in anaerobic incubation, that is, a low initial linear rate was followed by an increase in denitrification rate. It is tempting to attribute this pattern to the same causes in aerobic and anaerobic incubations. Although derepression is undoubtedly involved, other factors must be considered in the aerobic experiments and in field soils. The time required for the generation of anaerobic microsites accounts for the longer initial phase in the aerobic experiments. The difference between anaerobic phase I and phase II was usually less than an order of magnitude, while the rate increase subsequent to wetting of aerobic soils was significantly greater. This appears to be due to removal of O_2 inhibition of existing denitrifying enzymes

which would be expected to result in much greater rate increases than would derepression of enzyme synthesis.

The results of this research demonstrate the value of working with simplified soil systems, in this case stirred anaerobic suspensions. I have been able to isolate and identify the biological effects of reduced soil aeration by controlling physical variables, particularly O_2 and substrate diffusion.

My data provide some information about when and under what conditions denitrification could be expected to occur. I have demonstrated that denitrifying enzymes are present even in very dry soils. Since denitrifying enzymes are not constitutive (Payne, 1973) this implies that either denitrification can occur in anaerobic microsites of well aerated soils or that denitrifying enzymes may persist in functional form in the presence of O_2. I have also observed evolution of N_2O from the Miami sandy loam at 51% of maximum water holding capacity in an aerobic atmosphere. If it is assumed that the rates observed in our assays are similar to field rates, then a gross approximation of field N loss can be made. This is done by assuming that the net flux of N_2O from the soil in the tubes, i.e., the rate of accumulation in the headspace divided by the soil surface area, is equal to the flux of N from the soil in the field. Thus, the rate of N loss from the drier Miami sample (Figure 7) would be low, 69 mg N $\cdot ha^{-1} \cdot day^{-1}$. Some well-aerated soils I assayed aerobically evolved N_2O at significantly greater rates, up to 6 g $N \cdot ha^{-1} \cdot day^{-1}$. These "aerobic" rates are too small to be significant in the N economy of agricultural soils. Yet it is interesting that N_2O evolution was observed from virtually all of the well-aerated soils examined. I

have not yet demonstrated what fraction of this N_2O results from biological denitrification; several other mechanisms are possible. Bremner and Blackmer (1978) have suggested that nitrification causes low rates of N_2O production in aerobic soils, presumably by a process observed earlier in Nitrosomonas cultures (Yoshida and Alexander,1970). Non-biological reactions of NO_2^- also might contribute to N_2O production. It is also possible that the soil samples were super-saturated with N_2O. Denitrifying conditions in the field well before sampling might have caused accumulations of N_2O within aggregates which slowly equilibrate with the atmosphere. The relative contribution of each of these mechanisms will be difficult to determine. Preliminary experiments were not revealing due in part to variability within and among soil samples, and in part to the difficulty of experimentally isolating the various mechanisms of N_2O production.

In summary, nitrogen gases can be produced more or less continuously by soils. Several mechanisms could contribute to this, but only denitrification in very wet soils is likely to result in large N losses. Subsequent to a reduction in soil aeration, denitrification rate is greatly increased, but only after a lag period of several hours. Anaerobic or partially anaerobic conditions, established by respiration and reduced oxygen diffusion rate, eliminate O_2 inhibition then derepress the synthesis of denitrifying enzymes. Most nitrogen loss would occur during brief periods beginning a few hours after irrigation or a rainfall and lasting until NO_3^- is depleted or the soil water content decreases.

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CHAPTER IV

DENITRIFICATION IN THE RHIZOSPHERE

It is generally believed that increased available organic matter in the rhizosphere stimulates microbial activity, reduces O_2 concentrations, and thereby increases soil denitrification. This belief is based on numerous studies. In some of these (Woldendorp, 1962; Brar, 1972; Volz et al., 1976) denitrification has been measured indirectly, usually by NO_3^- disappearance. These methods are generally insensitive and may be confounded by non-assimilatory reduction of NO_3^- to NH_4^+ Stanford et al., 1975b), plant uptake of NO_3^-, or respiratory reduction of NO_3^- only to NO_2^-. Perturbation of the plant-soil system has been required in other studies (Bailey, 1976; Brar, 1972; Garcia, 1975). Removal of the shoot from the roots, the roots from the soil, or any alteration in the soil matrix would be expected to cause drastic changes in nutrient supply, soil metabolism, nitrogen sinks, and gas exchange. Only Stefanson (1970, 1972a, 1972b) has directly measured denitrification products from soil with intact plants. He did long-term analysis of N_2 and N_2O in elaborate sealed chambers. A consistent plant stimulation of denitrification was observed only under certain conditions: water contents near field capacity and N applied as NO_3^- rather than NH_4^+. The complexity of this equipment made replication difficult and limited manipulation of plant and soil variables.

It can be concluded from previous work that the rhizosphere is potentially a very important site for denitrification. Yet none of the methods previously used appeared promising for further investi-

gation of this relationship. This chapter presents the results of several new approaches to the study of denitrification in the rhizosphere.

MATERIALS AND METHODS

Soil treatments: The Miami sandy loam, Brookston loam, and Spinks loamy sand have been characterized in chapter 2. Corn, oats and orchard grass were grown in these soils either in the greenhouse or a growth chamber. In most experiments plants were grown in 125 g soil in styrofoam cups (9 oz) with a small hole in the bottom for drainage. Equal water and fertilizer was applied to soils with and without plants. Soils were treated with 1 mg K_2HPO_4/10 g soil at the time of planting. The amount of N added varied from one experiment to another. Treatments referred to as low NO_3^- received no N amendment. Most high NO_3^- treatments were leached with 100 ppm KNO_3-N at planting and then at monthly intervals for the grass, weekly for the corn. However, Brookston high NO_3^- soils received 1 mg KNO_3-N/10 g soil at planting and were leached with NO_3^- solution only before the aerobic assays. After some experiments, soil NO_3^--N was determined with an Orion NO_3^- electrode, following extraction with a 0.01 M $CaSO_4$, 1 mM $AgSO_4$ solution.

In most experiments I compared planted and unplanted soil, but in some cases I made a gross separation of rhizosphere soil from the non-rhizosphere soil in the planted cups. This was done by shaking the roots lightly to remove non-rhizosphere soil, then vigorously agitating to collect rhizosphere soil.

Fresh soil samples were 0 to 15 cm cores (5 cm diameter) collected from the Miami soil planted to corn. Subsamples were immediately assayed as anaerobic slurries for denitrification rate.

Split-plate experiments: This apparatus was based on a soil chamber designed for the study of endomycorrhizal fungi (Hattingh et al., 1973). My modification consisted of a small (5.4 cm diameter) plastic petri dish glued in the center of a larger (8.8 cm) one (Figure 8). A portion of both sides of the inner dish was cut away and covered by nylon mesh cloth (30 μm openings). The chamber was filled with Spinks soil, and both dishes were tightly covered. A germinated seed was planted through a hole in the side of the outer plate and the chamber was placed on its end allowing the roots to grow over the outer surface of the nylon mesh but not enter the inner dish. After one month the chambers were opened and those with roots in the center were discarded. Soil samples were carefully removed from the outside plate (root zone) and from the inside chamber at 5 mm increments from the root zone.

Anaerobic assays: The method of assaying denitrification anaerobically has previously been described in detail (chapter 2). Briefly: soils were made into a slurry and the appropriate substrates added. Serum bottles (25 ml) were used for the split-plate experiments because of the small amount of soil available. In all other experiments 125 ml Erlenmeyer flasks were used. The incubation vessels were twice evacuated for 5 min and flushed with He to achieve anaerobiosis. C_2H_2 (0.1 atm) was added to inhibit reduction of N_2O to N_2. A microthermistor detector was employed for N_2O analysis.

Figure 8. Split-plate apparatus used to determine spatial relationship between roots and denitrifying activity. Note that roots are restricted to the outer chamber.

Aerobic assays: Corn (2 plants per cup) was grown for 9 to 13 days and orchardgrass (4 plants per cup) for approximately 4 months in styrofoam cups. Three days before the assay the soil was briefly leached with a KNO_3 solution; 2 ppm-N for low NO_3^- treatments, 100 ppm for high NO_3^-. When C_2H_2 was used, the cups were placed in a dessicator containing 3% C_2H_2 for 12 hours before assaying for denitrification. At the beginning of the assay the soil was again briefly leached with 80 ml of the appropriate NO_3^- solution. C_2H_2 was bubbled through the leaching solution before the assay. Cups were allowed to drain in the dessicator for 1/2 to 1 hour. Thus the soil water content was reasonably uniform, approximating the maximum water holding capacity. A glass tube (7 cm x 5 mm i.d.) packed with Ascarite was taped to the side of each cup. This kept the CO_2 concentration low, but not below ambient, during the analysis and reduced interference with N_2O analysis. Cups, with plants and soil still intact were then placed in 30 x 15 cm Saran gasbags (Anspec, Ann Arbor, Mi.) which had an inflated volume of approximately 1 ℓ (Figure 9). The bags are manufactured with a tube, sealed by a serum stopper, which allowed introduction and sampling of gases. I modified the bags, cutting open the bottom, so the plant and cup could be placed inside. The bottom was resealed with a screw clamp made of wood and rubber. Enough C_2H_2 was immediately injected into the bag to give approximately a 5% concentration by volume. The volume of the gas space in the bag was determined by injecting 10 ml He then measuring the He concentration after 90 min with a microthermistor detector. In some experiments the rate of gas loss from the bag was measured by following the concentration change of introduced He or Ne. A ^{63}Ni

Figure 9. Saran gasbag used to measure denitrification rate of soils with and without intact plants.

electron capture detector was used for N_2O analysis in these experiments (chapter 2).

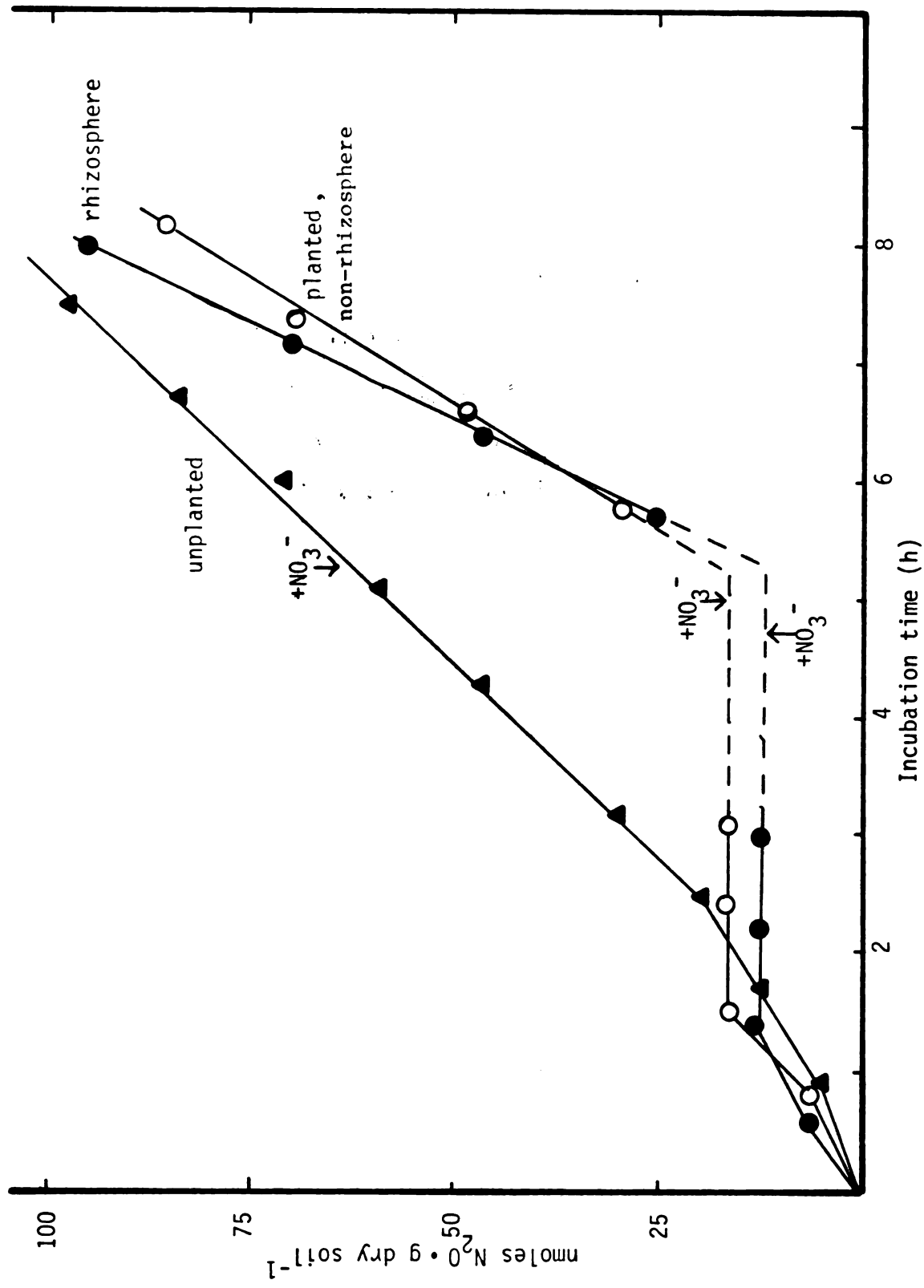
Enumeration of soil bacteria: Twenty g soil samples were blended for 1 min in 180 ml 0.85% NaCl with 1 drop of Tween 80. Dilutions were plated on nutrient agar (Difco, Detroit, Mi.) and grown at 30°C. The counts on these plates after 3 days were considered to be a measure of total aerobes.

Denitrifiers were enumerated by a most probable number procedure with 10 fold dilutions and 5 tubes per dilution. I used Hungate tubes (initially aerobic) containing 3.5 mM KNO_3 in nutrient broth (Difco). After 7 days, disappearance of NO_3^- and NO_2^- was determined with diphenylamine (Appendix A).

RESULTS

Anaerobic assays: The initial denitrification rate of soil which adheres to corn roots (rhizosphere) was greater than non-rhizosphere soil in the same cups (Figure 10). Soil from unplanted cups had the lowest rate. After this brief initial period endogenous soil NO_3^- was depleted, first in the rhizosphere soil, then in the planted soil. There was no indication of NO_3^- depletion from the unplanted soil. Only after 5 hours of anaerobic incubation were the soils amended with NO_3^- (1 ml of 0.1 M KNO_3). After the addition of NO_3^- the initial order of denitrification rates was quickly reestablished (Figure 10). i.e., rhizosphere greater than planted greater than unplanted. This result implies that competing NO_3^- sinks, presumably plant uptake, may reduce rhizosphere denitrification even though the potential for

Figure 10. Denitrification by non-rhizosphere planted, unplanted, and rhizosphere Brookston soil assayed anaerobically. Soils were amended with NO_3^- only after 5 hours of incubation. Points are means of 3 replicates.


rhizosphere
planted, non-rhizosphere
unplanted
+NO3-
+NO3-
+NO3-
nmoles N2O • g dry soil-1
Incubation time (h)
25
50
75
100
2
4
6
8

denitrification is greater in the rhizosphere. As was the case in previous work (chapter 3) denitrification rates became approximately linear (phase II) after an initial period (phase I).

I have suggested that the ratio of N_2O produced in the absence of C_2H_2 to N_2O produced with C_2H_2 is a valid approximation of the ratio $N_2O/(N_2 + N_2O)$. This approach was used to compare products from unplanted and planted (corn) soils (Table 9). In this, and all of the following experiments, planted soil refers to all of the soil in the cups with plants. The percentage of N_2O was at all times higher in the unplanted soil. In the planted soil N_2O reached a maximum concentration at about 10 h, then remained constant in the flasks with C_2H_2 but declined to 0 at 18 hours in the flasks with no C_2H_2. In the unplanted soil with no C_2H_2, N_2O reached a maximum at 35 h then slowly declined. As in the previous experiment, total N_2O production was higher (both with and without C_2H_2) from the planted soil than the unplanted.

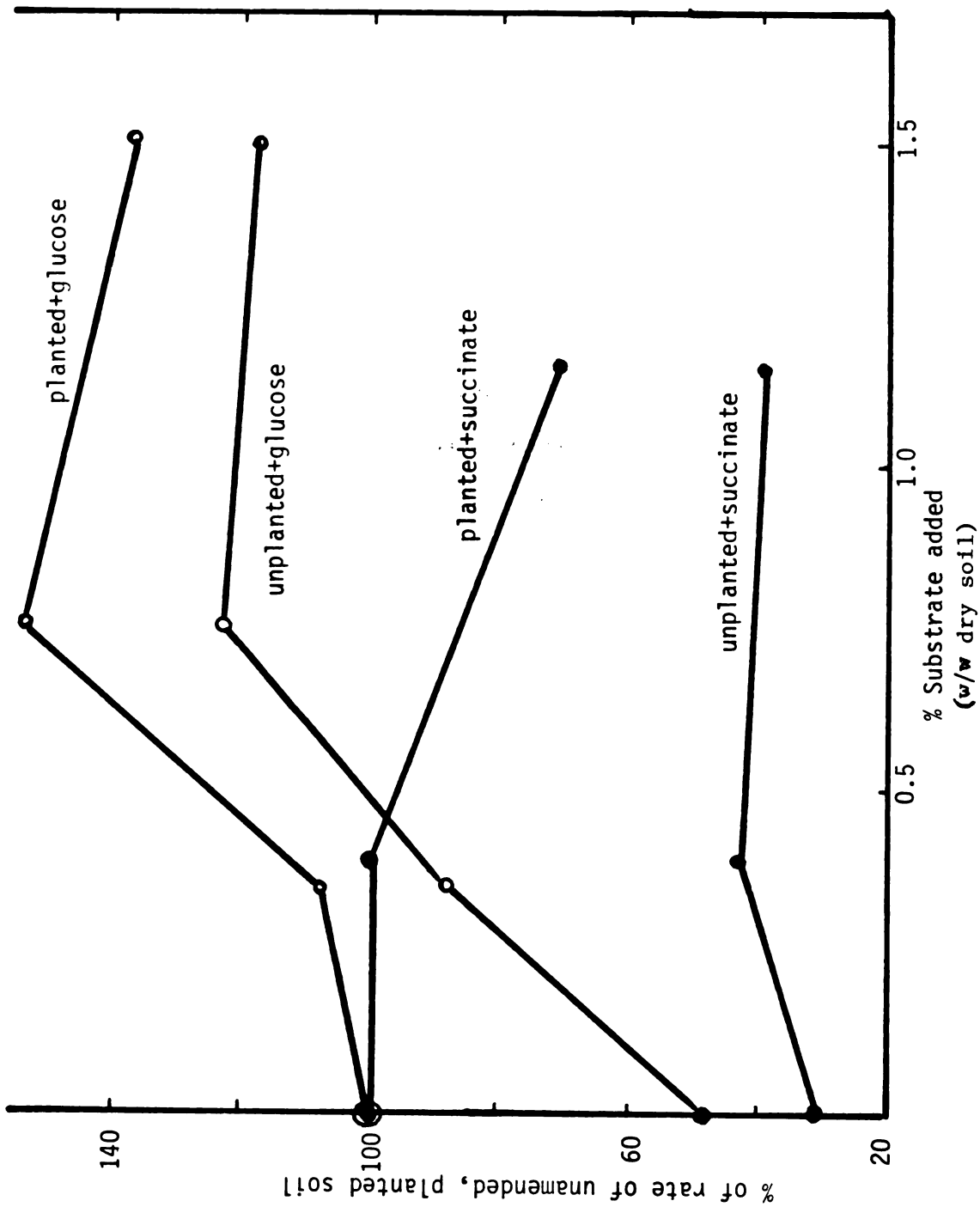
Addition of glucose to soils just before assaying increased the denitrification rate of both planted (corn) and unplanted Brookston soil (Figure 11). All soils were also amended with 10 ppm NO_3^--N (soil fresh wt. basis). Succinate slightly increased the rate of unplanted soil but not of planted soil. Reduced stimulation or inhibition was observed at high concentrations of added carbon. Significantly, amendment with both carbon sources reduced the difference between planted and unplanted soils. This indicates that at least part of the rate increase in planted soil may be due to supply of available carbon. However, both soils are apparently limited by the supply of electron donor under the assay conditions used, since

Table 9: Portion of N gas evolved as N_2O by anaerobic Brookston soil with and without plants.

Incubation time	$N_2O/(N_2 + N_2O)$*	
	Planted	Unplanted
hours		
2	0.30	0.58
6	0.42	0.49
10	0.30	0.48
14	0.09	0.45
22	0	0.40
30	0	0.36

* Rate of N_2O production without C_2H_2 over rate with C_2H_2.

Figure 11. Effect of glucose and succinate amendment on the phase II denitrification rate of planted and unplanted Brookston soil. Rates have been normalized relative to the rate of unamended, planted soil. There were 3 replicates per treatment.


planted+glucose
unplanted+glucose
planted+succinate
unplanted+succinate
% of rate of unamended, planted soil
140
100
60
20
0.5
1.0
1.5
% Substrate added
(w/w dry soil)

glucose amendment increased the rate of planted as well as unplanted soil.

The relationship between the presence of plants and anaerobic denitrification rate was examined with fresh soil samples. The two experiments shown (Table 10) were conducted at different times and with soil from different plots so no valid comparison can be made between the experiments. In experiment 1 soil between corn plants in a row was compared to soil from a border strip which had received the same fertilizer and water treatment. Soil was taken at the row and at various positions between rows in experiment 2. Samples were amended with 10 ppm NO_3^--N and phase II rate determined. In both experiments, soil closest to the corn plants denitrified most rapidly during the anaerobic assay.

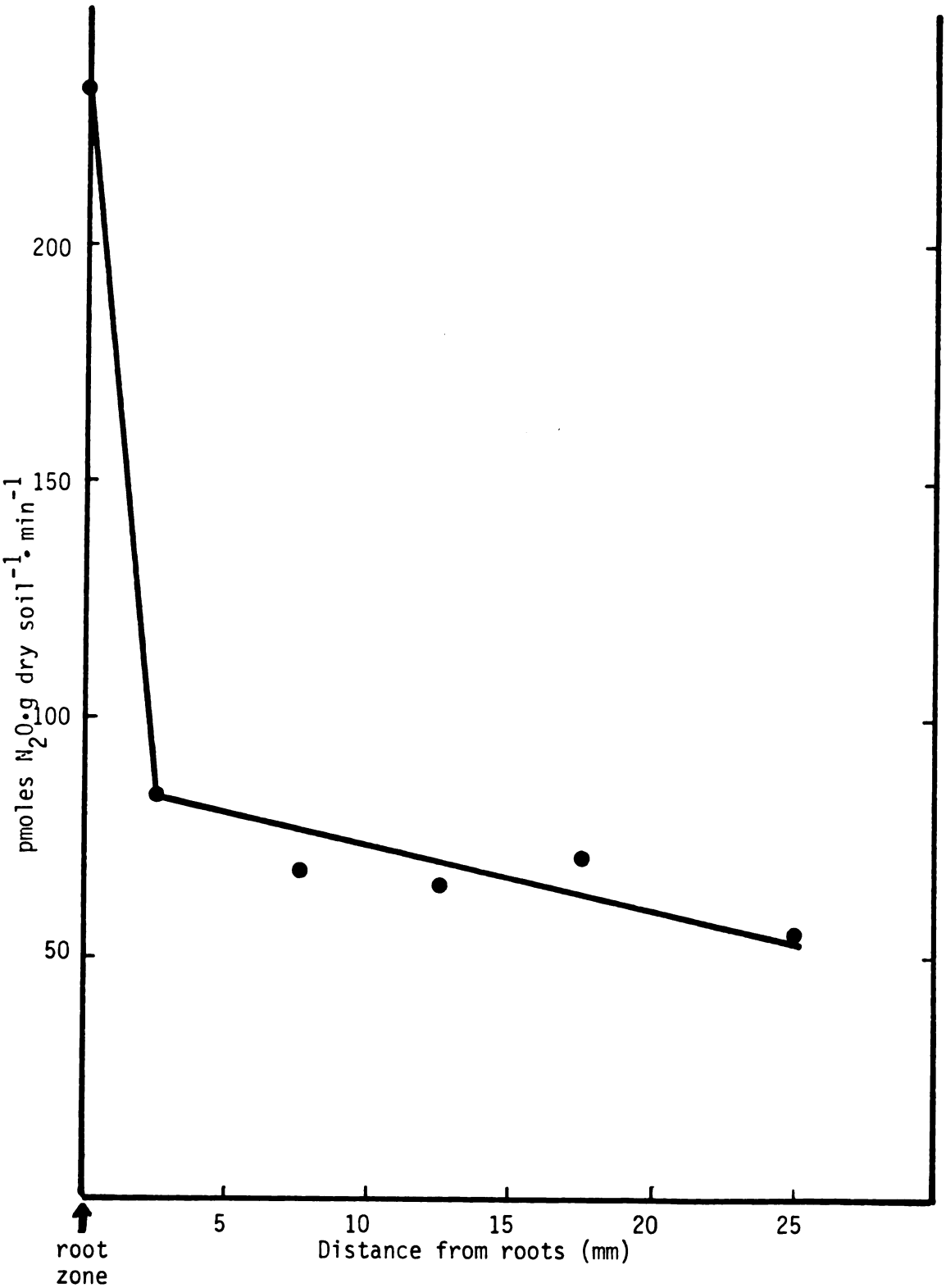
The spatial relationship between roots and soil denitrifying activity can be defined more precisely with the split-plate technique. Figure 12 shows the denitrification rate 200 to 400 min after the imposition of anaerobiosis. Nitrate (10 ppm) was added to all. The soil from the root zone (the outer chamber) denitrified more than twice as fast as any of the samples from the inner chamber. A gradual decrease in potential rate was observed with distance from the roots. The correlation coefficient of rate with distance for the samples from the inner chamber was -0.54, significant at the .05 level. Because of the design of the chamber, soil moisture was also negatively correlated with distance from the root. Although the difference was small, 17.5% gravimetric water content in the root zone vs. 14.7% at the center, this may have contributed to the observed differences in denitrification rate independently of a plant effect.

Table 10: Denitrification rate of Miami soil sampled at various distances from a corn row. The assays were anaerobic.

Experiment	Distance from row	Denitrification rate
	m	10^{-12} moles $N_2O \cdot g$ dry $soil^{-1} \cdot min^{-1}$
1	0	260 ± 50*
	3	110 ± 50
2	0	83 ± 7
	0.15	66 ± 7
	0.30	64 ± 12

* $\pm s_b t_{.05}$

Figure 12. Denitrifying activity related to distance from oat roots. Spinks soil was taken from split-plates. Rates were determined 200 to 400 min after the imposition of anaerobiosis. There were 3 replicates per treatment.


200
150
100
50
pmoles $N_2O \cdot g$ dry $soil^{-1} \cdot min^{-1}$
root
zone
5
10
15
20
25
Distance from roots (mm)

The number of isolatable aerobes and denitrifiers from several soils is shown in Table 11. These soils had been treated in the same manner as the soils used in the aerobic assays, they were kept in cups in a growth chamber. In 4 of 5 cases both aerobes and denitrifiers were more numerous in the soils with plants, although these differences were generally not statistically significant because of the large error inherent in MPN procedures (about 1 order of magnitude). The ratio of denitrifiers to aerobes was, in every case, higher in the planted soil. This implies that the presence of the plants specifically enriched for denitrifiers relative to aerobes.

The time course of N_2O production by saturated Brookston soil, with high NO_3^- and no plants is shown in Figure 13. After saturating the soil with water, the cups were placed in the Saran gasbags and 5% C_2H_2 was added. A lag period of about 4 hours was observed, consistent with earlier results (chapter 3). I have attributed this lag to the time required to consume soil O_2 and to derepress the synthesis of denitrifying enzymes. Between 8 and 11 hours the maximum rate of N_2O evolution was attained. The O_2 concentration in the headspace of the bags was only decreased by about 3% after 24 hours. The concentration of 20 ml Neon injected into the bags decreased by about 0.5%/hour, suggesting that leakage of evolved gases from the bags was slow.

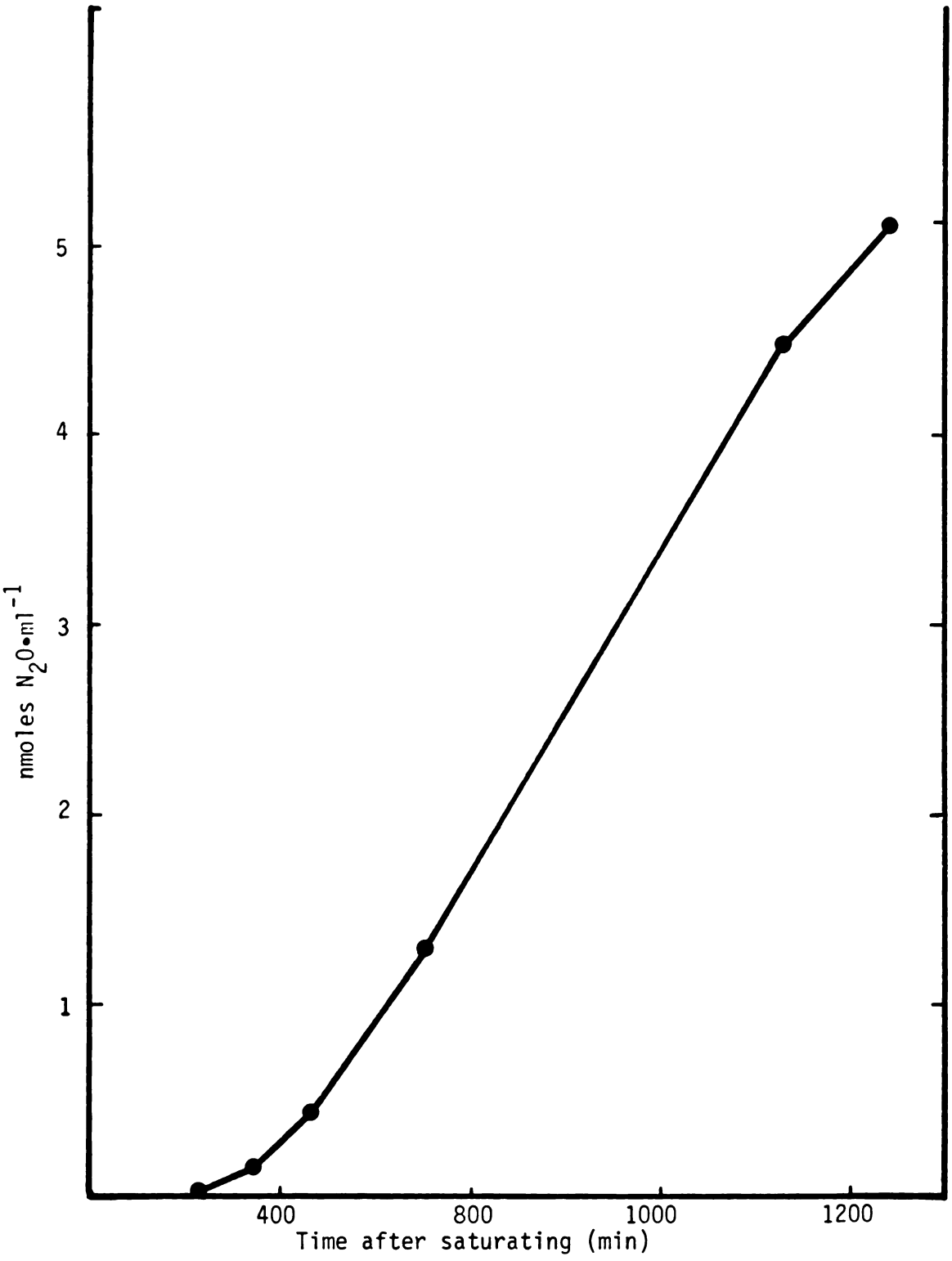
It was also observed that the denitrification rates of replicates of a treatment did not fit a normal distribution. Therefore the non-parametric Mann-Whitney test was used to determine statistical differences between treatments (Snedecor and Cochran, 1967).

Table 11: Number of denitrifiers in planted and unplanted soils.

Soil	Plant	Aerobes	Denitrifiers	Denitrifiers / Aerobes
		-log number·g^{-1}-		
Brookston (low NO_3^-)*	Corn	7.5	6.1	.033
	None	6.9	5.1	.015
Brookston (high NO_3^-)	Corn	7.7	6.5	.077
	None	7.6	4.5	.001
Brookston (high NO_3^-)	Grass	8.0	6.6	.037
	None	7.5	5.1	.004
Miami (high NO_3^-)	Grass	7.7	5.4	.006
	None	7.9	5.5	.005
Miami (low NO_3^-)	Corn	8.2	6.2	.011
	None	7.1	4.6	.005

* Refer to text for complete description of NO_3^- treatments.

Figure 13. Time course of N_2O production by unplanted high NO_3^- Brookston soil with C_2H_2 in aerobic gasbags. Points are means of 5 replicates.


5
4
3
2
1
nmoles $N_2O \cdot ml^{-1}$
400
800
1000
1200
Time after saturating (min)

Generally experiments were conducted only for an 8 to 10 hour period after saturation of the soils. This time was sufficient for differences between the treatments to develop yet leakage of N_2O from the bag was insignificant, and O_2 depletion and accumulation of product gases in the headspace was minimal.

The effects of NO_3^- concentration and plants on denitrification rate of two soils is shown in Table 12. With high NO_3^- the planted soils denitrified faster than the unplanted. Yet in the low NO_3^- treatments the planted soils denitrified at the slowest rates. This suggests that plant uptake can compete with denitrifiers for NO_3^-. The high NO_3^- treatments denitrified faster than the low NO_3^- treatments, except for the unplanted Brookston. This negative correlation of NO_3^- concentration and denitrification rate was repeated in separate experiments (data not shown) with unplanted Brookston.

The ratio of N_2O produced in bags without C_2H_2 to N_2O produced in bags with C_2H_2 is an approximation of the fraction of total gas evolved as N_2O, that is, $N_2O/(N_2 + N_2O)$. The fractions obtained for the Brookston and Miami soils are shown in Table 13. Most apparent are the large range of values, from 0 to ½ of the gas is released as N_2O. The ratios for the Brookston were always higher than the comparable treatment of the Miami. The concentration of NO_3^- also had a consistent effect. At low NO_3^- a smaller fraction of the gas was N_2O. After about 8 hours, there was no net production of N_2O by the low NO_3^- Miami soils without C_2H_2. At this time, the same soils blocked with C_2H_2 continued to evolve N_2O at an undiminished rate. The presence or absence of plants was not consistently related to the ratio.

Table 12. Denitrification rate of planted and unplanted soils assayed in aerobic gas bags.

Soil & Plant	NO_3^- Treatment#	Denitrification rate†		NO_3^- Measured	
		Planted	Unplanted	Planted	Unplanted
		-nmoles $N_2O \cdot bag^{-1} \cdot min^{-1}$-		———ppm* ———	
Brookston Corn	High	2.33a	0.33b	108.2	125.2
	Low	0.19b	1.14a	1.4	5.6
Miami Orchardgrass	High	10.89c	2.43a	25.0	56.6
	Low	0.22b	1.17a	1.2	1.3
Brookston Orchardgrass	High	24.30d	0.86a	not determined	

* Soil solution basis, as N.

† Average rate for 8 hours.
Rates with the same letter are not significantly different at the 5% level by the Mann-Whitney test.

See text for complete description of NO_3^- treatment.

Table 13. Portion of N gas evolved as N_2O by soils in aerobic gasbags.

Soil-Plant	NO_3^- Treatment†	$N_2O/(N_2O + N_2)$*	
		Planted	Unplanted
Brookston Corn	High	0.25	0.49
	Low	0.11	0.07
Miami Orchardgrass	High	0.08	0.04
	Low	0	0

* Rate without C_2H_2 over rate with C_2H_2, between 7 and 9 hours after soils were saturated.

† See text for explanation of NO_3^- treatments.

DISCUSSION

I have used several new methods to demonstrate that roots may increase denitrification rates. One approach was to determine the denitrification rate of soil slurries under totally anaerobic conditions. A rate increase was observed in soils from planted pots. The rate was also greater in rhizosphere soil than in non-rhizosphere soil from the same pots. Fresh field soils sampled near corn rows denitrified faster than soil at a distance from the row. The split-plate experiments revealed the spatial distribution of potential denitrifying activity relative to roots. The activity decreased very rapidly in the first few mm from the roots then declined slowly with increasing distance. Because I added NO_3^- to the soil in these experiments, the differences observed can be attributed primarily to variation in the number of denitrifiers and the supply of energy. This approach allows these important variables to be isolated and their effect determined independently of diffusion limitations, aeration state, and NO_3^- limitation. However, because direct effects of the latter set of variables have been eliminated these results cannot be directly related to field denitrification rates.

The number of isolatable denitrifiers was generally greater in planted than in unplanted soils. Yet this increase does not necessarily imply an increase in denitrification since denitrifiers are facultative aerobes. Their number can increase through respiration of O_2 as well as of N oxides. I also observed an increase in the ratio of denitrifiers to plate-count aerobes in planted soils, which is better evidence that denitrifying activity is enhanced. The relative

enrichment of a specific group of microorganisms should in general be better related to microbial activity than absolute numbers. However, it is possible that another mechanism could be responsible for the relative increase in number of denitrifiers in planted soils. For example, the substrate range of the pseudomonads (Bergey's Manual, 1974) corresponds fairly well to the compounds exuded by plant roots (Rovira, 1965).

The results with intact plants and soil in the aerobic gas bags are clearly best related to field denitrification rates. The physical conditions in the soil, the spatial relationship between roots and denitrifiers, and the sources and sinks of substrates are probably not significantly different from field soils under denitrifying conditions. Of previous methods, only Stefanson's (1970) satisfied these conditions. The method presented here requires a less complex incubation chamber and allows greater replication and manipulation of variables. Although all of these experiments have been conducted with saturated soils, it should be possible to study the effect of soil water content by slightly modifying the procedures. The slow diffusion rate of C_2H_2 in soils must be considered in the design of these experiments, however.

Long-term assays may increase the statistical uniformity of results and eliminate the problem of integrating rates to approximate total annual denitrification loss but they are time-consuming and greatly reduce the number of experiments possible. I have previously presented evidence suggesting that most denitrification occurs during brief periods immediately after soil wetting (chapter 3).

An approximation of denitrification rate in the field can be made

by assuming that the flux of N_2O from the soil in the gasbags is equal to the flux of N gases from the same soil in the field. The highest rate I observed was 36 nmoles $N_2O.bag^{-1} \cdot min^{-1}$, from the high NO_3^- Brookston with orchardgrass between 6½ and 8 hours after saturation. This is extrapolated to 4 kg $N \cdot ha^{-1} \cdot day^{-1}$. Therefore, large quantities of N may be denitrified during a brief period after irrigation or a rainstorm.

When NO_3^- concentrations were high, my results conformed to the prevailing opinion that roots increase denitrification rate. This was true of the Miami soil with orchardgrass, the Brookston soil with corn, and the Brookston soil with orchardgrass. Although I observed higher rates with orchardgrass than with corn, this may be due simply to the longer growth period of the orchardgrass. These methods will make it possible to examine differences between plant species or varieties. In one anaerobic assay not presented here, there were no significant differences between the anaerobic phase II (potential) rates of Miami soil from plots of corn, orchardgrass, or alfalfa.

Stefanson (1972a) observed a consistent increase in denitrification by planted soils only near field capacity. However, plant growth was greatly reduced by long-term exposure to saturated or near-saturated conditions. My short-term assays demonstrate that plants may also stimulate denitrification during the brief but critical period after soil is saturated by rain or irrigation.

The results provide some indications about the mechanism by which roots may increase denitrification rates. First, an increase in number of denitrifiers was observed in planted soils. Second, although both planted and unplanted soils were shown to be carbon-limited under

totally anaerobic conditions when NO_3^- was non-limiting, carbon amendment decreased the difference in rate between them. This suggests that part of the difference is due to a lower supply of energy source in the unplanted soils. The planted soils denitrified no more than 4 times as fast as unplanted soils in the anaerobic assays but 4 to 28 times as fast in the aerobic gas bags. This is indirect evidence for the involvement of a third factor, reduced O_2 concentration in the rhizosphere of intact soils due to increased oxidation of available organic matter. All of these mechanisms have been considered previously (Stefanson, 1972a; Woldendorp, 1962). I would also suggest another possibility, that mass flow of NO_3^- to the rhizosphere in the transpiration stream increases the supply of electron acceptor to the denitrifiers.

In spite of these considerations, I believe that denitrification will actually be reduced by the presence of roots under some conditions. This is supported by the rapid depletion of NO_3^- by the rhizosphere soil in the anaerobic assay; less NO_3^- was available to the denitrifiers. Concentrations of NO_3^- were consistently lower in the planted soils. Most convincing are the very low denitrification rates observed in the aerobic assays with the low NO_3^-, planted soils. More NO_3^- may have been depleted from these soils by enhanced denitrification prior to the assay. It seems more likely that plant uptake was the important NO_3^- sink. Therefore, competition between plants and denitrifiers is believed to be of great importance in determining the fate of soil NO_3^-. The long-term result of this competition is probably best determined by long-term studies, preferably with ^{15}N, rather than the 8 to 10 hour experiments I have used. It is

interesting in this regard that when Stefanson (1972b) amended soil with NH_4^+-N, rather than NO_3^--N long-term denitrification losses were in some cases significantly greater from soils without plants.

Nitrate concentration was thus seen to interact in a complex manner with the presence of roots. When roots were present, presumably meaning that energy supply was not the primary limitation, increased NO_3^- increased the denitrification rate. This relationship has been observed by others (Starr and Parlange, 1975; Stanford et al., 1975b). When NO_3^- was not limiting, the plants caused a consistent rate increase. In the absence of roots the rate was limited by carbon and no simple relationship between rate and NO_3^- concentration could be established. High NO_3^- did cause a reproducible decrease in the evolution of N gas from the unplanted Brookston soil. It is possible that in this instance the reduction of NO_3^- to NO_2^- was able to satisfy much of the small requirement for electron acceptor. This hypothesis is consistent with observations that pure cultures of some denitrifiers accumulate NO_2^- before producing N gas and that NO_3^- inhibits reduction of NO to N_2O and N_2 (Payne and Riley, 1969).

The concentration of NO_3^- did affect the percentage of gas evolved as N_2O, as determined by a comparison of uninhibited and C_2H_2 inhibited soils in the aerobic gasbags. In every case, a greater percentage appeared as N_2O in the high NO_3^- treatments. This agrees with our earlier results (chapter 2 and Firestone et al., 1977). It is believed that NO_3^- or NO_2^- may directly inhibit the reduction of N_2O to N_2 or alternatively, that the reduction of NO_3^- to NO_2^- and N_2O competes for electrons with the reduction of N_2O.

I expected that the presence of plants might also be related to

the ratio of $N_2O/(N_2 + N_2O)$. It was believed that the increased energy supply in planted soils would increase the demand for electron acceptor and drive the denitrification reactions more towards completion. This was, in fact, observed in the anaerobic experiments and in the high NO_3^- Brookston soil assayed aerobically. However, the ratio was slightly lower for the unplanted soils in the other aerobic experiments.

The large variation I have observed in the ratio of $N_2O/(N_2 + N_2O)$ is noteworthy. From half to none of the gas was evolved as N_2O. This emphasizes that current estimates of N_2O flux to the atmosphere must be considered tentative and subject to large errors. The ratio of N_2O to N_2 is a critical value in determining the effect of increased fertilizer use on stratospheric ozone stability (Johnston, 1977).

In summary, the total effect of the rhizosphere on denitrification depends on the concentration of NO_3^- and probably on soil conditions and stage of plant growth. The NO_3^- concentration and the presence of roots interact; simple independent relationships between these variables and the denitrification rate or the ratio of $N_2O/(N_2 + N_2O)$ should not be expected. The potential for denitrification is clearly greater in the rhizosphere. At high NO_3^- concentrations this potential will be reflected in the actual denitrification rate. However, when NO_3^- is limiting plant uptake will compete with denitrifiers for NO_3^-, thus, denitrification can actually be reduced in the rhizosphere.

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APPENDICES

APPENDIX A

ENUMERATION OF SOIL DENITRIFIERS

Several published methods of enumerating soil bacteria and original modifications of these methods have been evaluated. This is a summary of research conducted by Bill and Nancy Caskey and myself, to be published in full at a later date. Although numbers of denitrifiers cannot be directly related to soil denitrification rate (because denitrifiers can also grow by respiration of O_2) enumeration of denitrifiers is often of interest as related to other measurements of soil denitrification. Difficulty in our laboratory with commonly used methods led to the initiation of this study.

The most widely used method of determining numbers of soil denitrifiers is probably that of Focht and Joseph (1973). They used an MPN procedure with 9.9 mM NO_3^- in nutrient broth. We compared MPNs determined in this manner to MPNs determined in nutrient broth but with 3.5 mM NO_3^-. Table 14 shows that decreasing the NO_3^- concentration increased the recovery of denitrifiers. It is believed that the smaller quantities of NO_3^- were more rapidly and more completely reduced by denitrifiers and observation of their activity was less affected by competition or antagonism by anaerobes.

Volz (1977) has suggested that NO_2^- is a more specific electron acceptor for denitrifiers than is NO_3^- and should therefore be used in MPN procedures for these microorganisms. We encountered numerous difficulties wtih this approach, due in part to the apparent production of inhibitory products when NO_2^- is autoclaved in the closed

Hungate tubes we used. An additional problem with Volz's method is that the high concentrations of NO_2^- which he used (Table 15). In the experiments shown NO_2^- was sterilized separately and added to the tubes after autoclaving. In this case, 3.5 mM NO_2^- and 3.5 mM NO_3^- gave MPNs which were not consistently or significantly different. The MPNs were considerably reduced in 7.2 mM NO_2^- which was the lowest concentration used by Volz.

Table 16 shows the MPNs obtained when soil dilutions were inoculated into initially aerobic, but closed Hungate tubes, compared to truly anaerobic tubes. MPNs were significantly reduced in the anaerobic tubes. Thioglycollate (0.5%) was used as a reducing agent in these experiments. This concentration did not have a significant inhibitory effect on the growth of denitrifiers in pure culture. Furthermore, the same effect was observed in anaerobic tubes using titanium citrate as a reductant and in tubes, without a reductant, made anaerobic by flushing and evacuating with He. This result suggests that many soil denitrifiers are not able to grow when switched abruptly from aerobic to anaerobic conditions, presumably because no mechanism of energy generation is available for the synthesis of denitrifying enzymes. Denitrifiers which are already partially derepressed for denitrification would, of course, be able to continue growth when switched to total anaerobiosis.

MPNs obtained using tryptic soy broth were not significantly different from those determined with nutrient broth. Tryptic soy broth has been said to be the equivalent of soil extract media in the recovery of maximum numbers of aerobes from soil (Martin, 1975).

Patriquin and Knowles (1974) have suggested that the presence

of N_2O be used a test for the presence of denitrifiers rather than the disappearance of NO_3^- and NO_2^-. Using this approach, we found that some tubes from which all of the NO_3^- and NO_2^- had disappeared did not contain detectable amounts of N_2O. This problem could be eliminated by adding C_2H_2 to prevent the reduction of N_2O to N_2. Under these conditions, significantly higher MPNs were determined by N_2O detection than by absence of NO_3^- and NO_2^- (Table 17). That is, many more tubes showed production of N_2O than complete reduction of NO_3^- and NO_2^-. However, the validity of this approach was made questionable by the observation that NO_3^--respiring organisms such as E. coli, not normally associated with denitrification, produce significant amounts of N_2O. This reaction appears to be too slow, however, to completely reduce all NO_2^- during an MPN incubation period of 1or 2 weeks (Table 18). Because the significance of N_2O production by these bacteria in soil is unknown, N_2O production was rejected as a means of detecting the presence of denitrifiers.

Evolution of N_2O by E. coli appears to be dependent on enzyme activity. Autoclaved cell suspensions do not evolve N_2O from NO_3^- or NO_2^- (Table 18). N_2O is produced only near the end of log phase growth and production is terminated by the addition of arsenite, suggesting that chemical decomposition of accumulated NO_2^- is not responsible (data not shown).

Recovery of denitrifiers was further evaluated by adding a rifampicin resistant (see Appendix B) strain of Pseudomonas fluorescens to soil. After 15 min, recovery from the soil was determined by inoculating soil dilutions to MPN tubes containing 3.5 mM

NO_3^-, nutrient broth, and 50 µg/ml rifampicin. Dilutions of the inoculum were also plated directly onto rifampicin-containing agar. The number recovered from the soil was not significantly different from the number determined to be in the inoculum.

We conclude that 3.5 mM NO_3^- or NO_2^- with initially aerobic conditions gives excellent recovery of soil denitrifiers and is the best available method for enumeration of them.

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Table 14. Effect of NO_3^- concentration on MPN of denitrifiers.

Soil	Nitrate in nutrient broth	
	3.5 mM (7 days)*	9.9 mM (14 days)
	-log denitrifiers·g^{-1}-	
Miami A	6.28	4.65
Miami B	4.72	3.87
Miami C	5.85	3.54
Miami D	5.54	5.00
Brookston	3.49	2.83
Kranzburg	3.34	2.91
Conover	4.85	3.04

* Incubation time, temperature was 30 C.

Table 15. Comparison of MPNs obtained in low NO_3^-, low NO_2^-, and high NO_2^-.

Medium*	Soil			
	Brookston A	Brookston B	Miami	Conover
	-log denitrifiers·g^{-1}-			
3.5 mM NO_3^-	4.52	6.45	3.65	4.85
3.5 mM NO_2^-	4.66	6.34	4.88	4.45
7.2 mM NO_2^-	4.23	-	3.08	3.92

* All in nutrient broth, 7 day incubations at 30 C.

Table 16. Effect of initial aeration state of Hungate tubes on MPNs of denitrifiers.*

Soil	Initially aerobic	Initially anaerobic
	-log denitrifiers·g soil^{-1}-	
Miami	3.65	3.23
Brookston	4.52	3.34
Conover	4.85	3.69

* 0.5% thioglycollate as reductant, 3.5 mM NO_3^-, incubated 7 days at 30 C.

Table 17. Comparison of two tests for the presence of denitrifiers in MPN tubes.*

Soil	Disappearance of NO_3^- and NO_2^-	Presence of N_2O#
	-log denitrifiers.g^{-1}-	
Miami	4.15	5.84
Brookston	3.11	6.15
Kranzburg	2.65	5.23

* 9.9 mM NO_3^- broth, tubes read after 7 days.

Detection limit approximately 50 ppm (v/v).

Table 18. Production of N_2O by bacteria not commonly believed to be denitrifiers.

Additions to nutrient broth	%N recovered as N_2O after 7 days
NO_3^- #	0*
NO_2^-	0
autoclaved soil + NO_2^-	0
autoclaved *E. coli* + NO_2^-	0
E. coli	0
E. coli + NO_3^- (6.7)†	8.4
E. coli + NO_2^-	13.2
S. typhimurium + NO_3^- (6.5)	13.3
K. aerogenes + NO_3^- (6.4)	3.3

3.5 mM NO_3^- and NO_2^-

* Detection limit about 1 ppm (v/v)

† final pH of medium.

APPENDIX B

A RIFAMPICIN RESISTANCE MARKER TO STUDY SOIL DENITRIFIERS

I have begun to investigate the feasibility of using a rifampicin resistance marker to study the ecology of soil denitrifiers. The presumed advantages of this approach are that the marker would allow selective recovery of a specific denitrifier from soil. Rifampicin is suited for this application because it has little or no clinical usefulness and rifampicin resistance has generally been found to be a stable trait. Rifampicin inhibits DNA-dependent RNA synthesis in prokaryotes. (Wehrli and Staehelin, 1971; Weller and Saettler, 1978.)

Naturally occurring resistant strains have been isolated from pure cultures of the soil denitrifiers, Pseudomonas fluorescens* and P. aureofaciens*. With 50 µg/ml rifampicin (Rifampin B, Calbiochem, San Diego, Ca.) the frequency of resistance was $1/1.8 \times 10^8$, in agreement with published values for other bacterial genera. Resistance was determined to be a stable trait by repeated transfers of the resistant strains in rifampicin-free media, followed by determination of frequency of resistance. After 10 transfers the number of cells capable of growth on rifampicin plates was 70 to 100% of the number on rifampicin-free plates, i.e., no significant difference.

The background of rifampicin resistant bacteria in soil was low; less than 10^2/g under anaerobic conditions†, less than 10^3/g under aerobic conditions. Furthermore, these naturally occurring resistant organisms grew very slowly in the presence of rifampicin and could almost always be distinguished from the selected strain on the basis of colony size and morphology. It was necessary to include 50 µg/ml cycloheximide in the media to prevent the growth of soil fungi.

A preliminary experiment suggested that this technique would indeed be useful. A rifampicin resistant strain of P. fluorescens was added to soil at a density of 10^4 cells/g. The soil was saturated with water and after 6 days dilutions of the soil were plated on NO_3^- agar containing rifampicin and cycloheximide. The plates were incubated anaerobically. P. fluorescens had increased to a density of 3.3×10^4/g of soil.

Another experiment involving the use of a rifampicin resistant denitrifier has been described in Appendix A. These experiments indicate that the use of a rifampicin resistance marker will make it possible to precisely determine small changes in population size of a specific microorganism under natural soil conditions.

Some results of these preliminary experiments are summarized in the table on the following page.

LITERATURE CITED

1. Wehrli, W. and M. Strehelin. 1971. Action of the rifamycins. Bacteriological Reviews 35(3):290-309.

2. Weller, D. M. and A. W. Saettler. 1978. Rifampin-resistant Xanthomonas phaseoli var. fuscans and Xanthomonas phaseoli: Tools for field study of bean blight bacteria. Phytopathology (in press).

* P. fluorescens strain number 72, P. aureofaciens strain number 59.

† Anaerobic incubations in glove box with NO_3^- agar.

Table 19. Summary of results with rifampicin resistant denitrifiers.

Frequency of resistant organisms in culture of *P. fluorescens*	$1/1.8 \times 10^8$
Stability of resistance, frequency of resistance after 10 transfers	70 to 100%
Naturally occurring resistance, background in soil	
aerobes	$\leq 10^3$ cells/g
anaerobes + denitrifiers	$\leq 10^2$ cells/g
Estimated minimum detectable frequency of an introduced resistant denitrifier in soil	10^3 cells/g