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dissertation entitled

NITROGEN CYCLING IN SOILS:
SIMULTANEOUS ESTIMATION OF TRANSFORMATION RATES,
DIFFUSIONAL CONTROL OF DENITRIFICATION, AND
ESTABLISHMENT OF DENITRIFICATION CAPACITY

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Soil Microbiology

Major professor

Date 6 February 1984

NITROGEN CYCLING IN SOILS: SIMULTANEOUS ESTIMATION OF TRANSFORMATION RATES, DIFFUSIONAL CONTROL OF DENITRIFICATION, AND ESTABLISHMENT OF DENITRIFICATION CAPACITY

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSPOPHY

Department of Microbiology and Public Health

1984

ABSTRACT

NITROGEN CYCLING IN SOILS:
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Rates of mineralization, immobilization, nitrification, and denitrification were simultaneously estimated in three dissimilar soils. The estimation process included elements of mathematical modeling, nonlinear parameter estimation, and the use of $^{15}NH_4^+$ as a tracer. Analysis of the sensitivity coefficients showed that good estimates of the mineralization, immobilization, and nitrification parameters could be obtained, but denitrification parameters could not be as well defined. The results suggested that biomass N estimated by the $CHCl_3$ fumigation method is the major component of the active organic N pool over short time periods (< 3 weeks). N cycling in forest soil studied was best fit with zero order kinetics. In this soil, mineralization and immobilization were the dominant processes, with rates of 1.0 and 0.67 $\mu g\ N\ g^{-1}\ d^{-1}$, respectively. Two agricultural soils were used, one high in organic matter, the other low in organic matter. In both agricultural soils, N cycling was best described with the first order model. nitrification, which had a rate constant of 1.3 d^{-1}. Nitrification was also rapid in the low organic matter soil (1.6 d^{-1}), however, this soil also had a high rate of immobilization (1.7 d^{-1}).

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A model of NO_3^- reduction and diffusion was developed and used along with the Thiele modulus (a dimensionless parameter) to determine the conditions under which denitrification would and would not be limited by NO_3^- diffusion. Results from this exercise suggest that, under anaerobic conditions, only aggregates greater than 0.2 cm will experience a NO_3^- diffusional limitation. In aerobic soils, only large aggregates have anaerobic centers, and under these conditions NO_3^- diffusional limitations are more likely. Experimental results with a clay loam soil showed no effect of a NO_3^- diffusional limitation which was in agreement with model predictions.

Experiments were conducted to determine the effect of carbon, water, and NO_3^- additions on the development of denitrification capacity in soil. There was no effect of either NO_3^- or water additions on denitrification capacity. However, added carbon caused a significant increase in denitrification capacity. This response to added carbon was paralleled by a similar increase in microbial ATP. These results suggest that the increase in denitrification capacity was due to a proportionate increase in denitrifier and non-denitrifier biomass.

To Jackie and Kirk

and

in memory of

Herman Edwin Myrold

ACKNOWLEDGEMENTS

Many individuals have added depth and richness to my four and one half year experience at Michigan State. I thank the members of my graduate committee for their support: John Breznak and Frank Dazzo for helping me learn some microbiology; Erik Goodman for introducing me to the mathematical aspects of biology; Peter Vitousek for giving me an ecosystem perspective of microbial activity.

The interactions with the other members of Jim Tiedje's lab are treasured experiences. I learned and benefited from all those I worked with and hope that they may have gained some things from me. In particular, I thank Joe Robinson, who was an excellent sounding board for my mathematical ideas and whose work encouraged me to continue pursuing parameter estimation problems. I also thank Alan Sexstone, who was never too busy to talk about ^{15}N, denitrification, or other less scientific (but perhaps more important) subjects. I hope that the friendships started here will last a lifetime.

My deepest gratitude goes to Jim Tiedje, who I admire greatly. I thank him for allowing me to devise and pursue my own research plans--even when they may not have been particularly germane to the denitrification project and especially when the initial results did not look very promising. His dedication, incisive thinking, concern for others, and relationship with his family will always serve as a model for me.

Perhaps my greatest thanks goes to my family for their love and support. I am grateful to my mother and father who taught me that all adversities in life can be overcome through love, faith, and patience.

I thank Jackie, my wife, for her unfailing support, encouragement, and love through the rigors, delays, and long hours of graduate study; for sharing the joys and sorrows of this experience with me. I thank my son, Kirk, whose cheerful countenance and laughter helped me remember the truly important things in life.

Lastly, I think it is fitting to acknowledge the inspiration and guidance of the Lord in this work. After reflecting upon the few months it took to bring this dissertation to fruition, I think that my feelings are best summarized by the following passage: "...it is by faith that miracles are wrought..." (Moroni 7:37).

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INTRODUCTION

Nitrogen is a constituent of the nucleic acids which serve as the blueprints for living cells; nitrogen is a component of the enzymes which construct living cells; and nitrogen is a part of the polymers which form the structure of living cells. Indeed, with the exception of carbon and water, nitrogen constitutes the largest fraction of living cells. It is no small wonder that several volumes have been devoted to the physics, chemistry, and biology of nitrogen.

In terrestrial ecosystems, nitrogen is often found to be limiting for plant growth. Consequently, much work in the biological sciences, particularly in agriculture, has been focused on the transformations of nitrogen in nature. In the past few years several books have been published to review what is known about the nitrogen cycle and the dynamics of its transformations (Nielsen and MacDonald, 1978; Clark and Rosswall, 1981; Stevenson, 1982).

One of the most thought-provoking representations of the nitrogen cycle is that given by Jansson and Persson (1982), in which they divide the nitrogen cycle into three subcycles: the elemental, autotrophic, and heterotrophic cycles (Figure 1). The elemental cycle connects the large reservoir of atmospheric N_2 to living organisms through the microbially mediated processes of N_2 fixation and denitrification. The autotrophic cycle involves plant photosynthesis and concomitant assimilation of inorganic nitrogen from the soil solution and the subsequent return of organic nitrogen to the soil. The heterotrophic cycle is dominated by the activities of microorganisms performing the processes of mineralization, immobilization, and nitrification. From

Figure 1. The terrestrial nitrogen cycle. Modified from Jansson and Persson (1982).

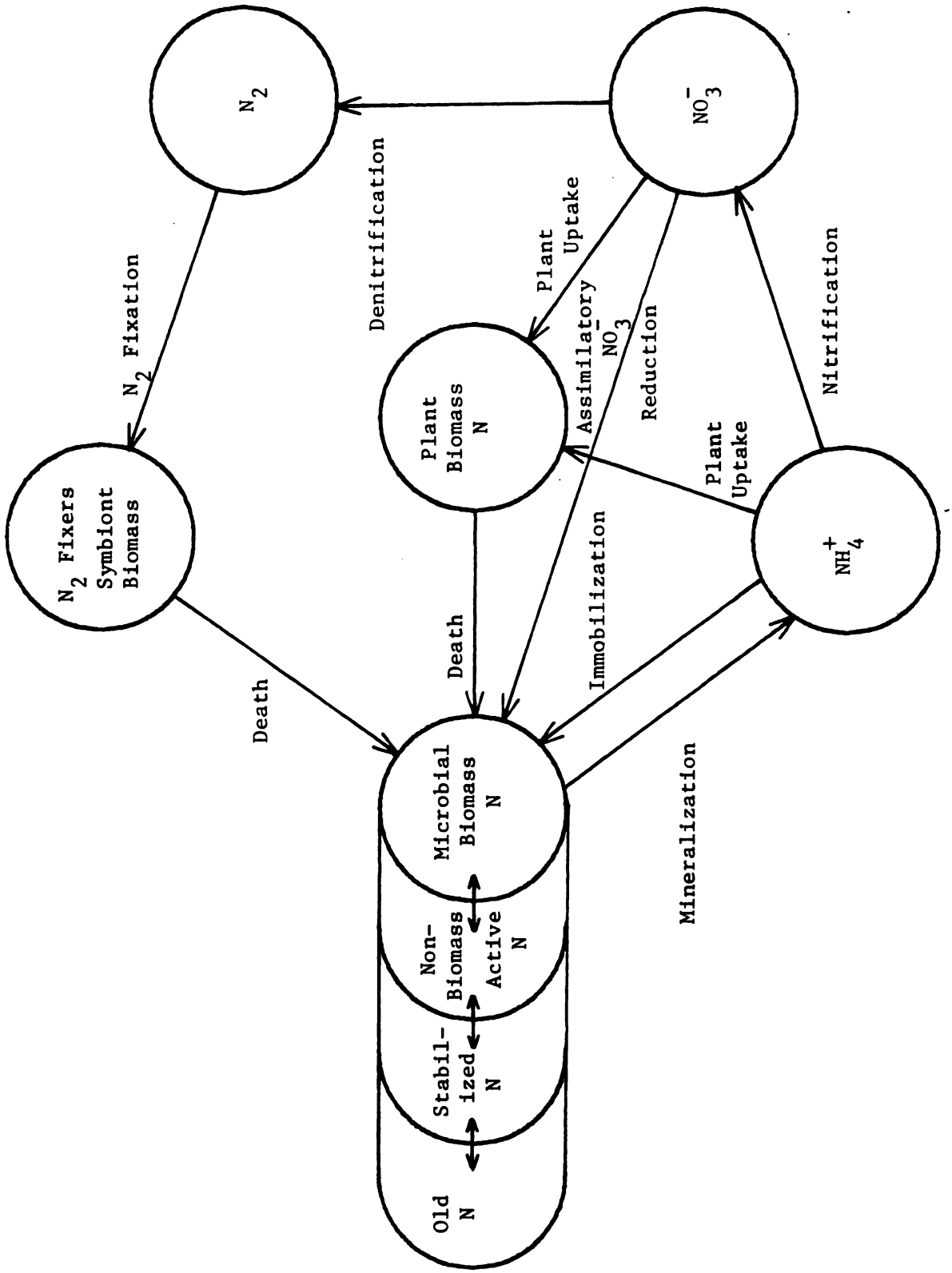
N_2
NO_3^-
N_2 Fixation
Denitrification
Plant Uptake
Assimilatory NO_3^- Reduction
Nitrification
N_2 Fixers Symbiont Biomass
Plant Biomass N
Plant Uptake
NH_4^+
Death
Death
Immobilization
Microbial Biomass N
Non-Biomass Active N
Stabil-ized N
Old N
Mineralization

Figure 1

this conceptual framework it is obvious that the activities of microorganisms are central to the cycling of nitrogen.

There are three distinct pools of nitrogen in soil--organic N, NH_4^+, and NO_3^---all of which are interconnected by microbially mediated processes (Figure 1). This interconnectedness not only provides the redistribution of nitrogen necessary for life, but also contributes to the stability of the system through feedback mechanisms. In conjunction with this feedback phenomenon, two branch points exist which are susceptible to regulation--the NH_4^+ and NO_3^- pools. With this in mind, it is not surprising that the concentrations of NH_4^+ and NO_3^- in soil are usually quite low, at least in comparison to the organic N pool.

Regulation of nitrogen cycling in soil is affected primarily through microbial competition for the various forms of nitrogen, within the constraints of the environment (Rosswall, 1982). The final outcome of this competition is determined by the biomass of the competing populations, their affinities for the substrate competed for, and the amount of substrate available (Tiedje et al., 1982). Each of these factors, in turn, may be modulated by physical or chemical processes. These interactions between the physics and chemistry of the environment and the biology are not likely to be static, but rather dynamic in nature.

In order for the nitrogen cycle and its regulation to be studied as a whole, it is necessary to be able to measure nitrogen cycle processes as an integrated unit. This requires the use of a tracer, which allows one to follow the labelled nitrogen through the circles and along the arrows of the nitrogen cycle. Nitrogen-15 is the tracer of choice for any studies longer than a few tens of minutes and has been used as a

qualitative, or semi-quantitative, indicator for several decades. The interested reader is referred to several excellent reviews which cover the historic uses of ^{15}N in biology and agriculture (Hauck 1973; Hauck and Bremner, 1976; Faust, 1982).

In addition to being able to qualitatively follow the fate and partitioning of nitrogen in the soil system, one would also like to be able to quantitatively measure the fluxes of nitrogen between the various pools of the nitrogen cycle and how rapidly these pools are turned over. Mathematical models or kinetic analyses are generally needed to address this problem. Jansson (1958) did the pioneering work along these lines, applying the simplified model of Kirkham and Bartholomew (1955) to mineralization-immobilization dynamics in soil.

The next major push forward came in the late 1970's when Koike and Hattori (1978) used the principles of isotope dilution to simultaneously measure nitrification and nitrate reduction in marine sediments. Subsequently, this technique has been successfully applied to mineralization and immobilization in sediments (Blackburn, 1979) and water column studies (Gilbert et al., 1982) and to nitrification and nitrate reduction in rice paddy soils (Watanabe etal., 1981). Thus far, the most ambitious application of isotope dilution to nitrogen studies has been the simultaneous measurement of rates of denitrification, dissimilatory NO_3^- reduction to NH_4^+, mineralization, and immobilization in anaerobic soil slurries (Tiedje et al., 1981).

The logical extension of this work seems to be the application of more sophisticated mathematical modeling and rate estimation techniques. One such approach was used by Van Cleve and White (1980) who applied the principles of compartmental analysis (cf., Jacquez, 1972)--which has

long been used in studies of animal metabolism--to nitrogen cycling in soils of the Alaskan bush. Using ^{15}N as tracer, they were able to calculate total fluxes of nitrogen among the organic N, NH_4^+, and NO_3^- pools, as well as the turnover times of these pools. Unfortunately their approach required them to assume that the system they worked with was at a steady state--a situation not likely to occur often in nature. This should not be a limitation, however, since methods of analysis are available to study non-steady state systems. Winkler and Hubner (1979) have applied the principles of nonlinear parameter estimation and ^{15}N labelling to measure protein turnover in plants. In the first chapter of this thesis a similar approach will be used to simultaneously estimate rates of nitrogen cycle processes in soil.

One way in which nitrogen processes are regulated is through the physical process of diffusion, which determines the rate of substrate supply and thus influences the concentration of the substrate that is available to a microorganism. On a very macroscopic scale, this principle has been recognized in the large nitrogen transport and transformation models developed by soil physicists (cf., Tillotson, 1980). Indeed, Reddy et al., (1978 and 1980) have shown the importance of NH_4^+ and NO_3^- diffusion in determining the rate and reaction order of denitrification in flooded soils.

In well drained soils, much of the work on the interaction between the physics of diffusion and microbial activity has focused on oxygen diffusion in aggregates and its effect on microbial activity and the establishment of anaerobic microsites in otherwise aerobic soils (e.g., Greenwood and Goodman, 1964; Smith, 1980). This influence of oxygen diffusion and microbial respiration on denitrification was shown by

Greenwood (1962) for glucose and NO_3^- amended soil crumbs and has recently been demonstrated in natural soil aggregates (Sexstone et al., 1984).

It might be expected that the diffusion of NO_3^- might also play a role in determining rates of denitrification in well drained soils. Indeed, it has often been suggested that the high Km values for NO_3^- reduction measured in soils is an indication of NO_3^- diffusion limitations (cf., Firestone, 1982). The second chapter of this dissertation examines the question of whether or not NO_3^- diffusion is a limiting factor of denitrification in aggregated soils.

The relative size of competing populations is one component in determining the outcome of competition, and hence the regulation of nitrogen cycling. Whether or not a given group of organisms can multiply and survive in soil depends upon their tolerance of adverse environmental conditions and their ability to obtain and utilize substrates needed for growth.

Smith and Tiedje (1979) developed an assay system for quantifying the denitrifying capacity of a soil, which directly reflects the amount of active denitrifier biomass. When this method was used to survey soils from a range of habitats, denitrification capacity was found to be directly related to the moisture regime and carbon content of the soils (Tiedje et al., 1982). Other work has shown that denitrifier populations increase when soils are amended with NO_3^- and incubated anaerobically (Jacobson and Alexander, 1980). These observations led to the final chapter of this dissertation, which examines the effect of water, NO_3^-, and carbon additions on the establishment of denitrification capacity in soil.

To summarize, the work reported in this dissertation is built upon the foundation of the interacting microbial transformations of the nitrogen cycle and the regulation of these transformations. Chapter I focuses on the problems of measuring several of the interconnected nitrogen cycle rates in a single experiment. Using ^{15}N as a tracer and applying procedures long used in engineering and statistics, a method for simultaneously estimating mineralization, immobilization, nitrification, and denitrification rates is given. Chapter II addresses the area of environmental control of microbial activity. Specifically, the potential for NO_3^- diffusion to limit denitrification is studied from both a theoretical and experimental perspective. Finally, Chapter III examines factors which control the magnitude of the denitrification capacity of soil and attempts to elucidate the mechanism of this control. This is an example of how the environment can affect microbial biomass size and thereby influence the cycling of nitrogen.

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CHAPTER 1

SIMULTANEOUS ESTIMATION OF SEVERAL NITROGEN CYCLE RATES: THEORY AND APPLICATION

Nitrogen is constantly replenished throughout the biosphere by the interconnected transformations which constitute the N cycle. It is this cyclic quality of N transformations which make them both ecologically beneficial and difficult to investigate. Most of the research on N in soils has focused on the activity of a single process and factors which affect its activity. Considerably less effort has been expended to examine the interactions among several N cycle processes and effects of environmental perturbations on these interacting transformations. In order to examine N transformations as an interacting unit--or even to measure gross rates of N cycle processes--it is necessary to trace N through the various compartments of the N cycle. This can be done by using ^{15}N.

Research using ^{15}N to measure the dynamics of N cycling can be partitioned into three, somewhat overlapping categories: (1) ^{15}N as a tracer, (2) isotope dilution experiments using ^{15}N, and (3) the application of mathematical models to ^{15}N dynamics. Most frequently ^{15}N has been used as a tracer. This application generally involves the addition of ^{15}N labeled NH_4^+ or NO_3^- (or $^{15}N_2$ in N_2 fixation work) and subsequent measurement of the ^{15}N content of the soil organic and inorganic N and plant N. In fertilizer recovery and N balance

experiments this change in ^{15}N is usually measured over the course of one growing season (e.g. Carter et al., 1967). It can also be used to measure N cycling over shorter time periods in the laboratory (Jansson, 1958; Ross et al., 1964; Jones and Richards, 1977 and 1978). These types of experiments are useful in determining the relative fates and partitioning of added ^{15}N, but provide only a qualitative estimate of process rates.

Isotope dilution experiments involve the addition of ^{15}N into a product pool. The subsequent dilution of the atom % ^{15}N in this pool by natural abundance N from a precursor pool is monitored over time. This principle, with numerous modifications, has been successfully used to study nitrification and nitrate reduction in sediments (Koike and Hattori, 1978; Nishio et al., 1983) and mineralization and immobilization in sediments (Blackburn, 1979) and water columns (Glibert et al., 1982). Nitrification and NO_3^- reduction have been measured by isotope dilution in rice paddy soils (Watanabe et al., 1981) and Tiedje et al. (1981) used $^{15}NH_4^+$ and $^{15}NO_3^-$ in a double labeling experiment to simultaneously measure rates of denitrification, dissimilatory NO_3^- reduction to NH_4^+, mineralization, and immobilization in anaerobic soil slurries. The isotope dilution method is good for simultaneously measuring short term rates of N cycle processes, but assumes that the process rates are constant over each time interval. The rate estimates are also quite sensitive to the data variability since differences in the data are taken, which is an error-amplifying process.

Models of the N cycle vary greatly in complexity. Different models have been used to fit experimental data (e.g., Mehran and Tanji, 1974) and to estimated rate constants for N cycle processes (Cameron and

Kowalenko, 1976), illustrating that no uniquely correct model for N cycling exists. Incorporating a ^{15}N label into experiments of N cycle dynamics should greatly enhance the estimation of N transformation rates and their corresponding kinetic rate constants by enabling gross rates of opposing reactions to be measured and also by allowing the separation of the organic N pool into reactive and unreactive fractions (Jansson, 1958; Juma and Paul, 1981).

Using ^{15}N along with total pool sizes in mathematical N cycle models was initiated by Kirkham and Bartholomew (1955) who examined mineralization and immobilization in a closed, two compartment system under steady state conditions. Jansson (1958) successfully applied their technique to mineralization-immobilization dynamics in soils receiving various organic amendments. The steady state condition has also been assumed by Van Cleve and White (1980) for a field study on N dynamics in a forest ecosystem. They applied the principles of compartmental analysis (cf., Jacquez, 1972) to their data and were able to determine total fluxes of N between the NH_4^+, $NO3^-$, and organic N pools and the turnover times of these pools. Under many (perhaps most) circumstances in nature the N cycle is not at a steady state; pool sizes are constantly changing, negating the usefulness of the steady state approach. Analysis of tracer data under non-steady state conditions is more difficult, but can be done by means of nonlinear parameter estimation. Winkler and Hubner (1977) have applied this method, along with ^{15}N labeling, to measure protein turnover in bean plants.

The application of mathematical modeling and nonlinear parameter estimation techniques to N cycling in soils would allow rates of several N cycle processes to be estimated simultaneously. Such an approach,

however, requires the assumption of an underlying kinetic mechanism for the N transformations occurring in the N cycle.

In this paper we use ^{15}N as a tracer in several soils and test the usefulness of nonlinear parameter estimation and mathematical modeling to simultaneously estimate mineralization, immobilization, nitrification, and denitrification rates in soil. We also examine the importance of heterotrophic nitrification in a forest soil and the effect of a C addition on the relative rates of immobilization and nitrification.

MODEL DESCRIPTION

The structure of the nitrogen cycle makes it amenable to description as a compartmental system (Figure 1). The compartments are the pools of chemically or biologically distinct forms of nitrogen and the flows among these pools are the rates of the various nitrogen cycle processes.

In our work we were primarily interested in N mineralization, immobilization, nitrification, and denitrification, since these are generally the dominant processes in unvegetated soils. The process of heterotrophic nitrification is included in Figure 1, since it appeared to be comparatively large in one soil studied. The organic N pool was divided into two components--the passive and active fractions--according to the work of Jansson (1958). It was assumed that flow between these two organic fractions, or between the passive fraction and inorganic N pools, would be insignificant over the relatively short time span of our experiments (less than three weeks).

The compartmental model shown in Figure 1 is described by the following differential equations, which can be derived from the mass balance of total N ($^{14}N + {}^{15}N$) and ^{15}N for each pool.

$$\frac{dY_1}{dt} = f_1 - (f_2 + f_3) \qquad [1]$$

$$\frac{dY_2}{dt} = f_3 + f_5 - f_4 \qquad [2]$$

$$\frac{dY_3}{dt} = f_2 - (f_1 + f_5) \qquad [3]$$

Figure 1. Compartmental model of the N cycle.

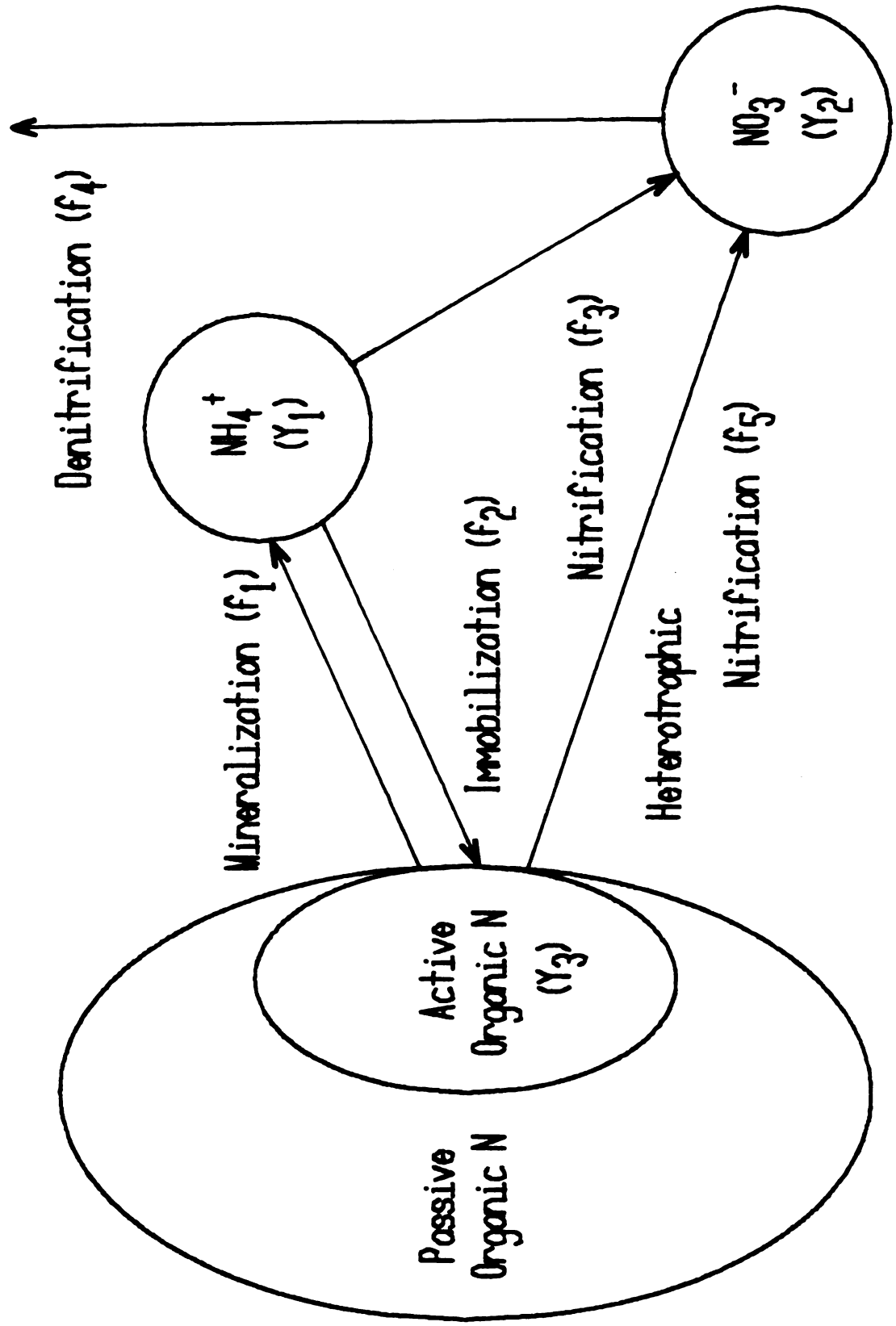
Denitrification (f_4)
NH_4^+ (Y_1)
NO_3^- (Y_2)
Mineralization (f_1)
Immobilization (f_2)
Nitrification (f_3)
Heterotrophic Nitrification (f_5)
Active Organic N (Y_3)
Passive Organic N

Figure 1

$$\frac{dy_1}{dt} = A_3 f_1 - A_1(f_2 + f_3) \qquad [4]$$

$$\frac{dy_2}{dt} = A_1 f_3 + A_3 f_5 - A_2 f_4 \qquad [5]$$

$$\frac{dy_3}{dt} = A_1 f_2 - A_3(f_1 + f_5) \qquad [6]$$

In these equations, Y_i is total N, y_i is ^{15}N, and A_i is the atom % ^{15}N in the i^{th} pool, where i = 1 for NH_4^+-N, i = 2 for NO_3^--N, and i = 3 for active organic N. The reaction rates of the processes, or flows, are designated by f_j where j = 1,2,3,4,5 for mineralization, immobilization, nitrification, denitrification, and heterotrophic nitrification, respectively. No kinetic interpretation has been given here to the reaction rates. However, zero order, first order, and Michaelis-Menten kinetics can all be implemented by simply inserting the appropriate rate equation for the f_j terms.

Since Y_i and A_i are the experimentally measured variables, Equations [1-6] can be combined to form the following:

$$\frac{dA_1}{dt} = \frac{f_1(A_3 - A_1)}{Y_1} \qquad [7]$$

$$\frac{dA_2}{dt} = \frac{f_3(A_1 - A_2)}{Y_2} + \frac{f_5(A_3 - A_2)}{Y_2} \qquad [8]$$

$$\frac{dA_3}{dt} = \frac{f_2(A_1 - A_3)}{Y_3} \qquad [9]$$

Equations [1-3] and [7-9] were solved with a Runge-Kutta integration scheme and used to model the dynamics of nitrogen cycling on the soils used in this experiment. For the work reported in this paper, we examined first and zero order models, which can be thought of as two subsets of Michaelis-Menten kinetics. Conceptually, Michaelis-Menten kinetics should best describe the microbial N transformations, under non-growth conditions. However, incorporation of Michaelis-Menten kinetics has at least two practical limitations: (1) doubling the number of parameters to be estimated--and good estimates of K_m and V_{max} for a single microbial reaction in pure culture are difficult to obtain because they are inherently correlated (Robinson, 1984)--and (2) many other processes, like diffusion, influence reaction rates in soil (Reddy et al., 1978).

MATERIALS AND METHODS

Soils. The feasibility of estimating the rates of several N cycle processes simultaneously was tested using three soils from different habitats, with different physical and chemical properties (Table 1). These soils were collected from the field, sieved to < 2mm, and stored at 4°C until used.

Experiment 1. Onaway loam was amended with 3.4 μg $^{15}NH_4^+$-N g^{-1}soil as 99 atom % $(^{15}NH_4)_2SO_4$. The $^{15}NH_4^+$ was applied to the soil as a fine spray using a syringe with a 22 gauge needle and mixed into the soil to promote even label distribution. The labeled soil was adjusted to a water content of 0.28 g g^{-1} and incubated in Parafilm covered beakers at 20°C. (Several holes were punched in the Parafilm covers to insure aerobic conditions, while minimizing water loss.) Five replicates were sampled at 0, 1/2, 1, 2, 4, 7, 10, 14, and 21 days. Total concentrations and atom % ^{15}N of the NH_4^+ and NO_3^- pools were determined at each sampling time.

Experiment 2. Capac clay loam was amended with either ^{15}N labeled NH_4^+ or NO_3^-. Treatment 1 received approximately 7 μg $^{15}NH_4^+$-N g^{-1} soil (99 atom % $(^{15}NH_4)_2SO_4$) and a corresponding amount of natural abundance NO_3^--N, while treatment 2 received about 7 μg 15 NO_3^--N g^{-1} soil (99.4 atom % $K^{15}NO_3$) and the same amount of natural abundance NH_4^+-N. The label was added as described in experiment 1 and the water content was adjusted to 0.20 g g^{-1}. Soil was packed into plastic cylinders to a bulk density of 1.5 g cm^{-3}, covered with plastic wrap, and incubated at

Table 1. Soil characteristics.

Soil series	Classification	Vegetation	Texture	pH	% Total N
Onaway	Alfic haplorthod	Northern hardwoods	Loam	5.9	0.16
Capac	Aeric ochraqualf	Corn/soybean rotation	Clay loam	6.8	0.28
Spinks	Psammentic hapludalf	Corn/soybean rotation	Sandy loam	6.5	0.053

25°C. Four cores from each treatment were sampled at 0, 1/2, 1, 3, 4, 8, 12, 15, 19, and 22 days for ^{15}N and pool size analysis.

Experiment 3. Preincubated Spinks sandy loam was amended with about 2.5 μg $^{15}NH_4^+$-N g^{-1} soil (99 atom % $(^{15}NH_4)_2SO_4$) and the moisture was adjusted to 0.13 g g^{-1}. In experiments 3, 4, and 5, the ^{15}N label was added using a chromatographic sprayer, which produced a very fine spray which promoted uniformity of label addition, and was then mixed into the soil. The soil was incubated in closed, Erlenmeyer flasks which were aerated daily to prevent anaerobiosis. Five replicate samples were taken at 0, 1/2, 1, 2, 3, 5, and 7 days, extracted for NH_4^+ and NO_3^-, and measured for ^{15}N and inorganic N concentration. Biomass C and N were also measured at each time point by the $CHCl_3$ fumigation method.

Experiment 4. Three treatments of preincubated Onaway loam were set up to evaluate the presence and magnitude of heterotrophic nitrification and the effect of added carbon on the rates of immobilization and nitrification. All treatments received 2.5 μg $^{15}NH_4^+$-N g^{-1} soil as 99 atom % $(^{15}NH_4)_2SO_4$ and were adjusted to a water content of 0.28 g g^{-1}. Treatment 1 served as a control while treatment 2 received finely ground maple leaves at a rate of 3 mg g^{-1} soil--this is equivalent to typical litterfall values for Northern hardwood forests (Nadelhoffer et al., 1983). Treatment 3 received 10 μg nitrapyrin g^{-1} soil; prepared according to the procedure of Bremner et al., (1978). These treatments were incubated and sampled according to the schedule given for experiment 3.

Experiment 5. Preincubated Capac clay loam was used to examine the effect of straw addition on the relative rates immobilization and

nitrification. Both treatments received about 2.5 μg $^{15}NH_4^+$-N g^{-1} soil (99 atom % $(^{15}NH_4)_2SO_4$). Treatment 2 also received finely ground alfalfa straw at the rate of 1 mg straw-C g^{-1} soil. These treatments were incubated and sampled as described in experiment 3.

Analytical procedures. NH_4^+ and NO_3^- were extracted from soil with 2 N KCl at either a 10:1 (experiments 1 and 2) or 5:1 (experiments 3-5) extractant to soil ratio. The 5:1 ratio provided greater sensitivity for the ^{15}N ratio analysis.

Biomass N and C were determined for experiments 3, 4, and 5 on separate subsamples of soil by the $CHCl_3$ fumigation method (Jenkinson and Powlson, 1976). The N flush (N_f) after 10 days of incubation was measured by extracting the accumulated NH_4^+ with 2 N KCl using a 5:1 extractant to soil ratio. The CO_2 flush (C_f) was measured by analyzing the headspace gas on a microthermister equipped GC. A conversion factor, kc, of 0.41 (Anderson and Domsch, 1978) was used to estimate biomass C and the nitrogen conversion factor, $kn = 0.39 - 0.014(C_f/N_f)$, of Voroney and Paul (1984) was used to estimate biomass N.

NH_4^+, NO_3^-, and biomass N (as NH_4^+) were prepared for mass spectrometer analysis using steam distillation as described by Bremner (1965). ^{15}N ratio measurements were made using a Micromass 602 isotope ratio mass spectrometer. Concentrations of NH_4^+ and NO_3^- (after conversion to NH_4^+) were measured using the Solorzano (1969) method (experiments 1 and 2) or using a Technicon autoanalyzer (experiments 3, 4, and 5).

Model Evaluation and Parameter Estimation. To obtain unique parameter estimates it is necessary that the sensitivity coefficients be linearly independent (Beck and Arnold, 1977). A sensitivity coefficient

is defined as the first derivative of a measured variable with respect to a model parameter (e.g., $\partial Y_i/\partial f_i$ or $\partial A_i/\partial f_i$). Sensitivity coefficients are linearly dependent when they are a constant multiple of one another. The degree of linear independence among the sensitivity coefficients can be assessed by plotting them and examining their relationships. Plotting sensitivity coefficients also yields information about optimal experimental design, which parameters the model is most sensitive to, and how well the parameters will be determined. When a sensitivity coefficient is large in absolute value, the respective measured response contains much information about the parameter, while a sensitivity coefficient of zero contains no information about the parameter. Consequently, the best experimental design concentrates measurements during the time when sensitivity coefficients are large in absolute value.

Sensitivity coefficients for both a zero order and first order model were calculated using parameter values close to those expected for the soils used in this study. In addition to the sensitivity coefficients for the rate parameters, the sensitivity coefficient for $Y_3(0)$--the initial concentration of N in the active fraction--was calculated. These sensitivity coefficients were determined for the four normally measured responses: the concentration and atom % ^{15}N of the NH_4^+ and NO_3^- pools.

Rates or rate constants of the N cycle processes were estimated using a nonlinear regression technique. A Gauss minimization method (Beck and Arnold, 1977) was used in conjunction with the interpolation-extrapolation step size routine of Bard (1974). Inequality constraints in the form of penalty functions were used to

insure that only reasonable (non-negative) parameter estimates were obtained (Bard, 1974). The sensitivity coefficients were obtained using the finite difference method suggested by Beck and Arnold (1977), and the model equations were integrated using a Runge-Kutta technique. The objective function (i.e., residual sum of squares) was minimized according to the least squares criterion.

The nonlinear parameter estimation program was written in BASIC and implemented on a microcomputer. In addition to calculating parameter estimates, the program also calculated the parameter correlation matrix and provided approximate 95% confidence intervals for the parameters.

RESULTS AND DISCUSSION

Model Evaluation. Sensitivity coefficients for the zero order model are shown in Figures 2-5. The sensitivity coefficients for the mineralization, immobilization, and nitrification rates change linearly with time and are therefore linearly dependent, while all other sensitivity coefficients are zero when measuring the NH_4^+ pool (Figure 2). If the NH_4^+ pool is the only response measured, the mineralization, immobilization, and nitrification rates could not be uniquely identified. As one would intuitively expect, only a net rate could be obtained. When the sensitivity coefficients for the atom % $^{15}NH_4^+$ response is examined, the mineralization rate is no longer linearly dependent with respect to the immobilization and nitrification rates, thus it is uniquely determined (Figure 3). However, the immobilization and nitrification rates are still almost linearly related. Adding the NO_3^- response allows one to uniquely estimate the immobilization and nitrification rates, since the sensitivity coefficient for immobilization is zero (Figure 4) rather that approximately twice that of the nitrification sensitivity coefficient as it was in the previously described responses (Figures 2 and 3). The final response--atom % ^{15}N enhances the linear independency among the immobilization, mineralization, and nitrification rates (Figure 5). Close examination of Figures 2-5 will show that the denitrification rate is also uniquely determined. However, since its sensitivity coefficients are all small (< 5%) compared to the others, the denitrification rate will be poorly determined. This is caused, at

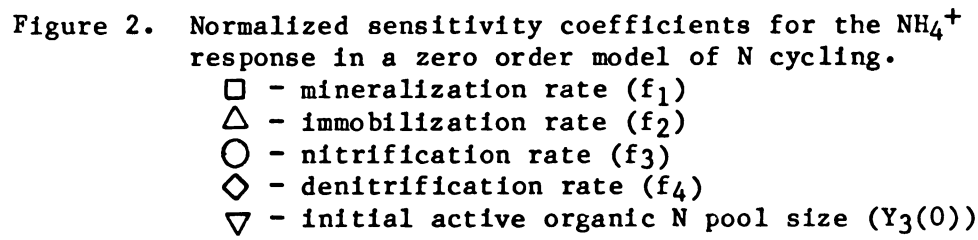
Figure 2. Normalized sensitivity coefficients for the NH_4^+ response in a zero order model of N cycling.
□ - mineralization rate (f_1)
△ - immobilization rate (f_2)
○ - nitrification rate (f_3)
◇ - denitrification rate (f_4)
▽ - initial active organic N pool size ($Y_3(0)$)

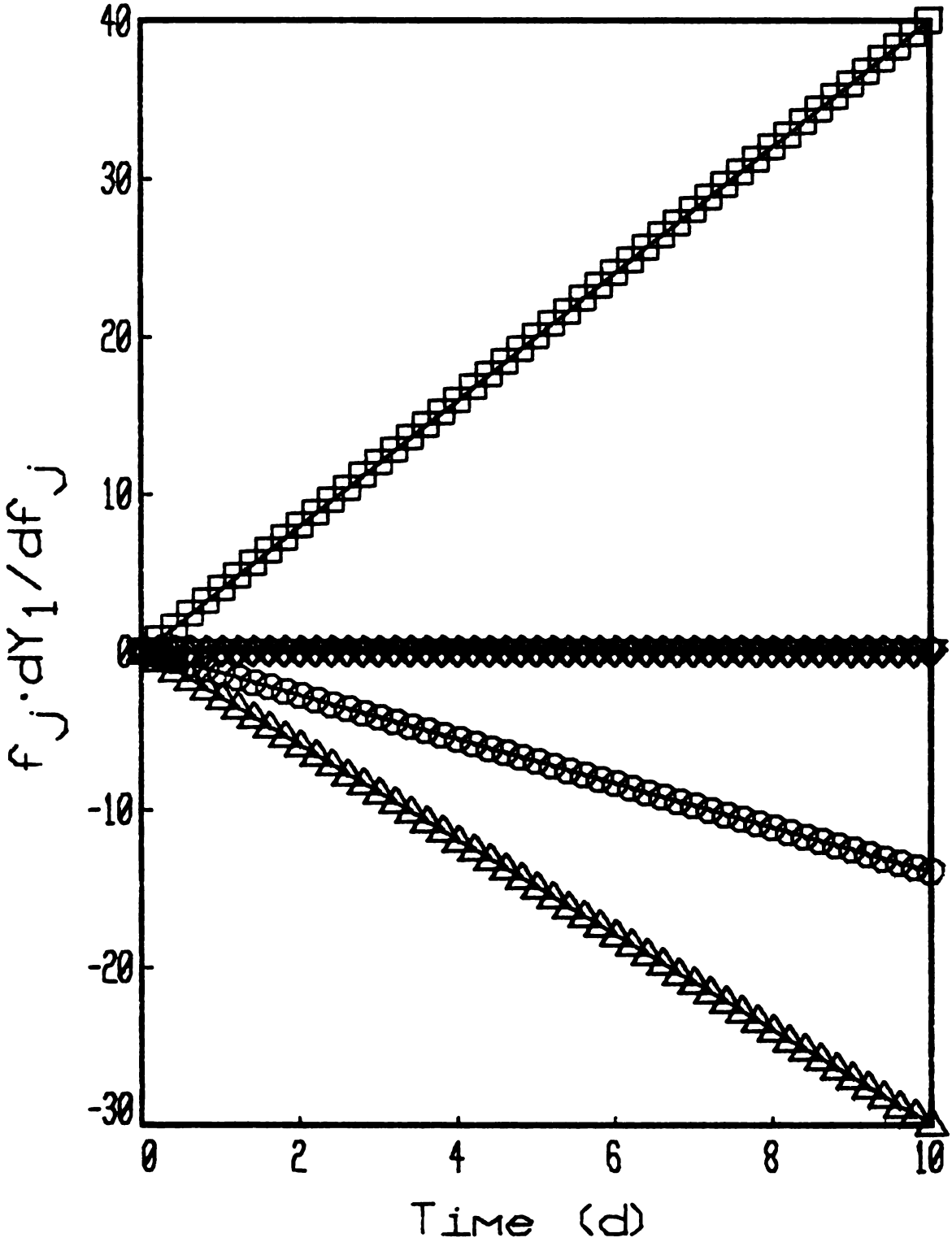
40
30
20
10
0
-10
-20
-30
0
2
4
6
8
10
Time (d)
$f_j \cdot dY_1/df_j$

Figure 3. Normalized sensitivity coefficients for the atom % $^{15}NH_4^+$ response in a zero order model of N cycling. Symbols as in Figure 2.

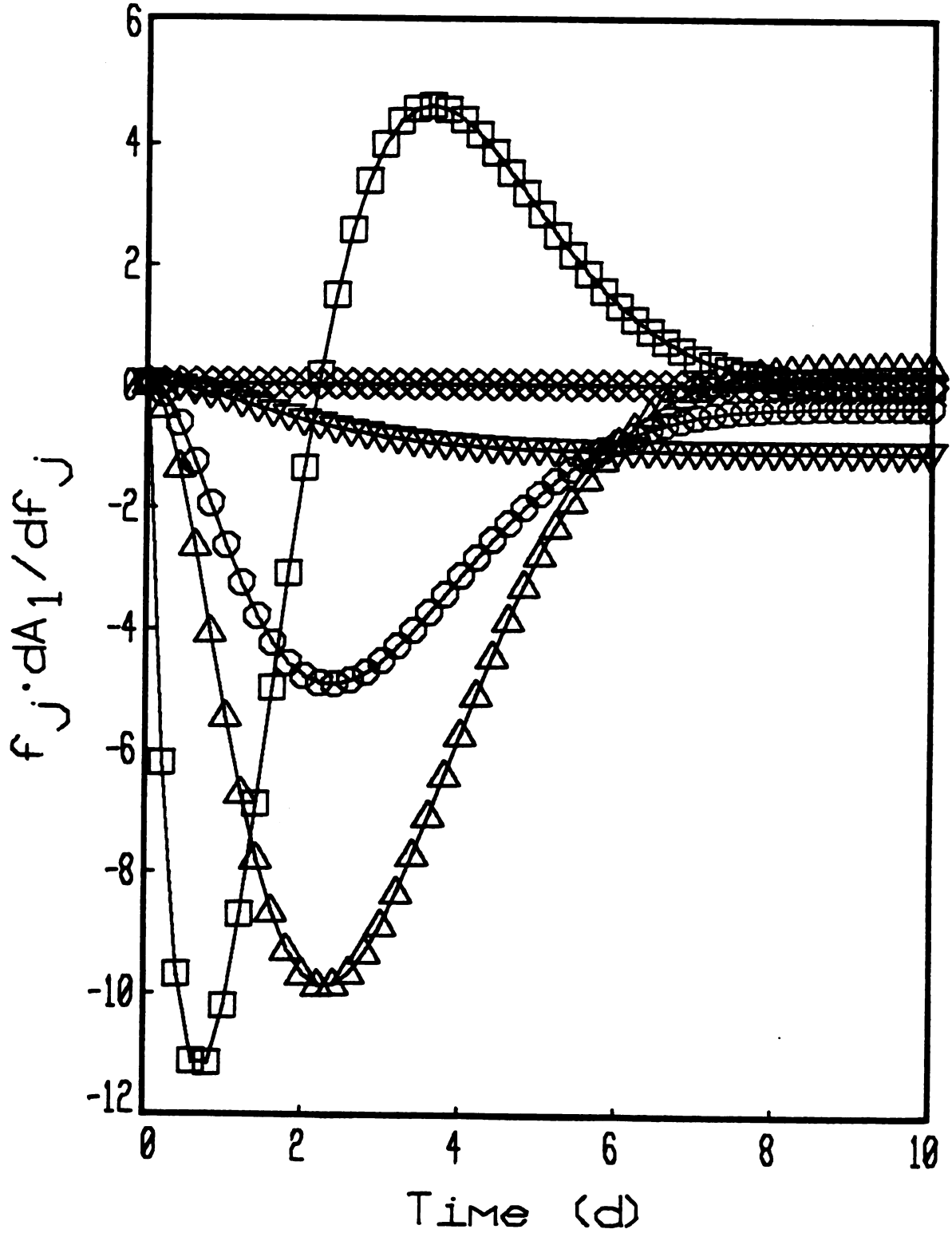

Figure 3

Figure 4. Normalized sensitivity coefficients for the NO_3^- response in a zero order model of N cycling. Symbols as in Figure 2.

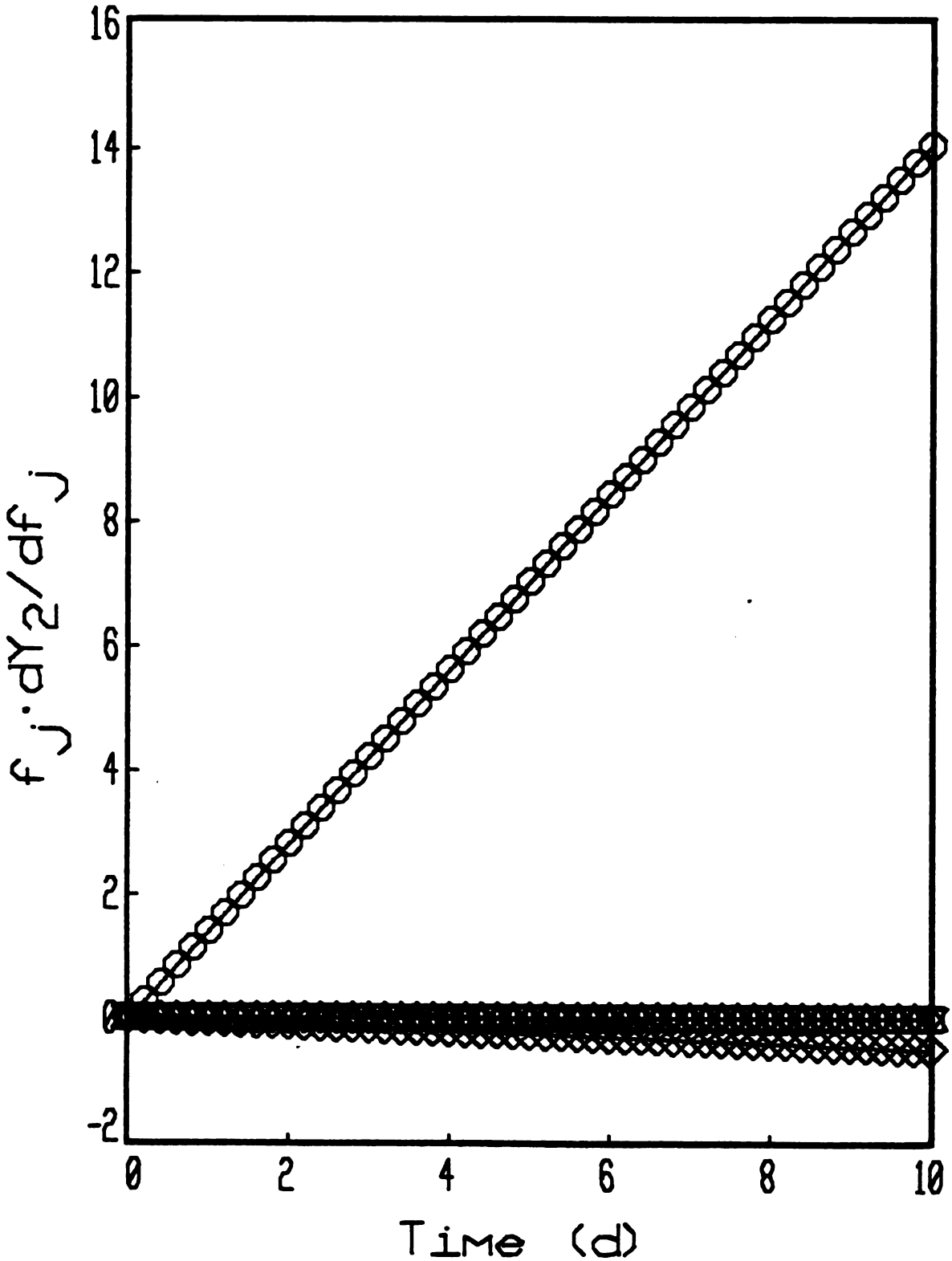

Figure 4

Figure 5. Normalized sensitivity coefficients for the atom % $^{15}NO_3^-$ response in a zero order model of N cycling. Symbols as in Figure 2.

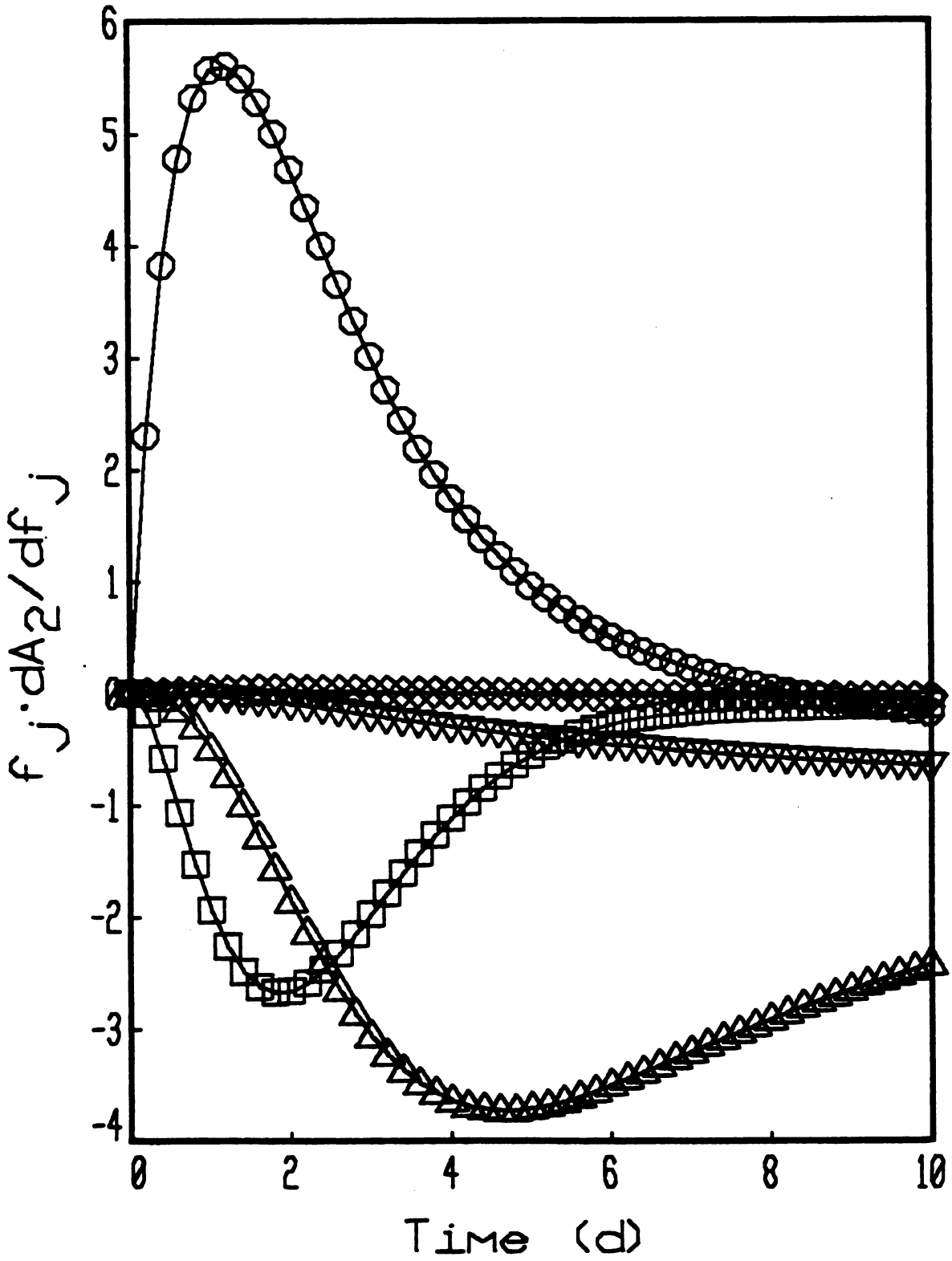

Figure 5

insure that only reasonable (non-negative) parameter estimates were obtained (Bard, 1974). The sensitivity coefficients were obtained using the finite difference method suggested by Beck and Arnold (1977), and the model equations were integrated using a Runge-Kutta technique. The objective function (i.e., residual sum of squares) was minimized according to the least squares criterion.

The nonlinear parameter estimation program was written in BASIC and implemented on a microcomputer. In addition to calculating parameter estimates, the program also calculated the parameter correlation matrix and provided approximate 95% confidence intervals for the parameters.

RESULTS AND DISCUSSION

Model Evaluation. Sensitivity coefficients for the zero order model are shown in Figures 2-5. The sensitivity coefficients for the mineralization, immobilization, and nitrification rates change linearly with time and are therefore linearly dependent, while all other sensitivity coefficients are zero when measuring the NH_4^+ pool (Figure 2). If the NH_4^+ pool is the only response measured, the mineralization, immobilization, and nitrification rates could not be uniquely identified. As one would intuitively expect, only a net rate could be obtained. When the sensitivity coefficients for the atom % $^{15}NH_4^+$ response is examined, the mineralization rate is no longer linearly dependent with respect to the immobilization and nitrification rates, thus it is uniquely determined (Figure 3). However, the immobilization and nitrification rates are still almost linearly related. Adding the NO_3^- response allows one to uniquely estimate the immobilization and nitrification rates, since the sensitivity coefficient for immobilization is zero (Figure 4) rather that approximately twice that of the nitrification sensitivity coefficient as it was in the previously described responses (Figures 2 and 3). The final response--atom % ^{15}N enhances the linear independency among the immobilization, mineralization, and nitrification rates (Figure 5). Close examination of Figures 2-5 will show that the denitrification rate is also uniquely determined. However, since its sensitivity coefficients are all small (< 5%) compared to the others, the denitrification rate will be poorly determined. This is caused, at

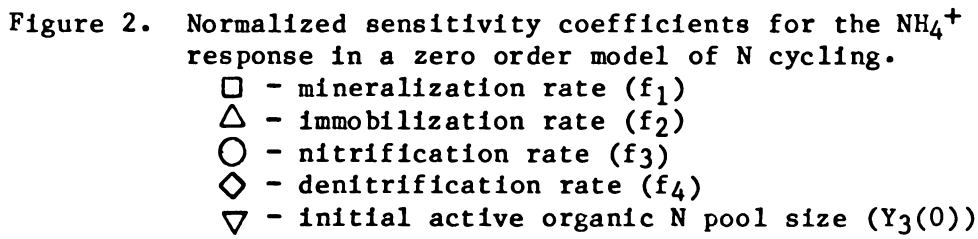
Figure 2. Normalized sensitivity coefficients for the NH_4^+ response in a zero order model of N cycling.
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△ - immobilization rate (f_2)
○ - nitrification rate (f_3)
◇ - denitrification rate (f_4)
▽ - initial active organic N pool size ($Y_3(0)$)

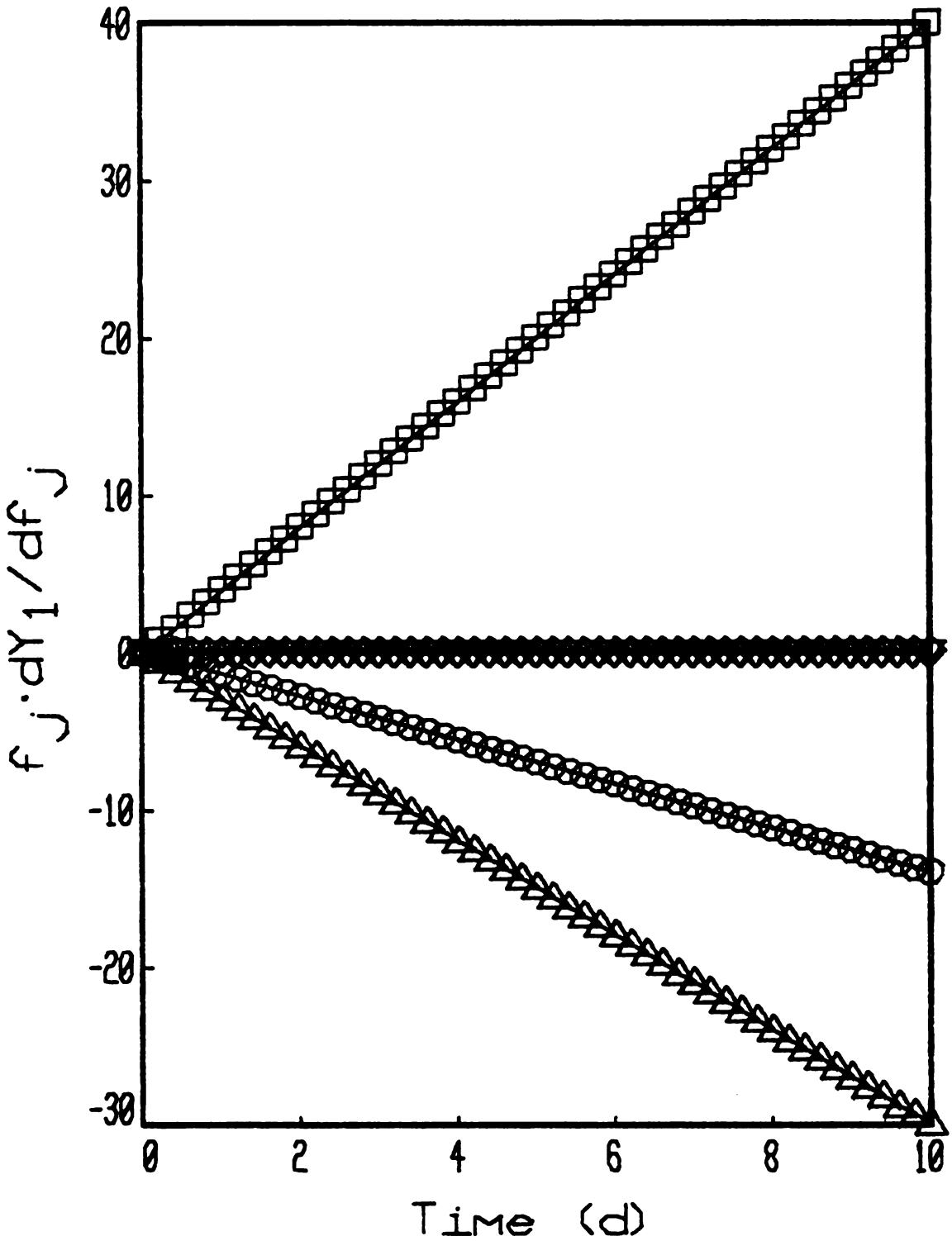
40
30
20
10
0
-10
-20
-30
0
2
4
6
8
10
Time (d)
$f_j \cdot dY_1/df_j$

Figure 3. Normalized sensitivity coefficients for the atom % $^{15}NH_4^+$ response in a zero order model of N cycling. Symbols as in Figure 2.

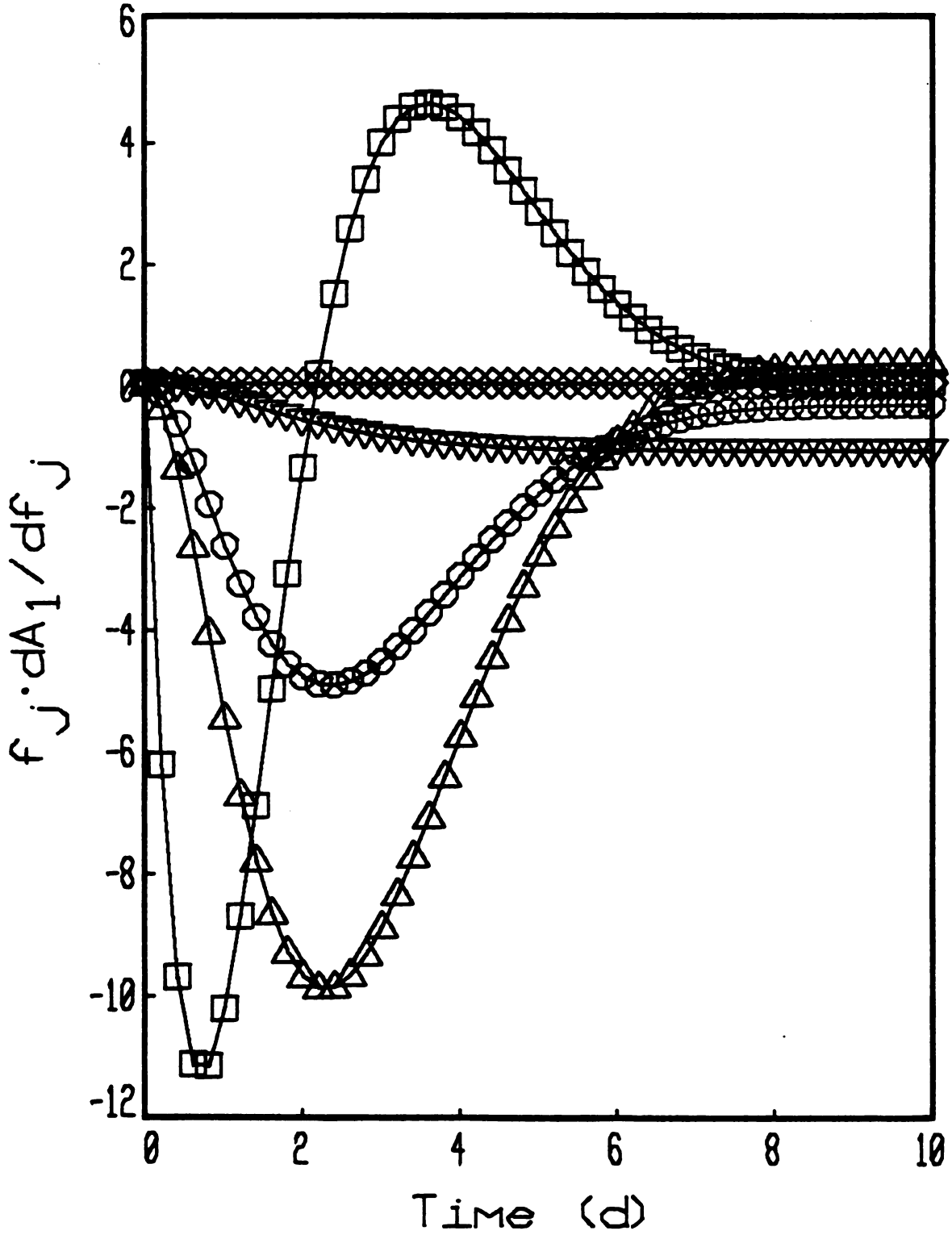

Figure 3

Figure 4. Normalized sensitivity coefficients for the NO_3^- response in a zero order model of N cycling. Symbols as in Figure 2.

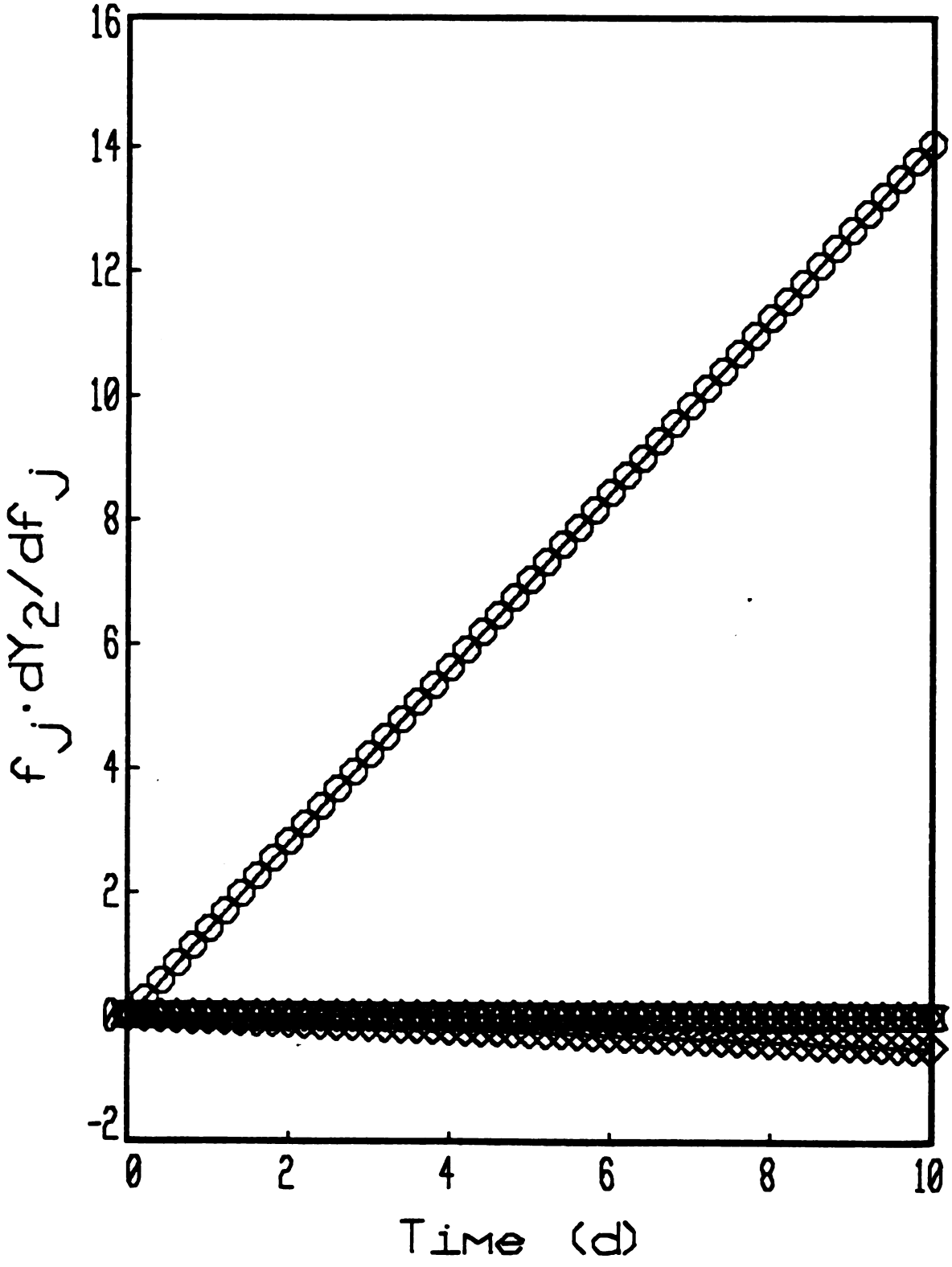

Figure 4

Figure 5. Normalized sensitivity coefficients for the atom % $^{15}NO_3^-$ response in a zero order model of N cycling. Symbols as in Figure 2.

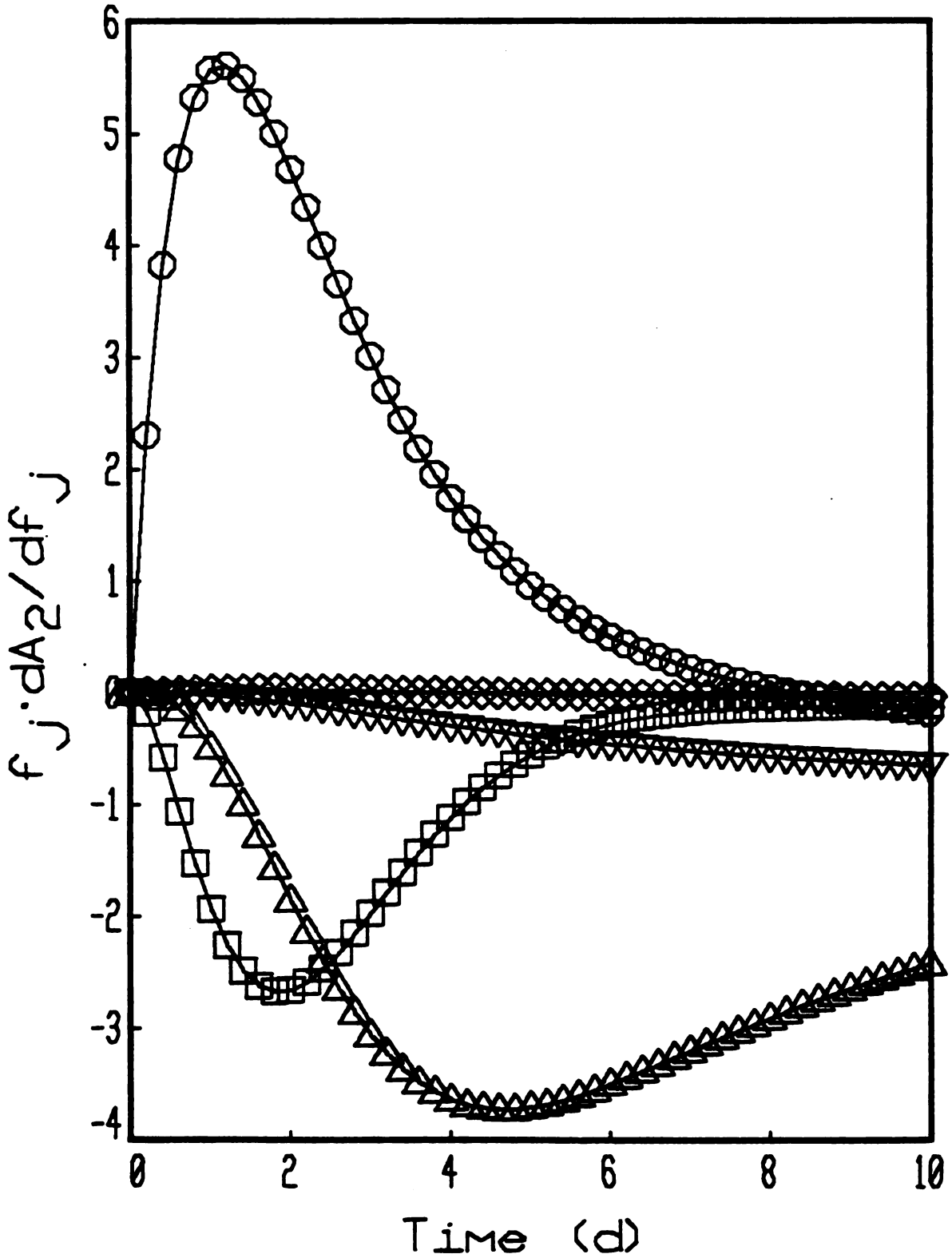

Figure 5

least in part, by the fact that the denitrification rate is small relative to the rates of other N cycle processes. Similarly, a good estimate of the initial size of the active organic N fraction cannot be obtained because of the small magnitude of its sensitivity coefficient.

The results of examining the sensitivity coefficients for the first order model (Figures 6-9) are quite similar to those of the zero order model. Linear dependence among mineralization, immobilization, and nitrification rate constants is strong when only the NH_4^+ pool is measured (Figure 6). Adding the atom % $^{15}NH_4^+$response separates out the mineralization rate constant from the other rate constants (Figure 7), and the NO_3^- response allows the immobilization and nitrification rate constants to be uniquely identified (Figure 8). Once again, the denitrification rate constant will not be well estimated. The behavior of the active organic N pool is quite different in the first order model. The magnitude of the active organic N sensitivity coefficient is large, however, it is now linearly dependent with the mineralization rate constant. Thus, these two parameters cannot be uniquely determined; one must be measured by an independent method for the other to be determined.

Plotting the sensitivity coefficients also reveals the optimal sampling strategies. With either model, the best information is obtained during the first five days (Figures 3, 5, 7, and 9), at least for the atom % ^{15}N measurements. Although the sensitivity coefficients generally continue to increase with time for the total pool size responses (Figures 2, 4, 5, and 8), information is only provided for the nitrification rate since the immobilization and mineralization rates are approximately linearly dependent for these responses.

Figure 6. Normalized sensitivity coefficients for the NH_4^+ response in a first order model of N cycling. Symbols as in Figure 2.

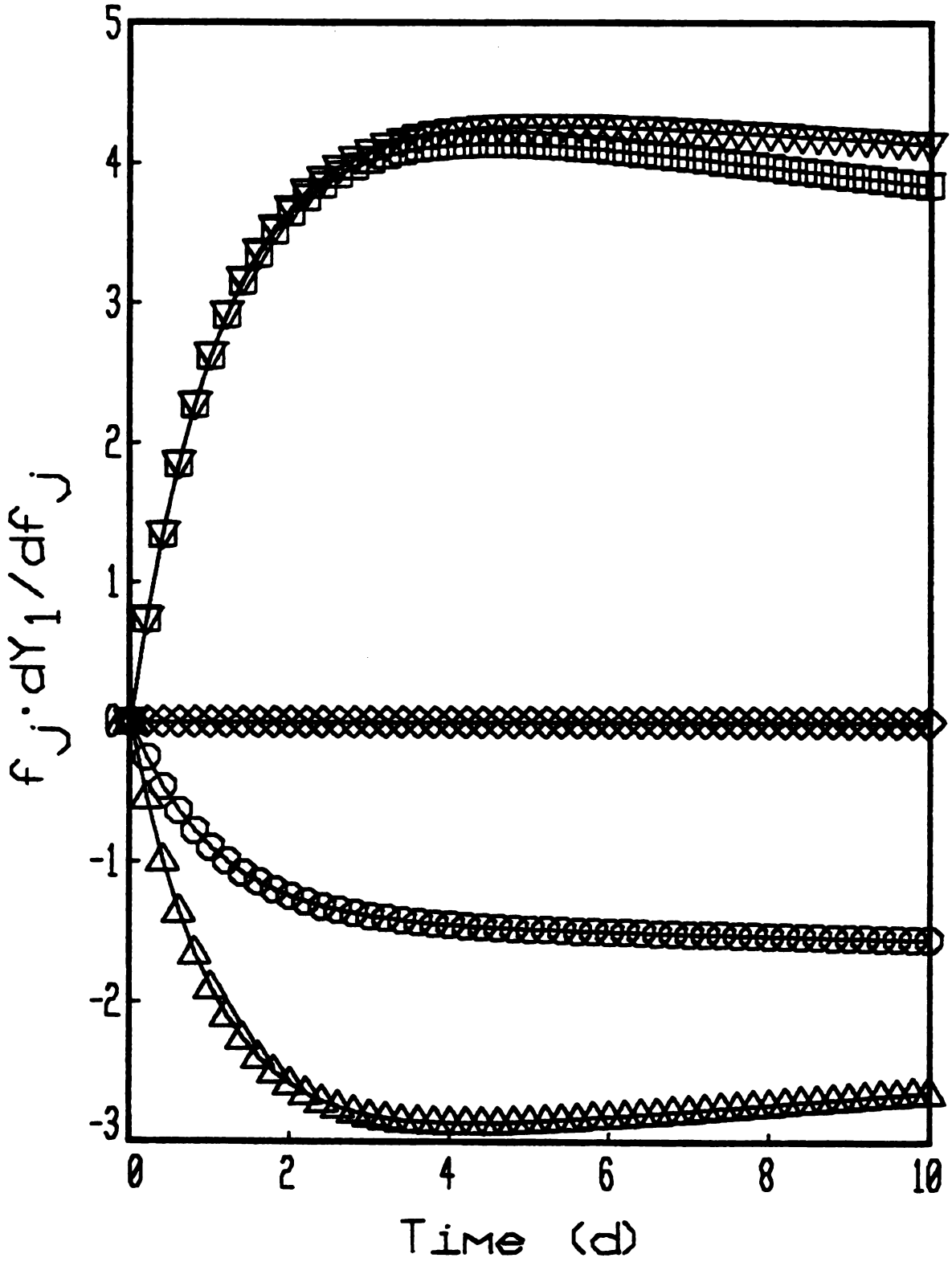

Figure 6

Figure 7. Normalized sensitivity coefficients for the atom % $^{15}NH_4^+$ response in a first order model of N cycling. Symbols as in Figure 2.

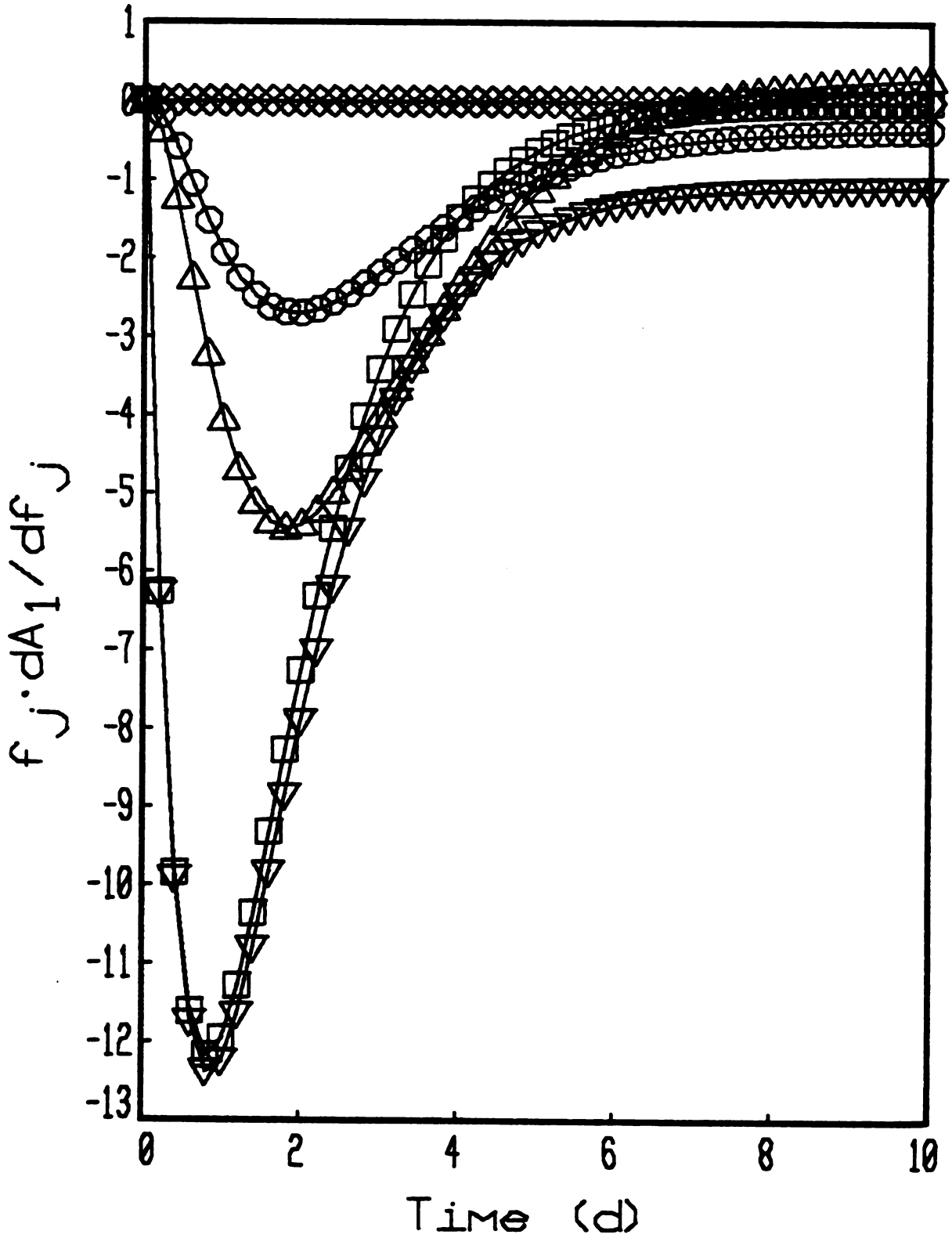

Figure 7

Figure 8. Normalized sensitivity coefficients for the NO_3^- response in a first order model of N cycling. Symbols as in Figure 2.

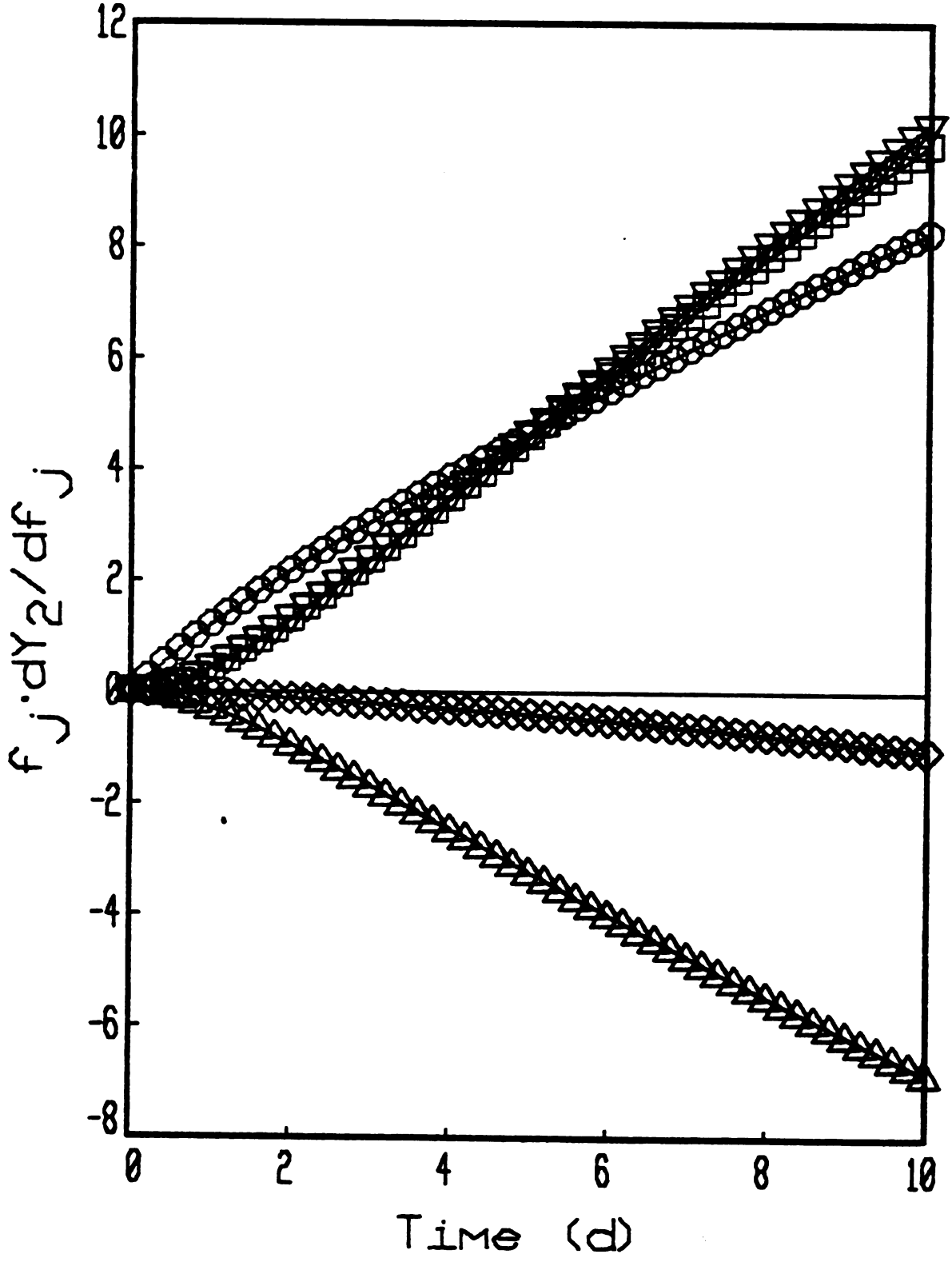

Figure 8

Figure 9. Normalized sensitivity coefficients for the atom % $^{15}NO_3^-$ response in a first order model of N cyling. Symbols as in Figure 2.

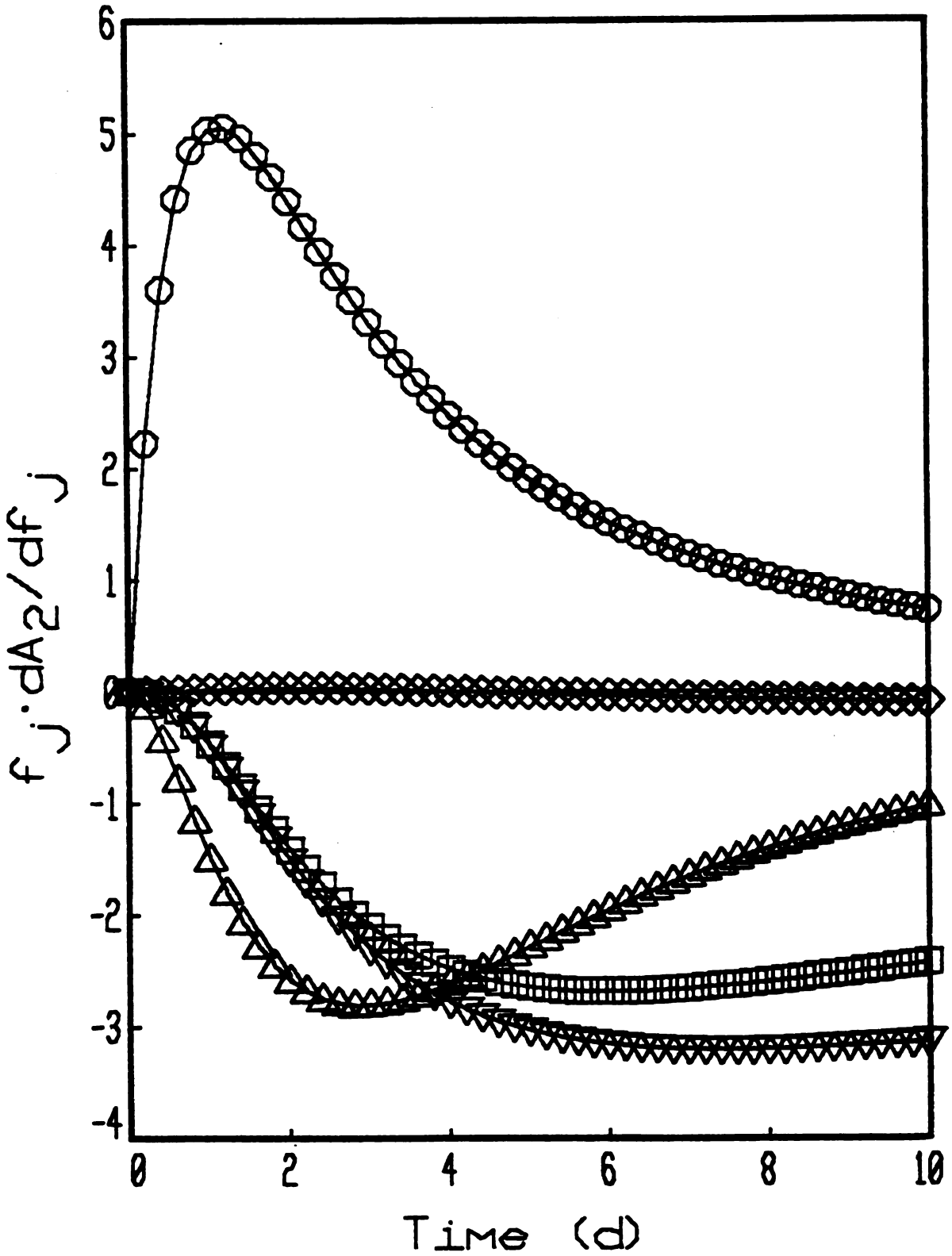

Figure 9

Estimation of Active Organic N. In order to estimate N cycle rates by fitting data with our model it was necessary to measure or estimate the initial size and atom % ^{15}N of the active fraction of the organic N pool. In all cases this pool was assumed to have an atom % ^{15}N of 0.3663 %, or natural abundance. The small departures ($< \pm 1\%$) from natural abundance found in soil organic N (Hauck, 1973) do not significantly affect the parameter estimation process.

The size of the active fraction is more difficult to estimate. We used two different approximations to obtain our estimates. In experiments where we used the $CHCl_3$ fumigation method, we estimated biomass N and assumed that it reflected the size of the active organic N over the short length of our experimental incubations. We also employed the isotope dilution principle, calculating the active fraction as did Jansson (1958). The active fraction was set equal to the ^{15}N lost from the NH_4^+ and NO_3^- pools during the incubation period (losses of ^{15}N via denitrification were minor) divided by the atom % ^{15}N of either the biomass N at the end of the incubation (when this was measured) or of the NH_4^+ pool. When the atom % $^{15}NH_4^+$ was used, it was assumed that the atom % ^{15}N of the active organic N and NH_4^+ pools was close to equilibrium. In most cases these two methods agreed quite well (Table 2), although the isotope dilution method did give a slightly higher estimate of active organic N in the Capac soil. This suggests that biomass N represents most of the active organic N, at least over one to three week time span. Juma and Paul (1981) found biomass N made up 40% of the active organic N, but their experiments were of several months duration, during which a larger pool of organic N would be expected to become involved in mineralization-immobilization dynamics.

Table 2. Range of active organic N fraction estimated by two different methods.

Soil	Active organic N	
	Biomass N	Isotope dilution
	- - - - - - $\mu g\ N\ g^{-1}$ soil - - - - - -	
Onaway loam	170–190	150–170
Capac clay loam	145–165	190[+]
Spinks sandy loam	25–35	25–40

[+]Single observation.

The sample of Onaway loam used in experiment 1 was collected a year earlier than that used to estimate the active N fraction in this soil (Table 2). Since the NH_4^+ and active organic N pools were still far from equilibrium in experiment 1 (after 21 days the NH_4^+ pool was still highly labeled), and the size of the active N could change due to fluctuations in environmental conditions, we fit the data using a wide range of organic N pool size estimates (Figure 10). The best fit was obtained with an active N pool size of about 120 $\mu g\ N\ g^{-1}$ soil, which is lower than the estimates obtained for other samples of the same soil (Table 2).

Estimation of N Process Rates. The N cycle model presented in this paper (Figure 1) fit the measured dynamics of N cycling in the three soils studied in several experiments. However, neither the zero order nor the first order model fit all the data in some of the experiments, particularly when a carbon source or nitrapyrin was added. Presumably in these cases other processes were functioning in the soil that were not accounted for in the model. In these cases, a portion of the data could sometimes be fit when the entire data set was not well described by the model. The following examples illustrate the usefulness, as well as the limitations, of the parameter estimation approach used in this study.

Nitrogen dynamics of the Onaway loam soil in experiment 1 were well described by a zero order kinetic model which included the heterotrophic nitrification process (Figures 11 and 12). Without the inclusion of the heterotrophic process the residual sum of squares (RSS) increased markedly from 17.3 to 399. The zero order model fit the data better than the first order model, which had residual sums of squares of 49 or

Figure 10. Reduction in the residual sum of squares for experiment 1 with Onaway loam as a function of the initial active organic N pool size.

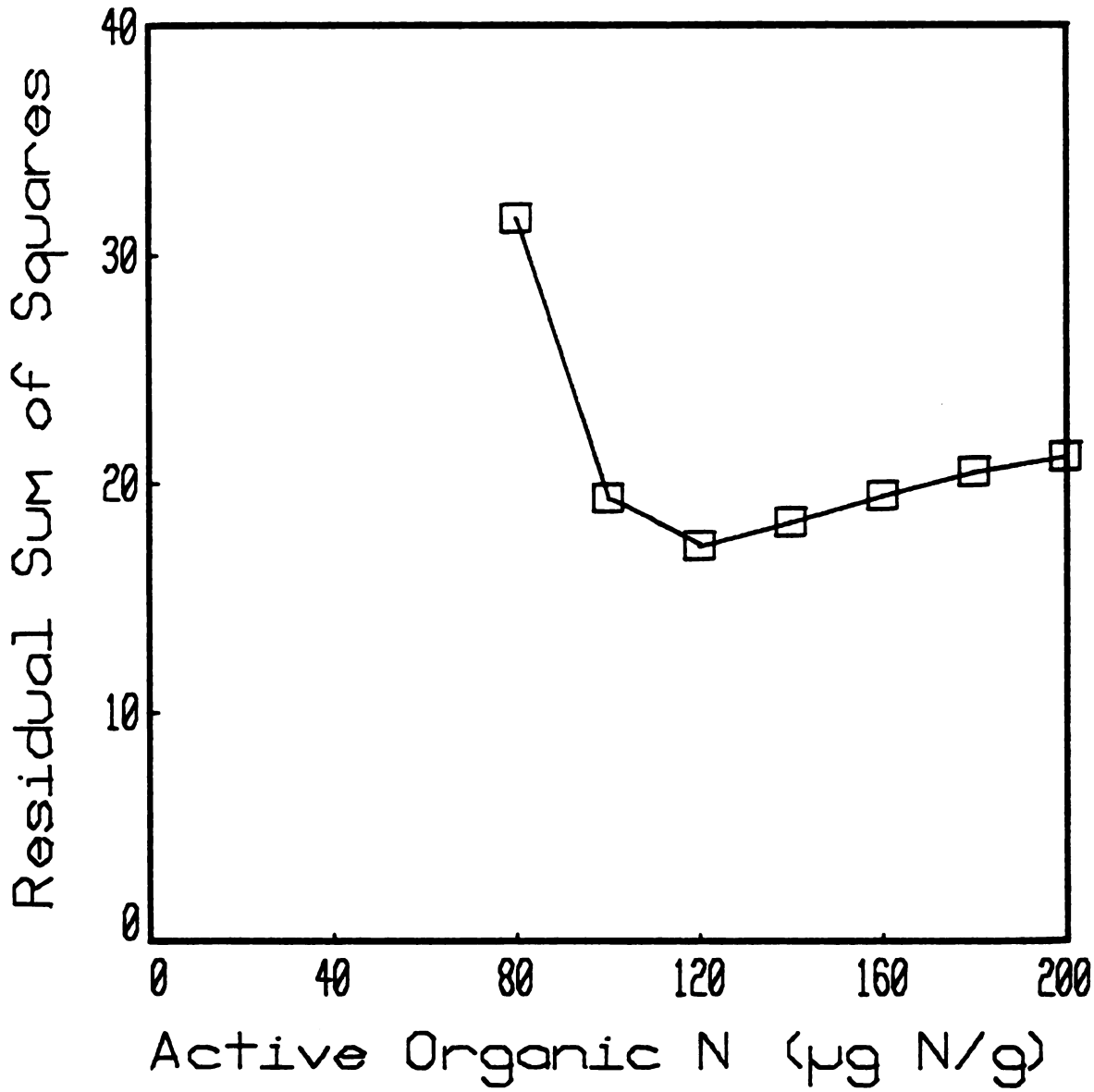
Residual Sum of Squares
40
30
20
10
0
0
40
80
120
160
200
Active Organic N (µg N/g)

Figure 10

Figure 11. Experimental and simulated pool size data for Onaway loam soil.
□ - NH_4^+
△ - NO_3^-
—— - simulated using estimated parameters

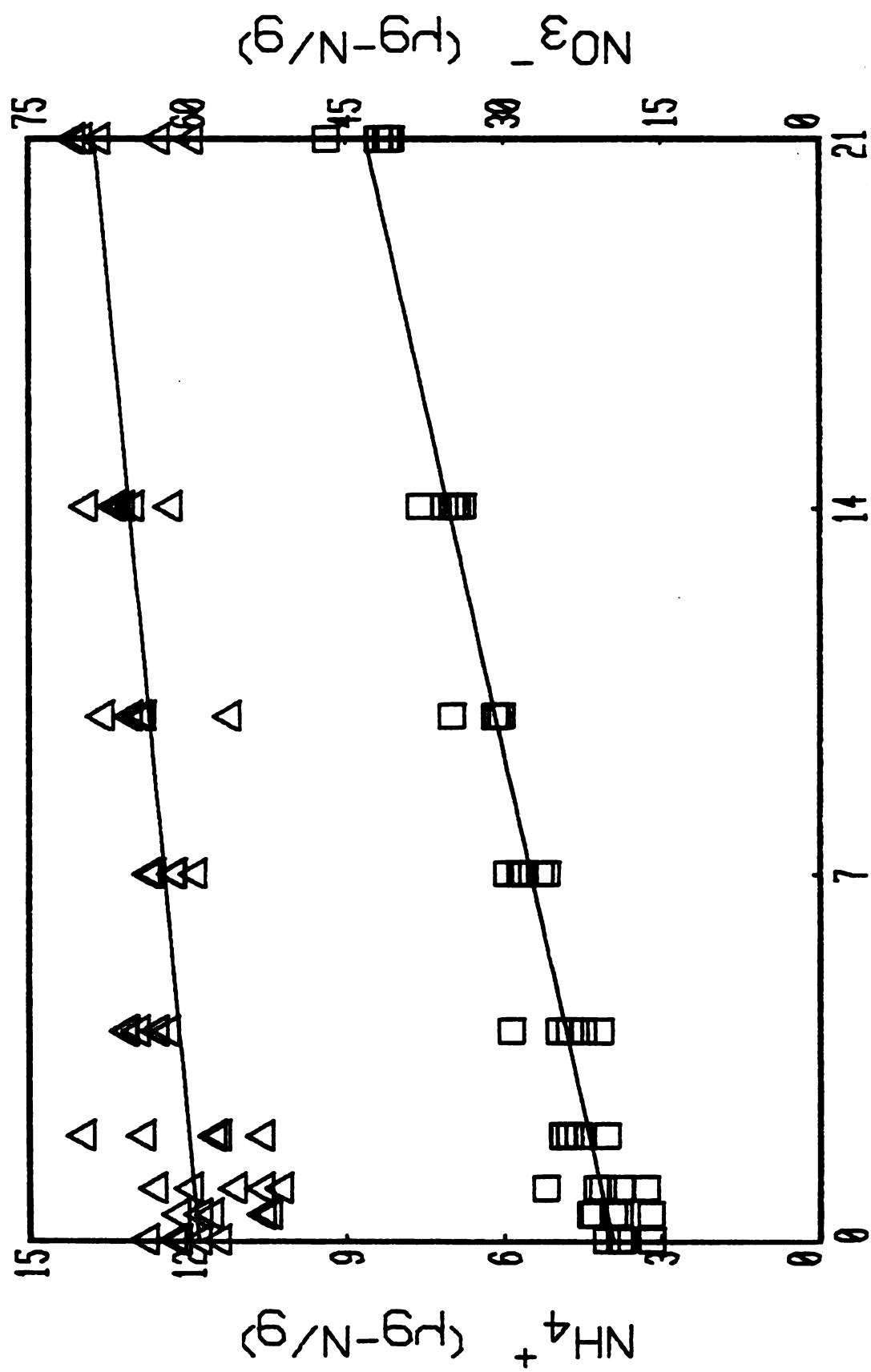

Figure 11

Figure 12. Experimental and simulated atom % ^{15}N data for Onaway loam soil. Symbols as in Figure 11.

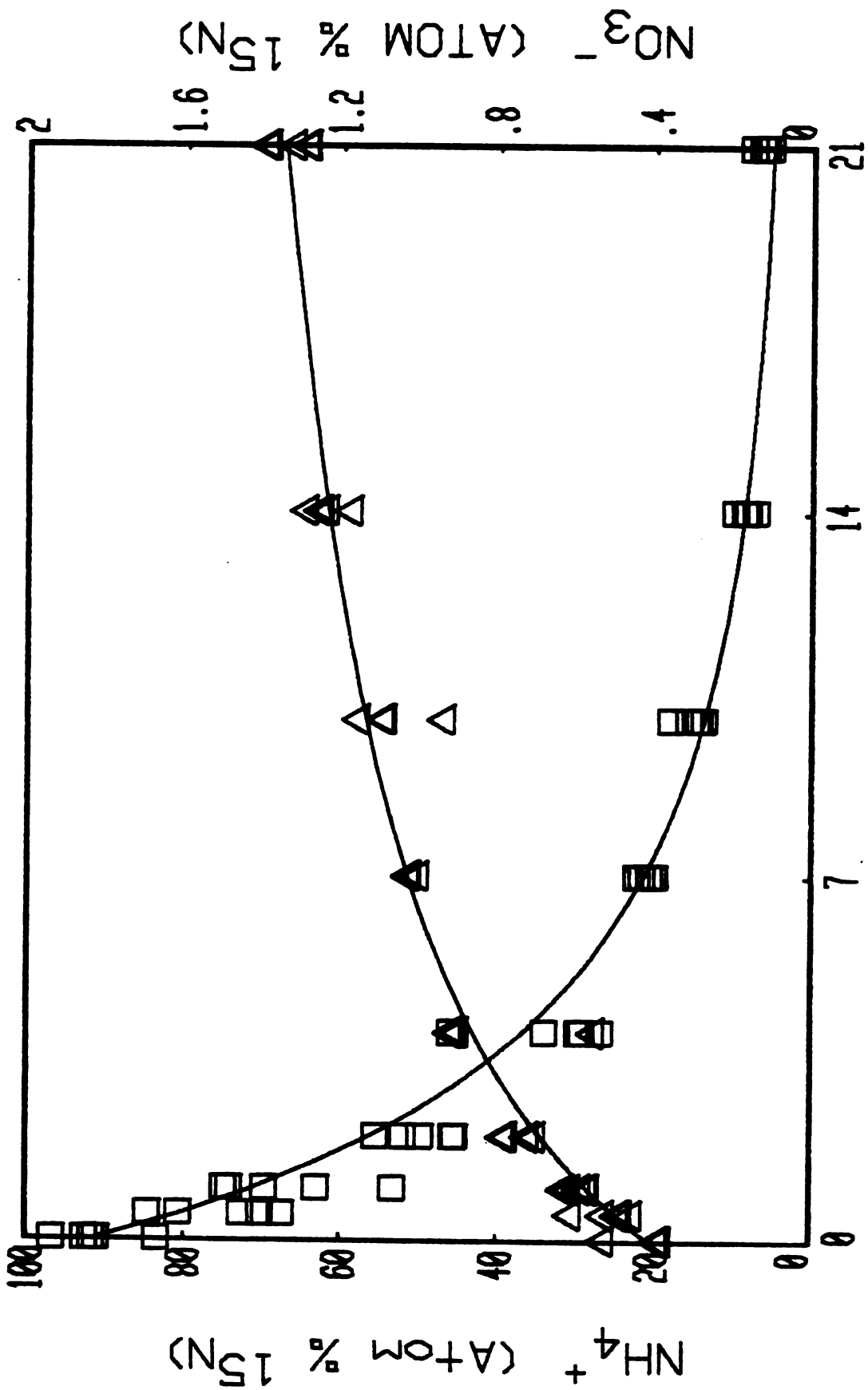
NO_3^- (ATOM % ^{15}N)
2
1.6
1.2
.8
.4
0
21
14
7
0
Time (d)
100
80
60
40
20
0
NH_4^+ (Atom % ^{15}N)

Figure 12

1660, with or without the heterotrophic nitrification process, respectively.

Rate estimates for the Onaway loam range from 17 ng N g^{-1} d^{-1} for denitrification to over 1 μg N g^{-1} d^{-1} for mineralization (Table 3). The rates of mineralization and immobilization are comparable to those given by Jansson (1958). However, the mineralization rate obtained was generally higher than those measured by the standard incubation procedure without the ^{15}N label (Tabatabai and Al-Khafaji, 1980; Addiscott, 1983), probably because the standard technique only measures net mineralization.

Approximate 95% confidence intervals have been calculated for these N cycle rates, but it should be noted that the method used gives very optimistic values (narrower confidence intervals) and the actual variation is probably greater (Robinson, 1984). With this caveat in mind, it is apparent that the rates of mineralization, immobilization, and nitrification are estimated much more precisely than the denitrification and heterotrophic nitrification rates. This is a reflection of the earlier discussion about sensitivity coefficients. Nonetheless, the rates obtained seem quite reasonable compared to nitrification and denitrification rates typically found in forest soils (Robertson, 1982: Robertson and Tiedje, 1984).

First order kinetics best fit the data from the double labeling experiment with the Capac soil (Figures 13 and 14). This first order behavior is evident from the curvilinear trends of the NH_4^+ and NO_3^- pools over time. Nitrification was initially very rapid in this soil, with rates as high as 16 to 35 μg N g^{-1} d^{-1} during the first day of incubation. Denitrification rates varied from 450 to 890 ng N g^{-1} d^{-1}

Table 3. Rates and rate constants of N cycle processes in three dissimilar soils.

Process	Onaway loam[†]	Capac clay loam[§]	Spinks sandy loam[§]
	$\mu g\ N\ g^{-1}\ d^{-1}$	---------- d^{-1} ----------	
Mineralization	1.01 ± 0.06[¶]	0.0117 ± 0.0030	0.00745 ±0.00320
Immobilization	0.674 ± 0.054	0.146 ± 0.076	1.70 ± 0.39
Nitrification	0.113 ± 0.008	1.30 ± 0.14	1.60 ± 0.28
Denitrification	0.0166 ± 0.42	0.0114 ± 0.0047	0.00259 ± 0.0184
Heterotrophic nitrification	0.371 ± 0.384	-	-
Active organic N ($\mu g\ N\ g^{-1}$ soil)	120	150	40
RSS	17.3	147	72.8

[†] Mean ± 95% confidence interval.
[§] Zero order kinetics.
[¶] First order kinetics.

Figure 13. Experimental and simulated pool size data for Capac clay loam. Symbols as in Figure 11.

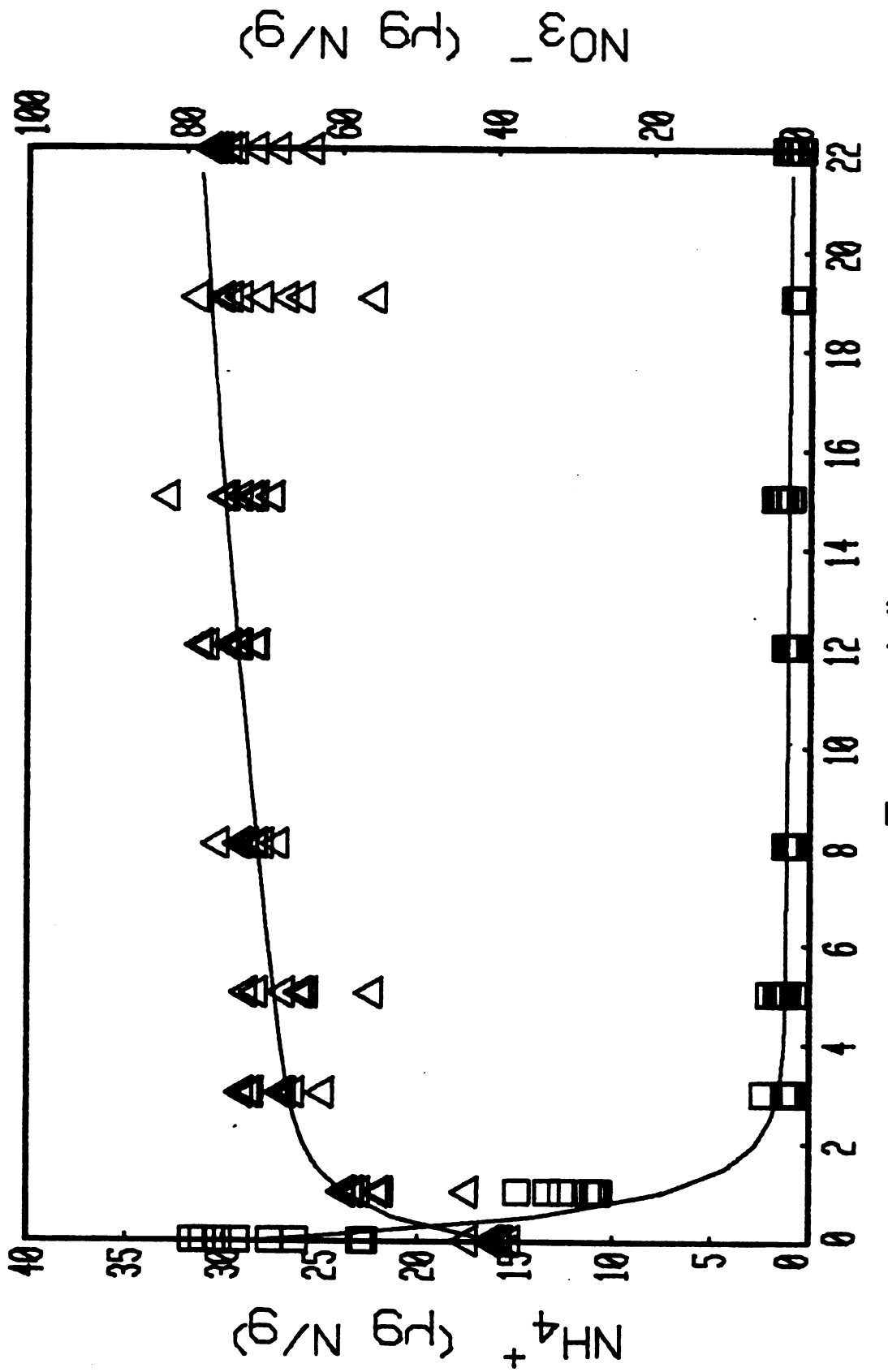
NO3- (µg N/g)
100
80
60
40
20
0
22
20
18
16
14
12
10
8
6
4
2
0
Time (d)
40
35
30
25
20
15
10
5
0
NH4+ (µg N/g)

Figure 13

Figure 14. Experimental and simulated atom % ^{15}N data for Capac clay loam.
□ - atom % $^{15}NH_4^+$ from $^{15}NH_4^+$ treatment
△ - atom % $^{15}NO_3^-$ from $^{15}NH_4^+$ treatment
◇ - atom % $^{15}NO_2^-$ from $^{15}NO_3^-$ treatment
—— - simulated using estimated parameters

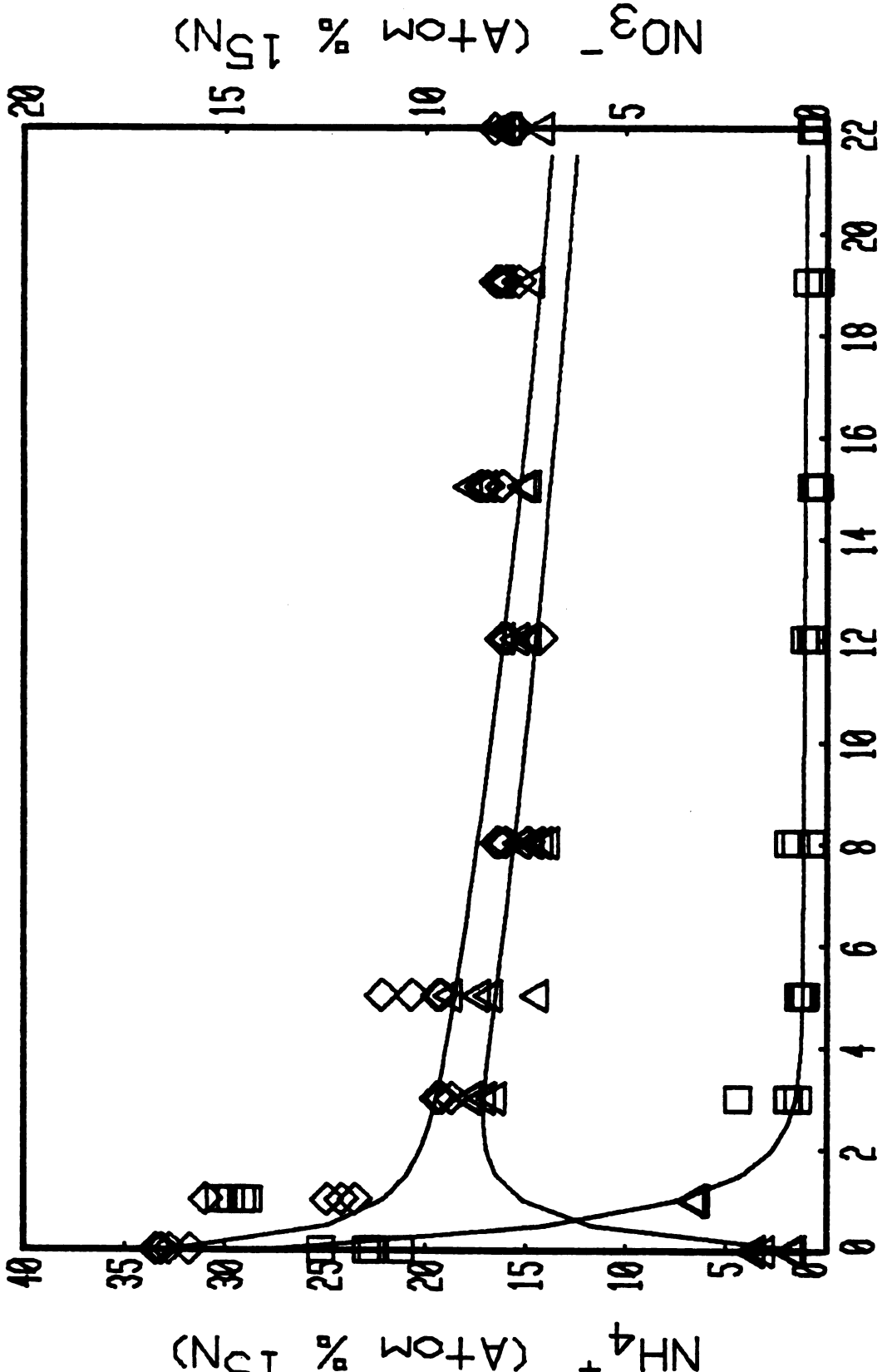
NO3- (Atom % 15N)
NH4+ (Atom % 15N)
Time (d)
0
2
4
6
8
10
12
14
16
18
20
22
5
10
15
20
25
30
35
40

Figure 14

over the course of the experiment, about four times the rate estimated by Parkin et al. (1984) for this soil using the C_2H_2 block method. The nitrification rate constant is comparable to those previously reported for agricultural soils (Cameron and Kowalenko, 1976; McLaren, 1976), while the denitrification rate constant is lower than those given by McLaren (1976). However, the difference in denitrification rate constants can likely be attributed to the higher water content (more anaerobic conditions) of the solution flow methods employed in the studies reported by McLaren (1976). The mineralization rate constant is high compared to those obtained using the method for determining potentially mineralizable N (Stanford and Smith, 1972; Campbell et al., 1981). As with the zero order rates obtained by this method, the lower rate constants obtained by others could be a reflection of net mineralization. However, the mineralization rate constant for the Capac soil is also twice as large as the biomass N decay rates determined by Paul and Juma (1983) using ^{15}N tracer methods. Few other measurements of this kind have been made, so this difference may simply be an expression of the inherent variability of net mineralization constants.

The Spinks sandy loam was also fit best by the first order model (Figures 15 and 16). Nitrification rates ranged from 10 to 15 $\mu g\ N\ g^{-1}\ d^{-1}$ in this soil, somewhat lower than those found in the Capac clay loam. Immobilization rates were considerably higher in the Spinks soil ($\sim$13 $\mu g\ N\ g^{-1}\ d^{-1}$) compared with either the Capac (0.1-4 $\mu g\ N\ g^{-1}\ d^{-1}$) or Onaway soil (0.7 $\mu g\ N\ g^{-1}\ d^{-1}$). This higher rate may reflect the lower N content of this soil (Table 1), suggesting that the Spinks sandy loam may be more N limited than the other soils studied. The mineralization rate constant for the Spinks soil is comparable to those

Figure 15. Experimental and simulated pool size data for Spinks sandy loam. Symbols as in Figure 11.

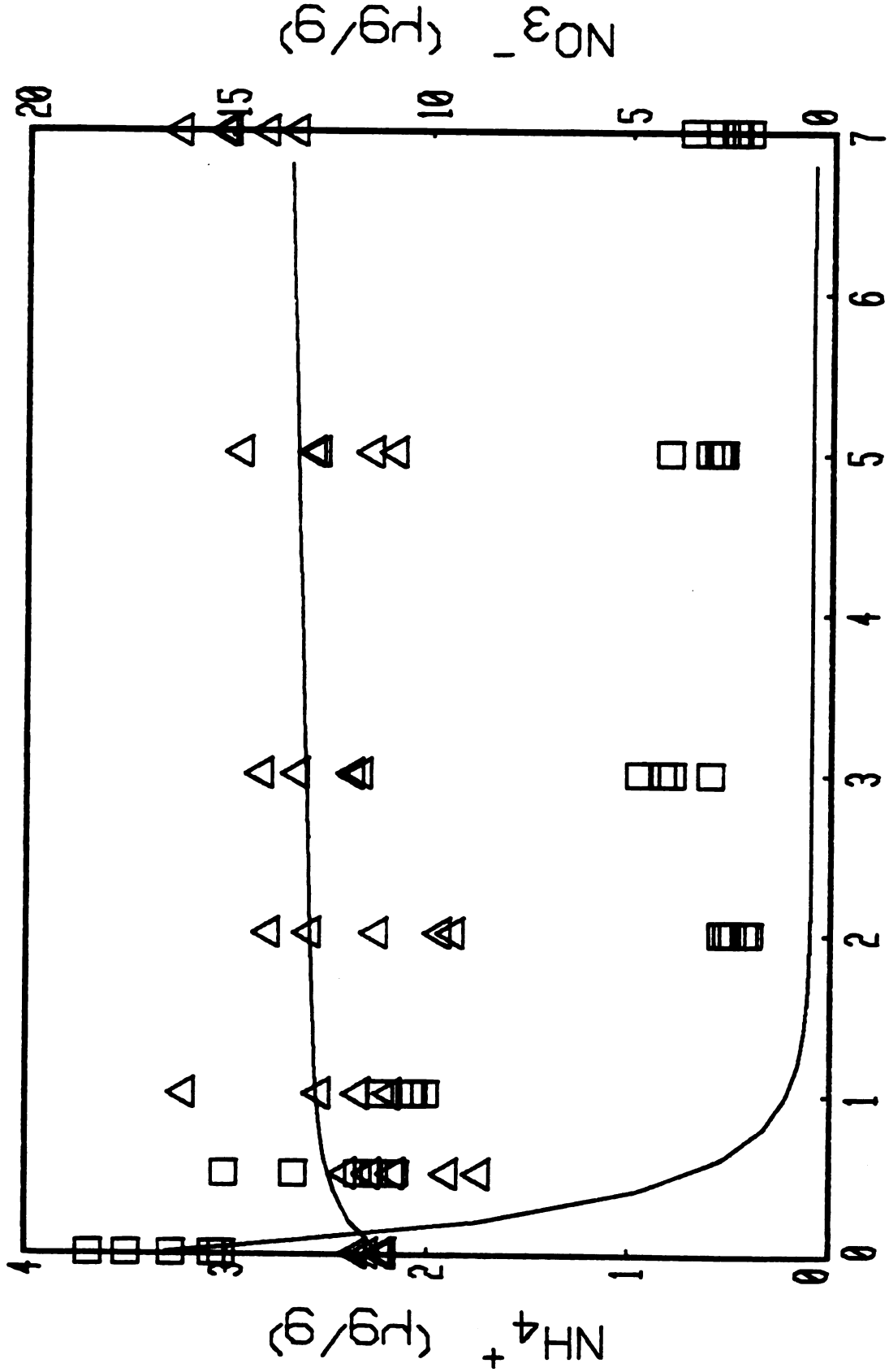

Figure 15

Figure 16. Experimental and simulated atom % ^{15}N data for Spinks sandy loam. Symbols as in Figure 11.

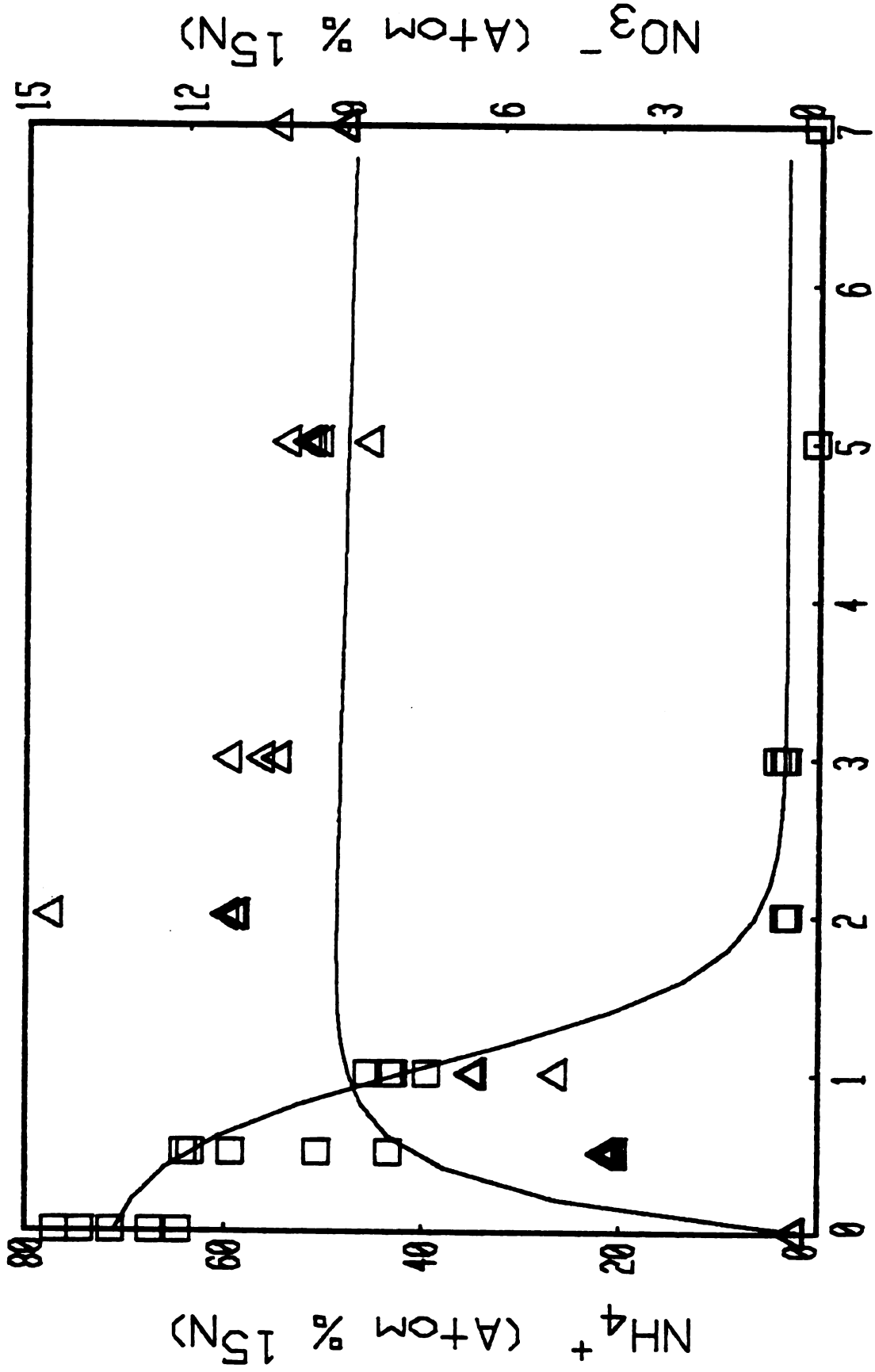
NO_3^- (Atom % ^{15}N)
15
12
9
6
3
0
7
6
5
4
3
2
1
0
Time (d)
NH_4^+ (Atom % ^{15}N)
80
60
40
20
0

Figure 16

found for biomass N decay (Juma and Paul, 1983). Denitrification rates (9-15 ng g^{-1} d^{-1}) were considerably lower than for the Capac soil, but similar to the rate found for the Onaway loam. The difference between the denitrification rates of the two agricultural soils is similar to that found by Sexstone et al. (1984) using the C_2H_2 inhibition method.

Evaluation of Heterotrophic Nitrification. The apparent presence of heterotrophic nitrification found with the Onaway loam soil in experiment 1 prompted a further examination of this phenomenon. To test whether or not heterotrophic nitrification was occurring, we incubated samples of Onaway loam with and without nitrapyrin, an autotrophic nitrification inhibitor. As in the previous experiment with this soil, the N cycling dynamics of these two treatments were well fit by zero order kinetics (Table 4). However, in order to obtain a good fit for the nitrapyrin amended soil, it was necessary to exclude the 0 and 7 day measurements. The atom % $^{15}NO_3^-$ increased from 0.65 to 2.22 during the first 12 hours, apparently because of a delay in inhibition of nitrification by nitrapyrin. After day five, the NO_3^- pool size increased markedly, perhaps because the effect of the nitrapyrin had diminished.

Addition of nitrapyrin affected only the nitrification rate, decreasing it about six-fold. Mineralization and immobilization rates were not significantly different between the two treatments. The magnitude of heterotrophic nitrification in experiment 4 was less than 1 ng N g^{-1} d^{-1}, thus the occurrence of this process in Onaway loam could not be confirmed. The apparent presence of heterotrophic nitrification found in experiment 1 may have been artifactual. It could have been caused by heterogeneous distribution of the added $^{15}NH_4^+$, which could

Table 4. Zero order N cycle rates of Onaway loam with and without nitrapyrin.

Treatment	Mineralization	Immobilization	Nitrification	Denitrification	Heterotrophic nitrification	RSS
	- $\mu g\ N\ g^{-1}\ d^{-1}$ -					
- Nitrapyrin	2.17 ± 0.56 [+]	2.62 ± 0.58	0.886 ± 0.349	<0.001	0.126 ± 0.371	10.3
	2.19 ± 0.54	2.60 ± 0.57	0.918 ± 0.295	<0.001	-	10.9
+ Nitrapyrin	2.86 ± 0.56	2.56 ± 0.59	0.138 ± 0.152	<0.001	<0.001	2.92
	2.90 ± 0.56	2.56 ± 0.59	0.144 ± 0.159	0.059 ± 0.367	-	2.76

[+] Mean ± 95% confidence interval.

result in concurrent mineralization and nitrification of less highly labeled organic N being modeled as heterotrophic nitrification.

Added Carbon Effect on Immobilization and Nitrification. Carbon was added to the Onaway soil in the form of maple leaves and to Capac clay loam as alfalfa straw. The data from the carbon amended treatments of these soils could not be fit with either a zero or first order model, presumably because microbial growth was occurring. (Under similar incubation conditions, Myrold and Tiedje (1984) found a 40% increase in total microbial biomass in straw amended Capac soil after four days.) This presumed biomass increase was not adequately reflected by changes in active organic N, since this pool also contains nonbiomass N compounds. Also, changes in biomass N reflect fluctuations of the total microbial population and do not account for growth or death of specific metabolic groups of microorganisms. Because of these difficulties, the effect of added carbon on the relative rates of immobilization and nitrification were assessed from the distribution of added ^{15}N at the end of the incubation (Table 5).

Immobilization was dominant over nitrification in Onaway loam, with 2.4 times as much ^{15}N incorporated into organic N than into NO_3^- after seven days of incubation. This agrees quite well with the magnitude of the estimated immobilization and nitrification rates for this soil (Table 4). The Capac soil, on the other hand, is a strongly nitrifying soil, with 7.3 times a much ^{15}N found in the NO_3^- pool compared to organic N after seven days. As with the Onaway soil, the ratio of the rate constants for immobilization and nitrification found in the Capac soil in experiment 2 (0.11) is close to the value of 0.14 for the

Table 5. Effect of carbon additions on the partitioning of $^{15}NH_4^+$ in two soils after a seven day incubation period.

Soil	Carbon added	$^{15}NH_4^+$	$^{15}NO_3^-$	Biomass ^{15}N	Biomass ^{15}N: $^{15}NO_3^-$ ratio
		- - - - - - - % - - - - - - - -			
Onaway	-	0.2	29.2	70.6	2.4
	+	0.7	14.8	84.5	5.7
Capac	-	0.0	87.9	12.1	0.14
	+	0.0	51.7	48.3	0.93

biomass $^{15}N:^{15}NO_3^-$ ratio. This suggests that assessing relative rates by examining the partitioning of added ^{15}N is a valid procedure.

In both soils, the carbon addition stimulated immobilization, or inhibited nitrification, however the mechanism could not be determined since the actual rates or rate constants could not be determined. Jones and Richards (1977) found similar changes in $^{15}NH_4^+$ partitioning when they added pine needles to soil. They suggested that this response may have been because nitrifiers are poor competitors for NH_4^+ compared to heterotrophic organisms. Straw additions have also been found to enhance immobilization rates (Jansson, 1958).

The effect of carbon addition was much greater in the Capac soil, where a greater than seven-fold increase in the biomass $^{15}N:^{15}NO_3^-$ ratio occurred. The greater response to carbon addition in the Capac soil was probably because ground alfalfa was a better carbon and energy source than the ground maple leaves used with the Onaway soil.

SUMMARY

Analysis of the sensitivity coefficients for the models used in this study demonstrated that the four parameters of interest--the rates or rate constants of mineralization, immobilization, nitrification, and denitrification--were estimable. This analysis also illustrated which sampling design would be best for the experiments performed. Since nonlinear parameter estimation is becoming more prevalent in soil science (cf., Cameron and Kowelenko, 1976; Smith et al., 1980; Campbell et al., 1981), the usefulness of calculating and examining the sensitivity coefficients cannot be underestimated.

We found that both zero and first order models could describe N cycling, with the model dependent upon the soil being studied. The rates or rate constants for mineralization, immobilization, nitrification, and denitrification compared favorably with those reported by others. The estimates obtained for mineralization, immobilization, and nitrification had relatively small confidence intervals; however, denitrification parameters were poorly estimated. This is due in part to the much lower rate of denitrification when compared to the rate of the other processes and also to the insensitivity of the measured responses to changes in the denitrification parameter.

Some experimental data could not be fit with zero or first order kinetics, however this was most likely because the model did not account for all of the processes occurring in these soils, rather than limitations to the parameter estimation procedure itself. Even with the

relatively high degree of experimental variability inherent in measuring N pool sizes and ^{15}N ratios, reasonably good parameter estimates were obtained in these laboratory incubations. Under field conditions, or with more heterogeneous soils, experimental variability would certainly hamper this estimation procedure.

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CHAPTER 2

DIFFUSIONAL CONSTRAINTS OF DENITRIFICATION IN SOIL

Rates of biochemical reactions are determined by the substrate concentration at the active site of the enzyme rather than the bulk solution concentration. Therefore, the processes which govern the transport of substrate molecules from the bulk solution to the enzyme influence the reaction rate. This interaction between biochemical activity and physical transport processes is described by reaction-diffusion equations.

To date, much of the work on coupled reaction-diffusion processes has been done in chemical engineering and with microbial biofilms where experimental variability is more easily controlled than in natural systems (Goldstein, 1976; La Motta and Shieh, 1978). Reaction-diffusion principles have also been applied to natural systems like marine sediments (Jorgensen, 1978; Jahnke *et al.*, 1982), the mud-water interface (Bouldin, 1968), and soils (Greenwood and Goodman, 1964; Reddy *et al.*, 1978; Phillips *et al.*, 1978). However, in these natural systems, the biological reactions have generally been described by simplified first- or zero-order kinetics rather than Michaelis-Menten kinetics which often describe biological activity.

Denitrification rates are controlled by the supply of at least three entities: O_2, NO_3^-, and available carbon. Several studies have examined the interaction of O_2 diffusion and consumption on the establishment of anaerobic zones in soil (Greenwood, 1962; Smith, 1980)

and the relationship between the anaerobic fraction of the soil and denitrification rates (Sexstone et al., 1984a and 1984b).

Fewer studies have examined the relationship between NO_3^- diffusion and denitrification rates in soil. The work that has been done studied flooded soils and demonstrated that NO_3^- diffusion from overlying aerobic floodwater to underlying anaerobic soil determined the rate and reaction kinetics of denitrification (Reddy et al., 1978; Reddy et al., 1980). It has been suggested that NO_3^- diffusion may also limit denitrification in aerated soils (Greenwood, 1962; Burford and Greenland, 1970), but this has not been experimentally proven. The high Km values reported for NO_3^- reduction in soils is often used as evidence to support this claim (Firestone, 1982), however, factors other than diffusion can also yield high Km values.

Studies on the effect of available carbon supply are hampered by the difficulty in measuring the available carbon pool in soil. Some evidence from relatively short term studies, where growth effects should be minimal suggests that denitrification may be carbon limited in soil (Bowman and Focht, 1974; Reddy et al., 1978), but other studies have not shown any carbon limitation (Smith and Tiedje, 1979).

In this study we have focused on the effect of NO_3^- diffusion on denitrification in unsaturated soils. This effect was both theoretically and experimentally examined, and we have attempted to describe the circumstances when NO_3^- diffusion is, and is not, a determinant of the rate of denitrification. In addition, we present evidence illustrating that the supply of substrate carbon can limit denitrification rates.

MATERIALS AND METHODS

Experimental Procedures. Two soils were used in this study (Table 1). Samples of the Medio soil came from an aspen watershed located in the Sante Fe National Forest in New Mexico (Gosz, 1978). Intact soil cores were obtained using the methods of Parkin et al. (1983) and transported to East Lansing, Michigan for denitrification measurements. The Capac soil was taken from an agricultural field near East Lansing, sieved to pass a 2 mm sieve, and stored at 4°C until used. Packed cores of Capac soil (bulk density of 1.5 g cm^{-3}) were amended with either distilled water, NO_3^- (100 μg N g^{-1} soil) or finely ground alfalfa straw (500 μg C g^{-1} soil) and incubated at 20°C for three weeks before denitrification measurements were made.

Anaerobic core rates of denitrification were determined by measuring the accumulation of N_2O in the presence of 10% C_2H_2 in an Ar gas phase using the gas recirculation and ECD-GC system of Parkin et al. (1983). Denitrification rates of anaerobic slurries were measured as described by Smith and Tiedje (1979) with the following modifications: (1) the chloramphenicol concentration was increased to 0.5 mg g^{-1} soil and (2) NO_3^- (100 μg N g^{-1} soil) and glucose (1 mg C g^{-1} soil) were added sequentially at one and two hours after the beginning of the incubation. The sequential NO_3^- and glucose additions made it possible to determine whether either an electron acceptor or electron donor limitation existed. Comparing the anaerobic core and anaerobic slurry rates provides information about the effect of electron donor and acceptor availability.

Table 1. Soil characteristics.

Soil Series	Classification	Vegetation	Texture	pH	Total N (%)
Medio	Typic cryochrept	Quaking aspen	Cobbly loam	6.4	0.38
Capac	Aeric ochraqualf	Corn/soybean rotation	Clay loam	6.8	0.28

The effect of electron acceptor and donor supply was further evaluated by adding solutions of NO_3^- and sodium succinate (a diffusible, nonfermentable carbon source in soil) to packed cores of preincubated Capac soil. Four treatments were used: distilled water, 100 μg NO_3^--N g^{-1} soil, 1 mg succinate-C g^{-1} soil, and 100 μg NO_3^--N plus 1 mg succinate-C g^{-1} soil. Soil was packed into 2.2 cm diameter by 13 cm acrylic tubes. Solutions (2.1 ml) were introduced by injecting them along the long axis of the tubes, and the amended cores were stored at 4°C for three days to allow the substrate to diffuse throughout the soil while limiting microbial growth. After a one hour equilibration at room temperature, anaerobic denitrification rates were measured, first in cores and subsequently in slurries.

Diffusion Model. A reaction-diffusion model was formulated for nitrate diffusion and reduction in aggregated soil. The following equation was used to simulate this process:

$$\frac{\partial C}{\partial r} = D\left[\frac{2\partial C}{r\partial r} + \frac{\partial^2 C}{\partial r^2}\right] - \frac{V(r)FC}{K_m + C} \qquad [1]$$

where C is the NO_3^- concentration, t is time, D is the intra-aggregate NO_3^- diffusion coefficient, r is the aggregate radius, V(r) is the maximum rate of NO_3^- reduction, Km is the half-saturation constant for NO_3^- reduction, and F is a carbon limitation factor. The F-value, which can vary from zero to one, converts the sink term into an implicit dual Michaelis-Menten relationship. V(r) was a function of the radius, which made it possible to simulate different denitrifier biomass distributions as well as the effect of aerobic and anaerobic zones. To simulate an

aerobic zone, V(r) was set equal to zero. The following boundary conditions were used with Equation [1].

$$C = C_o \text{ at } r = r_{an} \text{ for } t \geq t_o \qquad [2]$$

$$\frac{dC}{dr} = 0 \text{ at } r = 0 \text{ for } t \geq t_o \qquad [3]$$

where C_o is the NO_3^- concentration at the aerobic-anaerobic interface within the aggregate (r_{an}). This upper boundary was chosen to approximate a steady state of nitrification in the aerobic portion of the aggregate.

Equation [1] was solved for both transient and steady state conditions. The transient case was solved by using a finite difference approximation of Equation [1], incorporating time averaging of the diffusion terms. The sink term was not time averaged because of its inherent nonlinearity with respect to concentration. In the steady state, Equation [1] becomes a two point boundary value problem, which was solved using a finite difference method described by Keller (1968).

Both solutions were programmed in BASIC and implemented on a microcomputer. Aerobic and anaerobic denitrification rates and NO_3^- flux across the aerobic-anaerobic interface in aggregates were calculated along with the expected anaerobic slurry rate. Examples of NO_3^- concentration profiles within a diffusion limited aggregate and within an aggregate not limited by NO_3^- diffusion are shown in Figure 1. The marked difference in these two profiles is solely the result of a ten-fold difference in the maximum denitrification rate, V, which is

Figure 1. Simulated NO_3^- concentration profiles in an anaerobic 0.4 cm aggregate under conditions with (□) and without (Δ) a NO_3^- diffusion limitation. Parameter values used (Table 5): $D = 5 \times 10^{-6}$ cm^2 g^{-1}; $F = 1$; $Km = 0.04$ μg N g^{-1}; $Co = 0.15$ μg N g^{-1}; and $V = 2.0$ or 0.2 μg N g^{-1} h^{-1} for the diffusion limited and non-diffusion limited cases, respectively.

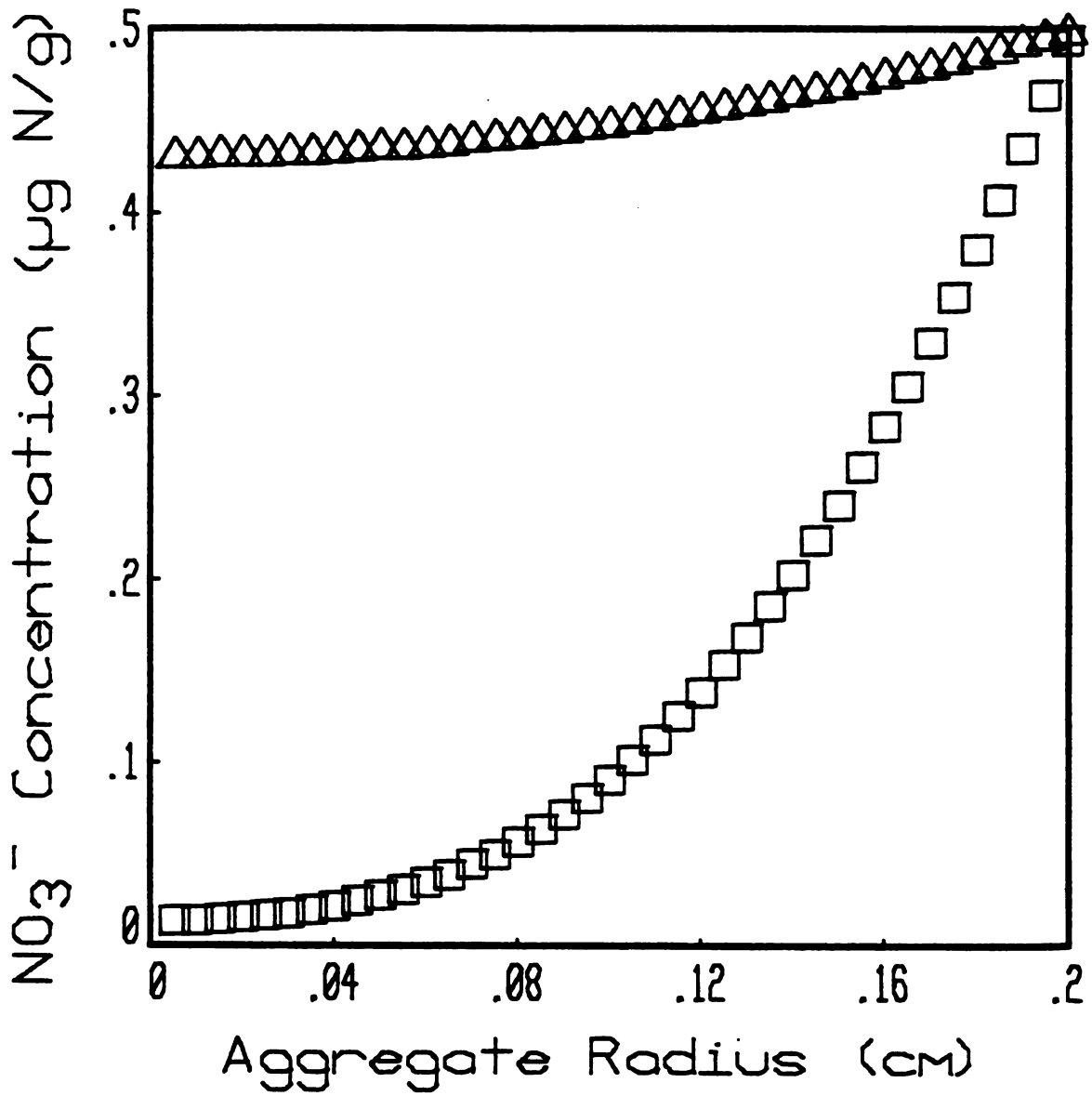
NO_3^- Concentration (µg N/g)
.5
.4
.3
.2
.1
0
0
.04
.08
.12
.16
.2
Aggregate Radius (cm)

Figure 1

within the range found in soils (Tiedje et al., 1982). In both cases the mass balance of NO_3^- is conserved since the denitrification rate is equal to the flux of NO_3^- across the aerobic-anaerobic interface.

RESULTS

The effect of supplemental NO_3^- and glucose on denitrification rates in anaerobic slurries differed between the two soils (Figure 2). Neither addition significantly altered the denitrification rate in the Medio soil, while both NO_3^- and glucose additions increased the denitrification rates in anaerobic slurries of all treatments of the Capac soil (Table 2). Anaerobic slurry rates of the Capac soil increased asymptotically as NO_3^- concentration increased (Figure 3). When these data were fit by the Michaelis-Menten relationship, a Km of 17 $\mu g\ N\ g^{-1}$ soil (450 μM) was obtained. The addition of glucose further increased denitrification rates in all treatments of the Capac soil. Individual soil cores did not always follow the response to NO_3^- and glucose described above so the maximum anaerobic slurry rate obtained during the course of the slurry measurement was used for all other comparisons.

Anaerobic slurry rates were much greater than anaerobic core rates for both soils (Table 3). The core to slurry ratio was significantly lower in the Medio soil (0.3%) than for the treatments of Capac soil (1.1-3.4%). A similar comparison of anaerobic core to anaerobic slurry rates was made for packed cores of Capac soil which were equilibrated with NO_3^- and succinate solutions (Table 4). The slurry rates of the control and NO_3^- amended soil in the cold room experiment were similar to those obtained in the long term incubation experiment (Table 3), while the anaerobic core rates were 4 to 17 times higher. Increased NO_3^- availability was not the cause of this difference because NO_3^-

Figure 2. Effect of NO_3^- and glucose additions on the denitrification rate of anaerobic slurries. NO_3^- added at first arrow, glucose added at second arrow. □ - Medio soil, △ - Capac soil.

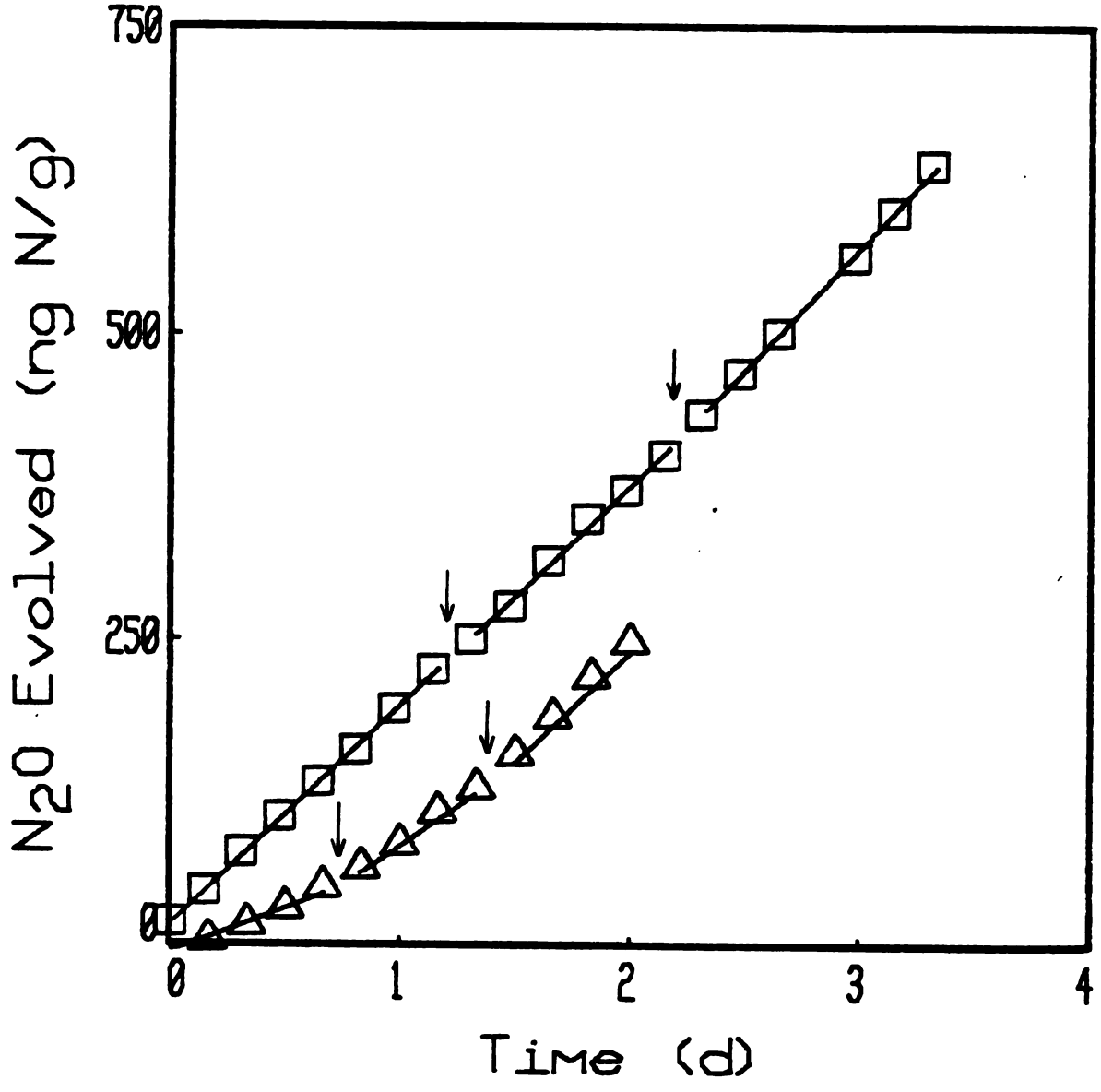

Figure 2

Table 2. Effect of NO_3^- and glucose additions on denitrification rates in anaerobic slurries.

Soil + pretreatment	Unamended	+ NO_3^- (100 μg-N g^{-1})	+ Glucose (1 mg-C g^{-1})
	ng N_2O-N g^{-1} h^{-1}		
Medio	268 ± 143[†]	282 ± 190	265 ± 144
Capac	256 ± 53	401 ± 72	540 ± 125
+ NO_3^-	405 ± 105	439 ± 91	528 ± 100
+ straw	300 ± 43	406 ± 72	487 ± 86

[†] Mean ± 95% confidence interval.

Figure 3. Effect of NO_3^- concentration on the denitrification rate of anaerobic slurries of Capac soil.

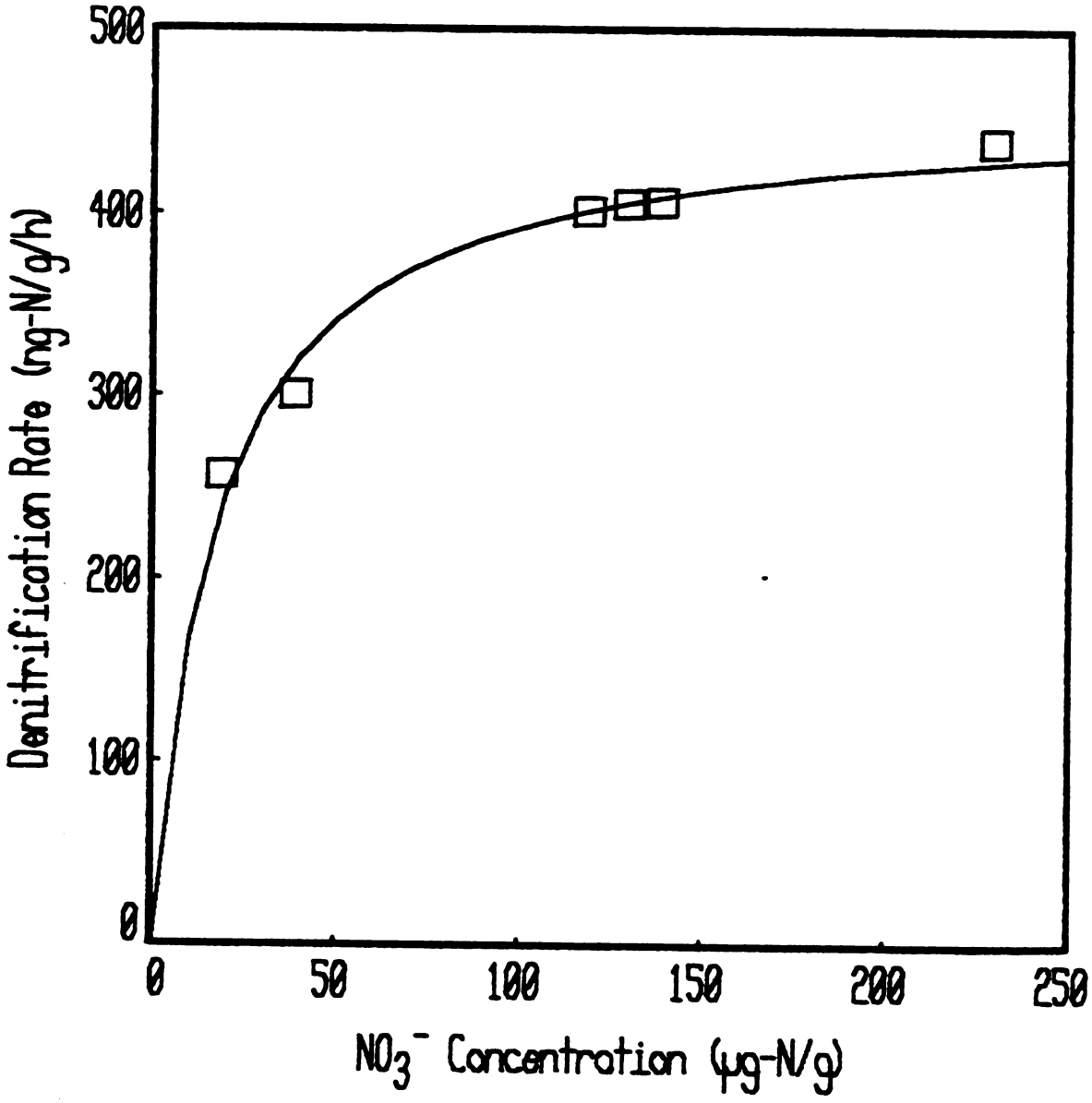
500
400
300
200
100
0
Denitrification Rate (ng-N/g/h)
0
50
100
150
200
250
NO_3^- Concentration (μg-N/g)

Figure 3

Table 3. Denitrification rates of anaerobic cores and anaerobic slurries.

Soil pretreatment	Anaerobic core	Anaerobic slurry	Core:Slurry ratio
	----- ng N_2O-N g^{-1} h^{-1} -----		--- % ---
Medio	0.9 ± 0.53 [+]	340 ± 180	0.32 ± 0.12
Capac	17.4 ± 5.4	588 ± 174	3.43 ± 1.01
+ NO_3^-	5.0 ± 2.3	500 ± 158	1.10 ± 0.40
+ straw	15.7 ± 8.0	506 ± 62	3.01 ± 1.37

[+]Mean ± 95% confidence interval.

Table 4. Denitrification rate of Capac soil amended with NO_3^- and succinate.

Treatment	Anaerobic core	Anaerobic slurry	Core:Slurry ratio
	----- ng N_2O-N g^{-1} h^{-1} -----		---- % ----
H_2O	67.1 ± 50.6[†]	508 ± 89.0	12.3 ± 7.5
NO_3^-	87.0 ± 11.7	579 ± 38.5	15.0 ± 1.8
Succinate	554 ± 108	973 ± 129	57.3 ± 11.5
NO_3^- + succinate	400 ± 43.2	718 ± 48.0	55.9 ± 6.1

[†] Mean ± 95% confidence interval.

concentrations were the same in each experiment, about 20 μg NO_3^--N g^{-1} soil. The difference may have been due to greater carbon availability in the cold room experiment. Physical disturbance of soil by mixing and packing often results in an increase in available carbon (Rovira and Greacen, 1957), and one would expect more of this physically released carbon to be available for denitrification after three days incubation at 4°C than after 21 days at 20°C.

There were no significant differences in anaerobic core rates, anaerobic slurry rates, or core to slurry ratios between the NO_3^- amended and control soil (Table 4). However, the addition of succinate or NO_3^- plus succinate resulted in significant increases in all three of these measurements when compared to the control. Anaerobic core rates and anaerobic slurry rates were both significantly greater in the succinate treatment than in the NO_3^- plus succinate treatment, however, the core to slurry ratios were the same.

DISCUSSION

Differences in denitrification rates between anaerobic cores and slurries reflect the effect of the native soil structure on denitrification rates in nature. Slurried soils always gave markedly higher denitrification rates (Table 3 and 4), presumably because of enhanced distribution of denitrification substrates and denitrifying organisms. With the Capac soil NO_3^- supply shown was not to be limiting, since there was no difference between the core:slurry ratios of soil cores incubated with or without NO_3^- (Table 4). However, the supply of available carbon in the Capac soil was shown to limit denitrification since succinate additions greatly decreased core:slurry ratios (Table 4). The Medio soil had fairly high concentrations of NO_3^- ($\sim$5 μg N g^{-1} soil), so the low core:slurry ratio of this soil is also likely indicative of a carbon supply limitation.

Even with the addition of succinate, a core:slurry ratio less than 100% was obtained. This could be due to a phase transfer limitation of N_2O diffusion out of (or C_2H_2 diffusion into) the liquid phase of the anaerobic cores. Such limitations can be found even in well stirred solutions if the biological activity is large enough (Robinson and Tiedje, 1982).

Whether substrate diffusion is, or is not, important in determining the rate of a reaction is a function of several factors including: substrate concentration of the bulk solution, substrate diffusion coefficient, the path length for diffusion, the system geometry, and the biological kinetic parameters. One of the simplest ways to evaluate

these interacting factors is to calculate the Thiele modulus, which is a dimensionless parameter constructed from these interacting factors (Goldstein, 1976). For spherical particles, like soil aggregates, the Thiele modulus for a single Michaelis-Menten reaction is defined as:

$$\phi = \frac{r_{an}}{3}\left(\frac{V}{K_m \cdot D}\right)^{\frac{1}{2}} \qquad [4]$$

where ϕ is the Thiele modulus. The other constants have been previously defined, except that V is now assumed to be independent of the aggregate radius. The Thiele modulus indicates the degree of any diffusional limitation. Reactions are not limited by diffusion when $\phi \leq 1$, while diffusion becomes increasingly limiting as ϕ becomes larger (Goldstein, 1976). The actual reaction rate is a function of both ϕ and the bulk concentration of substrate (Figure 4). This figure illustrates that even diffusion limited reactions can proceed at maximal velocity if the substrate concentration in bulk solution is sufficiently high.

In soils, the values of each of the variables in Equation [4] for denitrification span one to over two orders of magnitude (Table 5). This variability, of course, leads to an even wider range of possible values of ϕ. Thus, whether or not NO_3^- diffusion is rate limiting to denitrification depends upon the parameter values of the particular soil of interest. For most soils, D and Km are probably quite close to the typical values given in Table 5, so ϕ will primarily be determined by V and r_{an}. It should be noted that ϕ is most sensitive to r_{an}, which it is directly proportional to, while varying with the square root of the other parameters.

Table 5. Soil properties and calculated Thiele moduli.

Parameter	Typical value	Range	Units	Reference
Aggregate radius (r)	0.2	0.02 - 5.0	cm	Gardner, 1956; Smith, 1980
NO_3^- diffusion coefficient (D)	5×10^{-6}	1×10^{-6} - 1×10^{-5}	$cm^2\ s^{-1}$	Smith, 1980
Maximum denitrification rate (V)	0.3	0.03 - 3.0	$\mu g\ N\ g^{-1}\ h^{-1}$	Kohl et al., 1976; Tiedje et al., 1982
NO_3^- Michaelis constant (Km)	0.04	0.01 - 0.1	$\mu g\ N\ g^{-1}$ †	Betlach and Tiedje, 1981
Thiele modulus (ϕ) §	1.4	0.019 - 480	-	-
NO_3^- concentration (Co)	1.0	0.1 - 60.0	$\mu g\ N\ g^{-1}$	Reddy et al., 1978; Vitousek et al., 1982

†Assumes 0.20 g g^{-1} water content.
§Assumes completely anaerobic aggregate.

Since NO_3^- concentrations in soil can vary from about Km to 6000 x Km (Table 5), it is important to examine the relation between and S_o presented in Figure 4. Under the low NO_3^- concentrations (< 1 μg g^{-1} soil) typically found in forest soils (Vitousek et al., 1982), there can be a response to NO_3^- additions, even when NO_3^- diffusion is not limiting. However, in agricultural soils, which generally have higher NO_3^- concentrations (> 5 μg N g^{-1} soil), a response to NO_3^- addition would be noticed only under diffusion limited conditions ($\phi > 10$; Figure 4).

If the typical parameter values of r_{an} V, Km, and D from Table 5 represent median values for aggregated soils, then NO_3^- diffusion should limit denitrification about half of the time under anaerobic conditions when carbon is not limiting, since ϕ is 1.4 for the median soil. Often, however, denitrification occurs within anaerobic microsites of a generally aerobic soil and under carbon limitation. The effect of carbon limitation on NO_3^- diffusion can be roughly approximated by multiplying V by the carbon limitation factor F, which varies from 0 to 1. This has the effect of decreasing ϕ by the square root of F. Thus, a carbon limitation effectively decreases the magnitude of a NO_3^- diffusion limitation. The Capac soil used in this study was carbon limited with an F of approximately 0.24. F was calculated from the quotient of the core:slurry ratios of non-carbon amended to carbon amended soil (Table 4). (This F value roughly corresponds to an available carbon concentration of Km/3 for carbon, if Michaelis-Menten kinetics are assumed). Applying this F to a V of 0.53 μg N g^{-1} h^{-1} (Table 3) and assuming typical values for r_{an}, D, and Km (Table 5), a ϕ of 0.89 for anaerobic Capac soil can be calculated. Thus, no NO_3^-

Figure 4. Normalized reaction rate as a function of the dimensionless bulk concentration ($S_o = C_o/Km$) for different values of the Thiele modulus, ϕ , shown for values from 0.1 to 500.

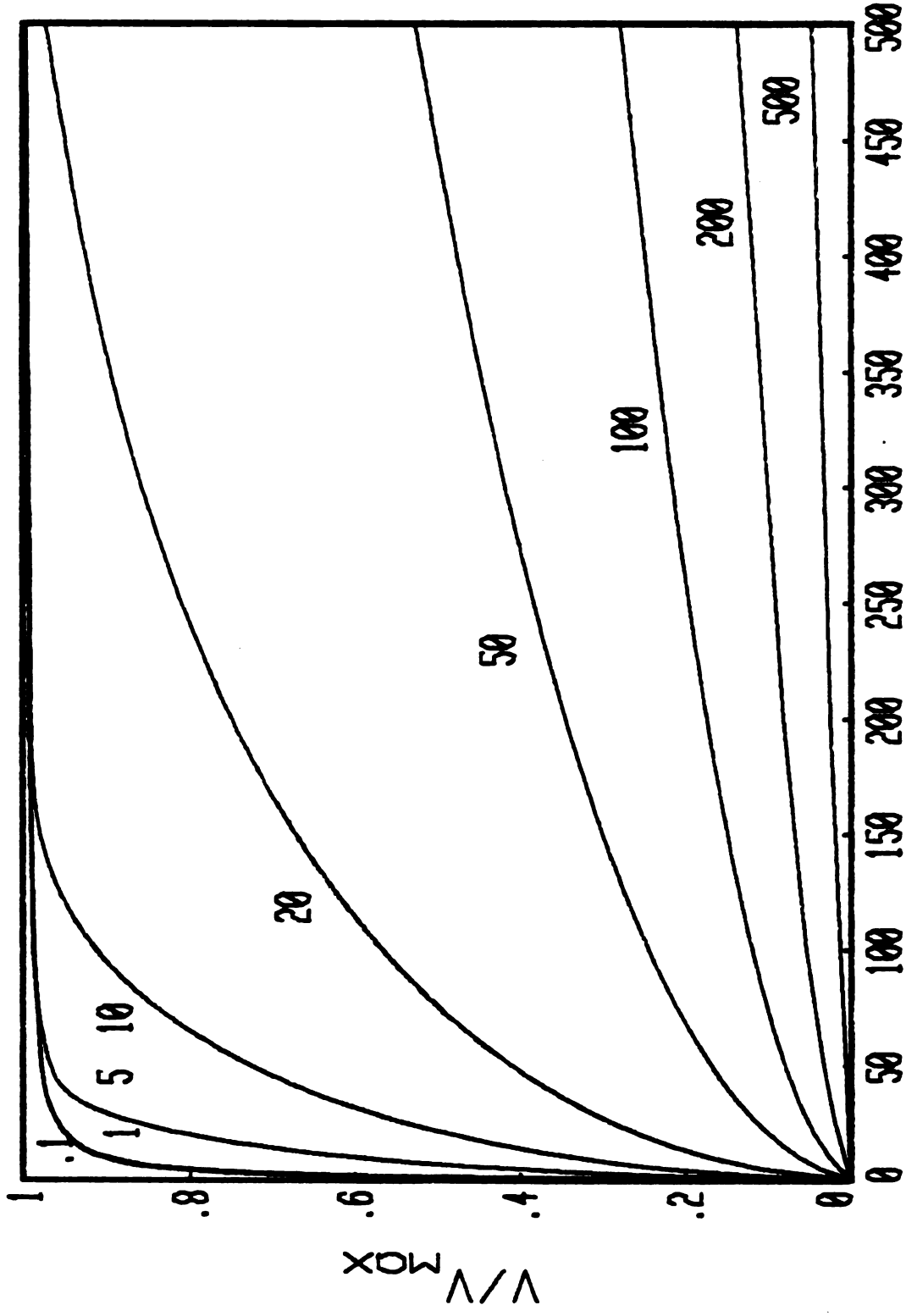
V/V_MOX
1
.8
.6
.4
.2
0
0
50
100
150
200
250
300
350
400
450
500
S_O
.1
1
5
10
20
50
100
200
500

Figure 4

diffusional limitation should exist in this soil, a contention supported by the lack of response to NO_3^- in the cold room experiment (Table 4).

The effect of anaerobic microsites within aerobic soil, on the other hand, would usually increase the likelihood of NO_3^- diffusional limitation. This effect can be studied using the zero order reaction-diffusion equation for oxygen developed by Greenwood (1962) and further extended by Smith (1980). A critical radius of 1.0 cm can be calculated at an oxygen concentration of 0.17 $cm^3\ cm^{-3}$, an O_2 diffusion coefficient of $1 \times 10^{-6}\ cm^2\ s^{-1}$, and respiratory activity of $1 \times 10^{-6}\ cm^3\ CO_2\ cm^{-3}\ s^{-1}$. Consequently, denitrification could only occur in aggregates larger than 1.0 cm. Aggregates of this size would usually have ϕ values in excess of 1.0, since r_{an} increases very rapidly as the aggregate radius exceeds the critical radius (Smith, 1978). Consequently aggregates larger than 1 cm will often be NO_3^- diffusion limited, even under most carbon limited situations. However, it should be noted that a diffusional limitation of NO_3^- may not be evident if the bulk solution NO_3^- concentration is several fold higher than the Km for NO_3^- reduction (Figure 4).

Aggregate size is not uniform in soils, so whether or not denitrification is diffusion limited also depends upon the aggregate size distribution. The log normal distribution generally describes soil aggregate distributions (Gardner, 1956) and has been used to model soil anaerobic microsites (Smith, 1980). The volume fraction of soil that experiences a diffusional limitation may be calculated using the equations presented by Smith (1980), by simply redefining the critical radius as the radius of the largest aggregate that is not diffusion limited. This critical radius for diffusion limitation can be

calculated from Equation [4] by setting ϕ equal to one and solving for r_{an}. The only limitation is that r_{an} must be as large or larger than the critical radius of anaerobiosis. This analysis was done with typical values of V, D, and Km (Table 5) for a totally anaerobic soil and for an aerobic soil with a critical radius of 1.0 cm (Figure 5). Two points can be made from the results shown in this figure: First, the fraction of anaerobic soil that is diffusion limited for NO_3^- is much greater under aerobic conditions, because only larger aggregates have anaerobic centers. Second, under anaerobic conditions, even when a soil has a mean aggregate radius at which denitrification is not diffusion limited (e.g., 0.1 cm), there is a certain fraction of the soil which does experience diffusion limitations (e.g., 30%).

Most previous work which suggests that NO_3^- diffusion limits denitrification rates is based upon the higher Km estimates obtained in soil systems compared to pure culture work (Table 6). Km values for soils are 13 to 1300 times those found in pure culture, although they are similar to the Km values obtained from cell free extracts. The high Km values found by Ryzhova (1979) and Kohl *et al.* (1976) for the 2% organic-C soil can probably be attributed to limiting NO_3^- diffusion from the water layer above the soil (Phillips *et al.*, 1978), however, a relatively low Km was found for the 2.2% organic-C soil incubated in the same manner (Kohl *et al.*, 1976). In studies which monitored N_2O evolution (Klemmedtson, 1977; Yoshinari, *et al.*, 1977), mass transfer of C_2H_2 into the soil and N_2O out of the soil may have inflated the estimated Km values (Robinson and Tiedje, 1984), however, only the glucose amended soil of Yoshinari *et al.* had a high Km. Internal diffusion of NO_3^- within the soil matrix is another factor which could

Figure 5. Relationship between aggregate size distribution and the extent of diffusion limitation in aerobic and anaerobic soils. Parameter values used: $V = 0.3\ \mu g\ N\ g^{-1}\ h^{-1}$, $D_{NO_3^-} = 5 \times 10^{-6}\ cm^2\ s^{-1}$, $Km = 0.04\ \mu g\ N\ g^{-1}$, and $r_{an} = 1.01$ cm.

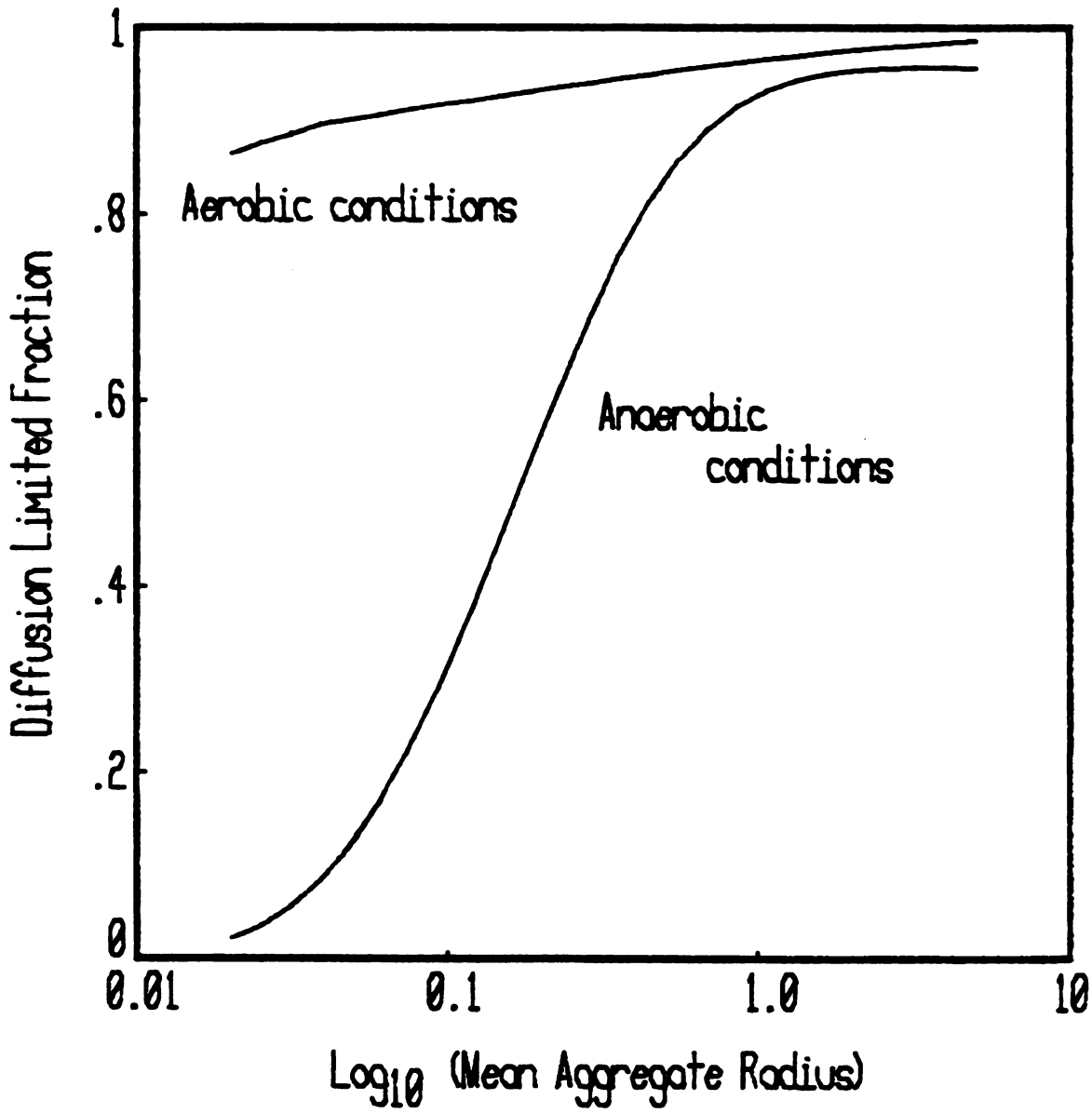
1
.8
.6
.4
.2
0
Diffusion Limited Fraction
Aerobic conditions
Anaerobic
conditions
0.01
0.1
1.0
10
Log10 (Mean Aggregate Radius)

Figure 5

Table 6. Comparison of K_m values for NO_3^- reduction obtained in various assay systems.

Assay system	Organism or soil characteristics	Experimental procedure	NO_3^- Km (μM)	Reference
Cell-free extract	Pseudomonas aeruginosa	e^- donor: NADH NO_2^- production	500	Fewson and Nicholas, 1961
	Micrococcus denitrificans	e^- donor: benzyl viologen NO_2^- production	250	Forget, 1971
	Micrococcus halodenitrificans	e^- donor: benzyl viologen NO_2^- production	1300	Rosso et al., 1973
	Pseudomonas aeruginosa	e- donor: benzyl viologen NO_2^- production	300	Carlson et al., 1982
Whole cells	Alcaligenes sp. Flavobacterium sp. Pseudomonas fluorescens	NO_3^- disappearance	<15	Betlach and Tiedje, 1981
	Pseudomonas fluorescens	NO_3^- disappearance	5–10	Edwards and Tiedje, 1981
Soil	sand 0.11% organic matter	NO_3^- disappearance anaerobic, 100% H_2O C amended, not shaken rate over (?) h	470	Bowman and Focht, 1974
	silt loam, < 2 mm 2.4% organic-C	NO_3^- disappearance anaerobic (?), 167% H_2O	2100	Kohl et al., 1976
	silt loam, < 2 mm 2.2% organic-C	not C amended, not shaken rate over 24 h	170	

Table 6 (cont'd.)

< 2 mm 5.2% organic-C	N_2O production with C_2H_2 anaerobic, 200% H_2O not amended, shaken rate over < 15 h	140	Klemmedtson, 1977
sandy loam, -glucose	N_2O production with C_2H_2 anaerobic, 35% WHC not shaken rate over 1 h	130	Yoshinari et al., 1977
sandy loam, +glucose		1200	
< 1 mm 3.2% organic matter	NO_3^- disappearance anaerobic (?), 500% H_2O not C amended, not shaken rate over 24 h	3100	Rhyzhova, 1979

have contributed to the inflated Km values found in soil. This influence can be estimated by calculating ϕ values for the soils using the maximum velocity values reported in the studies, a Km of 15 μM, and typical values of 0.2 cm for r_{an} and 5 x 10^{-6} cm^2 s^{-1} for D. These parameters give a range of ϕ from 0.53 to 2.68 or, in other terms, Km would have been overestimated by three fold at most. Taking into account, the soil Km values would range from 55 μM to 4 mM; still greater than whole cell values, but comparable to Km values of cell free extracts.

A possible explanation for the discrepancy between the Km for NO_3^- reduction of soils and cells is that more than one NO_3^- uptake system exists. Recent work with assimilatory NO_3^- reduction suggests that there may be both low affinity (1 mM) and a high affinity (5 μM) uptake mechanism (Thayer and Huffaker, 1981). Evidence for the similar situation with the dissimilatory system is lacking because whole cell studies have never been done at high enough NO_3^- concentrations to detect a low affinity system, while the NO_3^- concentrations used in soils have not been low enough to measure a high affinity system. From the organism's standpoint, it would be wasteful for a denitrifier in a carbon limited system like soil to (presumably) use ATP to scavenge NO_3^-, when it already exists in the soil solution at concentrations at or above the Km of the NO_3^- reductase enzyme. It would probably be a competitive advantage for denitrifiers to have two uptake systems; one with a relatively high Km which is energy independent, the second with a low Km powered by cellular energy. The existence of a low affinity system would decrease the importance of NO_3^- diffusion, since the higher Km would lower ϕ. However, a higher Km would make denitrification

more responsive to NO_3^- additions, since natural NO_3^- concentrations would be in the first order region.

The discussion has thus far centered on NO_3^- diffusion and denitrification in aggregated systems. Different conceptual and mathematical models would have to be used for non-aggregated soils. Presumably a non-aggregated soil would have "hot spots" of microbial activity more or less randomly distributed throughout the profile. These centers of microbial activity would likely be associated with organic carbon. In such a situation, one might envision the impact of NO_3^- diffusion to be greater since the path length for diffusion would probably be longer. This area of reaction-diffusion in non-aggregated soils has yet to be explored either experimentally or theoretically. It should also be noted that hot spots of microbial activity are likely to exist in aggregated soils, as well. Their presence would also tend to increase the importance of diffusive limitations, in this case primarily through an increase in the maximum velocity parameter.

CONCLUSIONS

1. Aggregate size, followed by the Vmax for denitrification, are the prime determinants of whether or not denitrification is limited by NO_3^- diffusion. However, a formal NO_3^- diffusional limitation may be ameliorated by high solution concentrations of NO_3^-.

2. Aerobic soils with anaerobic microsites are more likely to experience the effects of NO_3^- diffusion limitations.

3. Carbon limitation decreases the magnitude of any potential NO_3^- diffusion limitation by effectively decreasing the maximum denitrification rate.

4. The relatively high Km values for NO_3^- reduction in soil may be evidence for the presence of two NO_3^- uptake systems, since NO_3^- diffusion was not great enough to markedly inflate the Km values that have been reported.

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CHAPTER 3

EFFECTS OF CARBON, NO_3^-, AND MOISTURE ON THE ESTABLISHMENT OF DENITRIFICATION CAPACITY IN SOIL

Denitrification is a component of nitrogen cycling in soils of virtually all terrestrial ecosystems. However, the magnitude of this process varies greatly, both among and within ecosystems. This variability is undoubtedly a function of the previous environmental histories of the various habitats.

Environmental factors affect both the expression of denitrification by the existing population of denitrifying bacteria and the size of the active denitrifier biomass itself. Much work has been devoted to examining the effect of such factors as available carbon, NO_3^- concentration, pH, and aeration on denitrification rates. This work has recently been summarized in several excellent reviews (Firestone, 1982; Knowles, 1982). The effects of these variables on the establishment and maintenance of the denitrification capacity has been less well studied.

A previous survey of soils from a variety of habitats suggested that active denitrifier biomass, or denitrification capacity, was directly related to moisture and organic carbon, while pH had no consistent effect (Tiedje *et al.*, 1982). Smith and Tiedje (1979) observed a similar result when soils received either irrigation or a glucose amendment. Flooding soil with NO_3^- solution (Doner *et al.*, 1975; Volz *et al.*, 1975) or anaerobically incubating NO_3^- amended, saturated soil (Jacobson and Alexander, 1980) resulted in increased

denitrifier populations. However, no attempt was made in these studies to determine whether the population increase was due to the NO_3^- addition or to a change in aeration status.

Denitrification capacity could be controlled by environmental factors primarily by two mechanisms. The first involves the repression-derepression of the denitrification enyzmes by O_2 and the possible induction of these enzymes by NO_3^- (Firestone, 1982). This mechanism would not require microbial growth but simply the expression of the denitrifying potential of already existing, but inactive denitrifiers. The second mechanism involves the increase in denitrification capacity through cell division. These two mechanisms, which are illustrated in Figure 1, have been designated Phase IIa and Phase IIb by Smith and Tiedje (1979).

The purpose of this study was two-fold: (1) to examine the relative importance of carbon, moisture, and NO_3^- in controlling denitrification capacity, and (2) to attempt to elucidate the mechanism(s) functioning to control denitrification capacity.

Figure 1. Hypothetical soil biomass composition and anticipated response from two different mechanisms for increasing denitrification capacity.

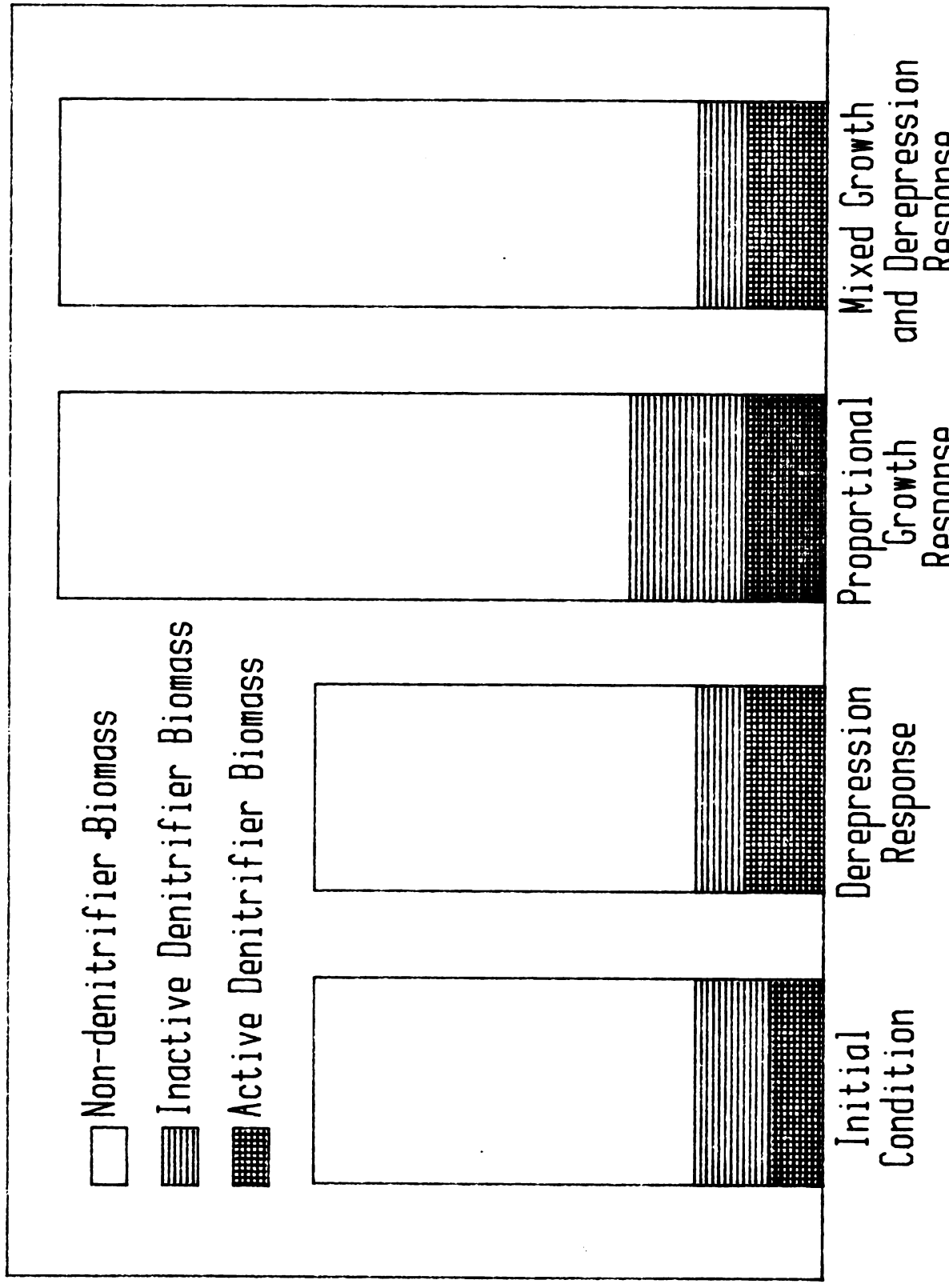
Non-denitrifier Biomass
Inactive Denitrifier Biomass
Active Denitrifier Biomass
Initial Condition
Derepression Response
Proportional Growth Response
Mixed Growth and Derepression Response

Figure 1

MATERIALS AND METHODS

Capac clay loam (Aeric ochraqualf; pH 6.8, 0.28% N) was collected from a field previously planted to corn, sieved to < 2mm, and stored at 4°C at field moisture until used. The sieved soil was preincubated in a polyethylene bag at room temperature for a few days prior to its experimental use. Finely milled, dried alfalfa straw (2.81% N) was used as the carbon source in this study.

A 2x2x2x4-way factorial arrangement of treatments was used in this experiment. Two moisture contents (23 and 28%; 0.2 and 0.01 MPa), two levels of carbon addition (0 and 1 mg C g^{-1} soil), and two levels of NO_3^- addition (0 and 100 μg NO_3^--N g^{-1} soil) were used. Estimates of denitrification capacity and total microbial biomass were measured at four time points (1,2,4 and 7 days).

The experiment was initiated by adjusting preincubated, moist soil to the desired water content with either distilled water or a NO_3^- solution. Straw was thoroughly mixed into the treatments receiving C at this time. Approximately 175 g of soil, on a dry weight basis was transferred to 250 ml Erlenmeyer flasks and packed to a bulk density of about 1.2 gm cm^{-3}. These incubation vessels were capped with a serum stopper and incubated at 25°C in the dark. Daily respiration rates were measured by analyzing headspace gas samples for CO_2 using a microthermistor equipped GC. The head space of the flasks was replenished daily, or as needed, to maintain aerobiosis.

At each time point in the experiment, one flask from each treatment was sacrificed for analysis. Six 10 g (dry wt.) subsamples were taken

for denitrification capacity measurements, seven 10 g (dry wt.) subsamples were removed for microbial biomass C measurements, and three 10 g (dry wt.) subsamples were taken for NH_4^+ and NO_3^- determinations. A Technicon autoanalyzer was used to colorimetrically determine NH_4^+ and NO_3^- concentrations. The remaining soil was used to determine gravimetric water content.

Denitrification capacity was determined using an anaerobic slurry technique similar to the Phase I assay of Smith and Tiedje (1979). Slurries were made by adding 25 ml of a solution containing glucose (1 mg C g^{-1} soil), NO_3^- (100 µg N g^{-1}) soil and chloramphenicol (500 µg g^{-1} soil) to 10 g of soil in a 160 ml serum bottle. The bottles were sealed with a Balch stopper, evacuated and flushed several times with Ar to remove any traces of O_2. The soil slurries were shaken on a rotary shaker (250 rpm) and N_2O production in the presence of 10% C_2H_2 was measured over the course of a one hour incubation. N_2O was quanitified using a GC equipped with a ^{63}Ni-electron capture detector (Parkin et al., 1984).

Total microbial biomass was measured by the $CHCl_3$ fumigation method (Jenkinson and Powlson, 1976). The amount of CO_2 produced by fumigated samples and an unfumigated control, after a 10 day incubation, was measured by gas chromatography.

Microbial biomass was also estimated in a separate experiment by measuring soil ATP. An incubation experiment similar to that described above was set up at 28% moisture with or without a 1 mg C g^{-1} soil straw amendment, without NO_3^- addition. Respiration was measured daily, as described above, and ATP was extracted from four subsamples of soil for each treatment by the method of Paul and Johnson (1977) and quantified

by bioluminescence with a Chem-Glo photometer equipped with an Aminco integrator-timer.

RESULTS

Microbial respiration was increased about ten-fold by the straw addition (Table 1). The effects of water content and NO_3^- addition were insignificant in the straw amended treatments. Respiration was 20% greater in the wetter, unamended soil, while the NO_3^- addition caused a 20% decrease in respiration in unamended soils.

Denitrification capacity measurements for the various treatments had a relatively high degree of variability, with a range of coefficients of variation from 8 to 52%. An analysis of variance of the data indicated a highly significant interaction between time of sampling and water content, as well as highly significant main effects of carbon amendment and sampling date. The NO_3^- addition had no effect on the the denitrification capacity of the soil. The effect of the straw addition was most dramatic, resulting in a 40 to 63% increase in denitrification capacity over the unamended soil (Figure 2).

The interactive effect of water content and sampling date was caused by the higher denitrification capacity at the lower water content on day 1, while the denitrification capacity was higher in the wetter soil at all other sampling times. However, the water content effect was not significant at any sampling date.

Increases in denitrification capacity over time were evaluated with respect to the denitrification capacity at the beginning of the incubation period. There was a significant increase in denitrification capacity with time when carbon was added at all sampling periods except day 1 for the 28% moisture treatment, when carbon was added. Without

Table 1. Cumulative CO_2 evolution over a seven day incubation period.

Carbon addition	23% H_2O		28% H_2O	
	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$
	- - - - - - - - - - $\mu g\ CO_2\text{-}C\ g^{-1}$ soil - - - - - - - - - -			
- straw	31 ± 0.73[+]	24 ± 0.98	37 ± 0.34	29 ± 0.37
+ straw	270 ± 3.6	270 ± 2.0	270 ± 3.0	270 ± 4.1

[+]Mean ± estimated standard deviation.

Figure 2. Changes in active denitrifier biomass over time of incubation. □ - 23%, no straw; ■ -23% H_2O, 1 mg straw-C g^{-1}; △ -28% H_2O, no straw; ▲ -28% H_2O, 1 mg straw-C g^{-1}.

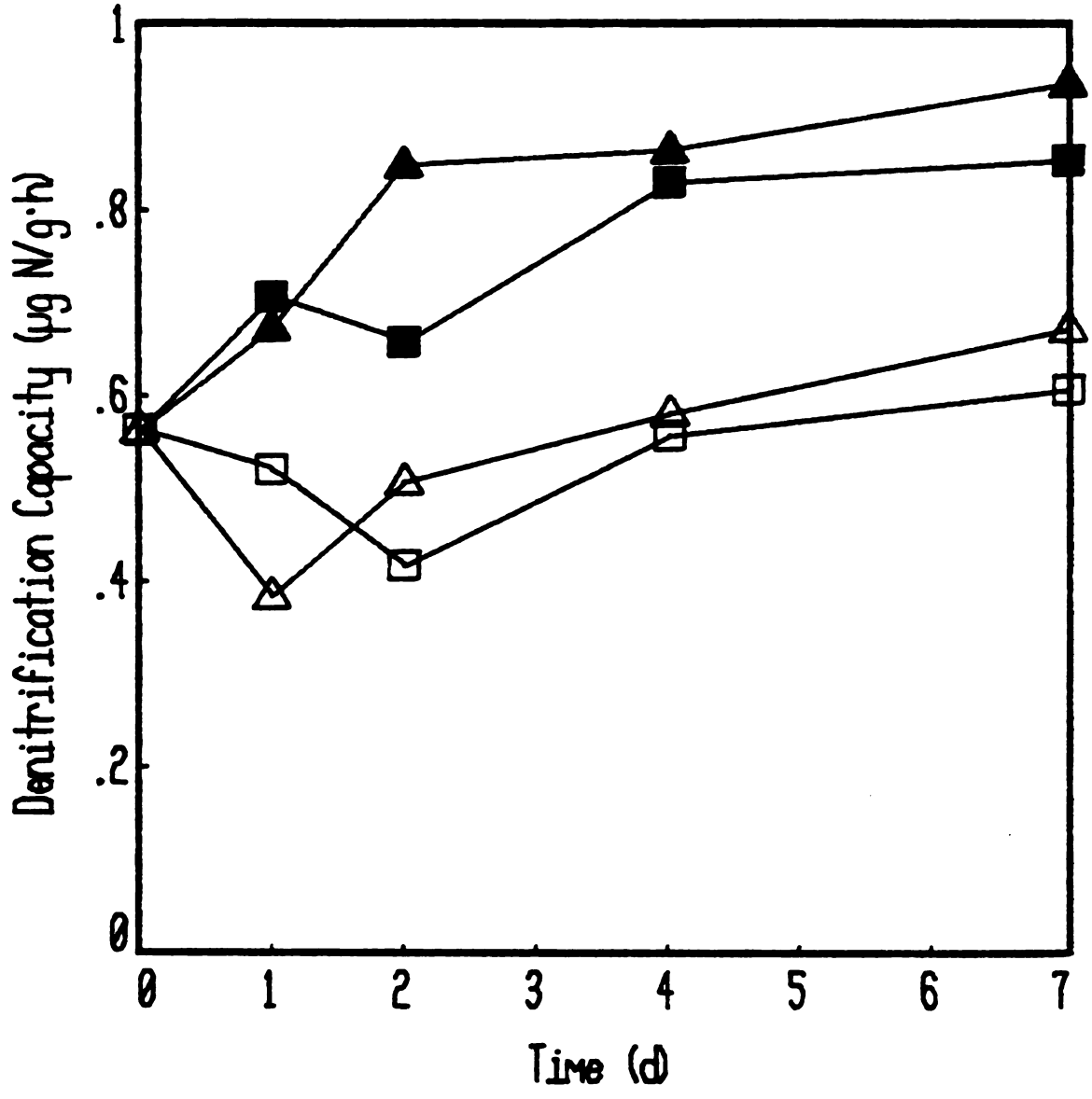

Figure 2

added carbon, the denitrification capacity did not change significantly from the start of the incubation, except at 28% moisture on day 1 (when it was lower) and day 7 (when it was higher).

The flush of CO_2 evolution was 46% greater when straw was added, while the other factors had little effect. A kc value of 0.41 (Anderson and Domsch, 1978) was used to estimate total microbial biomass from the CO_2 flush data. Microbial biomass in unamended soil was 455 μg C g^{-1} (354 μg C g^{-1} when unfumigated control was subtracted) and 667 μg C g^{-1} of microbial biomass in straw amended soil. Because the CO_2 evolved in unfumigated controls of the carbon amended soil was anomalously high--in one case even greater than the fumigated samples, they could not be used to provide a biomass measurement. This anomalous behavior in carbon amended soils has been previously observed (Sparling et al., 1981) and is apparently due to the inability of the surviving microorganisms to utilize the exogenous carbon source. It was because of this behavior that we used ATP as an additional indicator of microbial biomass.

Biomass ATP remained unchanged with time in the unamended treatment (Table 2). There was an increase in microbial ATP over time in the soil which received a carbon addition, presumably due to microbial growth. The new steady state level of biomass was 40% greater than that of the unamended soil.

The proportion of active denitrifer biomass to total microbial biomass was expressed by the ratio of denitrification capacity to microbial ATP. We chose to use ATP instead of biomass-C from fumigation because of the difficulties encountered with the control in the $CHCl_3$ fumigation method in carbon amended soils. The similarity in the temporal response of both the CO_2 flush and microbial ATP for the

Table 2. Changes in microbial ATP.

Time	No straw	Straw added
d	- - - - - - - μg ATP g^{-1} soil - - - - -	
0	0.93 ± 0.10[†]	0.93 ± 0.10
1	1.01 ± 0.10	1.19 ± 0.13
2	0.96 ± 0.12	1.36 ± 0.11
4	1.03 ± 0.11	1.42 ± 0.08
7	1.03 ± 0.18	1.40 ± 0.11

[†]Mean ± 95% confidence interval.

unamended soil supports the use of ATP for comparative purposes. The ratio of denitrification capacity to microbial ATP was not significantly affected by any of the treatments and remained relatively constant throughout the incubation period (Figure 3).

Figure 3. Changes in the ratio of denitrification capacity to microbial ATP over time of incubation. Symbols as in Figure 2.

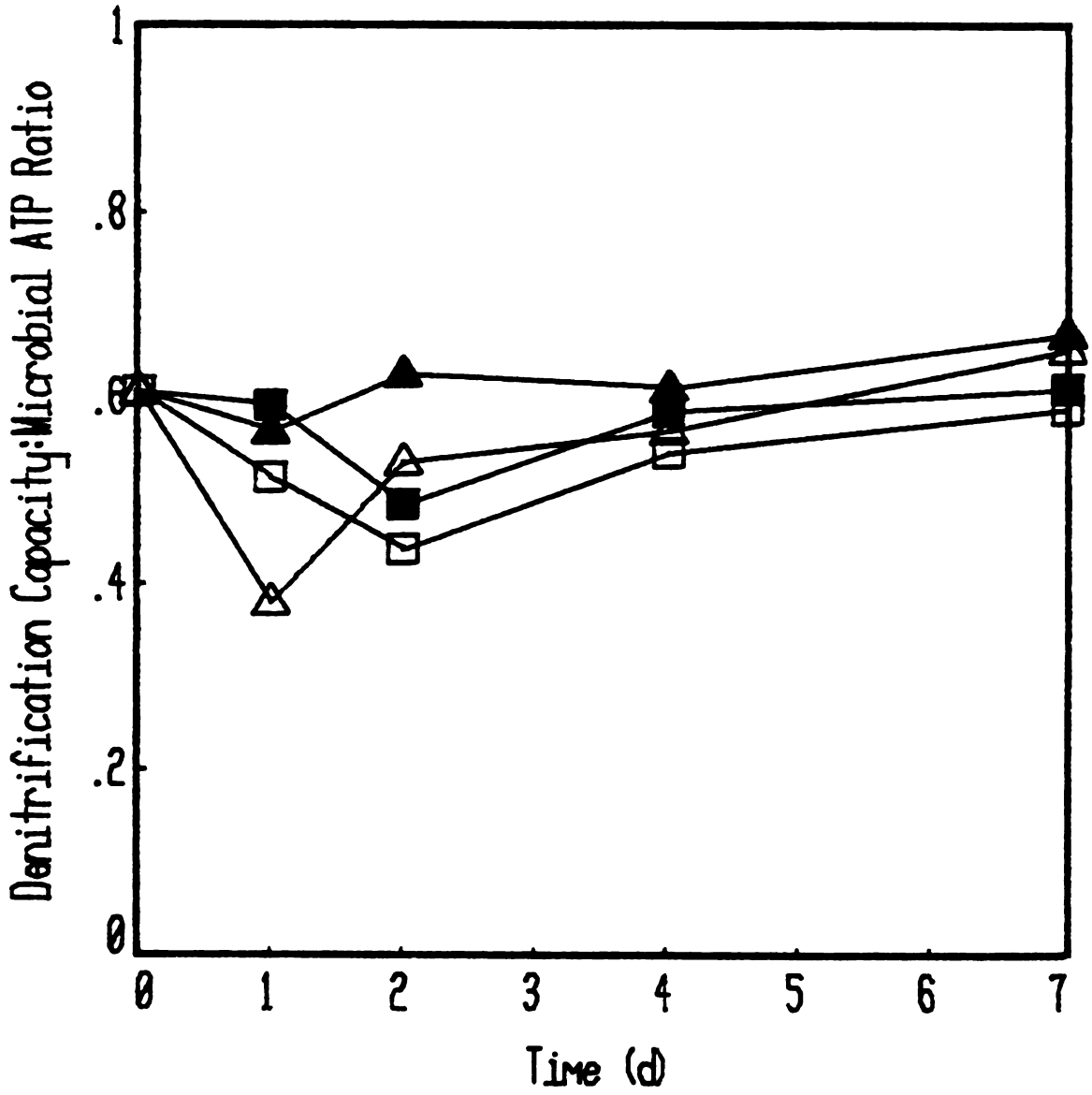
Denitrification Capacity:Microbial ATP Ratio
1
.8
.6
.4
.2
0
0
1
2
3
4
5
6
7
Time (d)

Figure 3

DISCUSSION

There was no effect of NO_3^- on the size of either denitrification capacity or total microbial biomass. This was probably because the Capac soil had a high NO_3^- content (> 20 μg N g^{-1}) even without the addition of 100 μg NO_3^--N g^{-1}. Denitrification would rarely be limited by NO_3^- in a soil with a NO_3^- concentration this high (Myrold and Tiedje, 1984). It would also be unlikely that NO_3^- would have been limiting for denitrifying enzyme induction. Under very low NO_3^- conditions (< 1 μg N g^{-1}), denitrifier biomass may be restricted by NO_3^- concentrations, since 2 to 8 μg NO_3^--N are needed to produce 10^6 denitrifiers in soil (Jacobson and Alexander, 1980).

Increasing the water content from 23 to 28% slightly increased denitrification capacity--but not significantly--and had no significant effect on total microbial biomass. This is consistant with the results of Sexstone *et al.* (1984), who found no significant change in the Phase I rate (denitrification capacity) of Capac clay loam at water contents of 19.6, 23.2, and 25.5%. However, using coarser textured soils, Smith and Tiedje (1979) and Sexstone *et al.* (1984) did find that denitrification capacity increased with increasing water content. These results are consistent with those of Doner *et al.*, 1975), who found increases in numbers of denitrifying bacteria, but no change in the total bacterial population when sandy loam soil columns were flooded with NO_3^- solution. The different responses to water content changes between fine and coarse textured soils may be the result of more complete derepression of denitrifying enzymes (i.e., a greater

proportion of active to inactive denitrifiers--Figure 1) in finer textured soils because of poorer aeration or a greater number of anaerobic microsites.

The potential effect of water content differences on the aeration status of the soils used in this experiment was evaluated by using a model which predicts the anaerobic volume of an aggregated soil (Smith, 1980). This model requires estimates of respiration rate and pore space O_2 concentration (which were measured), intra-aggregate oxygen diffusion coefficient, and log mean aggregate radius and dispersion constant. These last three parameters were estimated by to be 5×10^{-6} cm^2 s^{-1}, 0.2 cm, and 1.0, respectively, for the Capac clay loam (Sexstone et al., 1984). The anaerobic functions calculated from this model were less than 0.05% for the 23 and 28% water content treatments. The lack of a significant moisture effect was most likely due to the insignificant difference in anaerobiosis at the two water contents used in this study.

Unlike the other two factors, the addition of straw did cause an increase in the denitrification capacity and also in total microbial biomass. The carbon addition most likely caused an increase in active denitrifier biomass through growth, since the ratio of denitrification capacity to total microbial ATP remained constant. Smith and Tiedje (1979) also observed an apparently growth related response to their glucose addition to soil. This type of effect is not unlikely, since the majority of the denitrifiers in soil are chemoheterotrophs (Firestone, 1982). Thus, the active denitrifier biomass should increase in proportion to total microbial biomass, as long as denitrifiers can effectively compete with other heterotrophs for carbon. Indeed, Smith and Tiedje (1980) have shown that some denitrifiers are more capable

competing as aerobic heterotrophs than they are as denitrifiers. This contention is also supported by the work of Stanford et al. (1975) who found long-term denitrification activity measures to be highly correlated with extractable glucose-C, a parameter which has been correlated with total microbial biomass (Jenkinson, 1968).

It could, of course, be possible for derepression of inactive denitrifier biomass to occur to the same extent that the total microbial biomass increased due to growth (see Figure 1). This combination is, however, rather unlikely. In addition, when Smith's model of anaerobiosis was used with the respiration rates and pore space O_2 concentrations of straw amended soil, an anaerobic fraction of less than 0.7% was obtained. This is not much greater than those of the unamended soils and is not likely to have caused a disproportionate change in the derepression of denitrifying enzymes.

These results suggest that NO_3^- should not be important in establishing and maintaining denitrification capacity, at least at the generally high NO_3^- concentrations found in agricultural soils. In this study, carbon availability, through the mechanism of microbial growth was the dominant factor controlling denitrification capacity. However, in other soils or under different conditions of carbon availability and moisture, moisture could be a potentially important controlling factor and the derepression mechanisms could predominate.

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