AN EVALUATION OF CARBON MONOXIDE AND METHANE AS SUBSTRATES FOR THE DENITRIFICATION OF WATER

by

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Dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY
in
Civil Engineering

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March, 1990

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(ABSTRACT)

This study involved the use of soil and suspended growth microcosms to study the variation in groundwater denitrification rates using different substrates. Two gaseous substrates, carbon monoxide and methane, were studied and compared to a common liquid substrate, methanol.

Denitrification with carbon monoxide as a substrate was achieved using an acclimated seed of mixed activated sludge and anaerobic digester sludge. Kinetic studies of denitrification using carbon monoxide suggested a strong substrate inhibition effect. The observed maximum denitrification velocity of 0.026 mg N/d-mg VSS occurred at a carbon monoxide partial pressure of 0.10 atmospheres (2.8 mg/ℓ). At higher carbon monoxide partial pressures, denitrification velocities decreased. The denitrification velocities at various carbon monoxide concentrations were described by a modified form of the Haldane substrate inhibition model. The biomass yield using carbon monoxide was 1.1 mg VSS/mg VSS, the maximum specific growth rate was 0.03 mg VSS/d-mg VSS, and the half velocity constant was 26 mg-N/ℓ. Denitrification rates using carbon monoxide as a substrate were much slower than those obtained using methanol, and the cost of carbon monoxide was much higher.

Denitrification occurred readily, when methanol was provided as a substrate, in microcosms containing either a clay soil, a sandy soil, or activated sludge. Under the conditions of this study, denitrification was not achieved in clay soil or sandy soil microcosms using methane or carbon

monoxide as substrates. Denitrification was not achieved using methane as a substrate with an activated sludge seed.

Acknowledgements

I would like to thank my graduate committee for all of their advice, guidance, and patience. I studied under Bob Benoit as an undergraduate and in graduate school. He was a very good teacher and advisor, and he helped me "think more like a microbiologist". I have the deepest respect for Ray Reneau. If I had to do the impossible, I would want him on my team. Joe Sherrard planted the seed from which this research grew, however, equally important was his advice on the use of the word "however". John Novak helped me understand my unusual kinetic results and gave me much crucial advice, always at the right time. Greg Boardman was my advisor and friend. I cannot thank him enough for his assistance, guidance, and patience.

I would like to thank Marilyn Grender and Julie Petruska for their laboratory assistance, patience, and friendship. Marilyn taught me virtually everything I know about instrumental analysis of environmental samples, and more importantly how to responsibly interpret the results. Her attempt to teach me a flip turn was not as successful, but that was not her fault. Julie is an exception. She is a "Jack of all trades and a master of many". I could not have completed my laboratory work without her assistance.

Thanks also go to my fellow graduate students Deborah, Dale, Gary, Mark, Marcia, Phil, Dave and Dave, Kevin, Lisa, Jim, Jessi, Angela, Amy, Meg, Lynn, Don, Sam, Anne, Sheryl, and Trina. Their friendship helped me maintain my sanity throughout this work.

Finally, I would like to thank my parents for their support, patience, and love.

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Introduction

Until recently little research had been performed in the United States pertaining to the denitrification of drinking water supplies. Denitrification is the reduction of toxic nitrate to gaseous nitrogen products. In Europe research on denitrification has been ongoing for many years. One reason for this is that Europe has a much greater problem than the United States with nitrate contaminated aquifers. In some parts of Europe nitrate concentrations in groundwater reached serious levels 20 years ago and continue to increase (Barrenstein *et al.*, 1986). There are several commercial facilities currently providing denitrified drinking water to European communities. The most common denitrification substrates are methanol, ethanol, and acetic acid. Others include reduced sulfur compounds, hydrogen, methane, and carbon monoxide (Gayle *et al.*, 1989).

The use of gaseous substrates has certain advantages over the more common liquid substrates. If methanol is added to water as a substrate for denitrification, it is likely that some methanol may remain in the denitrified effluent. Removal of the excess methanol may be difficult, and the presence of this toxic amendment could render the water unfit to drink. With gaseous substrates, however, any excess substrate remaining in the effluent can be easily removed by aeration.

Of the gaseous substrates, hydrogen has been the most thoroughly studied. Methane has been studied to a lesser degree, and very little information has been published regarding carbon

monoxide. There is at least one commercial facility in West Germany currently using hydrogen as a substrate, while methane supported denitrification has been limited to research and pilot-scale applications (Gayle *et al.*, 1989). In the case of carbon monoxide, there is microbiological evidence that some denitrifiers can utilize this substrate (Park and Hegeman, 1984), but there is only one reported application of carbon monoxide supported denitrification (Fuchs, 1985). This application involves a process patented in West Germany and little background information is publicly available.

This study involved the use of soil and suspended growth microcosms to study the variation in groundwater denitrification rates using different substrates. Two gaseous substrates, carbon monoxide and methane, were studied and compared to a common liquid substrate, methanol. The objectives of this study were to:

1. Evaluate the use of carbon monoxide and methane as substrates for the denitrification of groundwater,

2. Compare the denitrification rates achieved with gaseous substrates to that with methanol,

3. Develop kinetic coefficients for methane, carbon monoxide, and nitrate.

Literature Review

Introduction

There is voluminous information in the literature regarding denitrification. The development of this information base has truly been a multidisciplinary effort. Numerous papers have been published in the fields of agronomy and soil science, which discuss denitrification in top soil and take the point of view of discouraging denitrification and the resulting losses of nitrogen as a plant nutrient. Limnologists have studied denitrification in lake sediments in relation to nitrogen budgets and eutrophication. Hydrologists have studied the extent and rate of denitrification in deep and shallow aquifers of varying geologic types. Microbiologists have studied the metabolism and physiology to gain a better understanding of the microbial processes involved. By working with and building upon this base of knowledge, environmental scientists and engineers have been able to develop approaches for the denitrification of water and in situ groundwater treatment.

In this review various aspects of denitrification are discussed including health aspects, microbiology and stoichiometry, the occurrence of nitrate in groundwater, denitrification kinetics, inhibition, denitrification in natural systems and in situ treatment, unit processes, and microcosm studies.

Nitrate concentrations may be expressed as nitrate or nitrate-nitrogen. In this presentation nitrate and NO_3 refer to quantities expressed as nitrate, while nitrate-N and NO_3-N refer to quantities expressed as nitrate-nitrogen. The convention used varies throughout the literature, and authors often do not adequately define their terminology. The drinking water standard for nitrate set by the U.S. Environmental Protection Agency (EPA) is 10 mg/ℓ as nitrate-nitrogen, and American authors tend to follow this convention. The drinking water standard set by the European Economic Community (EEC) for nitrate is 50 mg/ℓ as nitrate (11.3 mg/ℓ, as nitrate-nitrogen), and European authors tend to follow this convention. The reader is advised that both conventions are used in this literature review in an effort to be consistent with the authors cited.

Health Aspects

The consumption of nitrate or nitrite may cause serious health complications, for example, methemoglobinemia or "blue baby syndrome" in children under six months of age. This disease occurs when nitrate is reduced to nitrite in the gut of infants and the resulting nitrite oxidizes the ferrous iron in hemoglobin to ferric iron. This ferric iron compound, called methemoglobin, can not carry oxygen and asphyxiation may occur (Thomas, 1973, Lehninger, 1975).

Nitrate in well water was first linked to methemoglobemia in 1945 by an article in the Journal of the American Medical Association. Hunter H. Comly, M.D. (1945) reported on seven cases of methemoglobemia in infants receiving well water containing high nitrate concentrations (approx. 140 mg/ℓ nitrate-N). One of the infants died. It was observed that the parents of the infants, who also consumed the water containing nitrate, exhibited no ill effects. Comly proposed several reasons why infants may be more severely affected. Infants have less hemoglobin than adults, the conversion of nitrate to nitrite may be more complete in the gut of infants because of the type of bacteria present, and the high fluid circulation rate in infants may cause more complete absorption of nitrite through the intestine walls. Even though the reduction of nitrate to nitrite does not occur in the

gut of older infants, children, or adults, the ingestion of nitrite may cause similar health problems in all individuals. It is interesting to note that nitrate and nitrite may also cause methemoglobemia in animals. In addition it has been suggested that nitrate and nitrite may be converted in the gut into carcinogenic nitrosamines (Payne, 1981 after Crowe and Bouwer, 1987).

The USEPA has set a drinking water standard for nitrate at 10 mg/ℓ NO_3^- –N. Canada, the European Economic Community, and the World Health Organization have set similar standards (Sayre, 1988). The Virginia State Department of Health (SDH) has also recommended that drinking water contain no more than 1.0 mg/ℓ $NO_3^- - N$.

Microbiology and Stoichiometry

All microorganisms require nitrogen for protein synthesis and growth. Many may use either ammonium-N or nitrate-N , although ammonium is generally preferred since it is in a form most easily used for protein synthesis. The reduction of nitrate to ammonium for protein synthesis is called assimilatory nitrate reduction. The microbial reduction of nitrate to gaseous nitrogen products is termed dissimilatory denitrification or nitrate respiration. Throughout this paper the term denitrification refers to dissimilatory denitrification, unless otherwise stated.

Dissimilatory denitrification occurs when nitrate is used instead of oxygen as a terminal electron acceptor. The circumstances affecting the onset of denitrification and the extent to which the process proceeds are complex and variable. Generally denitrification is considered to be an anoxic process, occurring in the presence of nitrate and the absence of oxygen. The process is thought to proceed through a series of four steps from nitrate to nitrogen gas ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) in stepwise manner. Some investigators have suggested that a nitroxyl intermediate may be involved in the reduction of nitric oxide to nitrous oxide. This has not as yet been proven, however (Renner and Becker, 1970). Surprisingly, most of the organisms known to denitrify are not

anaerobic, but rather are facultative aerobes, which under anoxic conditions use nitrate as a final electron acceptor. Bacterial genera which are known to contain denitrifying species include (Yull-Rhee and Fuhs, 1978; Payne, 1976):

Achromobacter	*Paracoccus*
Alcaligenes	*Propionibacterium*
Bacillus	*Pseudomonas*
Chromobacter	*Spirillum*
Corynebacterium	*Thiobacillus*
Halobacterium	*Xanthomonas*
Moraxella	*Methanomonas*

Each step in the denitrification process is catalyzed by a separate enzyme system. The enzyme systems of some species have been studied in great detail. It appears, however, that the factors affecting induction and repression of these enzymes are not universal. Studies with *Paracoccus denitrificans* indicate that the production of nitrate reductase and nitrite reductase are induced by their respective substrates. The activity of nitrite reductase was strongly inhibited by the presence of oxygen, while nitrate reductase was not inhibited by oxygen or nitrite at 0.1 mM. Nitrate is used preferentially over nitrite even though both enzymes may be present (Lam and Nicholas, 1969). During aerobic and anaerobic cycling, there is a lag phase associated with the onset of denitrification, after which, nitrate is utilized readily and nitrite tends to accumulate. After nitrite accumulates to a sufficient concentration, nitrite reductase activity increases and the denitrification process proceeds (Waki *et al.*, 1980). The length of the lag phase is dependent on the degree of acclimation and the reaction conditions such as pH and nutrient availability (Kurt *et al.*, 1987).

Heterotrophic Denitrification

Denitrification is classically considered to be a heterotrophic process conducted by microorganisms which require a reduced organic substrate for energy and cell synthesis. Heterotrophic denitrifying microorganisms can utilize a variety of organic carbon sources. Most of the published research regarding the denitrification of water involves the use of methanol, ethanol, or acetic acid. The following stoichiometric relationships for these substrates have been formulated:

Methanol (Sherrard, 1988)

$$5CH_3OH + 6NO_3^- \rightarrow 3N_2 + 5CO_2 + 7H_2O + 6OH^- \quad [1]$$

Ethanol (Richard *et al.*, 1980)

$$5C_2H_5OH + 12NO_3^- \rightarrow 6N_2 + 10CO_2 + 9H_2O + 12OH^- \quad [2]$$

Acetic Acid (Frick and Richard, 1985)

$$5CH_3COOH + 8NO_3^- \rightarrow 4N_2 + 10CO_2 + 6H_2O + 8OH^- \quad [3]$$

Gaseous organic substrates such as methane and carbon monoxide can also be used as substrates for denitrification. Among gaseous substrates, methane is one of the most thoroughly studied. There remain some contradictions in the literature, however, regarding methane metabolism. There is evidence that methane can be used as a electron donor by some denitrifiers (Davies, 1973). Other investigators have suggested that methane oxidation requires aerobic or microaerophilic conditions, and that subsequent denitrification may be the result of a symbiotic relationship between two groups of organisms with different trophic requirements (Yull-Rhee *et al.*, 1978). It is conceivable that both phenomena occur, suggesting two possible mechanisms for methane utilization during denitrification.

There have been fewer studies published involving carbon monoxide. There is evidence, however, that it can be used as a substrate for denitrification (Park and Hegeman, 1984). A unit process utilizing carbon monoxide to denitrify groundwater has been patented in the Federal Republic of Germany (Fuchs, 1985). Stoichiometric relationships for methane and carbon monoxide utilization have been proposed:

Methane (Barrenstein *et al.*, 1986)

$$5CH_4 + 8NO_3^- + 8H^+ \rightarrow 5CO_2 + 4N_2 + 14H_2O \quad [4]$$

Carbon Monoxide (Sherrard, 1988)

$$2NO_3^- + 5CO + H_2O \rightarrow N_2 + 2OH^- + 5CO_2 \quad [5]$$

Autotrophic Denitrification

Denitrification can also be accomplished by autotrophic bacteria which may use hydrogen or various reduced sulfur compounds as an energy source. Under autotrophic growth conditions no organic carbon source is required but rather carbon dioxide or bicarbonate are used as a carbon source for cell synthesis. *Paracoccus denitrificans* and *Thiobacillus denitrificans* can denitrify using hydrogen and reduced sulfur compounds, respectively. Both can also grow heterotrophically, however, if an organic carbon source is present (Waki *et al.*, 1980; Kurt *et al.*, 1987; Claus and Kutzner, 1985a, 1985b, John and Whatley, 1977). The following stoichiometric relationships for hydrogen and sulfur have been cited.

Hydrogen (Kurt *et al.*, 1987)

$$2NO_3^- + 5H_2 \rightarrow N_2 + 4H_2O + 2OH^- \quad [6]$$

Thiosulfate (Claus and Kutzner, 1985)

$$5S_2O_3^{=} + 8NO_3^{-} + H_2O \rightarrow 4N_2 + 10SO_4^{=} + 2H^{+} \quad [7]$$

Sulfide (Barrenstein *et al.*, 1986)

$$5S^{=} + 8NO_3^{-} + 8H^{+} \rightarrow 5SO_4^{=} + 4N_2 + 4H_2O \quad [8]$$

Occurrence of Nitrate in Groundwater

The nitrate ion is very water soluble and poorly adsorbed and may enter groundwater aquifers via the various routes of groundwater recharge, as well as through surface water intrusion into poorly constructed wells. Sources of nitrate include agricultural fertilizers, human and animal wastes, and natural sources such as the oxidation of soil organic matter. There are many factors which affect the magnitude and extent of nitrate contamination of aquifers including the magnitude and areal distribution of the source, depth to groundwater, soil type, and rainfall, irrigation, and other hydraulic considerations.

Nitrate contamination of our groundwater resources is becoming an ever increasing problem. In some parts of Europe, nitrate concentrations in groundwater reached serious levels 20 years ago and have continued to increase (Barrenstein *et al.*, 1986). Nitrate in drinking water is also becoming a problem in other developing and third world countries (Burden, 1983). Numerous investigators have reported nitrate contamination of aquifers in various parts of the United States. The predominant sources of nitrate vary from region to region. Contamination resulting from agricultural fertilization and animal feedlots has been reported in Nebraska, New Mexico, and Washington (Exner and Spalding, 1985; Spalding *et al.*, 1978; Taylor and Bigbee, 1973; Spalding *et al.*, 1982). Septic tank drainfields have caused aquifer contamination in Delaware, Oregon, and Virginia (Kroehler, 1989; Quan *et al.*, 1974;). Elevated groundwater levels led to the leaching of natural soil

leaching of natural soil nitrate in Texas (Kreitler and Jones, 1975). Surface water intrusion into poorly constructed wells has been implicated in Nebraska and Virginia (Exner and Spalding, 1985; Chapman, 1983).

Irrigation has been shown to stimulate greater leaching of nitrate than dryland farming (Exner and Spalding, 1985). The problems caused by human and animal wastes vary with the density and magnitude of the source. Animal feedlots are much more likely to cause nitrate problems than are ranch operations with large grazing areas and low animal density (Taylor and Bigbee, 1973). Septic tank drainfields in low density areas with poor soils can cause localized problems, but nitrate contamination can be much more extensive in unsewered areas with higher population densities. One investigator estimated that 10 mgd of sewage was being discharged through subsurface systems in a 30 square mile area near Portland, Oregon (Quan *et al.*, 1974).

Identifying the source of nitrate contamination is often difficult. When groundwater surveys indentify large areas with uniformly high nitrate concentrations, nonpoint source contamination is suspected. While wide variations within a relatively small area are usually associated with point source contamination (Exner and Spalding, 1985). These trends can be further evaluated in conjuction with chloride and coliform data and information on land use.

A more objective method for the identification of nitrate contamination sources involves the use of nitrogen isotope ratios. The nitrogen isotope ratio ($\delta^{15}N$) is defined in equation 9 (Exner and Spalding, 1985):

$$\delta^{15}N = \frac{(^{15}N/^{14}N)sample - (^{15}N/^{14}N)standard}{(^{15}N/^{14}N)standard} \times 100 \qquad [9]$$

^{15}N is a stable isotope which represents 0.3663% of the nitrogen in the atmosphere. Nitrogen containing compounds may contain drastically different nitrogen isotope ratios because of isotope fractionation which occurs during chemical, physical, and biological processes (Marriotti *et al.*,

1981). Kreitler (1979) determined the nitrate-nitrogen isotope ratios for unfertilized cultivated land (+0.2% to +0.8%, avg. +0.49%), ammonium-fertilized fields (+0.2% to +1.4%, avg. +0.88%), and animal wastes (+1.0% to +2.2%, avg. +1.44%) and used these trends to identify the sources of nitrate contamination in central Texas. Others (Exner and Spalding, 1985 and Spalding *et al.*, 1982) have also used isotope ratios to identify nitrate sources in Nebraska and Washington.

Kinetic Models

There are several common kinetic models which may be used to describe denitrification kinetics, including Michaelis-Menten, Monod, and Haldane. The Michaelis-Menten model was derived to explain the kinetics of enzyme catalyzed reactions. A stepwise mechanism for product formation was proposed:

$$E + S \rightleftarrows ES \rightarrow E + P \qquad [10]$$

According to this model, enzyme (E) and substrate (S) combine reversibly to form an enzyme-substrate complex (ES). The ES complex breaks down irreversibly to form enzyme and product (P). This mechanism assumes that the rate of reaction is proportional to the concentration of a single, limiting substrate. The general form of the Michaelis-Menten equation is:

$$v = \frac{V_m S}{S + K_m} \qquad [11]$$

Where (v) is velocity, (S) is substrate concentration, (V_m) is maximum velocity, and (K_m) is the half velocity constant or the substrate concentration that yields a velocity equal to one half V_m. Mathematically K_m is the combined rate constant for the break down of the ES complex. If a reaction follows Michaelis-Menten kinetics the plot of velocity versus substrate concentration is in the form of Figure 1.

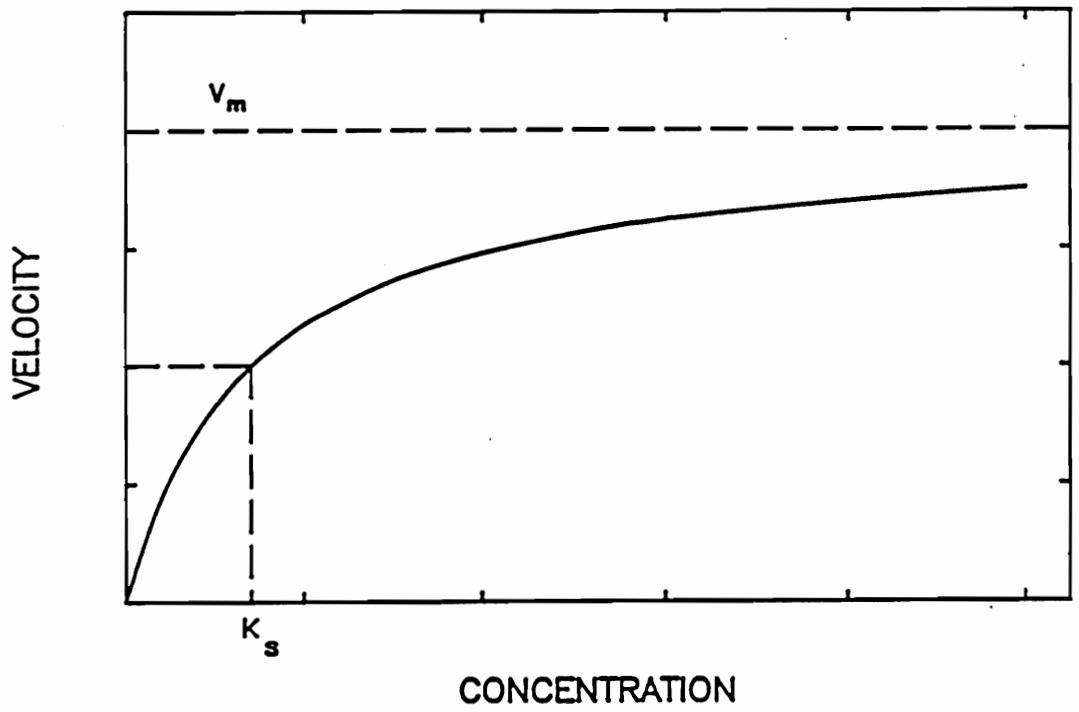

Figure 1. Effect of substrate concentration on the rate of an enzyme catalysed reaction: (after Lehninger, 1975).

At low substrate concentrations when $S << K_m$ the general form of the equation reduces to:

$$v = \frac{V_m S}{K_m} \qquad [12]$$

This rate equation is first order with respect to S and describes the initial rising limb of Figure 1 on page 12. When $S >> K_m$ the general form of the equation reduces to:

$$v = V_m \qquad [13]$$

This equation implies that v is constant and equal to V_m and describes the nearly horizontal portion of Figure 1. In this zone, velocity is constant and independent of substrate concentration. Such a relationship is zero order with respect to S.

If a reaction follows Michaelis-Menten kinetics, the constants K_m and V_m are sufficient to estimate the velocity at any substrate concentration. To experimentally determine V_m and K_m data must be collected over a broad range of substrate concentrations. This collection of data may be used to determine the kinetic constants using a Lineweaver-Burk double reciprocal plot (see Figure 2). The plot was derived by taking the recprocal of the Michaelis-Menten equation and rearranging to yield:

$$\frac{1}{v} = \frac{K_m}{V_m}\frac{1}{S} + \frac{1}{V_m} \qquad [14]$$

When 1/v is plotted versus 1/S the kinetic data are linearized and the resulting line has a slope of K_m/V_m and a Y-intercept of $1/V_m$.

Monod (1949) developed a kinetic model to descrbe bacterial growth and substrate utilization. The Monod model has a form mathematically similar to the Michaelis-Menten model. The two models differ in that the Michaelis-Menten model has a theoretical basis, whereas the Monod model is strictly empirical. The Monod relationship is described by the following equations:

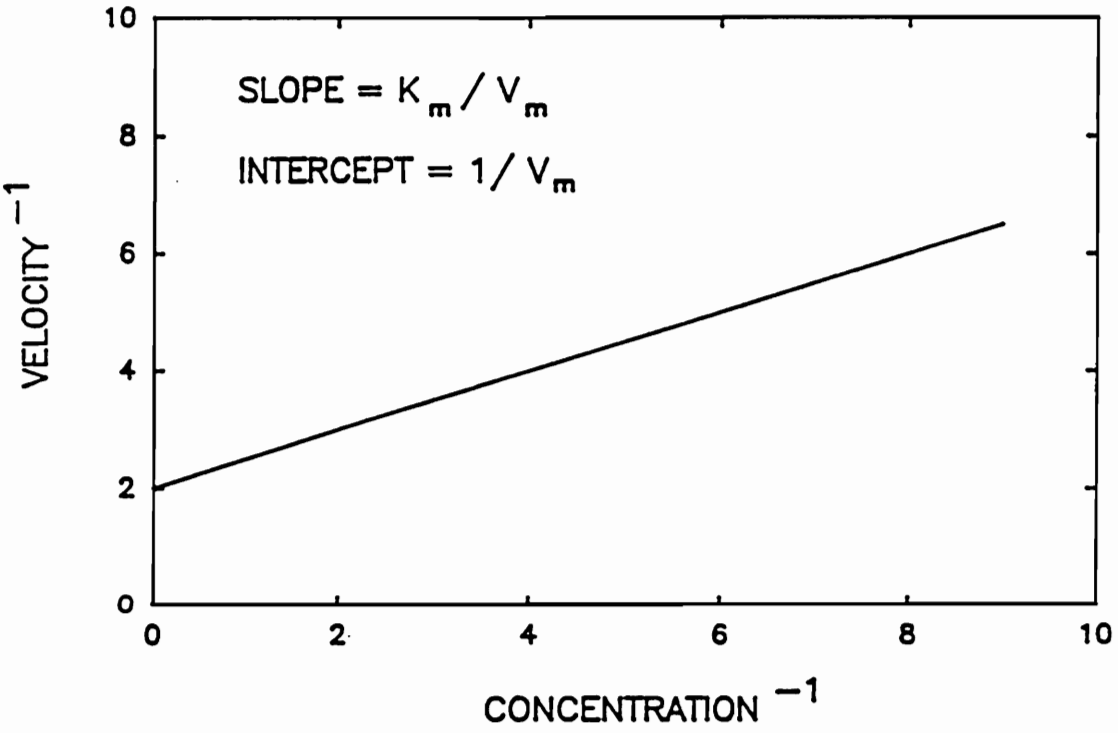

Figure 2. Lineweaver-Burk double reciprocal plot.

$$q = \frac{q_m S}{K_s + S} \qquad [15]$$

$$\mu = \frac{\mu_m S}{K_s + S} \qquad [16]$$

Where q is the specific substrate utilization rate, (dS/dt)/X, q_m is the maximum specific substrate utilization rate, μ is the specific growth rate, (dX/dt)/X, μ_m is the maximum specific growth rate, S is substrate concentration, and K_s is the half velocity constant.

In the Michaelis-Menten model the reaction is facilitated by an enzyme, whereas in the Monod model the reaction is carried out by microorganisms. In enzyme catalyzed reactions the assumption is made that the total concentration of enzyme remains constant, and as a result it is not neccesary to include enzyme concentration in the equation. The microorganism population which drives the Monod model is often not constant and must be included as a parameter in the model. To satisfy this, the mass of microorganisms (X) is included within μ and q terms.

Numerous models have been proposed to describe enzyme and bacterial kinetics under conditions of substrate inhibition. The most common of these is the Haldane model. The Haldane model was derived to describe enzyme catalyzed reactions in which high concentrations of substrate retard reaction rates. The proposed mechanism of inhibition builds upon the Michaelis-Menten enzyme-substrate model:

$$E + S \rightleftarrows ES \rightarrow E + P \qquad [17]$$

At higher S concentrations an inactive ES complex is formed involving two S molecules (Edwards, 1970):

$$S + ES \rightleftarrows SES \quad (inactive) \qquad [18]$$

In its most general form, this mechanism can be expanded to include inactive ES complexes containing three or more S molecules. The Haldane equation may be expressed as:

$$v = \frac{V_m S}{S + K_s + S^n/K_i} \qquad [19]$$

The parameters are as previously defined except K_i which is the dissociation constant for the inactive SES complex and the exponent n which is equal to the number of substrate molecules present in the inactive complex. Graphically the Haldane equation with $n = 2$ has the form shown in Figure 3. When K_i is small, inhibition will occur as substrate concentrations increase. When K_i is very large there is no inhibition and the Haldane and Michaelis-Menten models are equivalent (Godrej and Sherrard, 1988). D'Adamo *et al.* (1984) have shown that, when $n = 2$, the maximum velocity, $V^{\times}$, occurs at a substrate concentration, $S^{\times}$, where:

$$S^{\times} = (K_s K_i)^{1/2} \qquad [20]$$

Figure 4 compares the graphical forms of the Michaelis-Menten equation and the Haldane equation with $n = 2$ and $n = 3$. In each case $V_m = 80$, $K_s = 14$, and $K_i = 50$. As the exponent n increases, the onset of inhibition becomes more rapid and the magnitude of inhibition increases.

Studies of Denitrification Kinetics

Numerous investigators have studied the kinetics of denitrification reactions. A variety of substrates have been examined using pure culture cell suspensions and biological unit processes. Denitrification kinetics in soils, resulting from native organic matter, has also been studied. Unfortunately many investigators have not reported complete kinetic information, but rather disappearance rates or arbitrary rate constants that are not correlated with active biomass. When kinetic models were utilized, a Michaelis-Menten type kinetic approach has been most common.

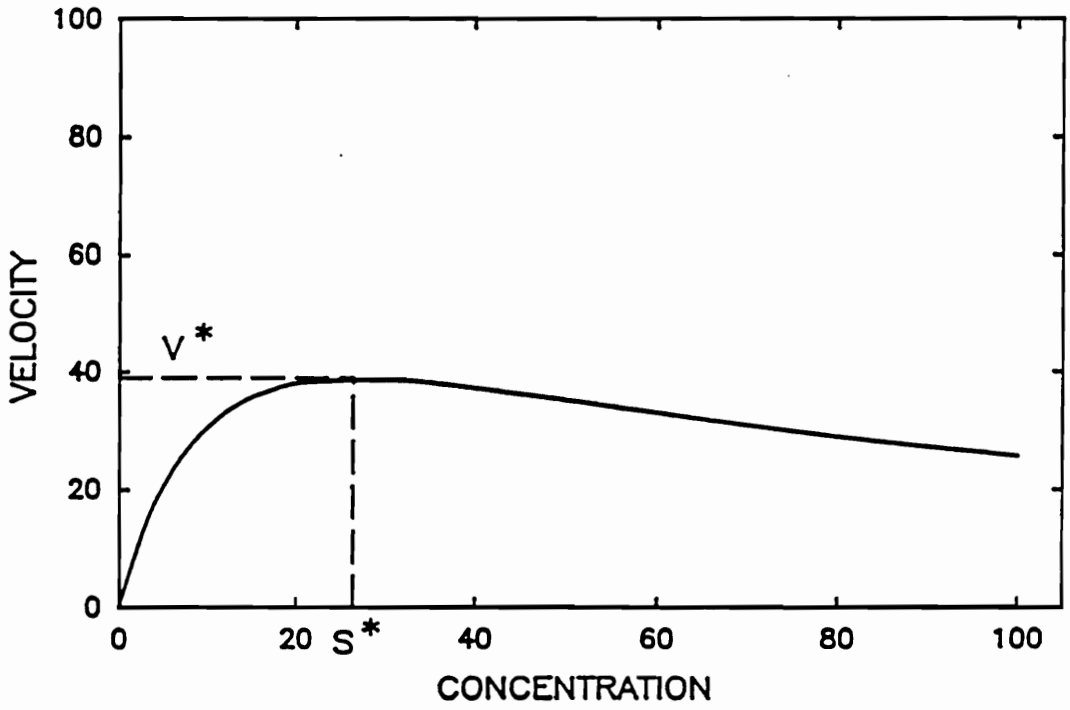

Figure 3. Example of a Haldane substrate inhibition plot.

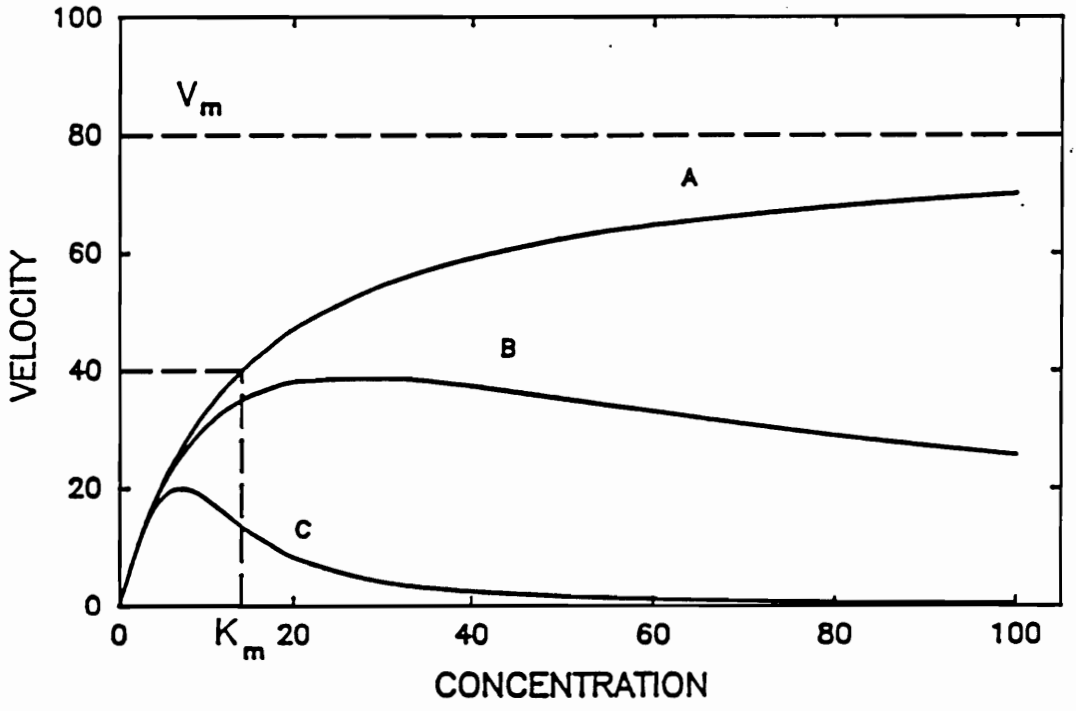

Figure 4. Comparison of the graphical forms of the Michaelis-Menten and Haldane models: (A) Michaelis-Menten, (B) Haldane with n = 2, Haldane with n = 3, V_m = 80, K_s = 14, and K_i = 50.

Betlach and Tiedje (1981) studied denitrification rates in pure cultures of *Alcaligenes* and *Pseudomonas* species and evaluated the relative rates of nitrate and nitrite reduction. They concluded that nitrite accumulation may be a result of differences in the reaction rates of the two steps. The Michaelis-Menten half velocity constants (K_m) for both nitrate and nitrite were found to be less than 15 μM (< 1 mg/ℓ) for these cell suspensions.

Denitrification rates in various soils were reported by McLaren (1976). In soil column studies, using naturally occurring organic matter as the substrate, the rates of disappearance of nitrate ranged from 0.003 to 0.006 h^{-1} and 0.036 to 0.056 μg/h/g soil. These values are considered arbitrary since the active biomass was not quantified.

Lee and Dahab (1988) studied denitrification of water using low solids, attached and suspended growth reactors. Acetate was used as a substrate and the initial COD was 400 mg/ℓ. A Michaelis-Menten kinetic approach was used and the assumptions were made that diffusion through the attached biofilm was complete and that the substrate flux was proportional to the biofilm thickness. The maximum specific substrate (nitrate) utilization rate (k) and K_m for suspended growth were 0.3 d^{-1} and 2.5 mg/ℓ, respectively, as compared to 0.2 d^{-1} and 14.8 mg/ℓ for attached growth. It was suggested that the differences in the K_m values may have been caused by poor diffusion of substrate through the biofilm.

Kurt *et al.* (1987) studied denitrification using hydrogen in a fluidized-bed reactor. The results were modeled using dual substrate (nitrate and hydrogen) Michaelis-Menten kinetics. The maximum denitrification rate was found to be 25 mg-N/gDW/h, while K_{NO_3} and K_{H_2} were 0.18 mg/ℓ and 0.002 mg/ℓ, respectively.

Several investigators have studied the effects of diffusion on reaction order. One study (Reddy *et al.*, 1978a, 1978b) found that denitrification in unshaken flooded soil microcosms followed first-order kinetics, while in saturated, but not flooded, soil microcosms the kinetics were zero-order. The slow rate of diffusion of nitrate from the floodwater into the soil was found to be affecting the

apparent reaction rate. Also, since the nitrate in the lower soil layers was quickly depleted and the diffusion rate was slow, most of the denitrification was thought to be occurring in the upper layers of soil and not throughout the entire soil mass. Cho (1982) determined that nitrate diffusion effects were overcome by shaking. Denitrification rates in shaken, flooded soil microcosms were zero order with respect to nitrate.

Cho (1982) also found that the aqueous diffusion of headspace oxygen in unshaken flooded soil microcosms was slow. Denitrification was shown to occur in flooded soils even when oxygen was present in the headspace. Oxygen was consumed by aerobic reactions sufficiently fast to maintain anoxic conditions in the lower soil layers.

The effects of gas diffusion rates may also be important when studying denitrification using gaseous substrates. There is interest in the use of hydrogen, methane, and carbon monoxide for *in situ* treatment of nitrate contaminated groundwaters. These gases are slightly soluble in water but their overall solubilities may not have as great an effect on denitrification kinetics as their aqueous diffusion rates.

Inhibition

The rate and extent of denitrification may be inhibited by pH, temperature, the presence of oxygen, and possibly by nitrite accumulation or substrate inhibition. The effects of pH on microbial reaction rates are thought to result from ionization of enzymes and substrates and changes in enzyme active sites. Generally, the relationship between enzyme activity and pH follows a classical bell-shaped curve with the optimum pH falling in a rather narrow pH range (Mahler, 1971).

Denitrification products such as carbon dioxide and alkalinity can cause pH shifts during treatment. In open systems, carbon dioxide is lost to the atmosphere, but alkalinity will accumulate. As a result

in poorly buffered systems, the pH will tend to rise. In closed systems the accumulation of carbon dioxide may cause the pH to fall.

The optimum pH for denitrification ranges between pH 7.0 and pH 8.0 (Knowles, 1981 after Koskinen and Keeney, 1982). Kurt (1987) found a pH of 7.5 to be optimum for autotrophic denitrification using hydrogen. Higher pH values up to pH 9.0 resulted in increasing accumulations of nitrite. Dodd and Bone (1975) found denitrification using acetate as a carbon source to have a optimum pH of 7.5, while at pH 9.2 denitrification ceased.

Koskinen and Keeney (1982) suggested that denitrifiers in soil were sensitive to pH changes but over the long term denitrifiers could be sucessful over a wide range of soil pH. Their studies also indicated that carbon availability, rather than pH, controlled denitrification rates in carbon limited soils.

Generally enzyme-catalyzed reactions proceed slower at low temperatures and faster at higher temperatures, up to some temperature optimum. Beyond the temperature optimum, enzyme activity falls off quickly and soon stops completely. The effects of temperature on enzyme-catalyzed reactions can be explained in two ways. Thermodynamics can be used to describe the variation in reaction rate with changes in temperature, whereas, at high temperatures , the decrease in enzyme activity is probably due to thermal denaturization of the enzyme (Mahler, 1971).

Kurt (1987) found denitrification using hydrogen as a substrate to have an optimum temperature of approximately 40 C. Stanford *et al.* (1975) cited reports of soil denitrification occurring in the temperature range from 2 to 65 C. Other investigators (Keeney *et al.*, 1979) have suggested that 65 C is too high for biological denitrification. They postulated that chemical, rather than biological, denitrification may be dominating at the higher temperatures. This could be occurring according to a chemical denitrification reaction studied by Christianson and Cho (1983) in which organic nitirogen was oxidized by nitrite to yield nitrogen gas.

In reality few denitrification processes will be operated at optimum temperatures. Because of energy costs, most unit processes will be operated at ambient temperatures which may range from 5-30 C, while in situ processes will operate at 5-10 C.

Most investigators consider oxygen an inhibitor of denitrification. From a redox point of view, oxygen is favored over nitrate as an electron acceptor. In addition, oxygen may inhibit the synthesis of the enzymes required for denitrification (Dodd and Bone, 1975). Waki (1980) found oxygen to inhibit nitrate reductase activity in *Parracoccus denitrificans*. In similar studies, Lam and Nicholas (1969) found oxygen to have no effect on nitrate reductase activity, but found nitrite reductase activity to be significantly reduced.

Despite these findings, some species have been reported to denitrify in systems with oxygen tensions as high as 153 mm of mercury (20% oxygen atmosphere). Also, there is evidence that in soil, nitrification and denitrification may occur simultaneously (Payne, 1976). Though not fully explained, these phenomena may be a result of anaerobic microzones in otherwise aerobic systems.

Numerous authors have reported nitrite accumulations during denitrification in batch and continuous flow systems (Kurt, 1987; Eggers and Terlouw, 1979; Requa and Schroeder, 1973). Yet there is very little information in the literature regarding nitrite inhibition of denitrification. There have been studies of nitrite inhibition of nitrification. Meyerhof (1916, after Boon and Laudelot, 1962) reported that nitrite-N concentrations over 280 mg/ℓ inhibited nitrite oxidation. Lee and Simpson (1957, after Boon and Laudelot, 1962) noted that these inhibitory effects may become measurable at nitrite-N concentrations as low as 56 mg/ℓ. Boon and Laudelot (1962) determined that this inhibition correlated with the concentration of undissociated nitrous acid. The equilibrium constant for nitrous acid is $10^{-3.4}$. Therefore, the inhibition caused by the undissociated acid would be expected to increase as pH decreases.

Many biological processes are known to be affected by substrate inhibition. Carbon monoxide, one of the substrates proposed for denitrification, is known to be inhibitory to various biological proc-

esses and higher life forms. In general, carbon monoxide is a metal complexing agent. It tends to bind with the metal co-factors of metallo-enzymes, especially metallo-porphyrins and copper enzymes (Mahler and Cordes, 1971). While there is some information in the literature regarding carbon monoxide inhibition of specific enzyme systems, the research regarding carbon monoxide inhibition of denitrification is incomplete.

There is direct evidence in the literature that carbon monoxide inhibits nitrous oxide reductase (Kristjansson and Hollocher, 1980). There is also direct evidence that carbon monoxide inhibits cytochrome oxidase, the electron-transferring protein that facilitates the reduction of nitric oxide to nitrous oxide (Mahler and Cordes, 1971).

Other possible modes of inhibition may be inferred based on the knowledge that carbon monoxide is a metal binding agent. All of the cytochromes in the electron transport system contain iron porphyrin groups. Carbon monoxide may bind to and inhibit these electron carriers, but there is no direct evidence in the literature. Nitrate reductase contains molybdenum, and is known to be affected by numerous inhibitors such as cyanide, azide, sulfide, thiourea, and metal chelators (Jain, 1982). Carbon monoxide may bind to and inhibit nitrate reductase, but there is also no direct evidence of this inhibition in the literature.

Denitrification in Natural Systems and In Situ Treatment

Edmunds and Walton (1983) studied denitrification and the hydrogeochemical evolution of a confined limestone aquifer in Lincolnshire, England over a ten year period. The aquifer was first studied in 1969 and then reevaluated in 1979. The location and concentration of many constituents (pH, O_2, HCO_3, Na, Mg, Sr, and F) were found to have remained surprisingly constant, while NO_3, SO_4, Cl, and Ca had increased in the up-gradient portions of the aquifer. In the down-gradient portions of the aquifer, at a point where redox potentials dropped below +200 mV, nitrate con-

centrations decreased to < 1 mg/ℓ, while SO_4, Cl, and Ca concentrations remained high. These observations led to the conclusion that nitrate reduction was occurring. The extent and rates of denitrification were not quantified.

A similar but more comprehensive study investigated the geochemistry and extent of denitrification in a confined limestone (chalk) aquifer near Grimsby, England and found denitrification to be negligible (Howard, 1985). This study examined major ions (Ca, Mg, Na, K, HCO_3, SO_4 , Cl, and NO_3), minor ions (Br, Sr, F, and I), redox conditions, and utilized carbon and tritium isotope dating. The author concluded that caution should be exercised when interpreting groundwater data related to denitrification because varying ages and origins of groundwater can cause the comparison of nitrate concentrations to be misleading. There were waters of four distinct ages in the aquifer, ranging from 120,000 years old to recent, each with a different origin. The older waters were low in nitrate, but conservative (non-biodegradable) ion concentrations suggested that the sources were originally low in nitrate. Based on this evidence, it was concluded that denitrification was not a significant factor leading to low nitrate concentrations in this aquifer.

In a South African groundwater study, denitrification rates were quantified in a confined sandstone aquifer (Vogel *et al.*, 1981). The investigators measured NO_3 , O_2 , N_2, and Ar and utilized N^{15}/N^{14} ratios and C^{14} dating. Denitrification was found to be occurring at a very slow rate. It was estimated to require approximately 27,000 years for the lowering of the nitrate concentration from 100 mg/ℓ to 3.7 mg/ℓ. An increase in dissolved N_2 was considered evidence of denitrification. Also, the groundwater remained aerobic for approximately 16,000 years prior to the onset of denitrification, suggesting low biological activity in the aquifer.

Heterotrophic denitrification resulting from soil organic matter was studied in a Canadian shallow sand aquifer (Trudell *et al.*, 1986). Two hundred liters of groundwater were withdrawn using a well point, spiked with 13 mg/ℓ NO_3—N and 5 mg/ℓ Br, and injected through the same well point into the aquifer. Samples were then withdrawn through the well point over a period of several days. The Br tracer was used to correct for advection and dilution. The decrease in NO_3—N in excess of di-

lution was considered evidence of denitrification. The measured rate of denitrification ranged from 0.0078 to 0.13 mg NO_3—N/h . It is not clear if a correction was made for assimilatory nitrate reduction.

Several investigators have evaluated the injection of various substrates and nutrients into aquifers in an effort to stimulate *in situ* denitrification. Liquid substrates such as acetic acid, ethanol, and treated wastewater have been used, and gaseous substrates including hydrogen and methane have been evaluated. In most cases phosphate was added as a microbial nutrient and in the case of autotrophic denitrification with hydrogen, carbon dioxide or some other inorganic carbon source was required.

Separate studies (Selenka *et al.*, 1986 and Barrenstein et al., 1986) evaluated underground treatment of nitrate contaminated ground water in Germany by infiltration with treated wastewater or methane gas. In both cases treated wastewater was found to stimulate faster rates of denitrification than methane. It was suggested that methane utilization by bacteria could occur only in the presence of oxygen and that a symbiotic relationship may be required to achieve denitrification with methane as a substrate.

A study conducted in Switzerland evaluated *in situ* denitrification using hydrogen (Ginocchio, 1983). Nitrate contaminated groundwater was withdrawn, amended with hydrogen, carbon dioxide, and phosphate and re-injected into the aquifer. The nitrate concentration in the aquifer was reduced from 92 to 11 mg/ℓ within four weeks.

In a somewhat related German study, nitrate contaminated groundwater was treated by infiltration through filter beds composed of soil and aquatic plants (Soeder *et al.*, 1986). Nitrate removals were found to be significant only during summer months, suggesting that plant uptake and not denitrification was the predominate removal mechanism.

Unit Processes for Dentrification

Various conventional unit processes have been adapted and used for the denitrification of drinking water. Fixed-film processes including fluidized-bed reactors, packed-bed reactors, and biofilters are most common. Other, more innovative fixed-film processes that use buoyant porous carriers or alginate beads have also been evaluated. In addition, an unusual hybrid system incorporating ion exchange and biological denitrification has been studied.

Heterotrophic Processes

The most studied and most widely used commercial processes for the denitrification of drinking water use organic carbon sources as electron donors. A wide variety of solid, liquid, and gaseous organic substrates has been studied. The most common substrates are methanol, ethanol, and acetic acid. Other lesser studied organic substrates include methane, carbon monoxide, cellulose, and whey.

Richard *et al.* (1980) evaluated denitrification of groundwater in France using a pilot-scale, fluidized-bed reactor with ethanol or acetic acid as the substrate. The reactor was 250 mm in diameter, 6 m high, and operated without effluent recycle. The system was modeled using Monod kinetics, considering carbon, nitrogen, and phosphorus as substrates. Under normal operating conditions, carbon and nitrogen were present in excess and phosphorus became rate limiting. The Monod half velocity constant for phosphorus was found to be 0.34 mg PO_4/ℓ, and the maximum specific nitrate utilization rate was 250 mg NO_3/h-g VSS. The actual denitrification rate, however, was strongly related to the flow velocity through the column. Denitrification rates were greater at lower velocities. The optimum velocity for denitrification and bed expansion was 20 m/h. At this velocity, the denitrification rate was approximately 33% of maximum, or 83 mg NO_3/h-g VSS.

The first commercial drinking water denitrification facility built in France was constructed in 1983 in Eragny. Philipot et. al. (1985) reported on the design and performance. The 80 m^3/h facility consisted of ethanol and phosphate addition, a biologically active clay filter, flocculant addition, activated-carbon/sand filtration, and disinfection. The nitrate concentration was reduced from 68 to 25 mg/ℓ, and the nitrite concentration in the effluent was maintained at less than 0.05 mg/ℓ.

Frick and Richard (1985) reported the performance of a denitrification facility that used acetic acid, which was constructed in 1983 in Chateau Landon, France. This 50 m^3/h facility consisted of acetic acid and phosphate addition, an up-flow, fixed-bed reactor, flocculant addition, carbon filtration, and chlorination. Nitrate concentrations were reduced from 80 to 30 mg/ℓ. Nitrite accumulated immediately after start-up but decreased to less than 0.1 mg/ℓ after process equilibrium was achieved. A similar facility was also constructed in Champfleur, France (Richard & Partos 1986). Several other denitrification studies have been conducted which evaluated the use of fixed-film processes with methanol, ethanol, or acetic acid as substrates (Boeckle *et al.*, 1984; Partos and Richard, 1985; Rudovsky *et al.*, 1986).

A German study (Sontheimer *et al.*, 1987) evaluated post treatment using $FeCl_3$ to reduce effluent nitrite concentrations. Following fixed-film denitrification using acetic acid, 0.3 mg/ℓ $FeCl_3$ reduced effluent nitrite from 18 to 0 mg/ℓ. This process is patented and the mechanism of the reaction has not been fully explained in the literature. It can be shown, however, that it is thermodynamically feasible to oxidize nitrite to nitrate using $FeCl_3$. The kinetics and the possibility of competing reactions have not been reported.

A fixed-film biological denitrification process using ethanol and buoyant carriers was developed in Germany and given the trade name Denipor (Roennefahrt, 1982, 1985, 1986). The process consists of ethanol and phosphate addition, a fixed-film filter packed with floating Styropor spheres, aerobic biological filtration (anthracite/sand), and chlorination. The operating temperature was 9-11 C, and a 95% nitrate removal efficiency was obtained at a filter loading rate of 1.0 kg NO_3/m^3-d and a re-

circulation rate of 200 % to 500 %. Effluent quality was not improved by additional post treatment with ozone/activated-carbon or flocculants.

Frank and Dott (1985) reported on the performance of a pilot-scale bioreactor packed with polystyrene beads, using methanol or ethanol as an energy source. Nitrate concentrations were reduced from 55 to 3 mg/ℓ. Most of the bacteria in the reactor were attached and of the genus *Pseudomonas.*

Denitrification was studied by Nilsson and Ohlson (1982) in a series of bench scale columns, packed with immobilized *Pseudomonas denitrificans* cells. The bacteria were encapsulated in a sodium alginate polymer and ethanol was used as a carbon source. Using four columns in series, the nitrate concentration was reduced from 104 to 0.1 mg/ℓ. Nitrite accumulated in the effluent from the first three columns but was reduced to 0.3 mg/ℓ in the effluent from the fourth column. Limitations cited included the rate of diffusion of substrates and products through alginate gels, undesirable leakage of cells from the gel matrix, and the relatively short (approx. 2 months) stability and activity of the gels and cells. The addition of calcium improved gel stability and the cells could be reactivated by incubation in a nutrient solution.

The costs associated with the use of methanol, ethanol, or acetic acid as denitrification substrates in a fluidized-bed reactor were evaluated by Croll *et al.* (1985). For operating conditions yielding effluent concentrations of 12 to 14 mg NO_3/ℓ, methanol was found to have the lowest cost. Acetic acid and ethanol were 50 % and 25 % more expensive than methanol, respectively.

Bacteria were isolated by Davies (1973) from settled sewage which were found to be capable of denitrification using methane as a sole carbon source and terminal electron acceptor. The genera identified included *Alcaligenes, Achromobacter, Methanomonas, Pseudomonas, and Bacillus.* All isolates were also capable of denitrification with methanol, ethanol, malate, or lactate.

Yull-Rhee and Fuhs (1978) studied denitrification with methane using two bench scale sand columns in series. The first column was seeded with a *Methylomonas* species and was sparged with methane and air. The *Methylomonas* oxidized methane under aerobic conditions but did not denitrify. The second column was seeded with *Pseudomonas stutzeri* and was supplied with the effluent from the first column but with no additional carbon source or air. The *Pseudomonas* was shown to denitrify using the metabolites produced from methane oxidation as its sole carbon source. These findings suggested that methane could be indirectly used as a substrate for denitrification as a result of a symbiotic relationship between organisms representing two different trophic groups.

Krantzenstein (1982) utilized methane for denitrifcation in a three stage process consisting of oxygen removal, denitrification in a biological filter, and reaeration. A patent was obtained in the Federal Republic of Germany by Fuchs (1985) for a denitrification process utilizing carbon monoxide saturated, flexible porous carriers. When depleted the carriers were resaturated with carbon monoxide and recycled back to the biological reactor. Birch wood was evaluated as a substrate for denitrification by Rauschmaier and Bardtke (1985). Cellulose was used readily by the bacteria and denitrification was achieved. Lignin was not utilized, however, and tended to accumulate. Bullermann and Keidel (1986) evaluated whey as a carbon source for denitrification in a fluidized bed reactor. Nitrate reduction was achieved, but the denitrification rate was slow.

A hybrid ion exchange/biological denitrification process was studied by van der Hoek and Klapwijk (1987,1988). This process utilized ion exchange for the removal of nitrate from groundwater and incorporated biological denitrification as part of the resin regeneration step. During regeneration a concentrated NaCl (10-15 g/ℓ) or $NaHCO_3$ (25-30 g/ℓ) solution was circulated in a closed loop between the ion exchange column and an upflow sludge bed (USB) denitrification reactor. Methanol was used as a substrate for the USB. The strong brine solution regenerated the the exchange resin and the USB removed the nitrate (700 mg NO_3^- — N /ℓ) that accumulated in the brine. Removal of the nitrate allowed for reuse of the brine through several regeneration cycles. A sand filter was used in the loop to minimize carry over of biological solids and organics to the exchange

resin. After regeneration the exchange resin was disinfected with 0.15% peracetic acid. Sulfate can interfere with nitrate removal since most anion exchange resins are more selective for sulfate than nitrate. A nitrate selective resin (Amberlite IRA 996 of Rohm and Haas) was found to minimize this interference. Ultimately sulfate accumulations in the regenerant necessitated brine disposal. In a pilot study the regenerant was replaced every six days, which resulted in an 80% reduction in brine disposal volume over conventional regeneration procedures.

Autotrophic Processes

Numerous investigators have studied autotrophic denitrification using hydrogen or various reduced sulfur compounds in fixed film reactors. Both fluidized-bed and packed-bed rectors have been used in studies of denitrification kinetics, stoichiometry, and inhibition.

Kurt *et al.* (1987) studied autotrophic denitrification of drinking water in Switzerland, using hydrogen in a bench-scale, fluidized-bed reactor. The process was modeled using a double Monod saturation function. Nitrate and hydrogen were assumed to be limiting substrates in the first denitrification step and nitrite and hydrogen were assumed limiting in the second step. The denitrification rate was shown to be more strongly dependent on the nitrate concentration than on hydrogen. Hydrogen is only sparingly soluble in water (approx. 1mg/ℓ) and the Monod half-velocity constant for hydrogen was found to be less than one percent of saturation. Stoichiometrically, 0.35 mg/ℓ H_2 were required for complete denitrification of 1.0 mg/ℓ NO_3— N. Nitrite tended to accumulate in batch tests, but complete denitrification occurred in continuous flow experiments with sufficiently long residence times. A residence time of 4.5 hours was required for complete denitrification of water containing 25 mg/ℓ NO_3— N. The optimum pH was found to be approximately 7.5 and the pH tended to rise as a result of denitrification under unbuffered conditions. If the pH was allowed to rise to the vicinity of pH 9.0, nitrite tended to accumulate.

Several other studies of laboratory scale fixed-film reactors using hydrogen as a substrate have been published (Gros, 1982a, 1982b; Ginocchio, 1984; Sekoulov, 1987). These report on various aspects of support media composition, hydrogen generation, degassing techniques, and post treatment methods for the removal of microorganisms and dissolved organic carbon.

Gros (1986a, 1986b) reported on the start up and performance of a commercial scale, biological denitrification plant utilizing hydrogen located in Monchengladbach, West Germany. The technology was developed by Sulzer Water and Wastewater Treatment and is given the trade name Denitropur. Start up began in early 1986 and the treatment plant reached full capacity (50 m^3/h) in approximately three months. The plant design incorporates indirect hydrogen saturation, phosphate addition, four packed-bed reactors in series, post aeration, flocculant addition, filtration, and UV disinfection. Carbon dioxide was added as an inorganic carbon source and to buffer against an alkaline pH shift. At the operating temperature of 10.5 C, the organism growth rate varied from 0.1 to 0.3 d^{-1}. The sludge production is approximately 0.2 kg/kg N removed, on a dry weight basis. Residence times of one to two hours are required to remove 50 mg/ℓ nitrate. The denitrification rate varied with the mass and activity of the biomass.

Several other articles regarding the Denitropur process have been published (Hellekes, 1986; Anonymus, 1987; Gaehrs *et al.*, 1986). Pilot plant performance and design and installation of the full scale plant at Moenchengladbach are discussed.

Overath and Haberer (1984, 1985, 1986) studied autotrophic denitrification using columns packed with elemental sulfur and activated carbon. The columns were 100 mm in diameter, 3 m long, and were operated at a volumetric loading rate of 30 ℓ/h. After a 15 day start up period, influent nitrate concentrations were reduced from 35 to 0 mg/ℓ.

Denitrification was studied in columns packed with various ratios of elemental sulfur and limestone marl by Blecon *et al.* (1983). Efficiency increased as the particle size of the packed material de-

creased. Reactors containing the lowest amounts of marl (0 to 10 %) gave the best results for a high alkalinity (305 mg/ℓ HCO_3) water.

Lewandowski *et al.* (1987) evaluated autotrophic denitrification using calcium alginate beads suspended in a bench-scale completely mixed batch reactor. The beads consisted of elemental sulfur, calcium carbonate, and *Thiobacillus denitrificans* encapsulated in a calcium alginate polymer. Mixing in the reactor was provided by compressed nitrogen gas. Nitrate was reduced from 27 mg/ℓ to 6 mg/ℓ in seven hours. The initial nitrogen removal rate was 1.6 mg N/ℓ-h and increased to 4.8 mg N/ℓ-h after approximately four hours. Nitrite tended to accummulate initially but was later reduced to less than 2 mg/ℓ.

Other authors have evaluated reduced sulfur compounds such as sulfide and thiosulfate for the denitrification of water and domestic and industrial wastewaters (Claus, 1985; La Motta Diaz, 1985; Martin, 1982; Batchelor, 1978). Sulfate is a by-product of denitrification using sulfur compounds (see stoichiometric equations above). In some cases denitrification of industrial wastewaters containing very high nitrate concentrations (up to 6000 mg/ℓ) may result in high sulfate concentrations and lead to sulfate inhibition. In water treatment where nitrate concentrations are lower, sulfate inhibition would not be expected. However, stoichiometrically, the denitrification of 152 mg/ℓ of nitrate using elemental sulfur would yield 250 mg/ℓ sulfate. In such cases, the potable quality of the water may be affected.

Batchelor and Lawrence (1978) developed a mathematical model to describe denitrification kinetics using elemental sulfur as a substrate. The model was developed for use with wastewater but is also applicable to water. Sulfur diffusion through the biofilm, nitrate diffusion through the bulk solution, and nitrate diffusion through the biofilm were considered as possible rate limiting steps. The influence of each of these steps on the overall reaction rate varies as the concentration of nitrate in the bulk solution varies.

A mathematical model was developed by LeCloirec *et al.* (1985) which described denitrification kinetics by *Thiobacillus denitrificans* on a sulfur calcium-carbonate filter. The model considers biomass growth, nitrate removal, nitrite evolution, and consumption of sulfur.

Microcosm Studies

The word microcosm was derived from the Greek *mikro* meaning small and *kosmos* meaning universe or world. A microcosm is, therefore a small world. It is possible that the first use of the word microcosm was by S. A. Forbes in his 1887 essay, *The Lake as a Microcosm* (Odum, 1971). Bengtsson (1985) defined a microcosm as " any part of an ecosystem that may be subject to laboratory control due to its reduction in size or complexity." Microcosms of varying size and complexity have been used for years to study a variety of natural biological processes. Microcosms studies offer many advantages. They allow for superior control of variables, provide for a high degree of reproducibility, and can be conducted at a much lower cost than most field studies. A major concern regarding the use of microcosms is that they may not accurately simulate the natural system.

Numerous investigators (Koskinen and Keeney, 1982; Reddy *et al.*, 1978a, 1978b; Cho, 1982; Stanford *et al.*, 1975) have used soil microcosms to study denitrification. The simplest type of soil microcosms which have been used to study denitrification have been comprised of air-tight containers, either test tubes or flasks, containing soil or soil and water. The headspaces may be purged with helium or argon to displace oxygen. Some microcosm experiments measure denitrification resulting from natural soil organic matter, while others may provide an additional energy source such as glucose or methanol. Other amendments such as nitrate, nitrite, and phosphorus may also be added. These microcosms are normally incubated at constant temperature without shaking.

Denitrification can be monitored by measuring the disappearance of nitrate and nitrite and by the appearance of the product gases nitric oxide, nitrous oxide, and nitrogen. Nitric oxide is usually a very brief intermediate and is difficult to detect. Acetylene may be used to inhibit the oxidation of nitrous oxide to nitrogen gas. Nitrous oxide is the easiest product gas to measure, so in some cases acetylene inhibition is used to block the formation of nitrogen gas. This procedure is only effective in short term tests, since acetylene may be degraded by some microorganisms (Reneau, 1989).

Materials and Methods

Introduction

This study involved the use of soil and suspended growth microcosms to study the variation in groundwater denitrification rates using different substrates. Two gaseous substrates, carbon monoxide and methane, were studied and compared to a common liquid substrate, methanol. This research effort consisted of a series of microcosm studies which proceeded in a stepwise fashion. The design of each subsequent experiment was influenced by information obtained from previous results. These studies are summarized in the list below and are more thoroughly discussed in the sections that follow:

1. Determination of Thermodynamic Feasibility

   Calculations to determine if denitrification using carbon monoxide and methane was thermodynamically feasible.

2. Soil Studies

Initial experiments with soil microcosms using both clayey and sandy subsoils.

3. Unacclimated Suspended Growth Studies

   Batch, suspended growth studies seeded with either activated sludge or anaerobic digester sludge.

4. Seed Acclimation

   Development of acclimated seed cultures for each substrate starting with a dilute mixture of activated sludge and anaerobic digester sludge.

5. Inhibition Studies

   Studies to evaluate the inhibitory effects of carbon monoxide on denitrification using methanol.

6. Comparison of Shaken versus Unshaken Kinetics

   Studies to compare the kinetics of denitrification with methanol in shaken versus unshaken microcosms.

7. Acclimated Suspended Growth Studies

   Suspended growth experiments with seed cultures acclimated to methanol, methane, and carbon monoxide.

8. Determination of Biokinetic Constants

   Kinetic studies with a mixed culture of carbon monoxide-utilizing denitrifiers.

Thermodynamics

Calculations were made to determine the thermodynamic feasibility of microorganisms using methane and carbon monoxide as substrates for the denitrification of water. The stoichiometric equations presented earlier (Equations [4] and [5]) were used and the calculations are shown in the Appendix. Denitrification with both methane and carbon monoxide was found to have a large negative free energy, when unit activities were assumed for all products and reactants. Calculations were also made using limiting concentrations of products and reactants. These considered the real-

istic values for parameters such as pH 7 and low aqueous solubilities of methane, carbon monoxide, and nitrogen. In all cases the thermodynamics remained favorable for denitrification using methane and carbon monoxide.

General Methods

All glassware was thoroughly washed with a low phosphorus liquid detergent, rinsed with distilled water, and sterilized by autoclaving for 20 minutes at 121 C and 15 psi. Reagents were prepared using deionized, distilled, Milli-Q water and were filter sterilized using a 0.22 micron membrane filter. Metal utensils used to handle soil were flame sterilized. Syringe needles were heat sterilized (250 C) using a Hamilton syringe cleaner.

Sample Collection

Two different soils were used in the microcosm studies. A clayey soil was obtained from a depth of ten feet in a forested area near Blacksburg, Virginia and a sandy soil was obtained from a depth of four feet in an agricultural field in Tidewater, Virginia. In each case a pit was dug to the desired level and contaminated soil was scrapped from an area of the pit bottom using a flame sterilized trowel. Subsoil samples were then aseptically collected and placed in sterile one quart Mason jars. Soil samples were packed in ice during transport to the laboratory where they were stored at 4 C until use.

Groundwater was obtained from a deep limestone aquifer near Blacksburg, Virginia. The samples were taken from the tap of a private water supply using a sterile, 20 ℓ, glass carboy. The groundwater characteristics are shown in Table 1.

Table 1. Characterization of the groundwater used in denitrification microcosms.

Parameter	Concentration
Hardness	280 mg/ℓ as $CaCO_3$
Alkalinity	200 mg/ℓ as $CaCO_3$
Cl^-	7 mg/ℓ
$NO_3^- - N$	2 mg/ℓ
$PO_4^{\equiv} - P$	< 1 mg/ℓ
$SO_4^{=}$	8 mg/ℓ
pH	7.2

Activated sludge containing 2100 mg/ℓ volatile suspended solids (VSS) was obtained from a pilot-scale, biological nutrient removal system. This facility was treating domestic wastewater using the University of Capetown (UCT) process. Anaerobic digester sludge was obtained from the Pepper's Ferry Regional Wastewater Facility near Radford, Virginia and contained 8360 mg/ℓ VSS.

Microcosms

All microcosms used in this study were established with sterile, 740 ml, septum-capped bottles (Wheaton) using 33 mm teflon-faced silicon septa. The bottles were partially filled with soil and groundwater, or groundwater and seed organisms, leaving approximately 350 ml of gas-filled headspace. The headspace was purged for approximately 20 minutes with either helium, methane, or carbon monoxide. The bottles were placed horizontally in a fume hood and the gases were introduced through the septa, below the water level using a syringe needle. A second syringe needle inserted through the septa above the water level acted as an exhaust. Standard amendments to the groundwater included 10 to 100 mg/ℓ nitrate-N (as sodium nitrate), and approximately 20 mg/ℓ ammonium-N, and 22 mg/ℓ phosphate-P (as dibasic ammonium phosphate). The purpose of the ammonium-N was to inhibit assimilatory denitrification. The phosphate was provided as a phosphorus source so that microbial activity would not be limited by this nutrient.

Initially the microcosms were established with excess substrate (100- 300 mg/ℓ methanol, 1.0 atm. methane, 1.0 atm. carbon monoxide) and varying concentrations of nitrate (10,25,50,100 mg/ℓ as N). Control microcosms were prepared which contained 100 mg/ℓ nitrate-N, no substrate, and helium in the headspace. In later experiments carbon monoxide was used at partial pressures varying from 0.005 to 0.75 atmospheres. In these cases helium was mixed with carbon monoxide to achieve a total pressure of one atmosphere. The gas mixtures were obtained by purging the headspace with the primary gas, withdrawing an appropriate volume with a gas-tight syringe, and then replacing the volume removed with the secondary gas, also using a gas-tight syringe.

Ten replicate microcosms were used in the first soil experiment (clay soil) for each combination of nitrate and substrate. Triplicate systems were used in the second soil study (sand), while duplicate systems were used in the suspended growth experiments. All microcosms were incubated in the dark at 20 C. Some were incubated without shaking, while others were shaken continuously using an oscillating shaker table (1.5 inch displacement, 150 cycles/min.).

Analytical Methods

The liquid in the microcosms and headspace gases were sampled through the septa using a 3.0 ml gas-tight syringe. Prior to sampling the shaken microcosms were allowed to settle for approximately 15 minutes. To obtain liquid samples, the microcosms were slowly tilted on their sides until the septa were below the liquid surface. Very little mixing of soil and water occurred when the microcosms were tilted during sampling, and in this position the syringe could be used to sample liquid without removing headspace gas or soil.

The liquid samples were analyzed for nitrate and nitrite with a Dionex Model 2010i Ion Chromatograph using a HPIC-AS4A column and a conductivity detector. The eluent was a carbonate-bicarbonate buffer (2.8mM sodium bicarbonate, 2.2 mM sodium carbonate, pH 9.5) and the eluent flow rate was 2.0 ml/min. The chromatograph was equipped with a 50 $\mu\ell$ sample loop. A total sample size of 2 ml was required, however, to adequately flush the sample loop between injections. Samples were filtered using 0.45 micron membrane filters (13 mm dia. with Swinney filter holders) and diluted as necessary prior to injection into the ion chromatograph.

Gas samples were analyzed for carbon monoxide with a Hewlett-Packard Model 5880 gas chromatograph using a 6 ft. x 1/8 in. packed column and molecular seive 13X packing. The carrier gas was helium at a flow rate of 80 cc/min.. A thermal conductivity detector was used and the de-

tector, injector, and column temperatures were 40 C. A sample size 0.5 cc was used and the retention time of the carbon monoxide peak was approximately 2.0 minutes.

Optical density measurements were made on liquid samples from some of the suspended growth microcosms using a Beckman DU-6 spectrophotometer. A scan of percent transmission versus wavelength was conducted from 200 to 700 nm and 500 nm was chosen as optimum.

Soil Studies

Microcosms containing two different soil types (sand and clay) were used to evaluate the potential for denitrification of groundwater using native microorganisms. Clay soil microcosms were established containing approximately 70 g dry weight of soil (100 g wet weight, 42.8% moisture) and 250 ml of groundwater, amended with 25 mg/ℓ nitrate-N, 20 mg/ℓ ammonium-N, and 22 mg/ℓ phosphate-P. The initial pH was 7.5.

In two sets of microcosms either methane or carbon monoxide were provided in the headspace as an energy source for denitrification. In a third set methanol (300 mg/ℓ) was added as a substrate and the headspace was purged with helium. Control microcosms were established with no added substrate and helium in the headspace. Ten replicates of each system were incubated in the dark, without shaking, at 20 C. The liquid in each microcosm was sampled at various intervals for a period of 70 days. Samples were analyzed for nitrate and nitrite using ion chromatography.

Sandy soil microcosms contained 95 g dry weight of soil (100 g wet weight, 5.7% moisture) and 200 ml of groundwater, and were amended with 100 mg/ℓ nitrate-N, 20 mg/ℓ of ammonium-N, and 22 mg/ℓ phosphate-P. Triplicate systems were established using three substrates (methanol, 300 mg/ℓ; methane, 1.0 atm; carbon monoxide, 1.0 atm) and four concentrations of nitrate-N (10, 25, 50, 100 mg/ℓ). Control microcosms contained no added substrate, 100 mg/ℓ nitrate-N, and helium in the headspace. These systems were incubated in the dark, on an oscillating shaker table, at 20

C. The liquid in each microcosm was sampled at various intervals for more than 100 days and was analyzed for nitrate and nitrite using ion chromatography.

Unacclimated Suspended Growth Studies

Suspended growth microcosms were established to screen activated sludge and anaerobic digester sludge for microorganisms capable of denitrification using methane and carbon monoxide. The activated sludge seed was obtained from a pilot-scale nutrient removal system and contained 2100 mg/ℓ VSS. The anaerobic digester sludge was obtained from the Peppers Ferry Regional Wastewater Treatment Facilty located near Radford, Virginia and contained 8360 mg/ℓ VSS.

The suspended growth microcosms contained either 10 ml activated sludge or 20 ml of anaerobic digester sludge in 350 ml of groundwater. Amendments to the groundwater included 100 mg/ℓ nitrate-N, 20 mg/ℓ ammonium-N, and 22 mg/ℓ phosphate-P. Triplicate systems were established using three substrates (methanol, 100 mg/ℓ; methane, 1.0 atm; carbon monoxide, 1.0 atm). Control microcosms were established with helium in the headspace and no added substrate. All of the suspended growth microcosms were incubated in the dark, on an oscillating shaker table, at 20 C. Samples were taken of the liquid in each microcosm at various intervals for 40 days and analyzed for nitrate and nitrite using ion chromatography.

Seed Acclimation

A dilute mixture of activated sludge and anaerobic digester sludge was used to develop acclimated seed cultures specific to each substrate. Four, 20 liter carboys were used as reactors. Each reactor contained 250 ml activated sludge, 250 ml anaerobic digester sludge, 100 mg/ℓ nitrate-N, 20 mg/ℓ ammonium-N, and 22 mg/ℓ phosphate-P in five liters of groundwater. The control seed was purged

with helium and sealed with helium in the headspace. Two hundred milligrams per liter of methanol was added to the methanol seed and the carboy was purged and sealed with helium in the headspace. The carbon monoxide and methane seeds were purged and sealed with carbon monoxide and methane, respectively. Each of these systems was incubated in the dark, without shaking, at 20 C, for 20 days. After one week, each of the seeds were purged again with their respective gases, and after two weeks, they were redosed with gases, nutrients, and methanol (methanol seed only).

Inhibition Experiments

Observations were made in earlier experiments that nitrite accumulations were much greater in soil and suspended growth systems when carbon monoxide was used as a substrate. Nitrite accumulations in microcosms with methanol as a substrate and helium in the headspace were always much lower. Inhibition caused by carbon monoxide was suspected and experiments were conducted to test this hypothesis. Microcosms were established containing 35 ml of acclimated seed in 350 ml of groundwater, amended with 100 mg/ℓ nitrate-N, 20 mg/ℓ ammonium-N, 22 mg/ℓ phosphate-P, and 300 mg/ℓ methanol. Duplicate systems were established using three combinations of seed type and headspace gas:

1. Methanol seed and helium in the headspace (MeOH seed/He),

2. Methanol seed and carbon monoxide in the headspace (MeOH seed/CO),

3. Carbon monoxide seed and carbon monoxide in the headspace (CO seed/CO).

All microcosms were incubated in the dark, on an oscillating shaker table, at 20 C. Samples were taken of the liquid in each microcosm at various intervals for 40 days and analyzed for nitrate and nitrite using ion chromatography.

Comparison of Shaken versus Unshaken Kinetics

Previous investigators (Cho, 1982) have found denitrification rates to differ between shaken and unshaken, flooded soil microcosms. Diffusion of nitrate through the liquid was found to be rate limiting in unshaken systems (Reddy *et al.*, 1978a,1978b). In the case of systems containing both nitrate and carbon monoxide, denitrification rates may be affected by the rate of diffusion of nitrate from the bulk solution to the location of biological activity, as well as the rate of carbon monoxide diffusion into and through the bulk solution. Preliminary experiments were conducted to measure denitrification rates in suspended growth microcosms under shaken and unshaken conditions. The results of these experiments were used to make decisions regarding subsequent experimental designs. Microcosms were established containing 35 ml of acclimated seed in 350 ml of groundwater, amended with 100 mg/ℓ nitrate-N, 20 mg/ℓ ammonium-N, 22 mg/ℓ phosphate-P, and 300 mg/ℓ methanol. Four replicate systems were established using three combinations of seed type and headspace gas:

1. Methanol seed and helium in the headspace (MeOH seed/He),

2. Methanol seed and carbon monoxide in the headspace (MeOH seed/CO),

3. Carbon monoxide seed and carbon monoxide in the headspace (CO seed/CO).

Two replicate microcosms of each combination of seed type and headspace gas were shaken during incubation, while the remaining two replicates were incubated without shaking. All microcosms were incubated in the dark, at 20 C. Samples were taken of the liquid in each microcosm at various intervals for 40 days and analyzed for nitrate and nitrite using ion chromatography.

Figure 5 through Figure 7 present data from experiments designed to compare denitrification rates in shaken and unshaken microcosms. Figure 5 on page 46 compares shaken and unshaken microcosms containing methanol as a substrate, a seed acclimated to methanol, and helium in the

headspace. The nitrate-N concentration in the shaken treatment decreased from 96 mg/ℓ to zero in less than one day. Identical microcosms that were incubated without shaking required more than six days to achieve complete denitrification. Figure 6 compares shaken and unshaken microcosms containing methanol as a substrate, a seed acclimated to methanol, and carbon monoxide in the headspace. The shaken treatment completely denitrified 103 mg/ℓ nitrate-N in 20 days, whereas the unshaken microcosms required over 36 days. Figure 7 compares shaken and unshaken microcosms containing methanol as a substrate, a seed acclimated to carbon monoxide, and carbon monoxide in the headspace. Once again the shaken treatment proceeded at a faster rate and experienced a shorter lag period than the unshaken microcosms.

Whether shaken or unshaken systems are more appropriate depends upon the objectives of experimental design. To determine optimum or maximum denitrification rates, shaken (or well mixed) systems would be necessary. To model in situ denitrification rates, unshaken systems would be more appropriate. The unshaken systems would exhibit slower rates, but would be more representaive of actual in situ conditions.

Acclimated Seed Studies

Suspended growth microcosms studies were conducted to determine if seed acclimation had been successful. Microcosms were established containing 35 ml of acclimated seed in 350 ml of groundwater, amended with 20 mg/ℓ ammonia-N, and 22 mg/ℓ phosphate-P. Duplicate systems were established using three substrates (methanol, 300 mg/ℓ; methane, 1.0 atm; carbon monoxide, 1.0 atm) and four nitrate-N concentrations (10, 25, 50, and 100 mg/ℓ). The acclimated seed added to each microcosm corresponded to the substrate being used in that microcosm. For example, the seed which had been acclimated to carbon monoxide was added to the microcosms using carbon monoxide as a substrate. Control microcosms contained no added substrate and helium in the headspace. All microcosms were incubated in the dark, on an oscillating shaker table, at 20 C.

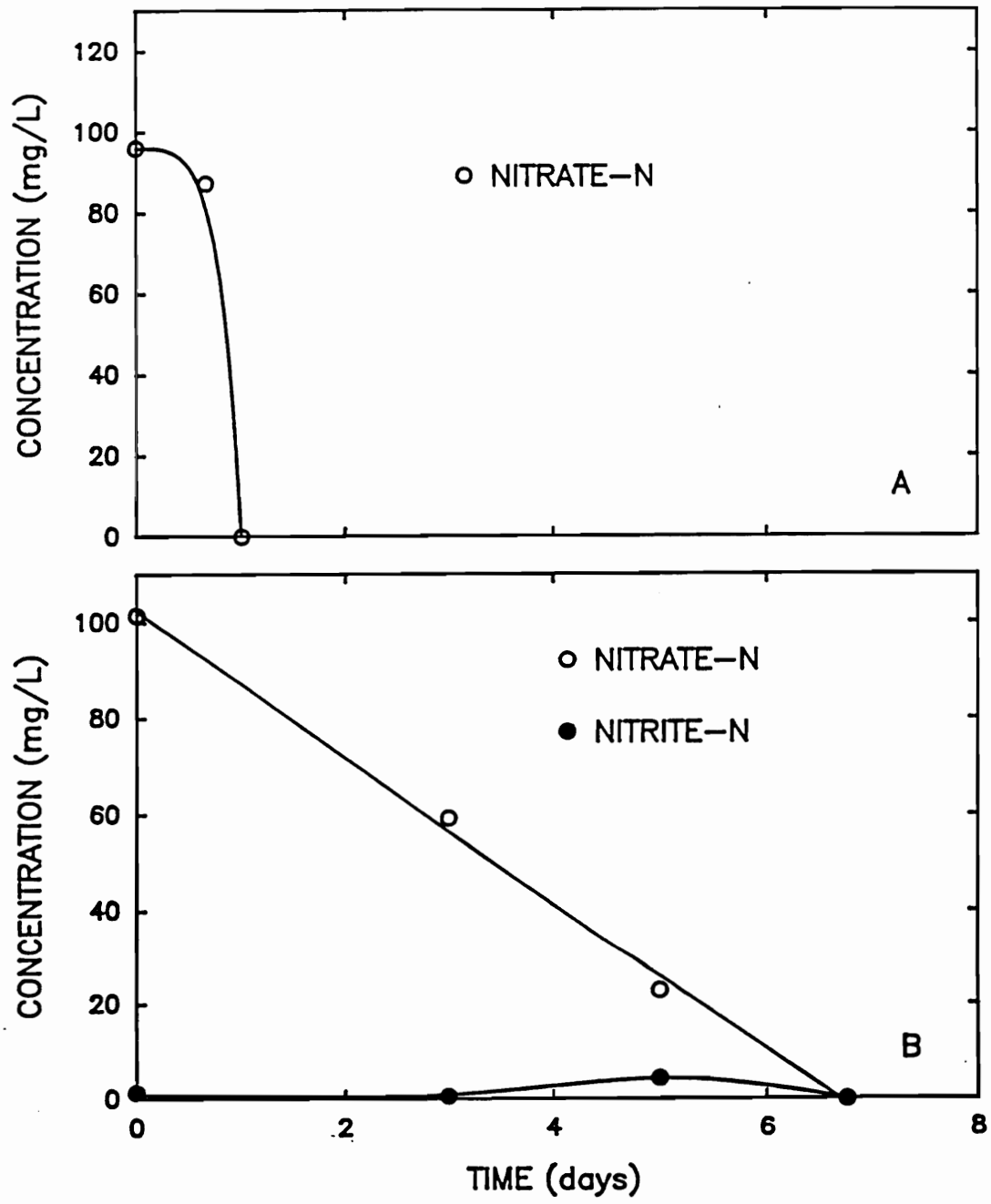

Figure 5. Shaken and unshaken, methanol-amended microcosms, containing a methanol-acclimated seed and He: Comparison of denitrification in (A) shaken and (B) unshaken, methanol-amended microcosms, containing a seed acclimated to methanol and He in the headspace.

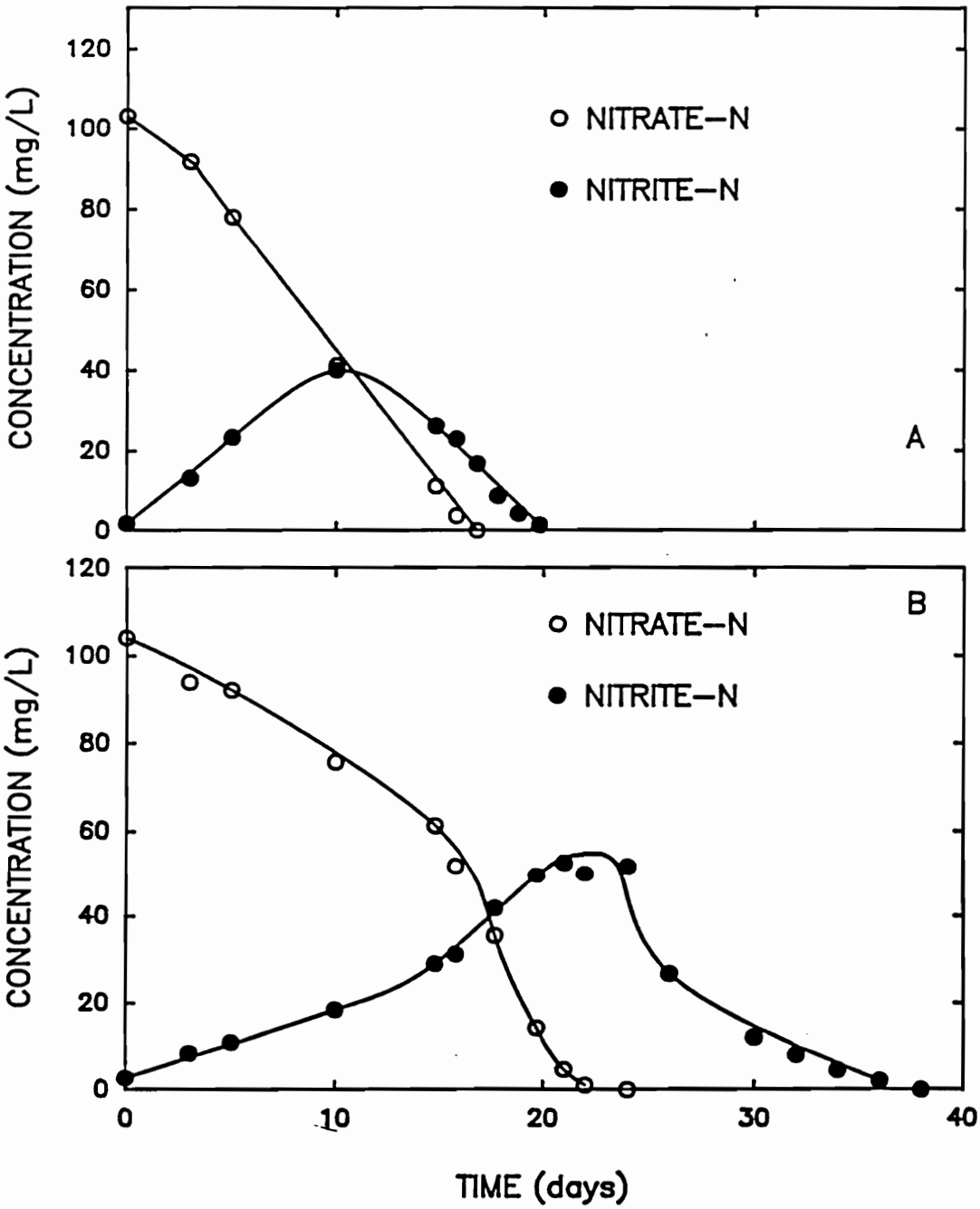

Figure 6. Shaken and unshaken, methanol-amended microcosms, containing a methanol-acclimated seed and CO: Comparison of denitrification in (A)shaken and (B) unshaken, methanol-amended microcosms, containing a seed acclimated to methanol and CO in the headspace.

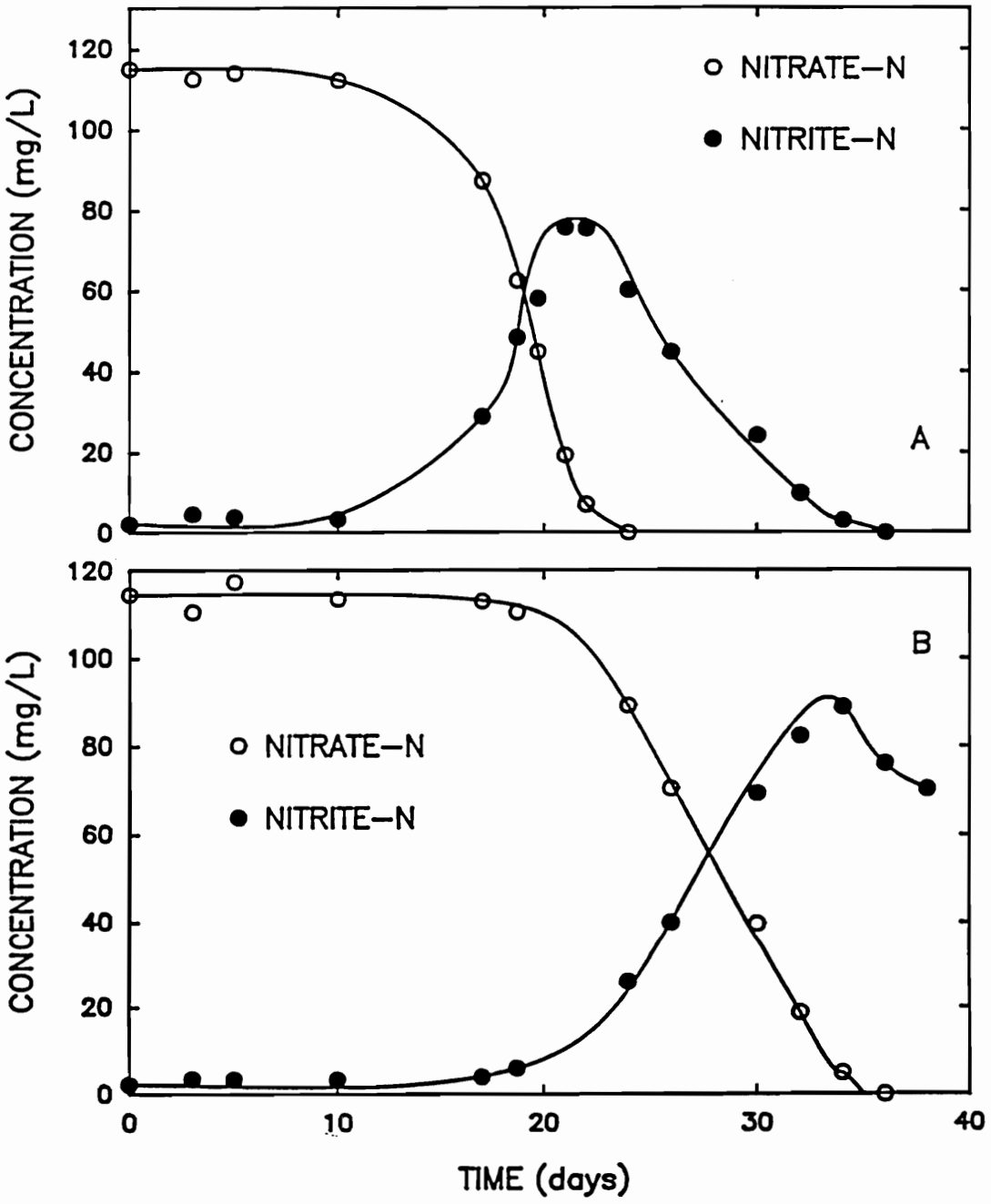

Figure 7. Shaken and unshaken, methanol-amended microcosms, containing a CO-acclimated seed and CO: Comparison of denitrification in (a) shaken and (B) unshaken, methanol-amended microcosms, containing a seed acclimated to CO and CO in the headspace.

Samples were taken of the liquid in each microcosm at various intervals for 40 days and analyzed for nitrate and nitrite using ion chromatography.

Determination of Biokinetic Constants

During the acclimated seed studies discussed above, a mixed culture of microorganisms was found that was capable of using carbon monoxide as a substrate for denitrification. A series of experiments were conducted using this acclimated seed to determine biokinetic constants for denitrification using carbon monoxide. The biokinetic constants of interest were the maximum specific substrate utilization rates (q) with respect to nitrate and carbon monoxide, the half velocity saturation constants (K_s) with respect to nitrate and carbon monoxide, the maximum specific growth rate (μ), and the biomass yield coeficient (Y). The total quantity of successfully acclimated carbon monoxide seed equalled approximately two liters. Microcosms were established containing 50 ml acclimated seed in 300 ml of groundwater, amended with 20 mg/ℓ ammonium-N and 22 mg/ℓ phosphate-P. Duplicate systems were established using four nitrate-N concentrations (10, 25, 50, and 100 mg/ℓ) and 1.0 atm of carbon monoxide in the headspace. In addition, duplicate systems were established using 100 mg/ℓ nitrate-N and four carbon monoxide partial pressures in the headspace (0.10, 0.25, 0.50, and 1.00 atm). Control microcosms contained 100 mg/ℓ nitrate-N and helium in the headspace. All microcosms were incubated in the dark, on an oscillating shaker table, at 20 C. Samples were taken of the liquid in each microcosm at various intervals and analyzed for nitrate and nitrite using ion chromatography.

For a non-inhibitory substrate these experiments would have been sufficient to determine the biokinetic constants for denitrification using carbon monoxide. Preliminary results indicated, however, that carbon monoxide at these concentrations was possibly causing substrate inhibition of denitrification. It was necessary to repeat the above experiments at several lower carbon monoxide

partial pressures. In total, experiments were conducted at 12 different carbon monoxide partial pressures ranging from 0.005 to 1.00 atmospheres (0.14 to 28.4 mg/ℓ).

Numerous attempts were made throughout this study to measure the biomass in the suspended growth microcosms. Gravimetric measurements of suspended solids (SS) and volatile suspended solids (VSS) were made using various sample sizes and filter configurations. Optical density was examined as a method of monitoring biomass growth. The use of direct microbial counts was also investigated.

Initial gravimetric measurements of biomass were made during the determination of nitrate and nitrite disappearance rates. There was a very limited quantity of active carbon monoxide using culture, and the growth rate was very slow. As a result it was necessary to use very small sample volumes. A 3 ml sample size was found to be adequate for the anion analysis. This sample was filtered prior to injection into the ion chromatograph. By drying and weighing the filter before and after sample filtration, it should be possible to determine the SS concentration in the liquid phase. Unfortunately, because of the low SS concentration in the microcosms and the small volume filtered, the dry weight of the filtered solids was only in the range of 0.0001 grams. This was too close to the detection limit of the balance to obtain an accurate measurement of SS.

In later tests the sensitivity of this measurement was improved by weighing several filters at once. It was not possible using this method to measure the SS concentration in each individual microcosm, but rather an average value for several microcosms. By weighing ten filters simultaneously, it was determined that the suspended growth microcosms contained an average of 125 mg/ℓ SS.

These SS measurements were too crude to to be used for the measurement of maximum specific growth rate and biomass yield, so optical density was evaluated as a method of monitoring biomass growth. Optical density could then later be correlated to SS and VSS measurements. A Beckman DU-6 spectrophotometer was used to measure optical density. A scan of wavelengths in the visible

range was conducted and 500 nm was found to be the most sensitive. Figure 8 illustrates the relationship between percent transmittance and dilution of the liquid fraction in the suspended growth microcosms. This relationship was fairly uniform and indicated that optical density could be correlated with SS concentration. Unfortunately, optical density measurements exhibited a large degree of variability over time. The variability in the optical density data for a typical microcosm is illustrated in Figure 9. The variation observed probably resulted from poor mixing and/or rapid settling of the suspended matter.

Problems were also experienced with direct microbial counts. The population in the suspended growth microcosms included gram negative rods, cocci, and filamentous organisms. The majority of the bacteria were present in large clumps and aggregates, making accurate counting impossible. The growth of filamentous organisms was also difficult to distinguish by microscopic observation. In addition, there was a significant amount of inert debris. A combination of surfactant (1%, sodium lauryl sulfate) and mixing was used to break up the clumps and facilitate counting. Little improvement was observed, however, and direct counts were abandoned as a method of biomass measurement.

Biomass yield, specific carbon monoxide utilization rate, specific biomass growth rate determinations were eventually made using data from a special experiment. After all of the denitrification rate experiments were completed, the biomass remaining in 20 microcosms was concentrated by settling and placed in two microcosms. Since this was the last experiment conducted using the carbon monoxide acclimated seed, larger sample sizes could be sacrificed during the SS and VSS analyses. A 20 ml sample size coupled with the higher SS concentration allowed for more accurate determinations of SS and VSS.

Six samples were taken over a period of 19 days and analyzed for VSS, nitrate, nitrite, and carbon monoxide. VSS was determined using standard gravimetric procedures, anion concentrations were measured using ion chromatography, and carbon monoxide partial pressures were measured using gas chromatography.

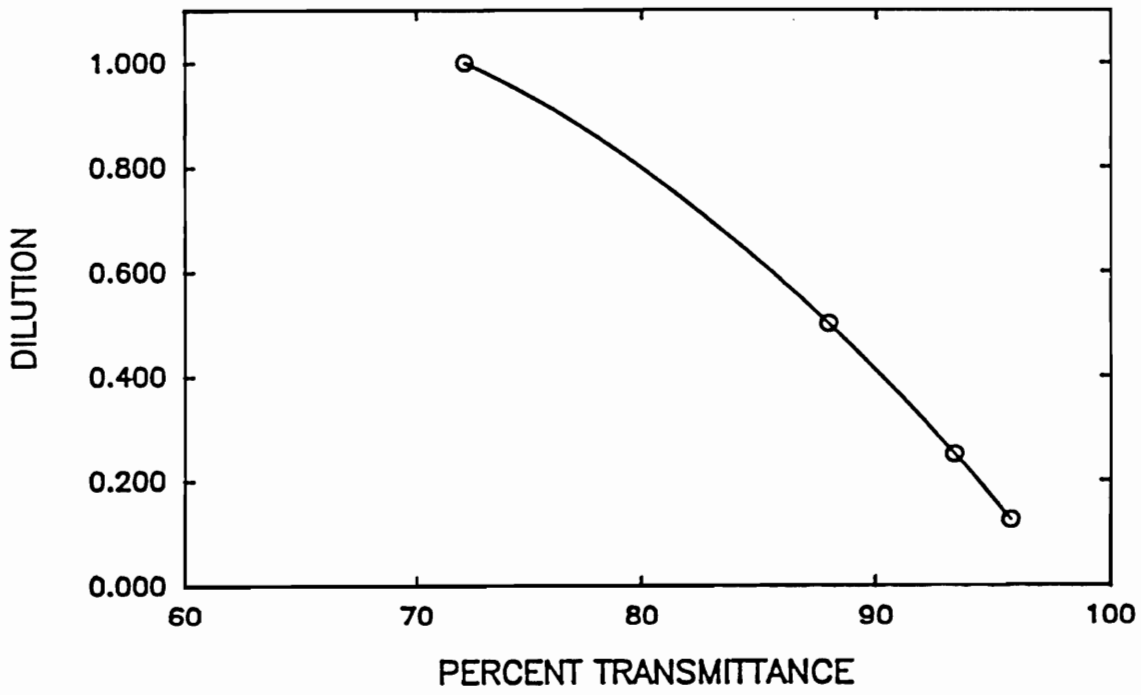

Figure 8. The effect of dilution on percent transmittance of microcosm mixed liquor.

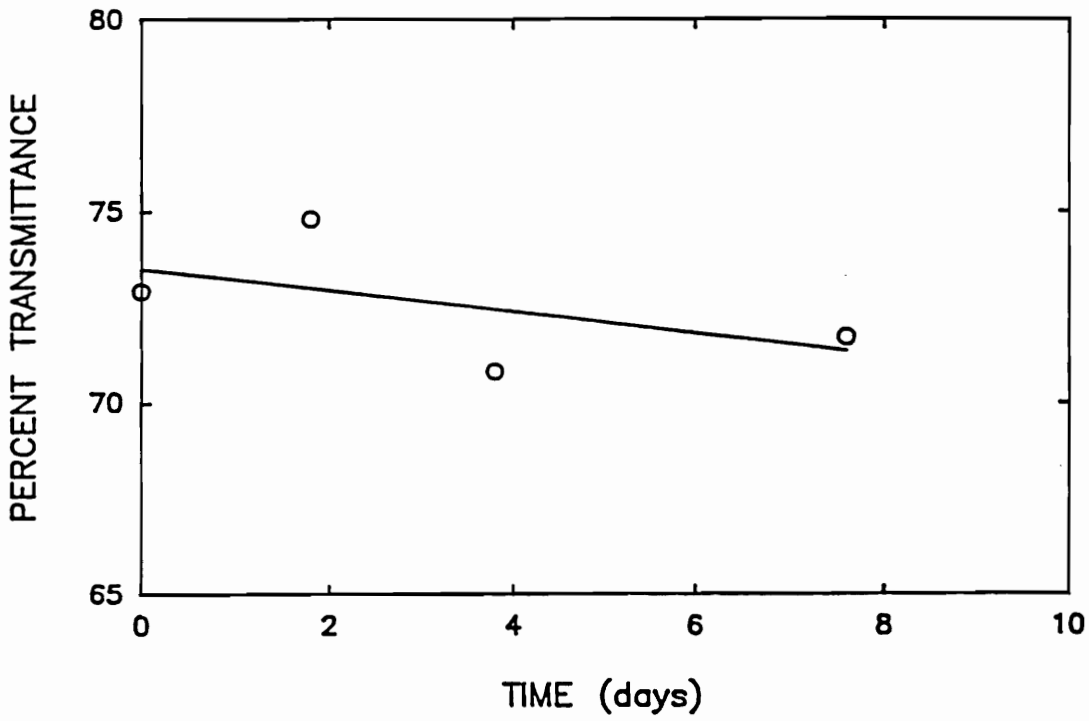

Figure 9. Variation of percent transmittance versus time in a suspended growth microcosm.

Biomass yield with respect to carbon monoxide was determined by plotting the increase in VSS versus carbon monoxide utilization. Biomass yield with respect to nitrate was determined by plotting the increase in VSS versus nitrate concentration. A linear, least squares regression was used to construct a line of best fit through these data. The yield in each case was the slope of these regression lines.

The specific substrate utilization rate (q) with respect to carbon monoxide is the mass of carbon monoxide consumed during denitrification each day, per unit mass of microorganisms (mg CO/d-mg VSS). The q was calculated by dividing the incremental change in the mass of carbon monoxide per time by the average biomass concentration during that time increment. The value obtained for q using this procedure was not the maximum specific carbon monoxide utilization rate because the experiment was not conducted using the optimum carbon monoxide partial pressure. A carbon monoxide partial pressure of 0.06 atmospheres was used in this experiment, whereas, subsequent experiments determined that a partial pressure of 0.10 atmospheres was necessary for optimum denitrification rates.

The maximum specific substrate utilization rate q_m with respect to nitrate was equal to the the maximum specific denitrification velocity. This quantity was obtained by inspection from the plot of specific denitrification velocity versus carbon monoxide concentration.

The specific growth rate, μ, is the increase in biomass per day, per unit biomass (mg VSS/d-mg VSS). The μ was calculated by dividing the incremental change in biomass concentration per day by the average biomass concentration.

The oxygen sensitivity of the successfully acclimated carbon monoxide culture was unknown. As a precaution, these microcosms were prepared under anaerobic conditions. All transfers were made in an anaerobic glove box, under an atmosphere of mixed nitrogen and carbon monoxide.

Two microcosms were sacrificed to test for oxygen sensitivity. These systems were exposed to aerobic conditions for one week. After this period, they were purged with helium and dosed with carbon monoxide. The initial carbon monoxide dose was 1.0 atmospheres, but the systems were later redosed at 0.25 atmospheres. These aerobically-stressed systems were incubated at 20 C, along with the other acclimated seed studies. Samples of the liquid in each microcosm were taken at various intervals and analyzed for nitrate and nitrite, using ion chromatography.

The denitrification rates in control and aerobically-stressed microcosms were found to be similar. The denitrification rate was slow in both cases, but it was apparent that the activity of the carbon monoxide utilizing culture was not adversely affected by exposure to aerobic conditions. These preliminary results indicated that it was not necessary to conduct subsequent sampling and redosing operations in a strict anaerobic environment.

Results and Discussion

Preliminary Acclimated Seed Studies

The results of the acclimated seed studies are shown in Figure 10 through Figure 12. Biodegradable organic matter that was present in the combined anaerobic digester sludge and activated sludge seed was mostly depleted during the 20 day acclimation period. As a result there was very little substrate present to drive denitrification in the controls (Figure 10). The nitrate-N concentration was nearly constant, varying from 108 mg/ℓ at time zero to 111 mg/ℓ at 38 days. The nitrite concentration, however, increased steadily to a high of 12 mg/ℓ at 38 days. The sum of the nitrate-N and nitrite-N concentrations increased from 108 mg/ℓ to 123 mg/ℓ during the study, suggesting that nitrification may have occurred. Why nitrite would accumulate to such an extent in these controls was not determined.

No positive results were obtained with the methane amended acclimated seed studies. Data for this treatment are not included in the text, but the raw data are presented in tabular form in the Appendix.

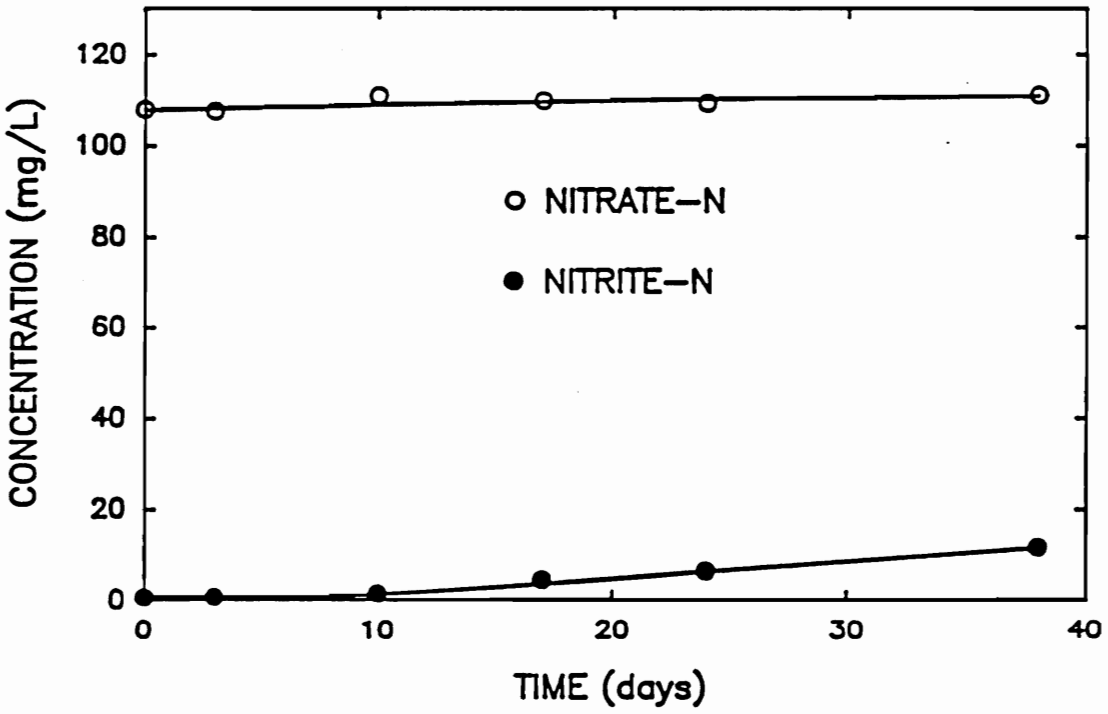

Figure 10. Denitrification in acclimated but unamended, control microcosms.

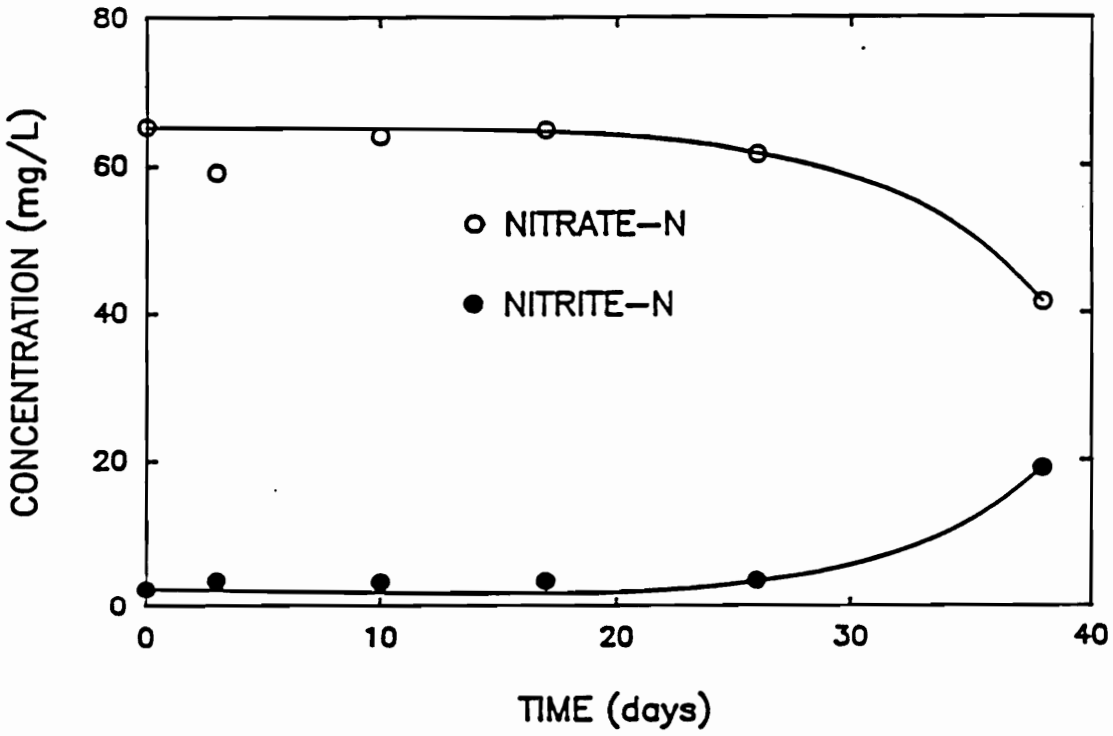

Figure 11. Denitrification in CO-amended microcosms, containing a CO-acclimated seed and low nitrate.

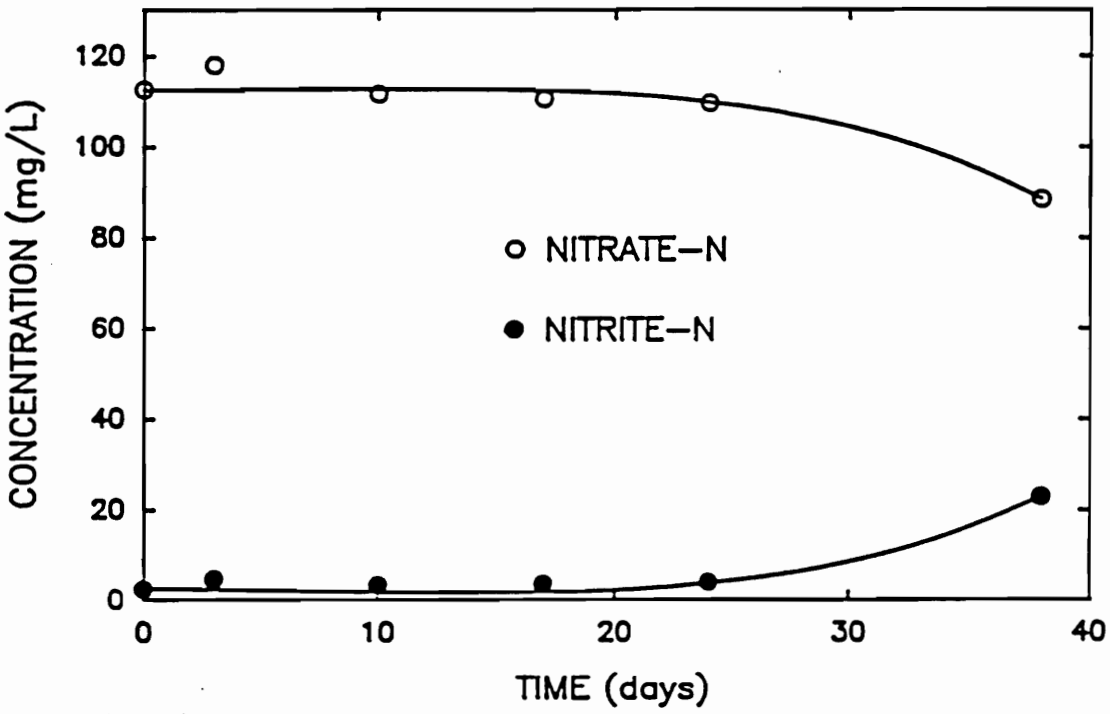

Figure 12. Denitrification in CO-amended microcosms, containing a CO-acclimated seed and high nitrate.

The results from the carbon monoxide amended microcosms are presented in Figure 11 and Figure 12. Very little nitrate reduction occurred during the first 26 days of this study. Between 26 days and 38 days the nitrate-N concentration in the treatment with the lower initial nitrate-N (Figure 11) decreased from 62 mg/ℓ to 41 mg/ℓ. During the same period the nitrite-N concentration increased from 4 mg/ℓ to 19 mg/ℓ This represents a net denitrification of 6 mg/ℓ. A similar amount of activity took place in the higher initial nitrate-N treatment (Figure 12). Between 24 and 38 days, 3 mg/ℓ of net denitrification occurred. Although this was a relatively small amount of net denitrification, the overall activity of these systems was more pronounced than any other previous experiment in which carbon monoxide was the sole energy source for denitrification. These were the first results obtained during this study that suggested carbon monoxide may be used as an energy source for denitrification.

Determination of Biokinetic Constants

Using the sucessfully acclimated carbon monoxide seed discussed above, a series of experiments were conducted to determine denitrification rates using carbon monoxide as a sole energy source. The data shown in Figure 13 illustrates the relationship between carbon monoxide use and nitrogen loss during denitrification using carbon monoxide. Nitrate-N and nitrite-N were measured using ion chromatography, while concurrently carbon monoxide was measured using gas chromatography. These results suggested that carbon monoxide was being used as an energy source for denitrification.

Microcosms containing four different nitrate-N concentrations (10, 25, 50, 100 mg/ℓ) and 12 different carbon monoxide partial pressures (0.005, 0.01, 0.02, 0.04, 0.06, 0.08, 0.10, 0.15, 0.25, 0.35,0.75,1.00 atm) were studied. The data gathered were used to determine biokinetic constants. Figure 14 illustrates the method of data reduction used to obtain net denitrification rates. Nitrate and nitrite concentrations in each microcosm were measured at various time intervals. Net

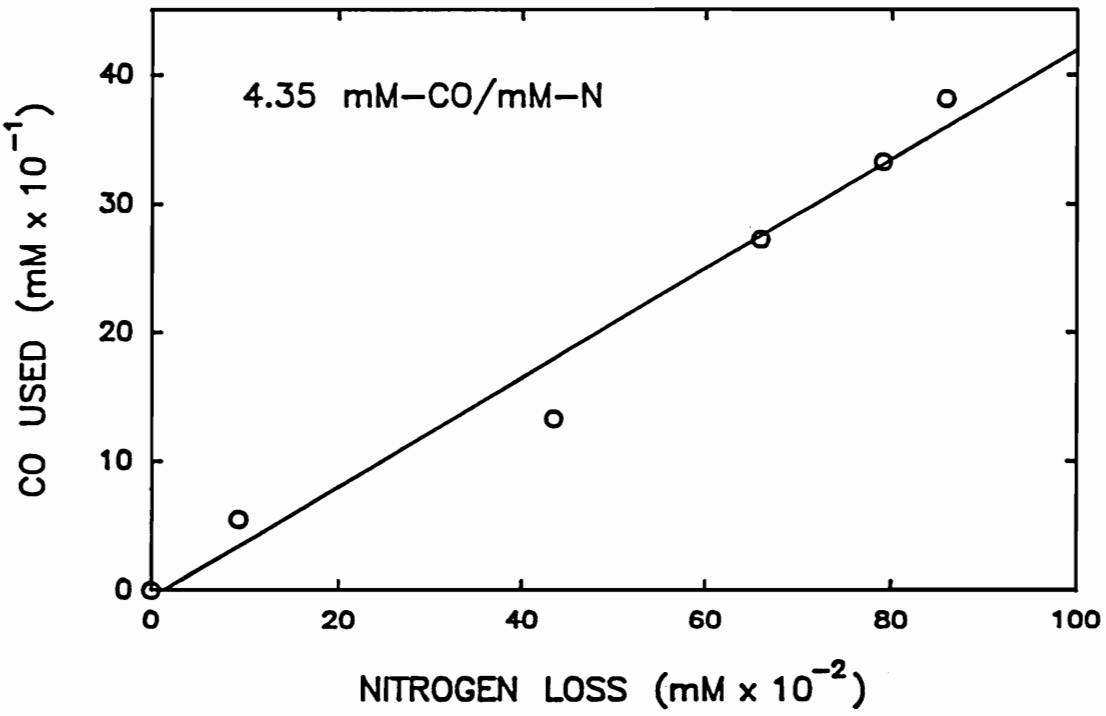

Figure 13. Relationship between CO use and N loss during denitrification using CO.

denitrification rate (mg-N/ℓ-d) was defined as the rate of decrease of the sum of nitrate-N and nitrite-N. The specific denitrification rate (mg-N/d-gVSS) was calculated by dividing the net denitrification rate by the average volatile suspended solids concentration in the microcosm.

Figure 15illustrates the variation in denitrification velocity at different initial nitrate concentrations. Figure 16 is a Lineweaver-Burk reciprocal plot of these velocity data. From the reciprocal plot it was determined that the $V_m^{NO_3}$ was 12.2 mg-N/d-gVVS or 0.01 d^{-1}. The $K_m^{NO_3}$ was found to be 1.88 mM or 26 mg/ℓ as nitrate-N.

The variation in denitrification velocity at different initial carbon monoxide concentrations is shown in Figure 17. The general form of this curve is characteristic of a substrate inhibition effect. At aqueous carbon monoxide concentrations below 10.14 x 10^{-5} M (2.8 mg/ℓ), velocity increased with increasing substrate concentration. At aqueous carbon monoxide concentrations above 10.14 x 10^{-5} M, velocity decreased with increasing substrate concentration. The curve in Figure 17 was fitted using a SAS non-linear, least squares regression and the Haldane substrate inhibition model. The program and output are included in the Appendix. The Haldane model was discussed earlier in the Literature Review section and is shown below:

$$v = \frac{V_m S}{S + K_s + S^n/K_i} \qquad [21]$$

Because of the substrate inhibition effect, the determination of biokinetic constants with respect to carbon monoxide could not be accomplished using a Lineweaver-Burk reciprocal plot. It was necessary to estimate the constants V_m^{CO}, K_s^{CO}, K_i, and n using the non-linear regression. The best fit of the experimental data was obtained with a V_m^{CO} of 54.7 mg-N/d-gVSS (0.055 d^{-1}), a K_s^{CO} of 7.7 x 10^{-5} M (2.2 mg/ℓ), a K_i of 88.6 x 10^{-5} M (24.8 mg/ℓ), and an n of 2.5. The correlation coefficient for this fit was 0.94.

Although the fit obtained using the Haldane model and the constants discussed above was reasonable, the Haldane model may not be the most appropriate kinetic model to describe denitrification

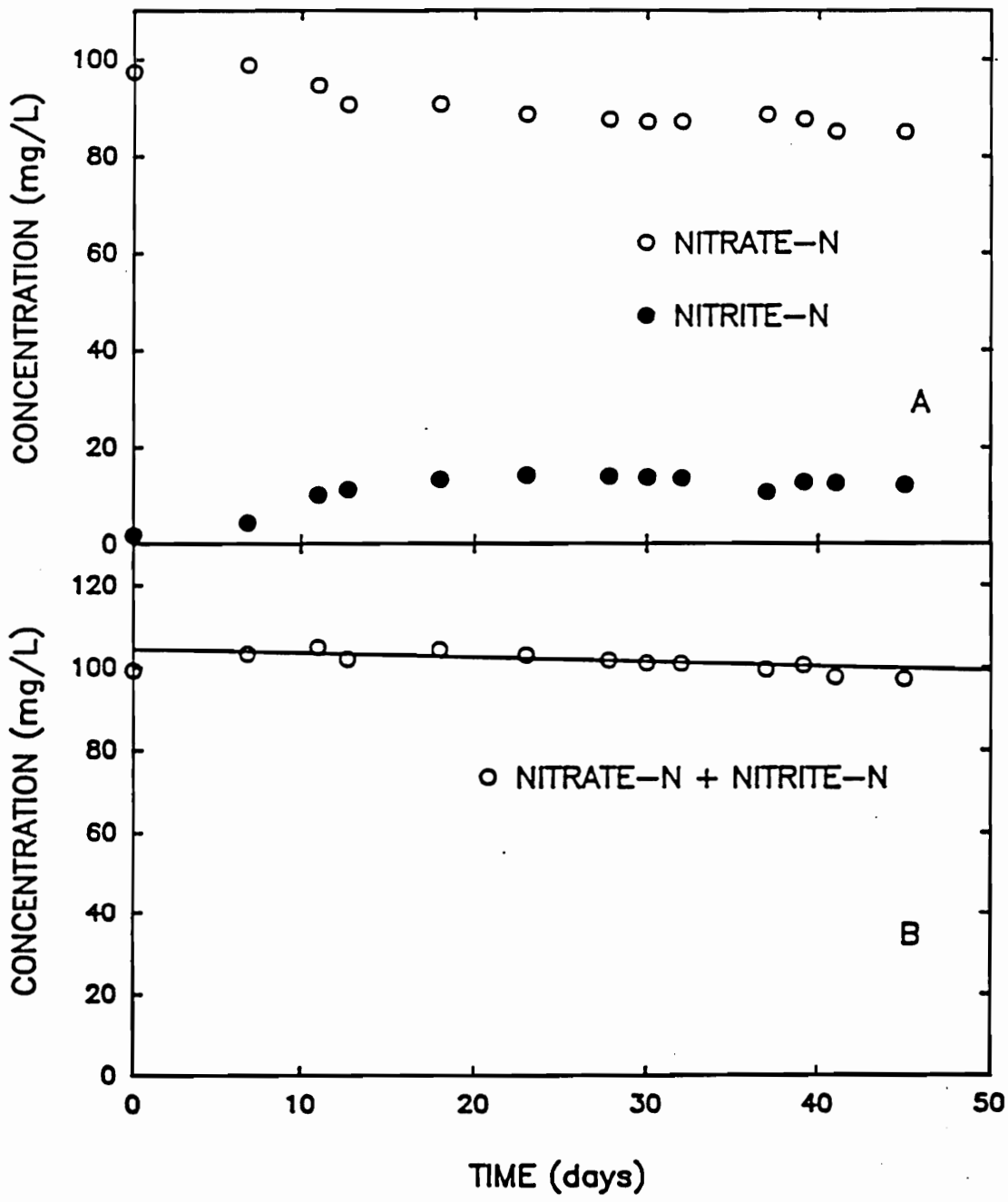

Figure 14. Comparison of (A) nitrate and nitrite reduction to (B) net denitrification.

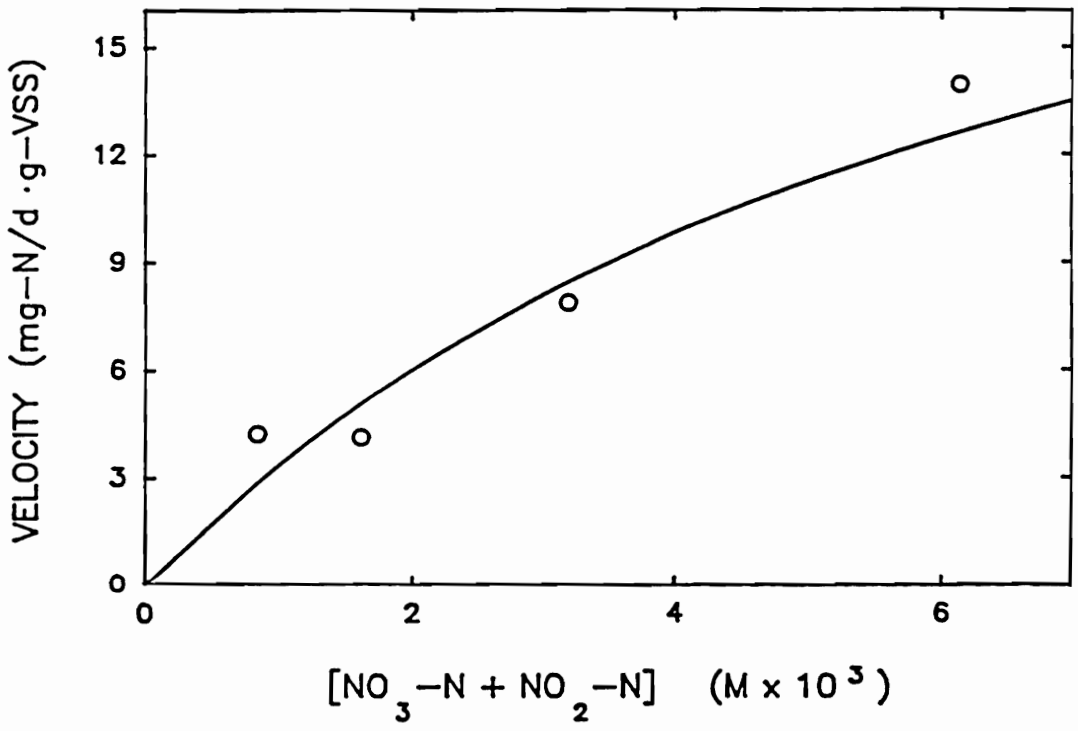

Figure 15. Effect of nitrate concentration on denitrification velocity, using CO.

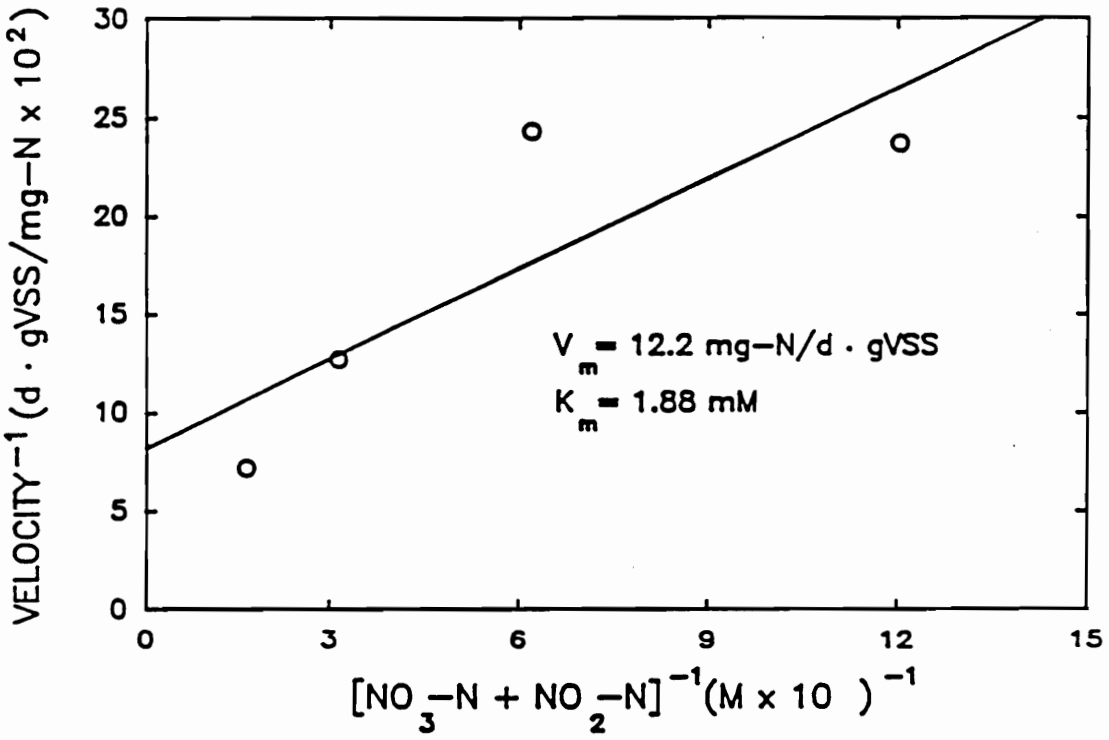

Figure 16. Lineweaver-Burk double reciprocal plot of denitrification using CO.

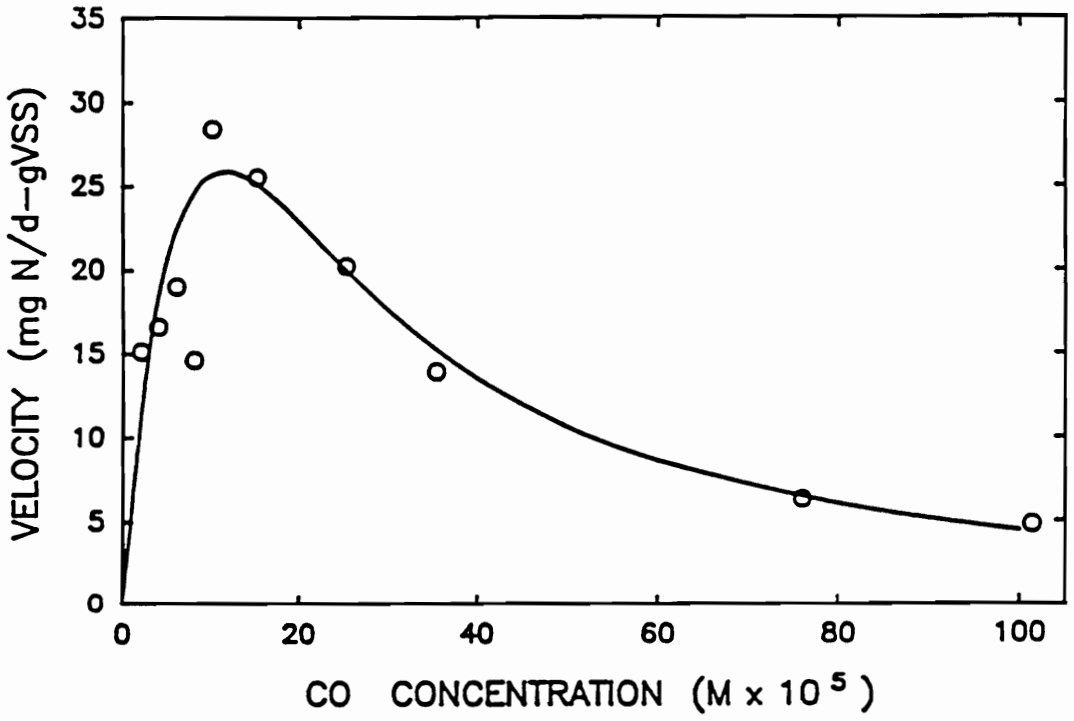

Figure 17. Effect of CO concentration on denitrification velocity.

kinetics using carbon monoxide. Before explaining the deficiencies of the Haldane model for this application, it would be helpful to review the mechanism upon which the model was based. The mechanism is shown below.

$$E + S \rightleftarrows ES \rightarrow E + P \qquad [22]$$

$$S + ES \rightleftarrows SES \quad (\textit{inactive}) \qquad [23]$$

It is proposed that an enzyme molecule (E) and a substrate molecule (S) combine to form an enzyme-substrate complex (ES). When the ES complex breaks down in the forward direction product (P) is formed. At high S concentrations, a second S molecule may combine with the ES complex to form a SES complex, which is inactive. In its most general form, the proposed Haldane mechanism may be expanded to included inactive ES complexes with three or more S molecules. The exponent n is equal to the number of substrate molecules in the inactive complex.

One problem with using the Haldane model in this situation involves the fractional exponent, n value of 2.5. According to the proposed Haldane mechanism, the exponent should always be an integer. Attempts were made to fit the model using integer exponents, but the goodness of fit was unacceptable in every case. The only acceptable fit was obtained using a fractional exponent.

A second problem with using the Haldane model in this case is also related to the proposed mechanism. The Haldane mechanism suggests that the inhibition results from two or more S molecules binding to the ES complex. There is no evidence that this is the mechanism by which carbon monoxide inhibits denitrification. All of the information available in the literature suggests that carbon monoxide inhibits biological processes by binding to metallo-enzymes and cytochromes (Mahler and Cordes, 1971; Kristjansson and Hollocher, 1980; Jain, 1982). If carbon monoxide were to bind to carbon monoxide oxidoreductase and inhibit the oxidation of carbon monoxide to carbon dioxide, then the mechanism of inhibition might be similar to that proposed by Haldane. The evidence in the literature suggests, however, that the inhibition occurs at the enzymes and

cytochromes involved with the reduction of nitrate to nitrogen gas. A proposed mechanism of inhibition is shown in Figure 18.

There is direct evidence in the literature that carbon monoxide inhibits nitrous oxide reductase (Kristjansson and Hollocher, 1980). There is also direct evidence that carbon monoxide inhibits cytochrome oxidase, the electron-transferring protein that facilitates the reduction of nitric oxide to nitrous oxide (Mahler and Cordes, 1971). Additional evidence in the literature implies that carbon monoxide may inhibit other steps in the denitrification process. In general, carbon monoxide is known as a metal complexing agent (Mahler and Cordes, 1971). It tends to bind to the metal co-factors of metallo-enzymes, especially metallo-porphyrins. Nitrate reductase contains a molybdenum co-factor and is known to be inhibited by metal chelators (Jain, 1982). It is therefore reasonable to speculate that carbon monoxide may inhibit nitrate reductase. In addition, all of the cytochromes involved in denitrification contain an iron-porphyrin group. Consequently, inhibition could occur at the cytochromes at each step of the denitrification process.

Because of the problems discussed above, the Haldane model does not accurately describe the mechanism by which carbon monoxide inhibits denitrification. When used in this case, it no longer has a mechanistic basis, but rather is strictly empirical in nature. The fit obtained using the modified Haldane model and fractional exponent does have some utility. It is useful as a predictive tool. Mathematically, it predicts well the denitrification velocities that result at various carbon monoxide concentrations. For design purposes that is sufficient. The empirical model provides sufficient information to choose the optimum carbon monoxide concentration for denitrification. The substrate concentration at the observed maximum denitrification velocity is the most important design parameter with respect to carbon monoxide. The other carbon monoxide kinetic constants are less significant since when designing a denitrification process, the objective is to optimize the removal of nitrate, not carbon monoxide. Once the carbon monoxide concentration has been optimized, the kinetic constants with respect to nitrate will determine the rate and economic feasibility of the denitrification process.

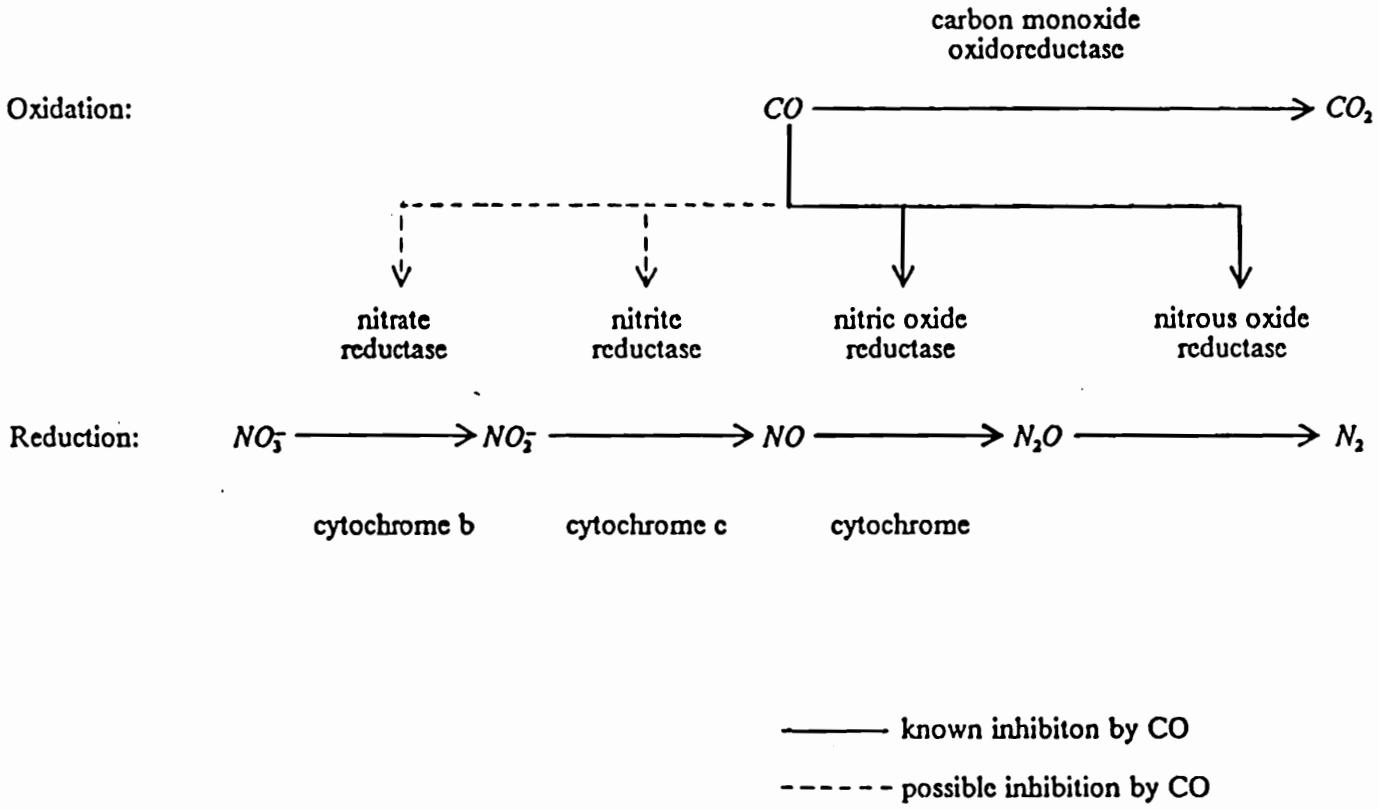

Figure 18. Flow chart of known and possible CO inhibition of denitrification.

Denitrification at Low CO Concentrations

The two lowest carbon monoxide partial pressures studied were 0.005 and 0.01 atmospheres (0.14 and 0.28 mg/ℓ aqueous). The results of these experiments are shown in Figure 19. The sum of nitrate-N and nitrite-N increased in the control microcosms (helium atmosphere) from 82.6 mg/ℓ to 97.2 mg/ℓ after 19 days. This sum of oxidized-N also increased in the 0.005 CO atmosphere systems from 82.6 mg/ℓ to 86.7 mg/ℓ. In the 0.01 CO atmosphere system, however, a small amount of net denitrification occurred. The sum of oxidized-N decreased from 83 mg/ℓ to 79.6 mg/ℓ.

The increase in oxidized-N in the control microcosms was similar to the trend seen in other control microcosms throughout this study. This increase could be a result of nitrification. To test this hypothesis, measurements were made of the ammonia-N remaining in each system. The results of these tests are shown in Table 2. The exact amount of ammonia-N initially present in each microcosm

was not known. Each system received the same initial dose (20 mg/ℓ) and all were redosed with equal quantities, but the actual amount of ammonia-N present was not measured until the end of the experiment.

The results indicated that the most ammonia-N was lost or consumed in the control microcosms, the least was lost from the 0.01 CO atmosphere systems, and an intermediate amount was lost from the 0.005 CO atmosphere systems. All of the microcosms in this experiment were at pH 7.2. At this pH, the losses of ammonia-N through volatilization would be very small. The most likely modes of ammonia-N loss were assimilation or nitrification. Assimilation would be greatest in the systems where the most net denitrification occurred and least in the controls. Since this is opposite from the observed trend, it is likely that assimilation was not the predominate mode of ammonia-N loss.

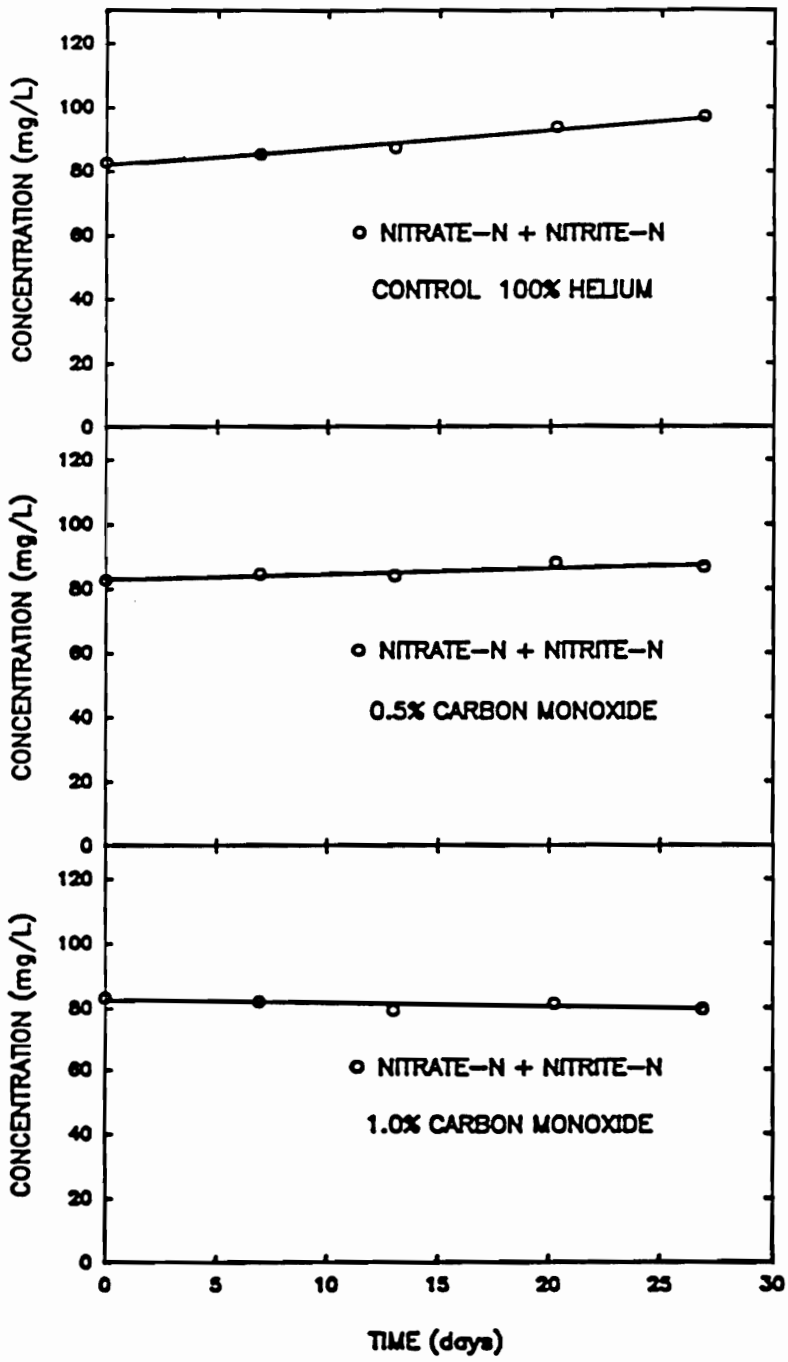

Figure 19. Denitrification at low CO concentrations, 0.005 and 0.01 atmospheres.

Table 2. Final ammonia-N concentrations in microcosms containing 0.00, 0.005, and 0.01 atmospheres of CO.

CO Dose (atm)	Ammonia-N Concentration (mg/ℓ)
0	38.4 37.0
0.005	41.2 41.2
0.010	44.3 43.2

Nitrification could have occurred in the microcosms if there was any oxygen present. It is possible that the process of purging the air from the microcosms was not completely efficient. If this was the case, however, it might be expected that an equal amount of nitrification and ammonia-N loss would occur in each of the microcosms. In this experiment a greater amount of nitrification occurred in the controls than in systems containing carbon monoxide. One explanation for this may be inhibition of nitrification by carbon monoxide. It was previously shown that carbon monoxide inhibits denitrification, and it is possible that nitrification may be inhibited in a similar manner. Consistent with this hypothesis is the fact that less nitrification occurred in the 0.01 CO atmosphere microcosms than occurred in the microcosms with the lower carbon monoxide dose (0.005 atm). The higher the carbon monoxide concentration the greater the inhibition.

The trend of increasing oxidized-N concentrations occurred in the controls throughout this study. Normally, the response of the controls is substracted from the response of the other treatments to correct for activity unrelated to the affect of the treatment. In this case, the nitrification which occurred in the controls may not have occurred in the carbon monoxide treatments. For this reason, throughout this study the responses of the controls were not used to adjust or correct the data from the carbon monoxide treatments.

Biomass Yield Measurements

The data used to determine biomass yield with respect to nitrogen are presented in Table 3. It is obvious that the VSS data at 2.2 days are much too high and are probably erroneous. It is likely that the microcosms were not mixed well prior to sampling. These data are plotted in Figure 20. The slope of this plot is the biomass yield with respect to nitrogen. The yield was found to be 1.12 mg-VSS/mg-N.

The relationship between biomass growth and carbon monoxide utilization is illustrated in Table 4 and Figure 21. The biomass yield with respect to carbon monoxide was found to be 0.13 mg-VSS/mg-CO.

The specific substrate utilization rate (q) with respect to carbon monoxide is the mass of carbon monoxide consumed during denitrification each day, per unit mass of microorganisms (mg CO/d-mg VSS). The q was calculated using the data shown in Table 4. A q was calculated for each time increment. There were five time increments, so there were five discrete values for q that ranged from 0.18 to 0.23 d^{-1}. The average was 0.203 d^{-1}.

This experiment was conducted using a carbon monoxide partial pressure of 0.06 atmospheres (1.7 mg/ℓ). As was previously shown in Figure 17 the optimum denitrification rate occurred at a carbon monoxide partial pressure of 0.10 atmospheres (2.8 mg/ℓ). As a result, the q calculated above is not the maximum specific substrate utilization rate (q_m). Figure 17 indicates that the denitrification rate at 0.06 atmospheres is approximately 87% of the maximum rate, which occurred at 0.10 atmospheres. Using this as a correction factor, the q_m may be estimated as 0.233 d^{-1}.

The q_m with respect to nitrate can be read directly from Figure 17. This quantity is equivalent to the observed maximum specific denitrification velocity 0.026 mg N/d-mg VSS. It is important to note that the V_m of 0.0547 mg N/d-mg VSS, which was estimated by the modified Haldane model, is not equivalent to the observed maximum denitrification velocity. The V_m is the maximum velocity that would be attained if no substrate inhibition occurred. Because of substrate inhibition, this theoretical maximum velocity is never attained.

It is interesting to note that, under some circumstances, one may use the Lineweaver-Burk reciprocal plot (Figure 16) to estimate the q_m with respect to nitrate. In this case the Haldane plot (Figure 17) shows q_m to be 0.026 d^{-1}, whereas calculations based on the reciprocal plot estimate q_m to be 0.012 d^{-1}. The difference in these two estimates is caused by a limiting substrate effect. To achieve maximum denitrification rates, both nitrate and the substrate (carbon monoxide) must

Table 3. VSS and N data used to determine biomass yield with respect to nitrogen.

Time (days)	VSS Concentration (mg-VSS/ℓ)	Nitrogen Concentration (mg-N/ℓ)
0	97.5 107.5	131.8 133.8
2.2	172.5 175.0	126.8 130.4
6.0	- -	109.6 111.1
13.4	137.5 150.0	94.9 97.4
16.1	155.0 157.5	86.2 85.7
19.3	162.5 162.5	78.4 79.4

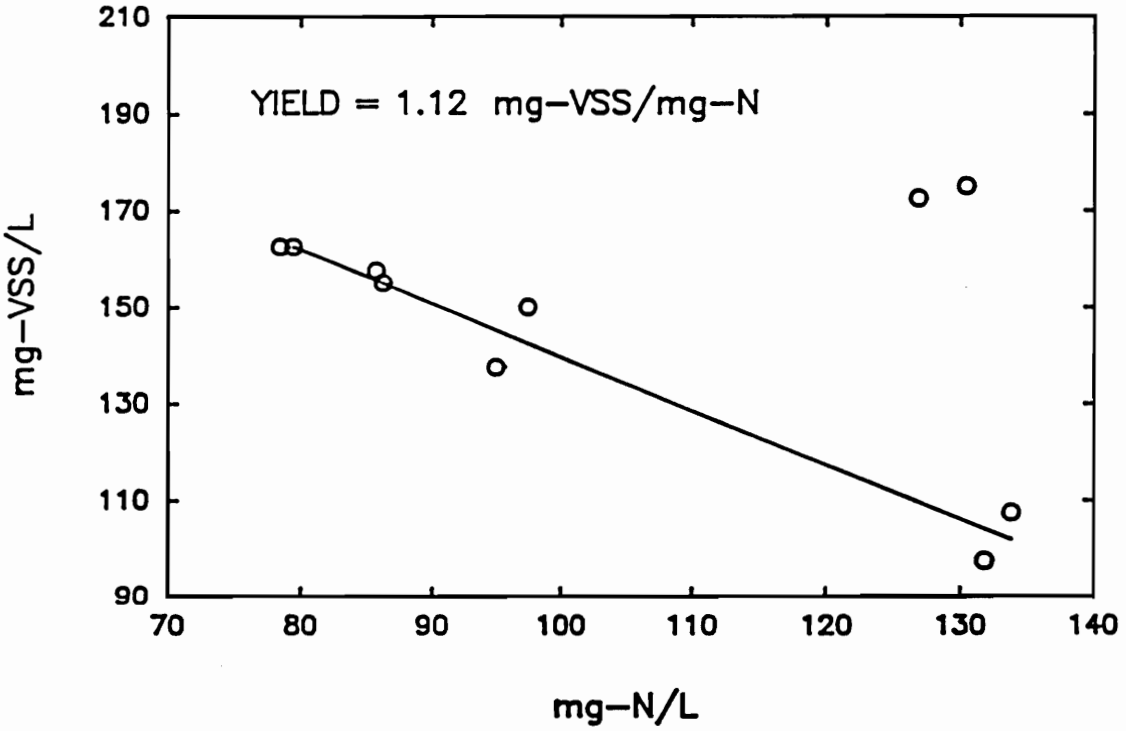

Figure 20. Biomass yield with respect to nitrogen during denitrification using CO.

Table 4. VSS and CO data used to determine biomass yield with respect to CO.

Time (days)	VSS Increase (mg)	CO Consumed (mg)
0	0	0
2.2	2.2	15.3
6.0	5.3	37.1
13.4	10.2	76.1
16.1	12.5	93.0
19.3	13.3	106.7

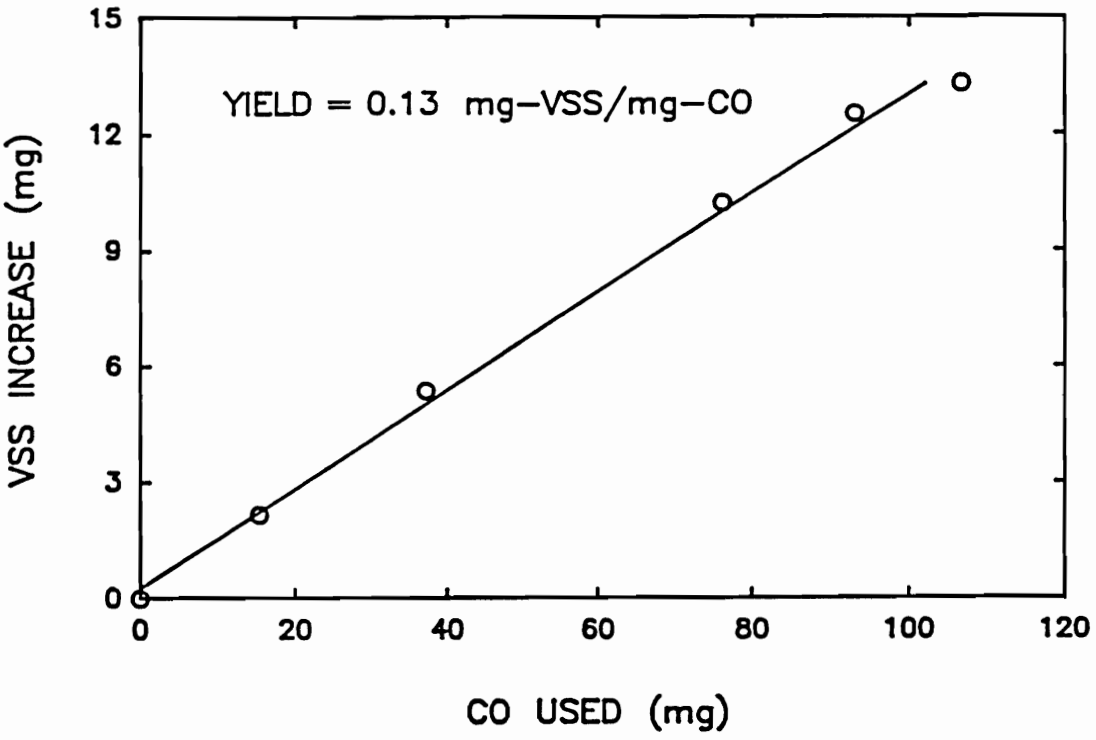

Figure 21. Biomass yield with respect to CO during denitrification using CO.

be present in excess. In the experiment which yielded the Haldane plot, nitrate was in excess and carbon monoxide was varied. At the observed optimum carbon monoxide concentration (2.8 mg/ℓ), the maximum denitrification velocity was achieved. In the experiment that yielded the reciprocal plot, carbon monoxide was constant at 0.06 atmospheres (1.7 mg/ℓ) and nitrate was varied. Carbon monoxide was limiting in this case, and as a result, the estimated q_m was lower.

The specific growth rate, μ, is the increase in biomass per day, per unit biomass (mg VSS/d-mg VSS). The μ was calculated using the data shown in Table 5. A μ was calculated for each time increment. There were five time increments, so there were five discrete values for μ. The values of μ ranged from 0.024 to 0.030 d^{-1} and averaged 0.0266 d^{-1}. As discussed above, this value for μ was determined at a substrate concentration (0.06 atm CO) which yielded less than optimal denitrification velocities. Using the same correction factor as above (87% of max.), the maximum specific growth rate, μ_m , may be estimated as 0.031 d^{-1}.

Comparison of Denitrification Kinetics

Table 6 lists kinetic coefficients for denitrification using methanol, which were obtained from the literature, and kinetic coefficients for denitrification using carbon monoxide, which were derived from this study. The maximum specific denitrification rate, q_m, using carbon monoxide is more than ten times smaller than the reported range of q_m for methanol. The half-velocity saturation constant, K_s, with respect to nitrate-N, using carbon monoxide, was very high at 26 mg/ℓ. The K_s for nitrate using methanol ranged from 0.06 to 0.16 mg/ℓ. The biomass yield coefficients were similar for both substrates. While the maximum specific growth rate, μ_m for carbon monoxide was, once again, more than ten times lower than that for methanol.

Table 5. VSS and CO data used to determine specific growth rate during denitrification with CO.

Time (days)	VSS Increase (mg)	CO Consumed (mg)
2.2	15.3	32.5
3.8	21.8	30.5
7.4	39.0	29.3
2.7	16.9	26.7
3.2	13.6	21.5

Table 6. Comparison of kinetic constants for denitrification using methanol and CO

(source: Moore and Schroeder, 1970, 1971; Kaufman, 1974; Schroeder, 1977; Gaudy and Gaudy, 1980).

Parameter	Methanol	Carbon Monoxide
k $(\frac{mg-N/d}{mg-VSS})$	0.3-0.9	0.03
$K_s^{NO_3}$ (mg/ℓ)	0.06-0.16	26
Y $(\frac{mg-VSS}{mg-N})$	0.6-1.2	1.1
μ_m $(\frac{mg-VSS/d}{mg-VSS})$	0.2-2.0	0.03

The implication of this kinetic comparison is that carbon monoxide is a much less favorable substrate for denitrification than is methanol. The maximum denitrification rate using carbon monoxide is relatively slow. This maximum denitrification velocity will be reduced by one half when the nitrate-N concentration decreases to 26 mg/ℓ. When denitrifying drinking water, it is desirable to attain an effluent nitrate-N concentration of much less than 10 mg/ℓ. At such low nitrate concentrations, the denitrification rates achieved using carbon monoxide as a substrate would be unacceptably low.

Economic Analysis

In previous sections denitrification using methanol and carbon monoxide have been compared from a biokinetic point of view. It is also important to compare these processes from an economic perspective. Capital and operation and maintenance costs should both be considered. Capital costs would include the costs of land, design, and construction. Operation and maintenance costs would include such items as chemical costs, electricity, sludge disposal, and labor.

The capital costs for alternatives using methanol or carbon monoxide would be slightly different. The costs for land and design may be similar, but the construction costs may be somewhat higher using carbon monoxide. An alternative using a gaseous substrate would require all of the equipment necessary for a liquid substrate alternative plus covered reactors, gas storage facilities, gas collection equipment, and compressors.

With respect to operation and maintenance costs, electricity and labor costs would be similar for either alternative. Sludge disposal costs would also be similar. The biomass yield with respect to nitrogen removed, is very similar for both substrates. The big difference in operation and maintenance costs lies in the cost of chemicals. According to Schroeder (1977), 2.2 to 3.2 pounds of methanol are required to remove one pound of nitrogen. The current bulk rate price for methanol

in Roanoke, Virginia is $0.55 per gallon ($0.0833/lb). Using these values, denitrification using methanol may cost $0.18 to $0.27 per pound of nitrogen removed.

Experimentally, it was determined that 4.35 moles of carbon monoxide were required to remove one mole of nitrogen (8.7 lb-CO/lb-N). The current price for carbon monoxide in Radford, Virginia is $118 per 240 cubic feet ($0.76/lb). Using these values, denitrification with carbon monoxide may cost $6.58 per pound of nitrogen removed. This is more than 24 times greater than the chemical costs associated with denitrification with methanol. Because of these high chemical costs, denitrification using carbon monoxide is not an economically attractive process for the denitrification of water.

In addition, carbon monoxide is a very toxic, odorless gas and represents a much greater safety hazard than does methanol. Consideration would have to be given to the safety of operators and maintenance personnel. Providing for safe working conditions would undoubtedly increase the capital and operation and maintenance costs of a carbon monoxide alternative.

Soil Studies

The results of denitrification studies using the clayey Blacksburg soil are shown in Figure 22and Figure 23. The data shown are the average of two replicate samples. There were ten replicates of each treatment in the clay soil studies. All replicates were sampled at zero time, but as few as two were sampled at each time thereafter. Figure 22 (A) shows the variation over time of the nitrate-N and nitrite-N concentrations in the liquid phase of the control microcosms. These controls contained no added substrate and helium in the headspace and were intended to provide a measure of heterotrophic denitrification resulting from native organic matter. The clay used in these microcosms was obtained from a depth of ten feet, and although no measurement of soil organic

matter was made, there appeared to be very little organic matter present. Nevertheless, it would not have been unusual to find a small amount of denitrification occurring in the controls.

The nitrate-N concentration increased slightly in the control microcosms. This trend of slightly increasing nitrate-N concentrations was observed in the control microcosms of several of the experiments in this study. As previously discussed, ammonia-N measurements suggested that a small amount of nitrification occurred in the control microcosms.

Figure 22 (B) shows the variation over time of the nitrate-N and nitrite-N concentrations in the liquid phase of the methanol amended microcosms. Three of the ten replicates denitrified readily using methanol, while seven of the ten remained inactive. The open circles and filled circles represent nitrate-N and nitrite-N concentrations, respectively, in the three active microcosms. The open triangles represent the nitrate-N concentration in the seven inactive systems.

If denitrification is defined as the reduction of nitrate to gaseous products, it is necessary to show that the sum of nitrate-N plus nitrite-N decreases in order to demonstrate net denitrification. Using this definition, the data presented in Figure 22(B) indicate that very little net denitrification occurred in the active microcosms during the first 30 days. While the nitrate-N concentration decreased from 22 mg/ℓ to 12 mg/ℓ during this period, the nitrite-N increased from zero to 9 mg/ℓ. The sum of the nitrate-N and nitrite-N concentrations varied from 22 mg/ℓ at zero time to 21 mg/ℓ at 30 days. After 30 days the nitrite-N concentration began to decrease sharply.

The accumulation of nitrite during denitrification in batch reactors has been observed by other investigators (Kurt *et al.*, 1987; Waki *et al.*, 1980). Some (Lam and Nicholas, 1969) have suggested that nitrate reductase and nitrite reductase are induced enzymes that are only produced in response to the accumulation of substrate (nitrate and nitrite). Others (Payne, 1976; Lam and Nicholas, 1969) have observed that denitrifying microorganisms may prefer nitrate as an electron acceptor and only use nitrite when nitrate becomes scarce.

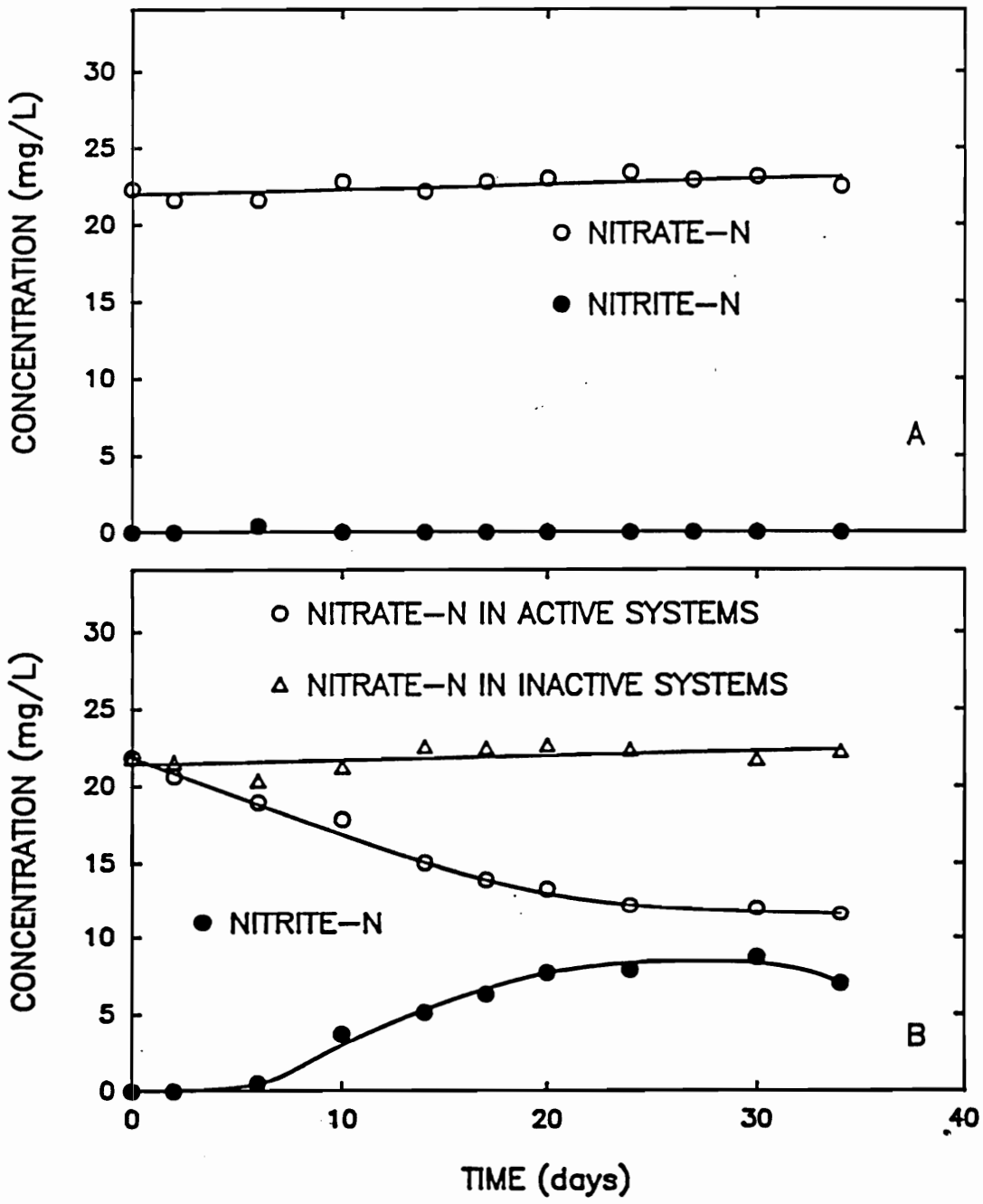

Figure 22. **Denitrification in control and methanol amended clay soil microcosms:** The variation over time of nitrate and nitrite concentrations in (A) control and (B) methanol amended microcosms.

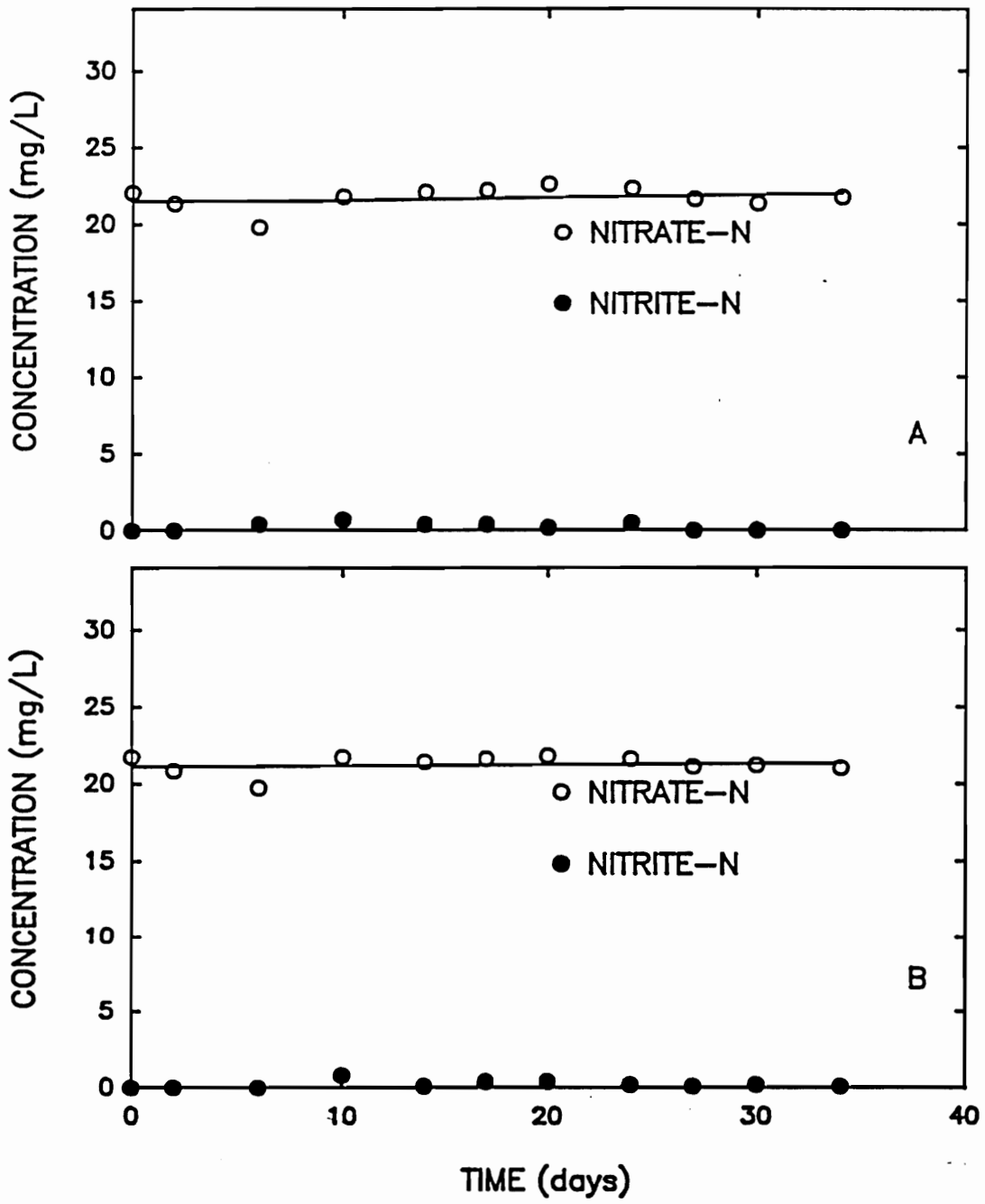

Figure 23. Denitrification in carbon monoxide and methane amended clay soil microcosms: The variation over time of nitrate and nitrite concentrations in (A) carbon monoxide and (B) methane amended microcosms.

It was considered unusual that seven of the microcosms failed to denitrify using methanol. Others (Hickman, 1988; Morris, 1988) studied soil microcosms containing a Blacksburg subsoil and found denitrification to occur readily and uniformly between replicates. The Blacksburg soil used in this study was obtained from the bottom of a freshly dug (empty) cow grave near the dairy barns at Virginia Tech. The actual cause of the inactivity in some of the replicates was never determined.

Figure 23 shows the variation over time of the nitrate-N concentration in the liquid phase of the methane and carbon monoxide amended microcosms. The data indicate that there were no microorganisms present in the Blacksburg soil capable of using methane or carbon monoxide as an energy source for denitrification. Figure 23 contains data collected during the first 34 days of this experiment. The microcosms were actually monitored for 70 days, but the last 36 days of data were misplaced. Laboratory notes indicated, however, that there was no change in either the methane or carbon monoxide systems during the 70 day study period. Except for slight accumulations of nitrite (< 0.5 mg/ℓ), there was very little difference between the behavior of the methane and carbon monoxide systems and that of the controls.

The results of the denitrification studies using the Tidewater sandy subsoil are shown in Figure 24 through Figure 28. Figure 24 shows the variation over time of the nitrate-N concentration in the liquid phase of the control microcosms. These controls contained no added substrate and helium in the headspace, and were intended to provide a measure of dentrification resulting from the presence of native organic matter. The soil was obtained from a depth of five feet and appeared to contain very little organic matter. The data shown in Figure 24 indicate that the nitrate-N concentration in the controls increased slightly during the study period of 128 days. As discussed above, it is likely that this increase represents a small amount of nitrification which may have resulted from a failure to completely purge the microcosms of oxygen.

All of the methanol amended microcosms denitrified readily. Figure 25 shows the variation over time of nitrate-N in these microcosms. The data shown are the averages of single samples taken from triplicate systems. Four initial nitrate-N concentrations were used (10, 25, 50, and 100

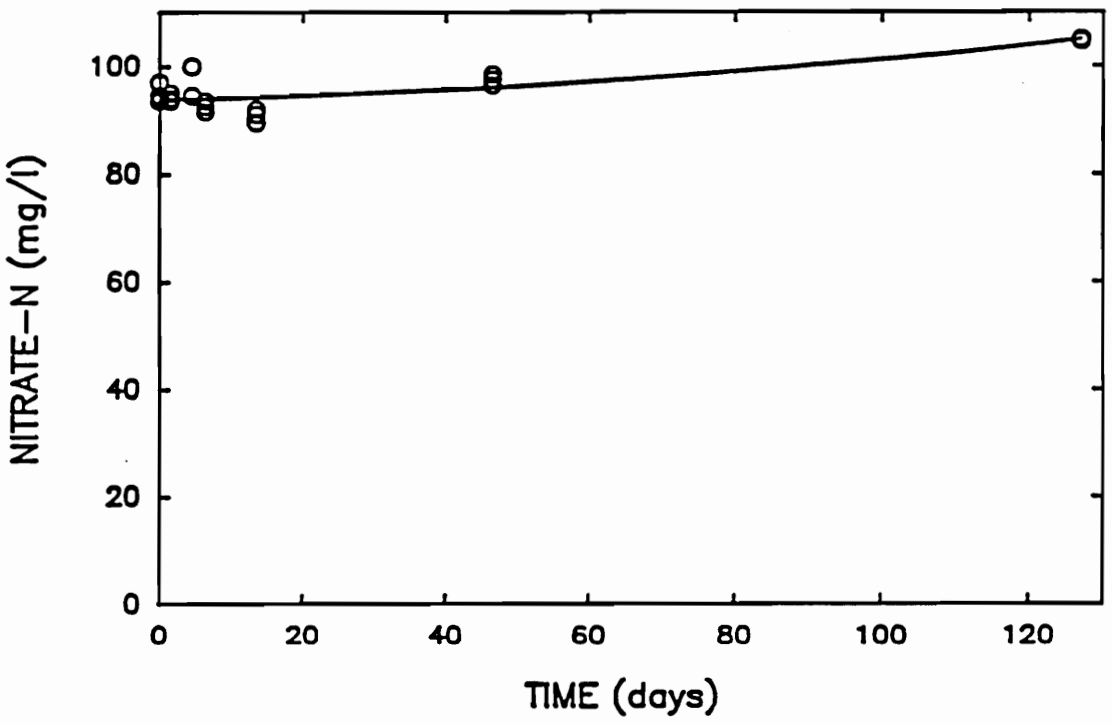

Figure 24. Denitrification in unamended, sandy soil microcosms: The variation over time of the nitrate concentration in control microcosms containing no added substrate.

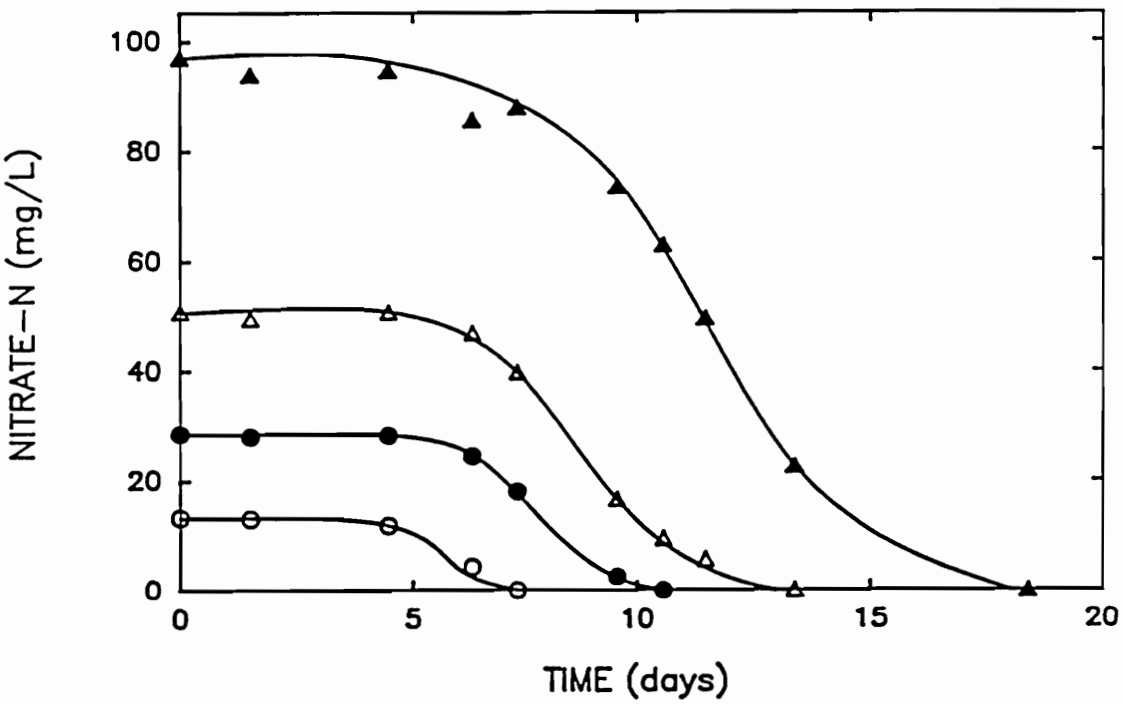

Figure 25. Nitrate reduction rates in methanol amended, sandy soil microcosms: The variation over time of nitrate concentrations in sandy soil amended with methanol.

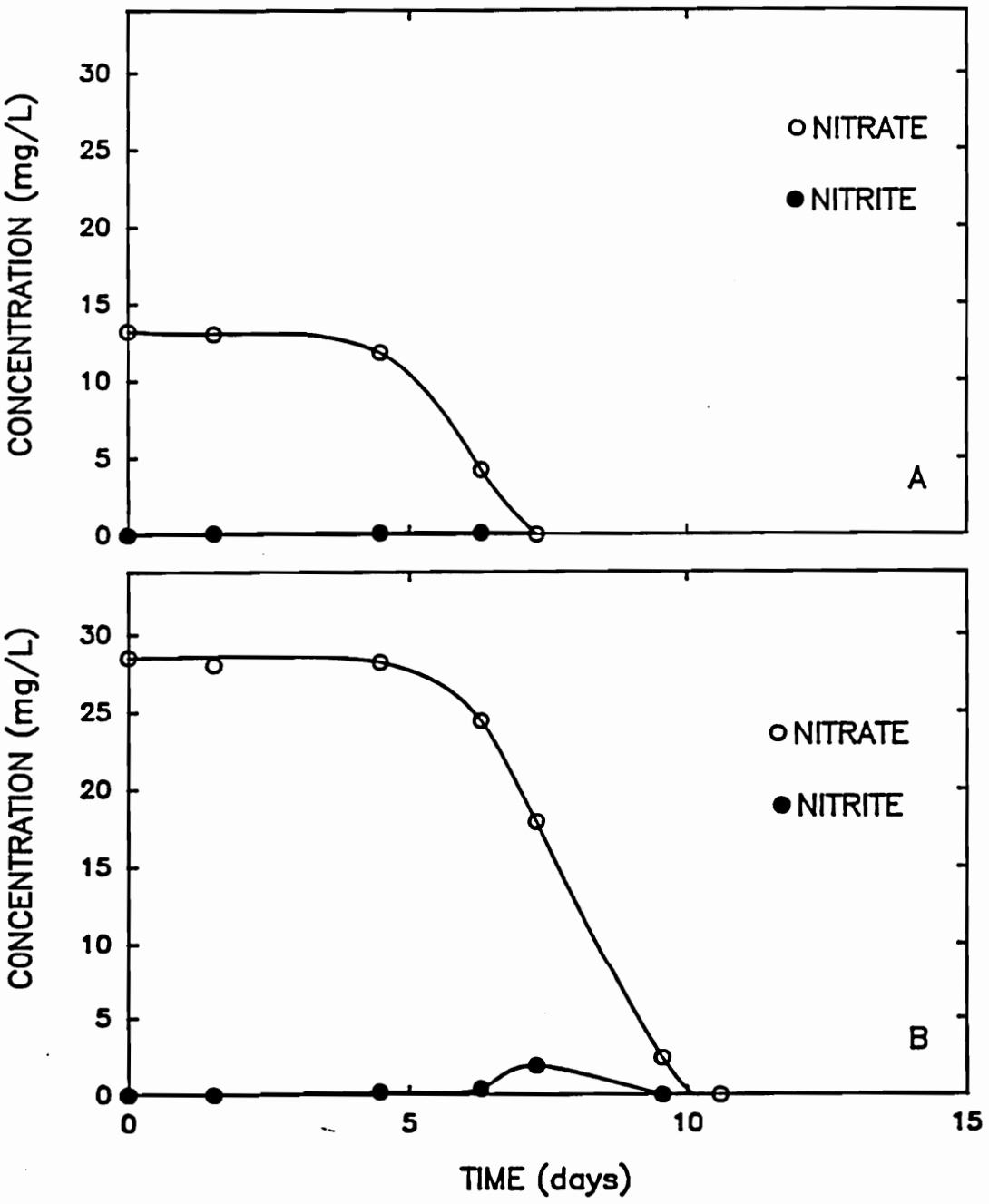

Figure 26. Denitrification in methanol amended, sandy soil microcosms, with a low initial nitrate concentration: The variation over time of nitrate and nitrite concentrations in methanol amended microcosms with low initial nitrate concentrations.

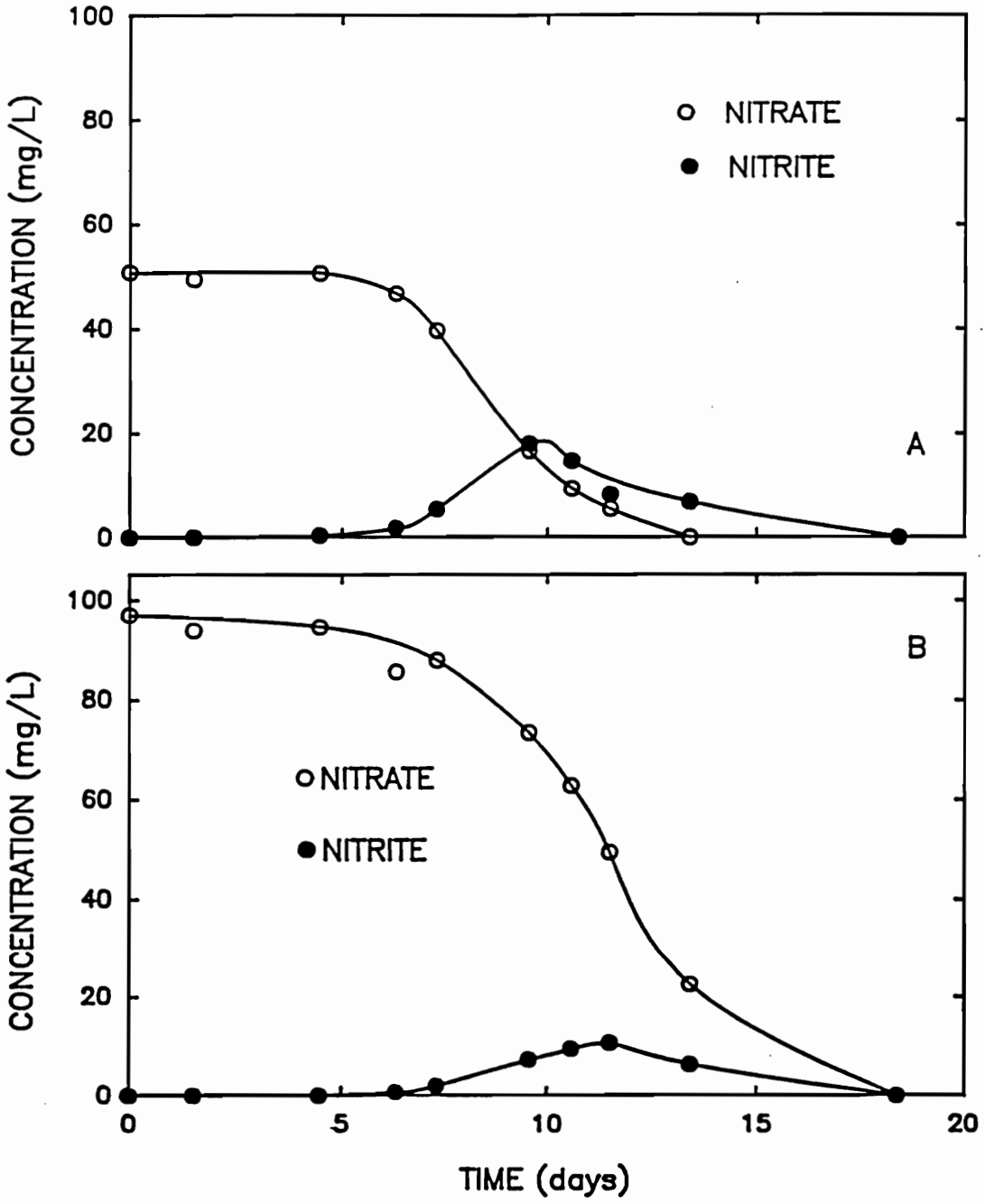

Figure 27. Denitrification in methanol amended, sandy soil microcosms with high initial nitrate concentrations.: The variation over time of nitrate and nitrite concentrations in methanol amended microcosms with high initial nitrate concentrations.

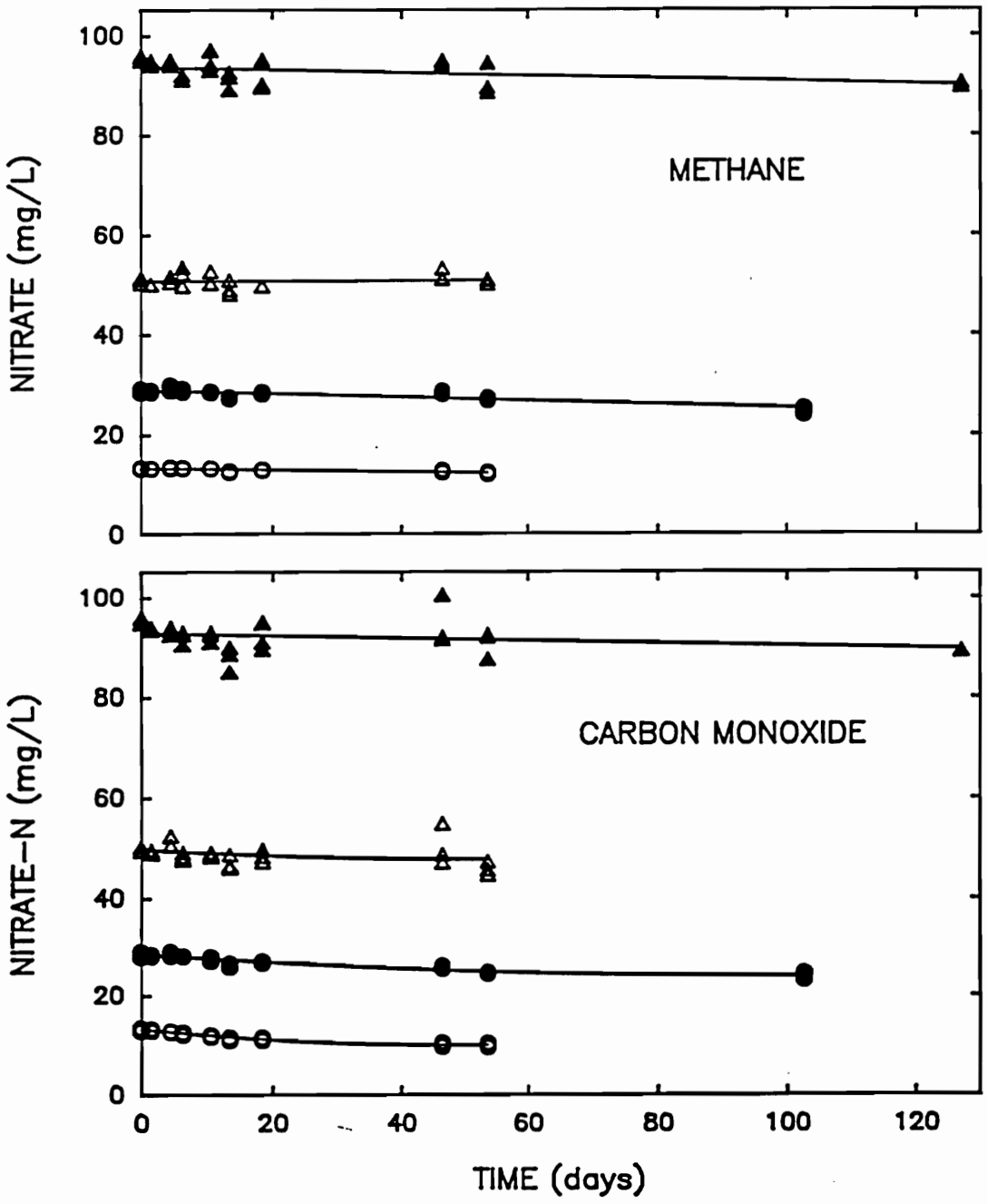

Figure 28. Denitrification in sandy soil microcosms using the gaseous substrates, methane and carbon monoxide.: The variation over time of nitrate concentrations in sandy soil microcosms amended with methane and carbon monoxide.

mg/ℓ). In each system there was a lag phase of approximately 5 days prior to the onset of denitrification. Apparently this period was required for the denitrifying population to reach a critical population size and to acclimate to the methanol substrate. After the onset of denitrification, all of the systems proceeded at a similar rate. Complete denitrification of 25 mg/ℓ nitrate-N required 10 days. This compares to more than 34 days in the methanol amended clay soil microcosms. One major reason for this was mixing. The sand microcosms were shaken during incubation, while the clay systems were incubated without shaking.

As shown in Figure 26, the nitrite-N accumulation was also much lower in the shaken sand systems (2.5 mg/ℓ) than in the unshaken clay microcosms (9 mg/ℓ). Nitrite-N accumulated up to 20 mg/ℓ in the systems with higher initial nitrate concentrations (see Figure 27).

Figure 28 shows the variation over time of nitrate-N in the methane and carbon monoxide amended microcosms. These systems were monitored for over 100 days. There was a slight decrease in the nitrate-N concentration in several of these systems. This indicated that denitrification was probably occurring, but it was difficult to determine if carbon monoxide and methane were being used as substrates, or if native organic matter was being used as an energy source.

The denitrification rates achieved under the conditions of this experiment were slow. There may be several reasons why these experiments were not sucessful. There may not have been microorganisms present that were capable of using carbon monoxide and methane as substrates for denitrification. If such microorganisms were present, the environmental conditions of the experiment may not have been favorable for the growth and activity of these organisms. Possibly, the organisms were present in low numbers and insufficient time was allowed for acclimation and growth. Others (Hickman, 1988; Morris, 1988) studying the degradation of organic compounds in soil microcosms have found that greater than 100 days may be required for acclimation and growth prior to the onset of microbial activity.

Denitrification Effects on pH in a Closed System

Alkalinity and carbon dioxide are products of denitrification. Others (Kurt *et al.*, 1987) have observed that in an open system, alkalinity production results in an increase in pH. The optimum pH for denitrification is approximately 7.5. As the pH rises, the velocity of denitrification decreases and nitrite accumulation increases. In a closed system, carbon dioxide would also accumulate, and tend to decrease the pH of the water.

The initial pH of the sandy soil microcosms was 7.2. After these experiments were completed, the pH of each microcosm was measured. Two measurements of pH were made, one immediately after opening the microcosm and again several hours later, after the dissolved carbon dioxide had equillibrated with the atmosphere. The results are shown in Table 7 on page 95. In each case, the final pH in the closed system was lower than the equillibrium pH. This was probably the result of dissolved carbon dioxide. In most cases, the equilibrium pH was higher than the initial pH (7.2) at the start of the experiment. This rise in pH was probably due to the production of alkalinity. The rise in pH was greater in the methanol amended microcosms, where more net denitrification occurred. It was clear from these results that the pH lowering effect of carbon dioxide was stronger than the pH raising effect of alkalinity production.

These finding may be important with respect to in situ denitrification. An aquifer may be considered as a closed system. In situ denitrification would produce carbon dioxide. If initial nitrate concentrations are high, dissolved carbon dioxide may significantly lower the ambient pH of the aquifer. At low pH's the velocity of denitrification may be reduced.

Table 7. Final and equilibrium pH of sandy soil microcosms.

Nitrate-N	Final pH/Equilibrium pH			
mg/ℓ	Helium	CO	Methane	Methanol
100	5.6/7.1	6.0/7.6	6.0/7.6	6.6/8.4
50		6.0/7.8	6.0/7.6	6.1/8.2
25		6.0/7.6	6.0/7.8	5.8/8.2
10		6.0/7.7	5.9/7.6	6.3/7.1

Unacclimated Suspended Growth Studies

After failing to achieve denitrification using methane or carbon monoxide in subsurface soil microcosms, a study was undertaken to screen anaerobic digester sludge and activated sludge for microorganisms capable of denitrification using these substrates. The variation over time of the nitrate-N and nitrite-N concentrations in the anaerobic digester sludge experiments are shown in Figure 29 through Figure 32. The anaerobic digester seed that was added to each microcosm (20 ml in 350 ml) contained a substantial amount of organic matter. As a result it was expected that some denitrification would occur in the controls. Figure 29 on page 97 shows the data from the control systems. The nitrate-N concentration decreased from 100 mg/ℓ to 20 mg/ℓ during the 40 day study period. Nitrate reduction was more rapid during the first ten days and then slowed later, presumably as the organic matter present in the seed was depleted. Nitrite-N accumulated to a high of 10 mg/ℓ at five days and decreased thereafter.

Data from the methanol amended systems is shown in Figure 30. Nitrate-N concentrations decreased from 95 mg/ℓ to zero in 28 days. The denitrification rate was very rapid during the first five days before leveling off. A comparison of the control system and the methanol amended treatment shows that the initial rate was much faster in the methanol microcosms, but the slopes of the final portions of each curve were similar. Methanol seems to be a more readily available substrate for denitrification than the more refractory organic matter present in the anaerobic digester seed. After the 100 mg/ℓ methanol was depleted, both systems were deriving energy from difficult to degrade organic matter.

The methane amended treatment denitrified readily, but the slope of the nitrate and nitrite curves are almost identical to the control (see Figure 31). There was no evidence that methane contributed to or had any effect on the denitrification rate in these systems.

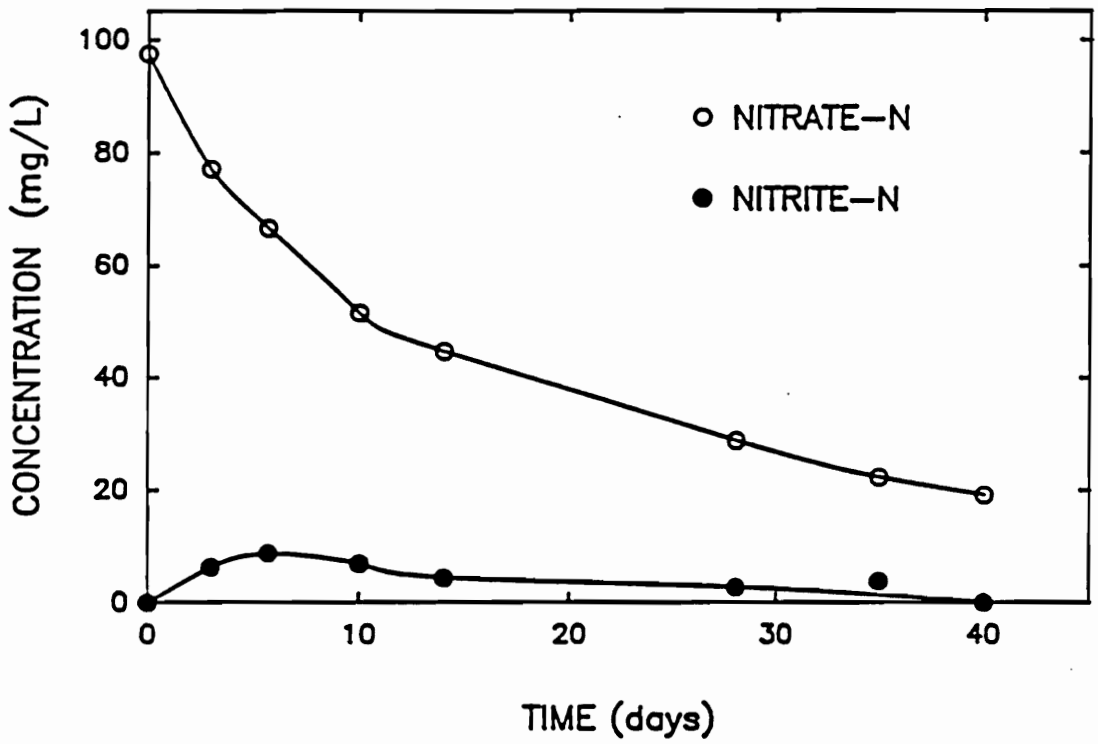

Figure 29. **Denitrification in unamended, anaerobic digester sludge:** The variation over time of nitrate and nitrite concentrations in suspended growth microcosms seeded with anaerobic digester sludge.

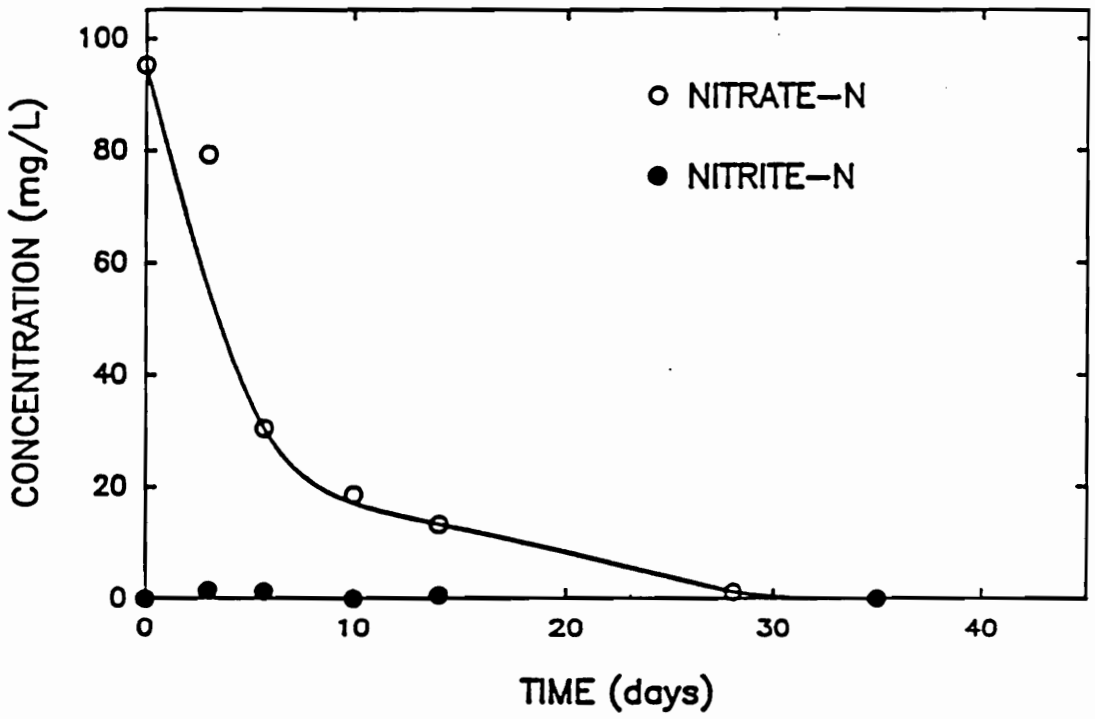

Figure 30. Denitrification in methanol amended, anaerobic digester sludge: The variation over time of nitrate and nitrite concentrations in suspended growth microcosms amended with methanol and seeded with anaerobic digester sludge.

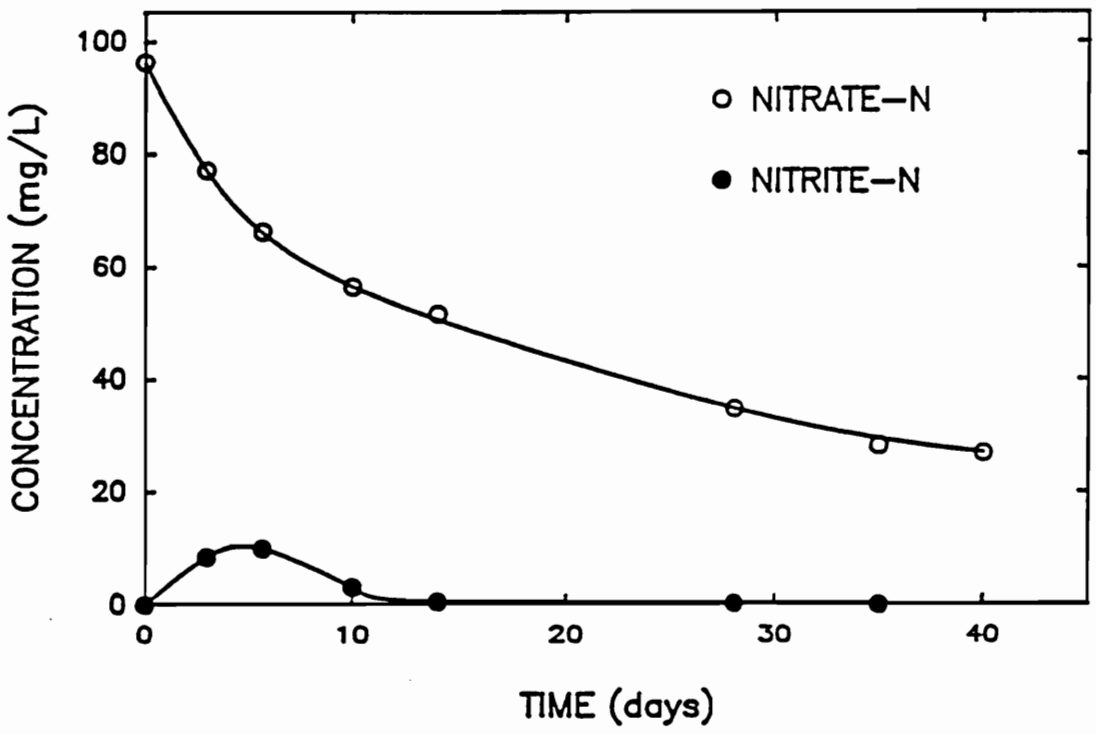

Figure 31. Denitrification in methane amended, anaerobic digester sludge: The variation over time of nitrate and nitrite concentrations in suspended growth microcosms amended with methane and seeded with anaerobic digester seed.

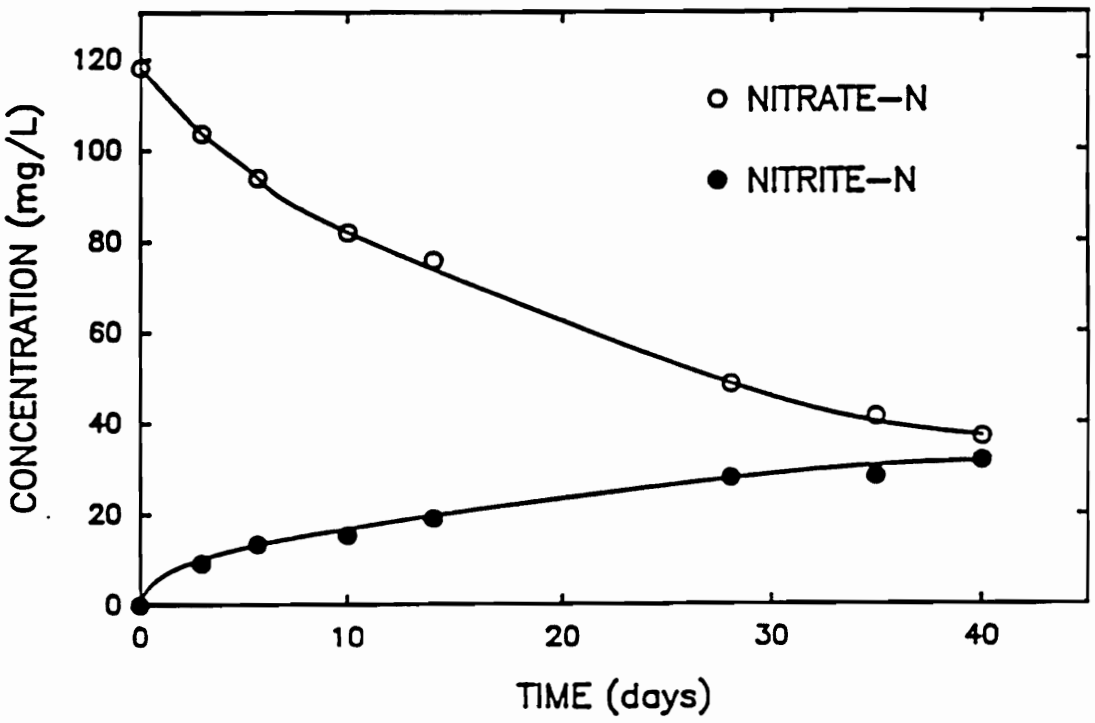

Figure 32. Denitrification in CO amended, anaerobic digester sludge: The variation over time of nitrate and nitrite concentrations in suspended growth microcosms amended with carbon monoxide and seeded with anaerobic digester sludge.

The carbon monoxide amended microcosms provided more interesting results which are illustrated in Figure 32. The slope of the nitrate curve is more shallow in the carbon monoxide treatment than in the control. More noticeable is the large accumulation of nitrite in the carbon monoxide system. The nitrite-N concentration increased steadily throughout the 40 day study period to a high of 30 mg/ℓ. The only difference between the control and the carbon monoxide treatment was the headspace gas. The controls contained helium. It was clear that the observed effect had to be caused by carbon monoxide. Carbon monoxide slowed the nitrate reduction rate, but it seemed to have a greater inhibitory effect on nitrite reduction.

The results of the studies using an activated sludge seed are shown in Figure 33 through Figure 36. After observing the large amount of denitrification that occurred in the anaerobic digester control, a smaller quantity of seed (10 ml in 350 ml) was used to establish the activated sludge microcosms. The variation over time of the nitrate-N and nitrite-N concentrations in the control microcosms is shown in Figure 33. The extent of denitrification in the activated sludge control was much less than in the previous experiment. The nitrate-N concentration decreased from 102 mg/ℓ initially, to 95 mg/ℓ at five days, and then rose slowly to 99 mg/ℓ at 47 days. The initial decrease was probably due to denitrification resulting from the organic matter present in the activated sludge seed. The subsequent rise follows the same trend observed in the previously discussed experiments and was thought to be due to nitrification.

The methanol amended activated sludge systems denitrified readily (see Figure 34). The nitrate-N concentration decreased from 102 mg/ℓ initially to 47 mg/ℓ in 10 days and remained constant at this level until the end of the study period at 47 days. The nitrite-N concentration rose to 13 mg/ℓ in days and decreased slowly to 9 mg/ℓ at 47 days. It was apparent that the 100 mg/ℓ of methanol had been depleted at 10 days. After this time no substrate remained to support nitrate or nitrite reduction.

The data collected from the methane amended microcosms are shown in Figure 35. The nitrate-N concentration decreased from 101 mg/ℓ initially to 92 mg/ℓ after 35 days and remained constant

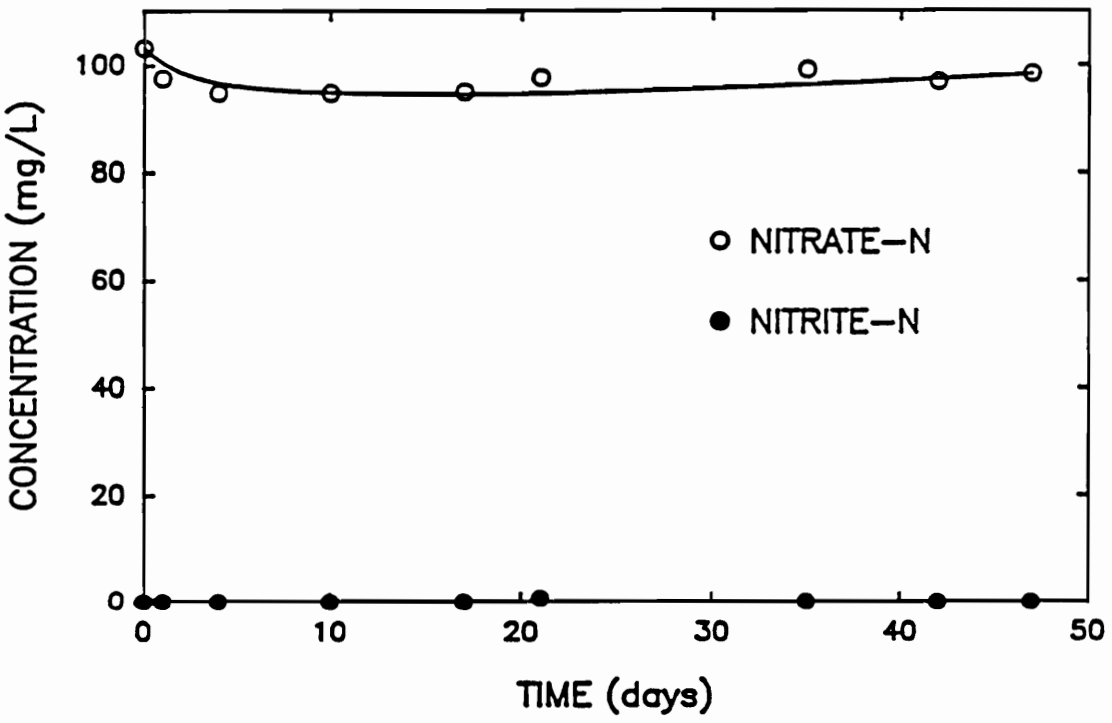

Figure 33. Denitrification in control microcosms seeded with activated sludge.

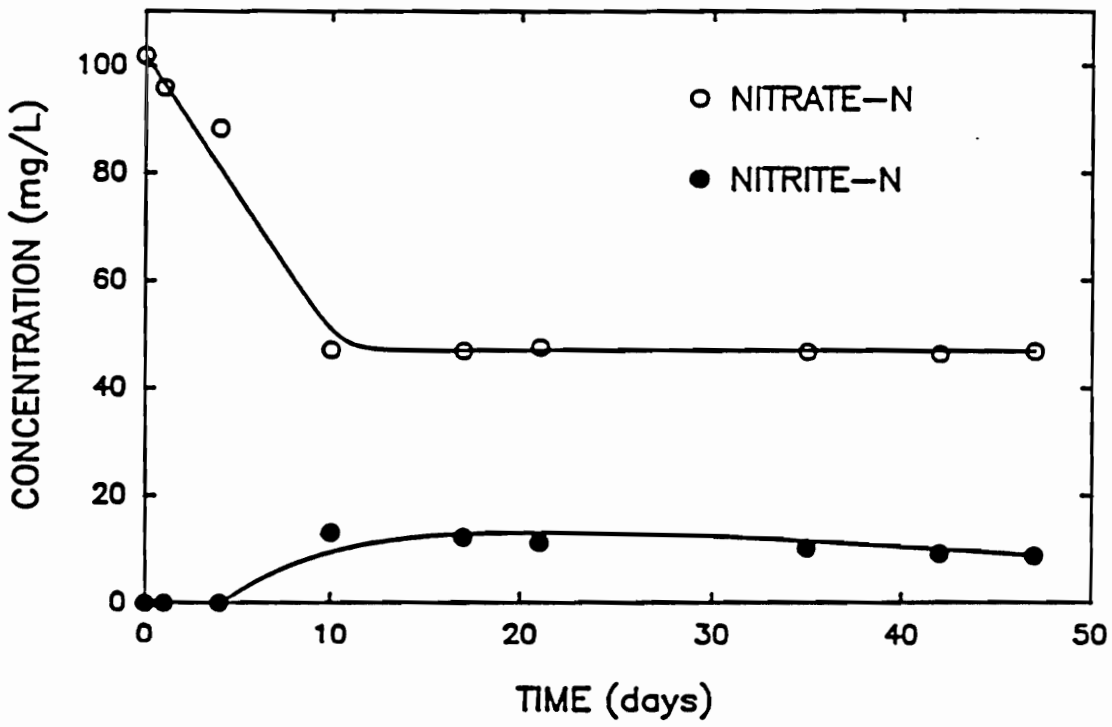

Figure 34. Denitrification in methanol-amended microcosms seeded with activated sludge.

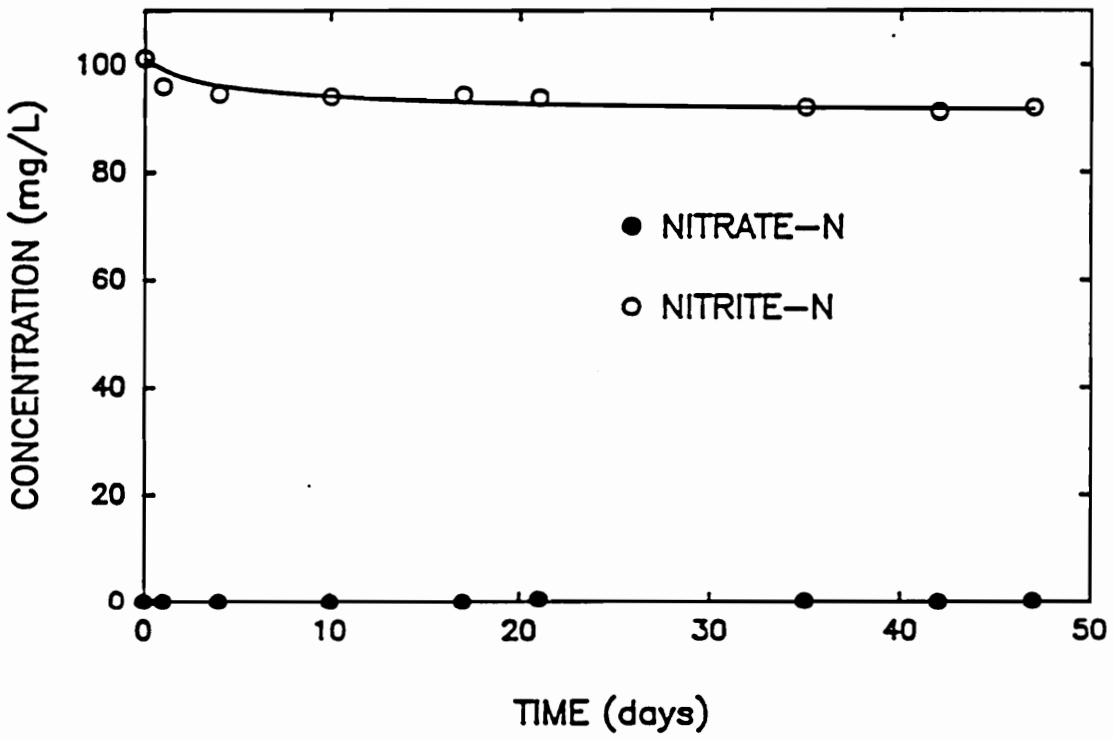

Figure 35. Denitrification in methane-amended microcosms seeded with activated sludge.

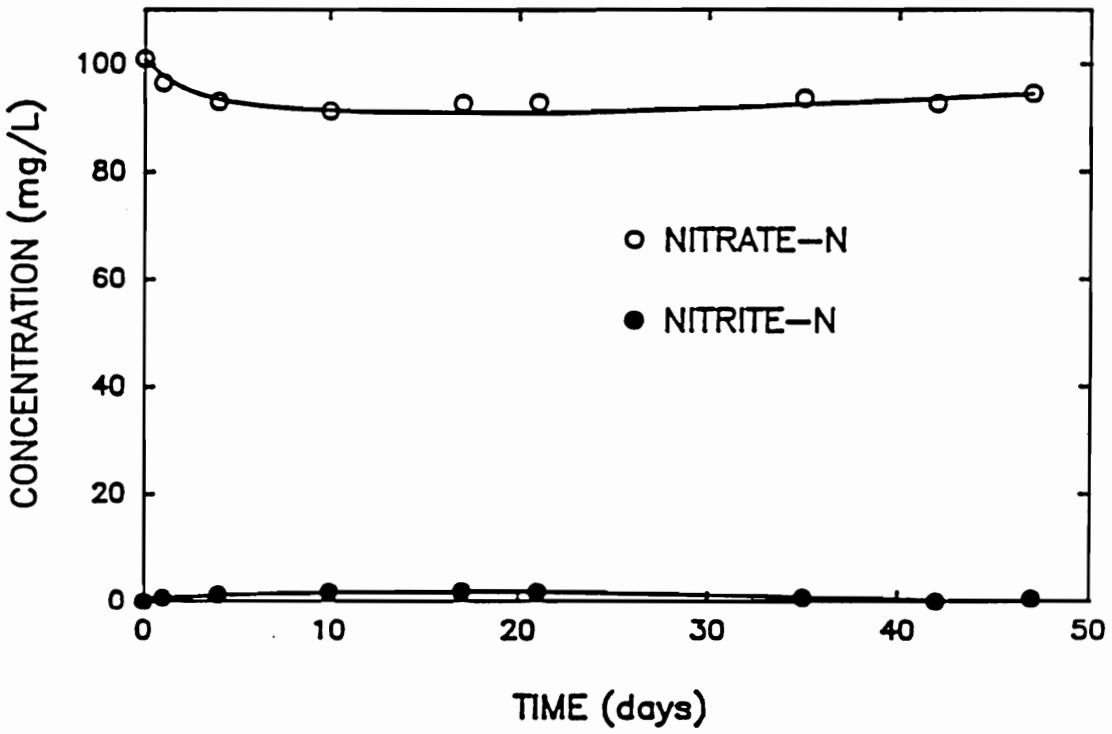

Figure 36. Denitrification in CO-amended microcosms seeded with activated sludge.

at that level until the end of the study period at 47 days. The behavior of the methane system was similar to the control except that there was no increase in nitrate near the end of the study. It was difficult to determine if this phenomena was caused by methane stimulating denitrification or inhibiting nitrification. The difference between the methane treatment and the control was too subtle to suggest that methane was being used as a denitrification substrate.

Figure 36 illustrates the variation over time of the nitrate-N and nitrite-N concentrations in the carbon monoxide microcosms. The behavior of the nitrate curve was similar to the methane treatment and the control. Nitrite, however, accumulated in the carbon monoxide systems up to 2 mg/ℓ after ten days, before decreasing slowly to zero at 42 days. This nitrite trend was much more subtle than that observed in the anaerobic digester sludge experiments, but was consistent with lower net denitrification that occurred due to organic matter present in the seed.

Carbon Monoxide Inhibition Experiments

The results of the carbon monoxide inhibition experiments are shown in Figure 37 and Figure 38. Figure 37 compares the denitrification rates in methanol amended microcosms containing helium in the headspace and carbon monoxide in the headspace. These microcosms were seeded with an acclimated culture of methanol using denitrifiers and were shaken during incubation. The systems containing helium denitrified very rapidly. The nitrate-N concentration decreased from 96 mg/ℓ to zero in less than one day with no measurable nitrite accumulation. Similar microcosms with carbon monoxide in the headspace required 20 days for complete denitrification and nitrite-N accumulations were significant. The nitrite-N concentration peaked at 40 mg/ℓ after 10 days. These data suggested carbon monoxide was inhibiting denitrification. Both nitrate and nitrite reduction rates were affected and high nitrite accumulations resulted.

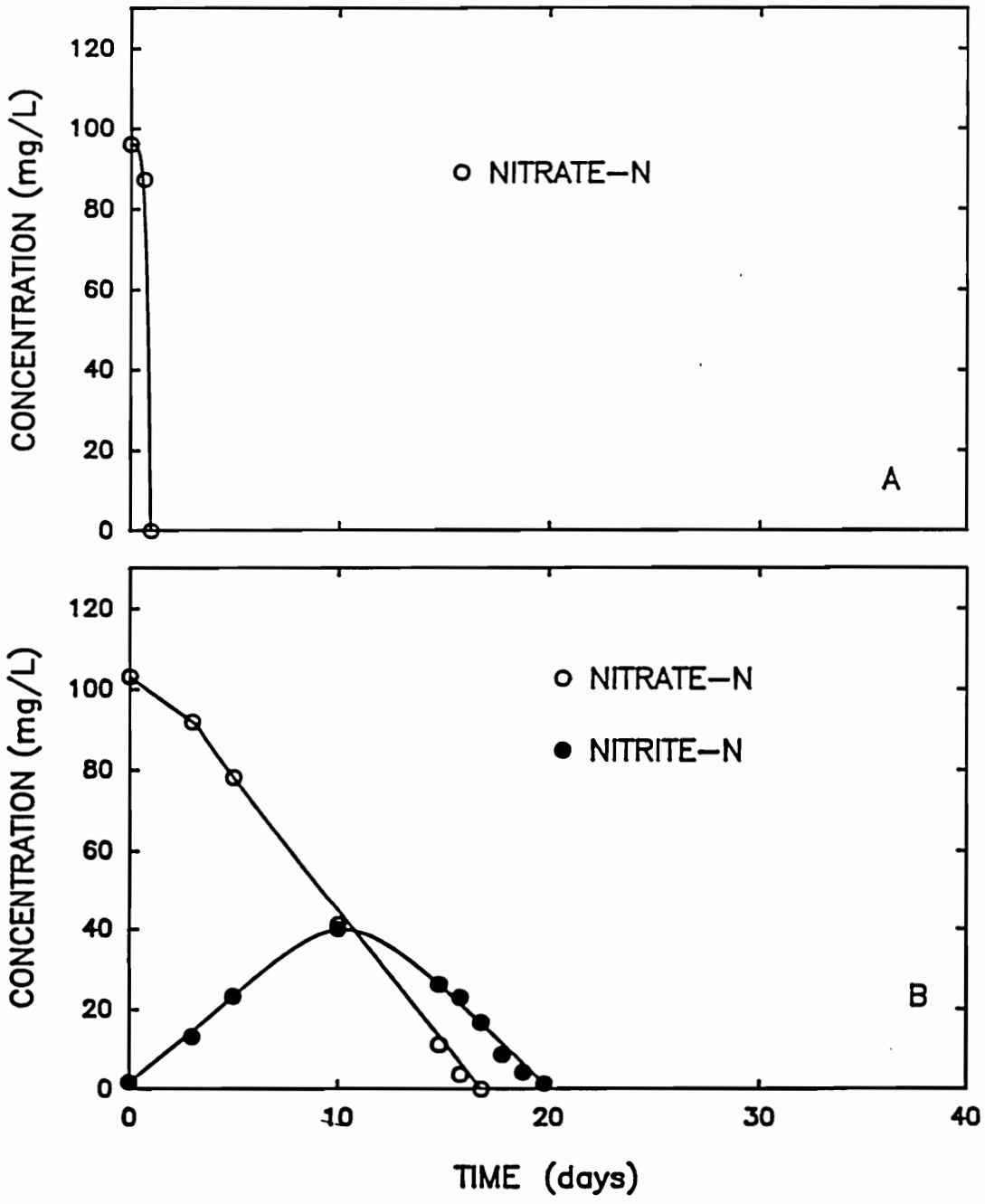

Figure 37. **Inhibition experiments using methanol-amended microcosms, with (A) He and (B) CO atmospheres.:** Denitrification in shaken, methanol-amended microcosms, using a seed acclimated to methanol, and with (A) He and (B) CO in the headspace.

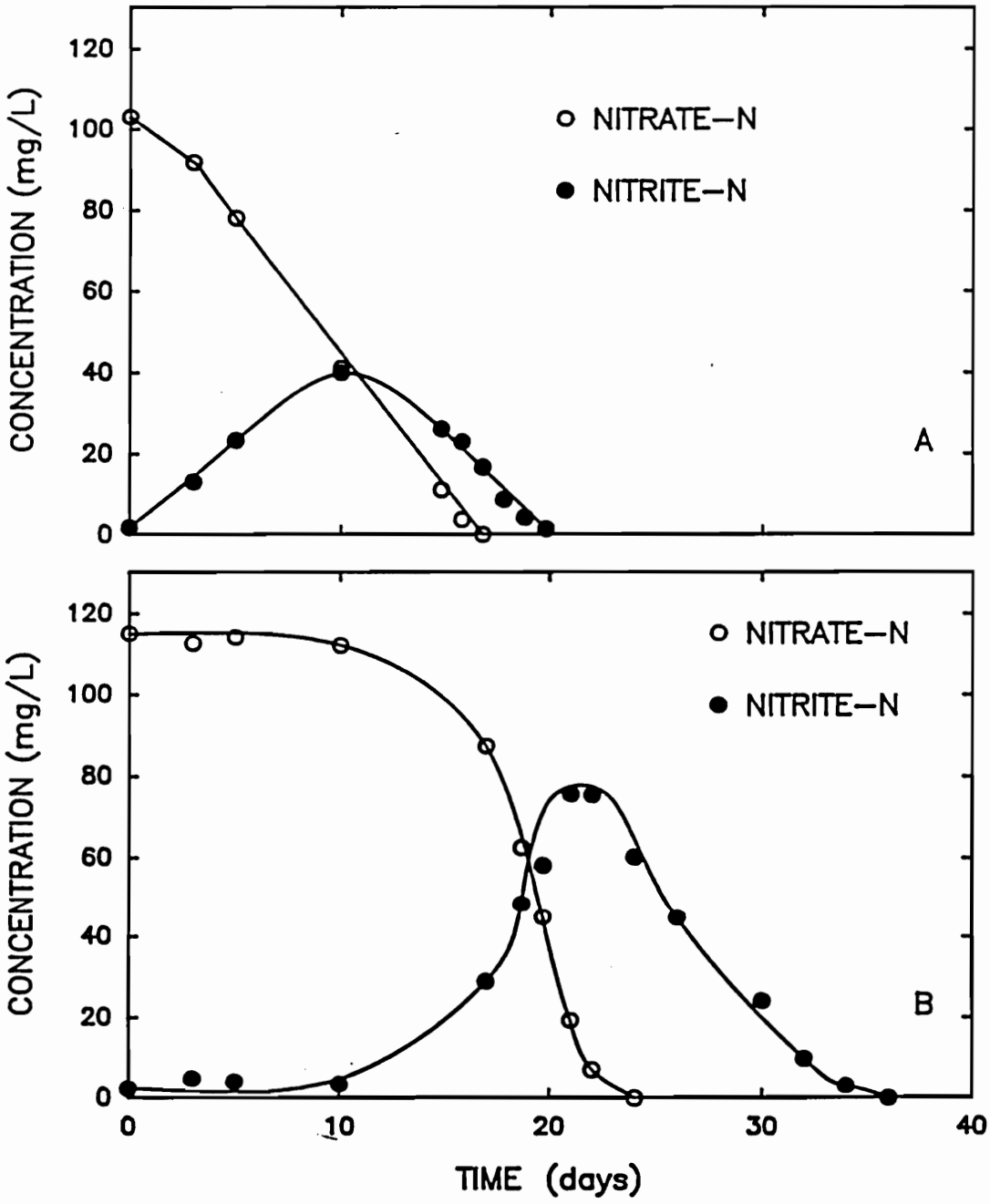

Figure 38. **Inhibition experiments using (A) methanol and (B) CO acclimated seeds and a CO atmosphere.:** Denitrification in shaken microcosms, containing (A) methanol and (B) CO acclimated seeds, and a CO atmosphere.

Figure 38 compares denitrification rates in methanol amended microcosms containing seed organisms acclimated to methanol and carbon monoxide. Both treatments contained carbon monoxide in the headspace and were shaken during incubation. The treatment containing the methanol acclimated seed began denitrifying immediately, while the treatment containing the seed acclimated to carbon monoxide experienced a lag period of approximately ten days prior to the onset of denitrification. Following the lag, however, nitrate reduction was most rapid in the carbon monoxide acclimated treatment. Nitrite accumulations were also much greater.

The lag period experienced by carbon monoxide acclimated treatment was typical of the behavior of biological systems adjusting to a new substrate. Although the acclimation period of ten days was unusually long considering how quickly other suspended growth microcosms initiated denitrification using methanol (see Figure 30 and Figure 34). The data suggest that during the previous 20 day acclimation of the seed to carbon monoxide, the number of methanol using denitrifiers was greatly reduced, and that the remaining organisms were inhibited during their acclimation to methanol by the presence of carbon monoxide.

After the acclimation period, the rate of nitrate reduction in the carbon monoxide seed was much greater than that achieved by the methanol seed. Nitrite accumulations were much higher (up to 75 mg/ℓ) in the microcosms containing the methanol seed. It was possible that both of these phenomena were caused by the combined affects of two different groups of microorganisms, methanol-using denitrifiers and carbon monoxide-using denitrifiers. This would result in a faster nitrate reduction rate than methanol-using denitrifiers alone. The increased nitrate reduction rate coupled with the inhibition of nitrite reduction caused by carbon monoxide would then lead to higher nitrite accumulations.

Summary and Conclusions

This study involved the use of soil and suspended growth microcosms to study the variation in groundwater denitrification rates using different substrates. Two gaseous substrates, carbon monoxide and methane, were studied and compared to a common liquid substrate, methanol. Denitrification using methanol occurred readily in both sand and clay soil microcosms, and in suspended growth microcosms seeded with activated sludge and anaerobic digester sludge. Denitrification did not occur in soil microcosms that were provided one atmosphere of carbon monoxide or methane as a substrate.

Denitrification using methanol was inhibited by the presence of carbon monoxide. The denitrification rate was 20 times slower in shaken, methanol amended, suspended growth microcosms containing one atmosphere of carbon monoxide, than in similar systems containing helium. It was not unusual for small accumulations of nitrite to occur in the early stages of denitrification. However, nitrite accumulations were much higher in systems stressed by carbon monoxide.

Denitrification rates were affected by mixing. Denitrification rates were seven times faster in shaken, suspended growth microcosms, than in identical, unshaken systems. Diffusion of nitrate through the bulk solution to the zone of microbial activity was thought to be rate limiting.

Denitrification with carbon monoxide as a substrate was achieved using an acclimated seed of mixed activated sludge and anaerobic digester sludge. The first evidence of carbon monoxide use occurred using a one atmosphere partial pressure of carbon monoxide. A similar acclimated seed provided with one atmosphere of methane did not denitrify.

Kinetic studies of denitrification using carbon monoxide suggested a strong substrate inhibition effect. Carbon monoxide was used as a substrate for denitrification, but at high concentrations it was inhibitory to denitrification. The observed maximum denitrification velocity of 0.026 mg N/d-mg VSS occurred at a carbon monoxide concentration of 0.10 atmospheres (2.8 mg/ℓ). The denitrification velocity at various carbon monoxide concentrations was described by a kinetic model that was mathematically similar to the Haldane substrate inhibition model. This model was useful as an empirical predictive tool, but the mechanism of carbon monoxide inhibiton was not thought to be similar to the mechanism upon which the Haldane model was based. A possible mechanism for carbon monoxide inhibition of denitrification was proposed. This mechanism was derived from information in the literature regarding carbon monoxide inhibition of other enzyme systems. There was evidence that carbon monoxide inhibited several of the enzymes and all of the cytochromes which facilitate the reduction of nitrate to nitrogen gas.

To better study carbon monoxide inhibition of denitrification, a different experimental design would be required. Future research should focus on carbon monoxide inhibition of specific enzymes in the denitrification process. It is likely that carbon monoxide acts as a competitive or uncompetitive inhibitor at the enzyme level. To measure this, it would be necessary to use a substrate that is independent from the inhibitor. A compound such as methanol should be used as a substrate and carbon monoxide should be added as an inhibitor. By measuring the effect of various combinations of methanol and carbon monoxide concentrations on denitrification velocity, one would be able to model the inhibition by applying conventional enzyme inhibition models. If the inhibition of each enzyme in the denitrification process was modelled in the manner, it would be possible to combine this information into a unified model of carbon monoxide inhibition of the denitrification process.

Both kinetic and economic comparisons indicated that methanol was much more favorable than carbon monoxide as a substrate for denitrification. The maximum specific denitrification rate and specific growth rate were ten times slower using carbon monoxide. In addition, the chemical costs of denitrification using carbon monoxide are 24 times greater than those using methanol.

It was surprising that methane was not used as a substrate for denitrification. There is much more energy available in methane than in carbon monoxide. Any additional research involving methane should consider two modifications in experimental design. Initial studies with carbon monoxide and methane used one atmosphere of each substrate. Later, it was determined that carbon monoxide was inhibitory and lower concentrations resulted in faster denitrification rates. Possibly lower concentrations of methane would be more appropriate. Secondly, fixed film systems should be evaluated. It is possible that the use of methane in denitrification requires a consorsium of organisms working together in close association with each other. A fixed film system would provide a better environment for the developement of such a population.

The conclusions of this study are:

1. Denitrification of water was achieved using an activated sludge seed and carbon monoxide as a substrate, but carbon monoxide partial pressures greater than 0.1 atmospheres resulted in a substrate inhibition effect.

2. Denitrification rates using carbon monoxide were much slower than those achieved with methanol and the cost of using carbon monoxide was much greater.

3. The maximum specific denitrification rate using carbon monoxide of 0.026 mg N/d-mg VSS occurred at a carbon monoxide concentration of 0.10 atmospheres (2.8 mg/ℓ).

4. The biomass yield using carbon monoxide was 1.1 mg VSS/mg VSS, the maximum specific growth rate was 0.03 mg VSS/d-mgVSS, and the half velocity saturation constant was 26 mg-N/ℓ.

5. Denitrification occurred readily, when methanol was provided as a substrate, in microcosms containing either a clayey Blacksburg subsoil, a sandy Tidewater subsoil, or activated sludge. Under the conditions of this study, denitrification was not achieved in clayey or sandy soil microcosms using methane or carbon monoxide as substrates. Denitrification was not achieved using methane as a substrate with an activated sludge seed.

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Appendix A. Thermodynamics

Methane

$$5CH_4 + 8NO_3^- + 8H^+ \rightarrow 5CO_2 + 4N_2 + 14H_2O$$

$$\Delta G^\circ = [5(-94.26)] + [14(-56.69)] - [5(-8.22)] - [8(-26.61)] = -1010.98\ kcal/mole$$

$$\Delta G = \Delta G^\circ + RT ln Q$$

For $20^\circ C$; $RT = (1.987x10^{-3}\ kcal/mole - {}^\circ K)(293^\circ K) = 0.582\ kcal/mole$

$$\Delta G = -1010.98 + 0.582(5ln[CO_2] + 4ln[N_2] - 5ln[CH_4] - 8ln[NO_3^-] - 8ln[H^+])$$

Assume unit activities for $[CO_2]$ *and* $[N_2]$, *pH 7, 100 mg/ℓ* NO_3^- *–N (7.14 x* 10^{-3} *M), one atmosphere of methane which yields 23.38 mg/ℓ (1.46 x* 10^{-3} *M)*

$$\Delta G = -1010.98 + 0.582[-5ln(1.46x10^{-3}) - 8ln(7.14x10^{-3}) - 8ln(10^{-7})]$$

$$\Delta G = -893.9\ kcal/mole$$

Carbon Monoxide

$$5CO + 2NO_3^- + H_2O \rightarrow N_2 + 2OH^- + 5CO_2$$

$$\Delta G^\circ = [2(-37.59)] + [5(-94.26)] - [5(-32.81)] - [2(-26.61)] - [-56.69] = -272.52\ kcal/mole$$

$$\Delta G = \Delta G^\circ + RTlnQ$$

For $20^\circ C$; $RT = (1.987x10^{-3}\ kcal/mole - {}^\circ K)(293^\circ K) = 0.582\ kcal/mole$

$$\Delta G = -272.52 + 0.582(\ln[N_2] + 2ln[OH^-] + 2ln[CO_2] - 5ln[CO] - 2ln[NO_3^-])$$

Assume unit activities for $[CO_2]$ *and* $[N_2]$, *pH 7, 100 mg/ℓ* NO_3^-*–N (7.14 x* 10^{-3} *M), one atmosphere of carbon monoxide which yields 28.38 mg/ℓ (1.02 x* 10^{-3} *M)*

$$\Delta G = -272.52 + 0.582(2ln[10^{-7}] - 5ln[1.02x10^{-3}] - 2ln[7.14x10^{-3}])$$

$$\Delta G = -265.5\ kcal/mole$$

Appendix B. Kinetic Data

Table 8. Denitrification in control microcosms containing sandy soil, helium, and nitrate.

Time (days)	Nitrate-N Concentration (mg/ℓ)	Nitrite-N Concentration (mg/ℓ)
0	104.3 111.5	0.7 0.8
3.0	107.8 107.2	0.8 0.8
10.0	110.6 111.2	1.3 1.7
17.0	108.5 111.1	4.2 4.8
24.0	108.3 110.1	6.6 6.3
38.0	111.5 110.5	11.8 11.4

Table 9. Denitrification in microcosms containing sandy soil, 1.0 atm. methane, and four different nitrate concentrations.

Time (days)	Nitrate-N Concentration (mg-N/ℓ)			
0	13.4	28.6	51.4	96.0
	13.1	29.2	51.0	95.0
	13.4	28.4	50.2	95.0
1.5	13.1	28.9	50.0	94.0
	13.2	28.5	50.0	95.0
	13.3	28.4	49.9	94.0
4.5	13.2	29.9	51.4	94.5
	13.3	28.8	51.6	94.0
	13.5	28.8	50.4	95.0
6.3	13.4	29.2	53.4	92.0
	13.2	28.6	49.6	91.0
	13.4	28.5	52.2	91.0
10.6	13.2	28.6	50.2	93.0
	13.2	28.4	52.6	94.0
	13.2	28.4	50.1	97.0
13.4	12.4	27.5	50.8	91.5
	12.4	27.3	48.0	92.5
	12.7	27.2	48.8	89.0
18.4	12.8	28.5	49.6	89.5
	12.8	28.2	-	95.0
	13.0	28.1	-	90.0
46.5	12.3	28.6	51.2	94.0
	12.4	28.6	53.2	95.0
	12.8	28.0	51.0	94.5
53.7	12.4	26.7	51.0	89.5
	11.9	27.2	50.0	94.5
	12.0	27.3	50.8	88.5
102.6	-	23.8	-	-
	-	25.1	-	-
	-	24.6	-	-
127	-	-	-	89.5
	-	-	-	90.3

Table 10. Denitrification in microcosms containing sandy soil, 1.0 atm. CO, and four different nitrate concentrations.

Time (days)	Nitrate-N Concentration (mg-N/ℓ)			
0	13.5	27.7	49.4	95.0
	13.2	28.2	50.2	95.0
	12.8	28.8	50.2	96.0
1.5	13.3	27.8	49.2	93.5
	13.0	28.0	49.6	93.5
	12.8	28.2	48.8	94.0
4.5	12.8	28.8	52.4	92.5
	12.6	27.9	50.4	94.0
	12.7	28.7	50.6	93.5
6.3	12.5	27.9	49.0	90.5
	12.6	-	48.2	92.5
	12.0	27.8	47.6	93.0
10.6	11.9	27.0	48.2	91.0
	12.0	26.9	49.0	92.5
	11.6	27.7	48.8	93.0
13.4	11.2	25.8	46.4	85.0
	11.6	25.7	46.0	90.0
	10.9	26.4	48.6	88.5
18.4	11.2	26.5	48.2	95.0
	11.5	26.5	49.6	91.0
	10.9	26.8	47.2	89.5
46.5	10.1	25.9	48.6	91.5
	10.5	25.3	47.0	100.5
	9.6	25.7	54.8	92.0
53.7	9.7	24.6	44.6	92.5
	10.4	24.3	45.6	92.0
	9.5	24.4	47.2	87.5
102.6	-	24.4	-	-
	-	23.1	-	-
	-	24.0	-	-
127	-	-	-	89.1
	-	-	-	89.2

Table 11. Denitrification in microcosms containing an activated sludge seed, 1.0 atm. methane, and 25 mg/ℓ nitrate-N.

Time (days)	Nitrate-N Concentration (mg/ℓ)	Nitrite-N Concentration (mg/ℓ)
0	24.1	0.7
	23.9	0.7
3	24.8	1.0
	25.1	0.6
10	23.6	0.5
	23.5	0.6
17	23.0	0.5
	23.5	0.4
24	21.1	1.4
	21.6	0.8
38	19.9	1.8
	20.2	1.6

Table 12. Denitrification in microcosms containing an activated sludge seed, 1.0 atm. methane, and 40 mg/ℓ nitrate-N.

Time (days)	Nitrate-N Concentration (mg/ℓ)	Nitrite-N Concentration (mg/ℓ)
0	39.7	0.7
	39.7	0.7
3	39.6	0.8
	39.3	0.6
10	40.4	0.5
	40.6	0.6
17	38.1	0.6
	37.7	0.9
26	36.5	1.4
	37.0	1.2
38	35.1	1.8
	36.6	1.3

Table 13. Denitrification in microcosms containing an activated sludge seed, 1.0 atm. methane, and 65 mg/ℓ nitrate-N.

Time (days)	Nitrate-N Concentration (mg/ℓ)	Nitrite-N Concentration (mg/ℓ)
0	65.3	0.7
	65.9	0.7
3	59.6	0.6
	60.4	0.8
10	65.5	0.6
	66.1	0.6
17	65.1	0.6
	65.6	0.9
24	54.3	1.8
	55.7	1.8
26	60.0	1.4
	61.5	1.3
38	59.4	1.5
	60.0	1.5

Table 14. Denitrification in microcosms containing an activated sludge seed, 1.0 atm. methane, and 115 mg/ℓ nitrate-N.

Time (days)	Nitrate-N Concentration (mg/ℓ)	Nitrite-N Concentration (mg/ℓ)
0	115.7	0.6
	113.5	0.7
3	119.4	0.8
	117.7	0.8
10	117.5	0.7
	115.5	0.6
17	113.1	1.1
	114.3	0.6
24	111.3	0.8
	111.4	1.3
38	110.9	1.7
	112.1	2.1

Table 15. Denitrification in microcosms containing an activated sludge seed, 1.0 atm. CO, and 25 mg/ℓ nitrate-N.

Time (days)	Nitrate-N Concentration (mg/ℓ)	Nitrite-N Concentration (mg/ℓ)
0	23.5	2.7
	23.1	2.7
3	19.8	2.6
	23.3	3.8
10	22.2	3.6
	21.9	3.8
17	21.9	3.6
	21.2	3.9
24	21.5	3.6
	20.3	3.4
38	12.6	0.6
	10.6	2.8

Table 16. Denitrification in microcosms containing an activated sludge seed, 1.0 atm. CO, and 40 mg/ℓ nitrate-N.

Time (days)	Nitrate-N Concentration (mg/ℓ)	Nitrite-N Concentration (mg/ℓ)
0	38.8	2.8
	39.4	2.7
3	40.0	4.0
	39.7	5.0
10	39.0	3.7
	39.8	3.6
17	36.5	3.6
	36.7	3.6
26	35.5	4.2
	36.2	3.2
38	24.2	6.0
	26.8	0.4

Table 17. Denitrification in microcosms containing an activated sludge seed, 1.0 atm. CO, and 65 mg/ℓ nitrate-N.

Time (days)	Nitrate-N Concentration (mg/ℓ)	Nitrite-N Concentration (mg/ℓ)
0	64.9	2.6
	65.4	2.7
3	58.8	3.8
	59.4	3.9
10	64.0	3.4
	64.0	3.7
17	64.7	3.5
	64.8	3.9
24	57.4	3.4
	58.5	4.3
26	61.0	3.7
	62.0	3.9
38	32.9	27.1
	49.9	10.7

Table 18. Denitrification in microcosms containing an activated sludge seed, 1.0 atm. CO, and 115 mg/ℓ nitrate-N.

Time (days)	Nitrate-N Concentration (mg/ℓ)	Nitrite-N Concentration (mg/ℓ)
0	111.6	2.5
	114.3	2.5
3	115.7	4.7
	119.6	4.4
10	109.8	3.6
	113.1	3.3
17	108.8	3.8
	111.6	3.4
24	110.6	4.5
	108.4	3.4
38	94.4	18.5
	82.4	27.3

Table 19. Denitrification in microcosms containing an activated sludge seed, 0.06 atm. CO, and 100 mg/ℓ nitrate-N.

TIME (days)	CONCENTRATION (mg-N/ℓ)
0	102.6 96.3
2.2	100.8 100.1
4.0	98.8 96.6
8.0	97.0 97.1

Table 20. Denitrification in microcosms containing an activated sludge seed, 0.06 atm. CO, and 50 mg/ℓ nitrate-N.

TIME (days)	CONCENTRATION (mg-N/ℓ)
0	52.6 52.6
2.2	52.6 51.9
4.0	51.0 50.9
8.0	51.5 51.3

Table 21. Denitrification in microcosms containing an activated sludge seed, 0.06 atm. CO, and 25 mg/ℓ nitrate-N.

TIME (days)	CONCENTRATION (mg-N/ℓ)
0	27.4 27.5
2.2	26.1 26.4
4.0	25.4 26.2
8.0	25.0 25.8

Table 22. Denitrification in microcosms containing an activated sludge seed, 0.06 atm. CO, and 15 mg/ℓ nitrate-N.

TIME (days)	CONCENTRATION (mg-N/ℓ)
0	16.8 15.7
2.2	15.7 15.0
4.0	15.0 14.7
8.0	14.8 14.2

Table 23. Denitrification in microcosms containing an activated sludge seed, 0.02 atm. CO, and 90 mg/ℓ nitrate-N.

TIME (days)	CONCENTRATION (mg-N/ℓ)
0	87.8 88.3
7.2	84.2 83.6
14.4	82.2 81.0

Table 24. Denitrification in microcosms containing an activated sludge seed, 0.04 atm. CO, and 90 mg/ℓ nitrate-N.

TIME (days)	CONCENTRATION (mg-N/ℓ)
0	88.0 89.1
7.2	85.3 85.7
14.4	80.6 82.3

Table 25. Denitrification in microcosms containing an activated sludge seed, 0.06 atm. CO, and 90 mg/ℓ nitrate-N.

TIME (days)	CONCENTRATION (mg-N/ℓ)
0	91.3
7.2	84.2
14.4	83.2

Table 26. Denitrification in microcosms containing an activated sludge seed, 0.08 atm. CO, and 90 mg/ℓ nitrate-N.

TIME (days)	CONCENTRATION (mg-N/ℓ)
0	89.2 90.9
7.2	84.9 86.0
14.4	82.8 84.8

Table 27. Denitrification in microcosms containing an activated sludge seed, 0.10 atm. CO, and 90 mg/ℓ nitrate-N.

TIME (days)	CONCENTRATION (mg-N/ℓ)
0	95.9 95.3
2.2	94.7 94.0
4.0	87.8 95.5
8.0	88.6 89.6

Table 28. Denitrification in microcosms containing an activated sludge seed, 0.15 atm. CO, and 100 mg/ℓ nitrate-N.

TIME (days)	CONCENTRATION (mg-N/ℓ)
0	99.9 98.8
2.2	96.6 95.7
4.0	93.7 95.6
8.0	91.8 94.2

Table 29. Denitrification in microcosms containing an activated sludge seed, 0.25 atm. CO, and 70 mg/ℓ nitrate-N.

TIME (days)	CONCENTRATION (mg-N/ℓ)
0	65.8 69.5
2.2	62.9 69.0
4.0	60.2 65.2
8.0	60.5 65.5

Table 30. Denitrification in microcosms containing an activated sludge seed, 0.35 atm. CO, and 100 mg/ℓ nitrate-N.

TIME (days)	CONCENTRATION (mg-N/ℓ)
0	101.2 98.5
2.2	96.1 95.0
4.0	97.2 94.5
8.0	96.7 94.8

Table 31. Denitrification in microcosms containing an activated sludge seed, 0.75 atm. CO, and 100 mg/ℓ nitrate-N.

TIME (days)	CONCENTRATION (mg-N/ℓ)
0	102.0 106.9
4.2	104.9 104.5
6.0	94.4 101.4
11.2	103.9 103.9
16.2	100.1 100.4

Table 32. Denitrification in microcosms containing an activated sludge seed, 1.00 atm. CO, and 100 mg/ℓ nitrate-N.

TIME (days)	CONCENTRATION (mg-N/ℓ)
0	103.7 101.4
4.2	103.1 103.7
6.0	102.0 100.5

Table 33. Denitrification velocities in microcosms containing an activated sludge seed, 100 mg/ℓ nitrate-N, and ten different CO

DENITRIFICATION VELOCITY (mg-N/d-gVSS)	AQUEOUS CO CONCENTRATION ($M \times 10^5$)	CO PARTIAL PRESSURE (atm)
15.1	2.03	0.02
16.6	4.06	0.04
19.0	6.08	0.06
14.6	8.11	0.08
28.4	10.14	0.10
25.5	15.21	0.15
20.2	25.35	0.25
13.9	35.49	0.35
6.3	76.05	0.75
4.8	101.4	1.00

Appendix C. Computer Model and Output

```
1          OPTIONS LS=80;
2             TITLE 'HALDANE LEAST SQUARES FIT';
3             DATA VEL;
4             INPUT V S;
5             LINES;
```

```
A232BPG  OUTPUT   A1 01/30/90 19:28   F 132   360 RECS 01/30/90 19:30 PAGE      4


NOTE: DATA SET WORK.VEL HAS 10 OBSERVATIONS AND 2 VARIABLES. 2346 OBS/TRK.
NOTE: THE DATA STATEMENT USED 0.07 SECONDS AND 212K.

16            ;
17
17            PROC PRINT;
18
NOTE: THE PROCEDURE PRINT USED 0.09 SECONDS AND 216K
      AND PRINTED PAGE 1.

18            PROC MEANS;
19
NOTE: THE PROCEDURE MEANS USED 0.09 SECONDS AND 232K
      AND PRINTED PAGE 2.

19            PROC NLIN METHOD = GAUSS;
20            PARMS VM=50  KS=7 KI=35;
21            MODEL V=VM*S/(KS+((S**2.5)/KI)+S);
22            DER.VM=(S)/(S+KS+((S**2.5)/KI));
23            DER.KS=(VM*S*(-1))/( ((S+KS+((S**2.5)/KI))**2) );
24            DER.KI=(VM*(S**3.5)/(KI**2))/((((S**2.5)/KI)+S+KS)**2);
25            OUTPUT OUT=RESULTS P=VHAT R=VRES;
26
NOTE: THE DATA SET WORK.RESULTS HAS 10 OBSERVATIONS AND 4 VARIABLES. 1304
      OBS/TRK.
NOTE: THE PROCEDURE NLIN USED 0.41 SECONDS AND 548K
      AND PRINTED PAGES 3 TO 4.

26            PROC PLOT DATA=RESULTS;
27            PLOT VHAT*S='*' V*S='+' /OVERLAY;
28
NOTE: THE PROCEDURE PLOT USED 0.12 SECONDS AND 228K
      AND PRINTED PAGE 5.

28            PROC PRINT DATA=RESULTS;
NOTE: THE PROCEDURE PRINT USED 0.09 SECONDS AND 216K
      AND PRINTED PAGE 6.
NOTE: SAS USED 548K MEMORY.
2         SAS(R) LOG   OS SAS 5.18          MVS/XA JOB A232BPG  STEP STEP1
                                                19:28 TUESDAY, JANUARY 30, 1990
NOTE: SAS INSTITUTE INC.
      SAS CIRCLE
      PO BOX 8000
      CARY, N C  27512-8000
```

HALDANE LEAST SQUARES FIT 1
19:28 TUESDAY, JANUARY 30, 1990

OBS	V	S
1	0.0	0.000
2	15.1	2.028
3	16.6	4.056
4	19.0	6.084
5	28.4	10.140
6	25.5	15.210

A232BPG OUTPUT A1 01/30/90 19:28 F 132 360 RECS 01/30/90 19:30 PAGE 5

7	20.2	25.350
8	13.9	35.490
9	6.3	76.050
10	4.8	101.400

HALDANE LEAST SQUARES FIT 2
19:28 TUESDAY, JANUARY 30, 1990

VARIABLE	N	MEAN	STANDARD DEVIATION	MINIMUM VALUE	MAXIMUM VALUE	STD ERROR OF MEAN
V	10	14.98000000	9.07595113	0	28.4000000	2.8700677
S	10	27.58080000	34.56469376	0	101.4000000	10.9303159

HALDANE LEAST SQUARES FIT 3
19:28 TUESDAY, JANUARY 30, 1990

NON-LINEAR LEAST SQUARES ITERATIVE PHASE

DEPENDENT VARIABLE: V METHOD: GAUSS-NEWTON

ITERATION	VM	KS	KI	RESIDUAL SS
0	50.000000000	7.000000000	35.000000000	369.949178964
.	0.662445958	-5.183014678	126.288424	2820.280312818
.	25.331222979	1.908492661	80.644212042	490.697208129
1	37.665611489	4.454246330	57.822106021	326.169876079
2	31.759849528	3.038234049	143.899290	121.127699827
3	44.280701368	5.333641728	111.058553	44.633085983538
4	50.819306567	6.755347944	96.352948455	38.661607504213
5	53.448289196	7.394512387	91.528224069	38.176647380689
6	54.338485601	7.617741599	89.396121813	38.148964156827
7	54.586931904	7.680751631	88.843956273	38.147165057089
8	54.651381503	7.697135477	88.695415738	38.147053854338
9	54.667426947	7.701227256	88.658157887	38.147047020616
10	54.671396454	7.702240051	88.648886442	38.147046603791
11	54.672375810	7.702489997	88.646602549	38.147046578426

NOTE: CONVERGENCE CRITERION MET.

NON-LINEAR LEAST SQUARES SUMMARY STATISTICS DEPENDENT VARIABLE V

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
REGRESSION	3	2947.2129534	982.4043178
RESIDUAL	7	38.1470466	5.4495781
UNCORRECTED TOTAL	10	2985.3600000	
(CORRECTED TOTAL)	9	741.3560000	

PARAMETER	ESTIMATE	ASYMPTOTIC STD. ERROR	ASYMPTOTIC 95 % CONFIDENCE INTERVAL LOWER	UPPER
VM	54.67237581	17.341290154	13.666411365	95.67834026
KS	7.70249000	4.177223823	-2.175154057	17.58013405
KI	88.64660255	45.334452003	-18.553202682	195.84640778

ASYMPTOTIC CORRELATION MATRIX OF THE PARAMETERS

CORR	VM	KS	KI
VM	1.0000	0.9773	-0.9641
KS	0.9773	1.0000	-0.9220
KI	-0.9641	-0.9220	1.0000

PLOT OF VHAT*KS SYMBOL USED IS *
PLOT OF VKS SYMBOL USED IS +

Benjamin Perry Gayle was born on September 4, 1954 and grew up in Sedley, Virginia. He received a B.S. degree in Biology in 1976 and an M.S. degree in Environmental Sciences and Engineering, in 1977, both from Virginia Polytechnic Institute and State University (VPI&SU). He worked for two and one half years with the Virginia State Water Control Board in Richmond, Virginia, before returning to VPI&SU to continue his studies. In May of 1990 he completed the requirements for a Ph.D. degree in Civil Engineering.