Plant and Soil 115, 261–284 (1989).

PLSO AL-25

Perspectives on measurement of denitrification in the field including recommended protocols for acetylene based methods

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Key words: acetylene inhibition, denitrifiers, geostatistics, kriging, nitrogen, nitrogen-15, nutrient cycling, soil cores, terrestrial ecosystems

Abstract

Of the biogeochemical processes, denitrification has perhaps been the most difficult to study in the field because of the inability to measure the product of the process. The last decade of research, however, has provided both acetylene and ^{15}N based methods as well as undisturbed soil core and *in situ* soil cover sampling approaches to implementing these methods. All of these methods, if used appropriately, give comparable results. Thus, we now have several methods, each with advantages for particular sites or objectives, that accurately measure denitrification in nature. Because of the general usefulness of the acetylene methods, updated protocols for the following three methods are given: gas-phase recirculation soil cores; static soil cores; and the denitrifying enzyme assay also known as the phase 1 assay. Despite the availability of these and other methods, denitrification budgets remain difficult to accurately establish in most environments because of the high spatial and temporal variability inherent in denitrification. Appropriate analysis of those data includes a distribution analysis of the data, and if highly skewed as is typically the case, the most accurate method to estimate the mean and the population variance is the UMVUE method (uniformly minimum variance unbiased estimator). Geostatistical methods have also been employed to improve spatial and temporal estimates of denitrification. These have occasionally been successful for spatial analysis but in the attempt described here for temporal analysis the approach was not useful.

Discussions of the importance of denitrification have always focused on quantifying the process and whether particular measured quantities are judged to be a significant amount of nitrogen. A second line of evidence discussed here is the extant genetic record that results from natural selection. These analysis lead to the conclusion that strong selection for denitrification must currently be occurring, which implies that the process is of general significance in soils.

Introduction

Denitrification is the process by which nitrogenous oxides, principally NO_3^- and NO_2^-, are reduced to dinitrogen gases, N_2 and N_2O. Most denitrification is carried out by respiratory denitrifiers that gain energy by coupling N-oxide reduction to electron transport phosphorylation (Tiedje, 1988). Nearly all respiratory denitrifiers prefer to use O_2 as their electron acceptor and will reduce N-oxides only when O_2 is not available. Denitrification is reasoned to be an important biogeochemical process since it appears to balance nitrogen fixation by recycling fixed nitrogen to the atmosphere. Most of the research on denitrification has been by agricultural scientists in an effort to understand and hopefully minimize loss of the nutrient that most often limits crop growth. Further reasons for in-

terest in denitrification are: in waste treatment it can remove excess nitrate; it can decrease nitrate contamination of groundwaters, it affects atmospheric composition through the production and consumption of N_2O and thus has impact on climate; and it can produce toxic intermediates, NO and NO_2^-, the latter which can lead to carcinogenic nitrosamines (Tiedje, 1988).

The first need to study any process is an assay method. This has been the major obstacle in the study of denitrification and is why progress in quantifying this process has not been extensive despite its discovery over a century ago. There are three reasons why methodology has been such a limitation to the study of denitrification. (1) The best assay method for a process is to measure its product, but the Earth's atmosphere is 80% N_2 which rules out the use of this approach except in certain sealed microbial or biochemical laboratory studies. An alternative approach is to measure substrate disappearance, which is usually unsatisfactory for denitrification because of the diverse sources and fates of nitrate. (2) Given the above, the next best approach is to use a radioactive isotope, but for nitrogen there are none convenient for frequent use. Hence alternative methods must be used and these require more assumptions or are less direct. (3) Of the biogeochemical processes, denitrification is the most dynamic with several environmental regulators *e.g.* oxygen, nitrate, carbon (Tiedje, 1988). The complex variation in and interactions of these regulators results in considerable variability of denitrification over time and space (Burton *et al.*, 1984; Folorunso and Rolston, 1984; Parkin *et al.*, 1985; Parkin *et al.*, 1987). This makes quantitation of denitrification imprecise, costly, and makes conclusions difficult to establish. To overcome these obstacles more effort has been required than for other biogeochemical processes to arrive at reasonable and reliable methods for study of denitrification in the field. We believe these methods now exist and that it is time to focus on studying the ecology of the process. As this chapter was derived from a talk which was to emphasize findings from our laboratory, this chapter reflects this perspective as well. Furthermore, we summarize recommended protocols for those acetylene methods for which we have considerable experience as this information is not available elsewhere.

How important is denitrification: the perspective from natural selection?

Discussions of the importance of denitrification always focus on quantifying the process and whether particular measured quantities are judged to be a significant amount of nitrogen. There is another line of evidence, however, that should be considered in evaluating the importance of denitrification. This evidence is based on whether the trait has left a significant impact, *i.e.* has it been selected, as evidenced in the genetic record of the extant natural communities. Evolution by natural selection is based on the principle that species, and their physiological processes, were derived from variants in the progeny of previous generations that were more fit for their environment, and thus reproduced more extensively. If we look at the selection and thus the historical importance of this process in the soil ecosystem. This evidence is as follows:

1. Among the soil biogeochemical processes, denitrification appears to be second only to respiration in the number of gene copies present. We base this statement on the fact that denitrifiers make up 1 to 5% of the culturable soil microbial population (Tiedje, 1988), which makes denitrifier genes more numerous than genes for nitrogen fixation, nitrification and even cellulose decomposition for example. Since it is costly to organisms to maintain unneeded gene sequences (and denitrification requires many genes), this large number of gene copies should not have been maintained unless the trait is of sufficient and frequent value to the populations that contain the genes. It is hard to envision that brief, sporadic denitrification events or low levels of denitrification, as is usually envisioned for most soils (Aulakh *et al.*, 1982; Duxbury *et al.*, 1986; Mosier *et al.*, 1986; Myrold, 1988; Rolston *et al.*, 1982; Sexstone *et al.*, 1985; Terry *et al.*, 1986), are enough to maintain this large quantity of denitrifying genes in the indigenous communities. The implication is that the essential use of the denitrification process is more extensive than this.

2. Denitrification is widely distributed among procaryotes, and is even found in archebacteria. In fact it is now easier to list those bacterial groups in which denitrification is not present (Tiedje, 1988). It is hard to imagine how a process could have such

a wide phylogenetic spread without it being a process under strong selection. The fact that denitrification is found in both the eubacteria and archebacteria (Tiedje, 1988) together with what is believed to be it's relatively recent evolutionary origin (Betlach, 1982) suggests that there must have been lateral transfer of these genetic sequences among genera in soil communities. Such a spread only becomes recognized and established if there is strong selection.

3. The key denitrifying enzyme, nitrite reductase, appears to have evolved twice since both Cu- and heme cd_1-based nitrite reductases are common in soil denitrifiers (Coyne *et al.*, ms submitted). The niche for denitrification must have been substantial for two separate enzyme systems to have evolved to carry out the same physiological reaction. The heme based enzyme may have evolved more recently but even if so, the Cu based enzyme is still readily found in soil denitrifiers today.

4. The denitrifying nitrite reductase, and particularly the heme cd_1-type, seem to be highly conserved structures. Of the more than 100 soil denitrifiers that we have surveyed by Western immunoblots, the immunological specificity and molecular weight suggest conserved protein structures (Coyne *et al.*, ms submitted). Nitrous oxide reductase and nitrate reductase appear to be even more highly conserved (Korner, 1987; Michalski and Nicholas, 1988). Proteins that carry out processes vital to cells tend to be highly conserved. Thus denitrification would seem to be an important process to microbial cells, and thus in ecosystems it must have been significant.

The above arguments all suggest that denitrification is a valuable process to microbial communities. It has long been known that denitrification is coupled to electron transport phosphorylation (ETP) and provides about 60% of the energy (ATP) of oxygen respiration (Koike and Hattori, 1975). This energy yield, while less than respiration, is considerably more than provided by fermentation. Thus, in anaerobic environments in which nitrate is present, organisms with the denitrifying capacity would clearly benefit. However, it is hard to imagine that nitrate-rich anaerobic niches are sufficiently frequent in time and space to account for the advantage of denitrification as evidenced by the genetic record.

One explanation might be that previous conditions on Earth were more conducive to denitrification than current conditions and that the present genetic record is historical and doesn't reflect current conditions, *i.e.* that denitrifying genes are currently being shed. However, there are two arguments against this explanation. First, the majority of denitrifiers freshly isolated from Nature readily loose part or all of their denitrifying capacity during cultivation on laboratory media (Abd-el-Malek *et al.*, 1974; Gamble *et al.*, 1977). In addition, in at least one case, denitrifier genes appear to be plasmid-borne (Romermann and Friedrich, 1985). The location of denitrifier genes is not yet well studied; but when it is we would expect the plasmid location to not be rare. Thus, many denitrification gene sequences seem not to be stably maintained and should be readily lost if they were not continually being selected for in the progeny that retained them. Hence, current conditions must be selecting for denitrification. Second, gene sequences even with a low negative selection coefficient, *e.g.*, such as for gene maintenance, should be lost in 100 to 1000 generations. Even if generations in soil are few, this negative selection should be apparent in 10 to 100 years. There is little evidence that soil conditions important to denitrification have changed significantly in recorded history to suggest that conditions conducive to denitrification have changed the selective pressure.

In support of the argument that current conditions are still selecting for denitrifiers, we found that the ratio of denitrification enzyme activity/microbial biomass C was consistently higher in poorly drained soils than in well drained soils, and in fine-textured soils than in course-textured soils (Groffman and Tiedje, 1989b). These data suggest that in environments more conducive to denitrifcation, selection favors denitrifiers relative to other soil microorganisms.

The conclusion from the above analysis is that the denitrification process is still significant enough to be selecting and thus maintaining diverse and extensive populations of denitrifiers in soil. In order to provide the energy benefit to the populations to explain this result, denitrification would seem to be more common than reported in most soil studies. One explanation may be that most agricultural studies are biased toward measuring denitrification

only during the cropping season when soil aeration is high and nitrate and carbon are low. The evidence to explain the apparent dilemma does not yet exist, but new nucleic acid and antibody probe methods and gene sequencing provide important tools to help resolve this issue.

Methods to measure denitrification

A number of different methods have been used to measure denitrification in the field. These include the acetylene and ^{15}N methods discussed here, nitrate/chloride ratios, nitrate disappearance, nitrogen balance, N gas production in sealed chambers, non-random isotope distribution, N production calculated from soil gas gradients, and micrometeorological methods (for recent reviews of these methods see Hauck, 1986 and Smith, 1988). The acetylene and ^{15}N methods are the most reliable and widely useful and these are further discussed here.

Acetylene inhibition methods

The ability of acetylene to cause denitrifiers to accumulate N_2O from NO_3^- was first noted by Fedorova *et al.*, (1973) and its use for denitrification assays was demonstrated in pure cultures in 1976 independently by Balderston *et al.* (1976) and Yoshinari and Knowles (1976). These contributions are major milestones in denitrification measurement and have led to an explosion of denitrification studies as well as to an improved understanding of the process. Some of the advantages of the acetylene method have been reviewed by Duxbury (1986). The major advantage is the tremendous improvement in sensitivity over previous methods: detection limits of 0.5 ng N/g soil·day (core)(Parkin and Tiedje, 1984) or 1 g/ha·day (field covers)(Duxbury, 1986). Other important advantages include (i) the use of the natural nitrate substrate pool, (ii) the large number of samples that may be assayed so that the spatial and temporal distributions can be analyzed and appropriate statistical analysis applied (Parkin *et al.*, 1987 (iii) the relatively low cost of the method (and especially for the analytical equipment) compared to the ^{15}N and ^{13}N methods, and (iv) the versatility of the method allowing lab, field, and remote site studies. It is these advantages that have made denitrification more widely studied and by a larger number of research groups.

There are some pitfalls with the acetylene method that the user must be aware of so that they can be avoided. These have been discussed by Keeney (1986); Rolston (1986) and Tiedje (1988). They are: (i) acetylene affects on other processes such as nitrification, sulfur cycling, and methanogenesis, (ii) acetylene inhibition can fail because not enough acetylene is present, which is particularly important when organic matter is high and/or nitrate concentrations are low. The acetylene can be biodegraded; the latter occurs only after the population is enriched by exposure to acetylene for approximately one week (Terry and Duxbury, 1985), (iii) contaminants in the acetylene may affect denitrifiers, and (iv) the dispersal of the acetylene, the recovery of N_2O, and the significant water solubility of N_2O are all important physical aspects that can lead to inaccurate results. Of these pitfalls the only one which cannot be overcome with appropriate care and design is the acetylene inhibition of nitrification. Fortunately this is an important consideration only for samples in which the nitrate concentration is very low. We have never found this to be a problem in agricultural soils but we have found it to be a problem in soils from unfertilized, natural ecosystems. In these latter cases, we have still found the acetylene method to be useful (*e.g.* Robert and Tiedje, 1984) when the gas-phase recirculation core (described below) is used.

The initial studies of denitrification on soil using this method were done with mixed, sieved or slurried soils. For measurement of natural rates, however, it is important to preserve the natural soil structure. Disruption of structure often stimulates denitrification by providing a new supply of carbon (Myrold and Tiedje, 1985; Parkin and Tiedje, 1984; Sexstone *et al.*, 1988), but in sandy soils it can cause a decrease in denitrification due to increased exposure of microsites to oxygen (Parkin and Tiedje, 1984). Thus, it is now generally accepted that maintaining the natural soil structure is required for measurement of natural rates of denitrification. The methods now used and described in this chapter accommodate this requirement.

Isotope methods

The use of ^{15}N in N cycling studies became popular during the 1960's, with denitrification being calculated by difference, i.e. denitrification was equated to the amount of ^{15}N unaccounted for at the end of the experiment. Since unaccounted for ^{15}N actually represents the sum of all experimental errors (Rolston *et al.*, 1979), as well as denitrification, the 'difference' method is not very accurate for denitrification studies. Although the first techniques for quantifying denitrification by direct measurement of $^{15}N_2$ were reported in the 1950's (Hauck *et al.*, 1958; Nommik, 1956), direct ^{15}N field measurements of denitrification did not begin until the mid to late 1970's (Rolston *et al.*, 1976). Early direct measurements, which had low sensitivity, have been improved upon (Siegel *et al.*, 1982), and fluxes as low as $50\,g\,N{\cdot}ha^{-1}\,d^{-1}$ can now be measured (Duxbury, 1986). Very sensitive measurements of denitrification can be obtained using ^{13}N ($> 10^6$ orders of magnitude more sensitive than ^{15}N, Tiedje *et al.*, 1981), but there have been few studies using this isotope due to its short half-life.

The ^{15}N difference methods were developed for assessment of fertilizer N losses, and not for denitrification measurement. To calculate denitrification, it is necessary to account for leaching and volatilization losses, for ^{15}N that is immobilized, and for unlabeled N that is mineralized during the course of the experiment. The errors associated with leaching, mineralization, and immobilization measurement are often greater than denitrification, greatly reducing the effectiveness of the difference method for calculating denitrification N losses.

Using ^{15}N isotope dilution techniques in combination with mathematical modeling allows for calculation of the gross rates of different N cycle processes, permitting more precise measurements of processes than those produced by simple mass balance (Juma and Paul, 1981). Myrold and Tiedje (1986) used mathematical modeling and nonlinear parameter estimation, along with ^{15}N, to simultaneously calculate estimates of denitrification, mineralization, immobilization and nitrification. Although rates could be determined for all the N cycle processes (Table 1), denitrification was the most poorly estimated because of the low 'sensitivity' of the denitrification parameter, due to the low rate of denitrification activity relative to other N cycle processes under the conditions of this study. Nonetheless, this is the only approach that allows quantitation of all the rates of the N cycle processes as they naturally interact.

The most important recent advancement in ^{15}N, field-based measurements has been the direct measurement of denitrification by the $^{30}N_2$ mass. The basis for this method is the sensitivity provided by measurement of the $^{30}N_2/(^{28}N_2 + {}^{29}N_2)$ ratio since this ratio at natural abundance is low (Duxbury, 1986; Siegel *et al.*, 1982; Smith, 1988). While superior to the difference method, the $^{30}N_2$ method also has disadvantages. A primary problem is the necessary enhancement of the NO_3^- pool by the ^{15}N addition, which increases denitrification rates unless the *in situ* NO_3^- pool is high enough to make rates independent of NO_3^- concentration. A second problem is determing the source of the ^{15}N gas produced. The methods of Rolston *et al.* (1976) and Siegel *et al.* (1982) require a uniform distribution of ^{15}N in the soil NO_3^- pool. Since the NO_3^- pool is dynamic, with production and consumption occurring in diverse soil microenvironments, it is unlikely that uniform ^{15}N distribution can be maintained over moderate to long time periods. Heinemeyer *et al.* (1988) in a phytotron study comparing the $^{30}N_2$

Table 1. Simultaneous estimation of several N cycle rates using isotope dilution, modeling and non-linear parameter estimation

Process/Pool	First order rate constants (day^{-1})		N fluxes ($mg\ N\ kg^{-1}\ day^{-1}$)	
	Clay loam	Sandy loam	Clay loam	Sandy loam
Mineralization	0.0104	0.016	1.5	0.56
Immobilization	0.108	1.27	0.1–3.0	0.7–4.2
Nitrification	1.23	1.39	1–34	0.7–4.6
Denitrification	0.0132	0.001	0.54–0.96	0.04–0.015
Active N fraction ($mg\ N\ g^{-1}$ soil)	145	35		

From Myrold and Tiedje, 1986.

method with the ^{15}N balance method, found close agreement between the methods for up to 30 days but losses by ^{15}N balance were higher over longer time periods.

A second ^{15}N method that directly measures *in situ* denitrification is the isotope dilution of the product pool. In this case the sample atmosphere is switched to $^{15}N_2$ and any denitrifier product ($^{14}N_2$) dilutes the ^{15}N enrichment of the $^{15}N_2$ (Limmer *et al.*, 1982). This method avoids problems with substrate alteration and uniform label distribution, but it is difficult to remove all $^{14}N_2$ from a sample without either extensive alteration of its physical structure, or holding the sample for extended time periods.

The high sensitivity of ^{13}N based methods makes it possible to minimize substrate enhancement while measuring denitrification (Smith *et al.*, 1978; Tiedje *et al.*, 1981). However, the short half-life (9.6 minutes) and the requirement of access to a cyclotron or Van de Graaff accelerator to produce the isotope, has limited application of these methods to only a few laboratories (reviewed in Tiedje *et al.*, 1981). It is impossible to do field studies with ^{13}N, and its use is best reserved for questions that cannot be addressed by other techniques. Therefore, it will not be discussed in this chapter.

Comparison of methods

Acetylene vs ^{15}N

One of the most important aspects of any method for measurement of a biochemical process is whether the natural substrate concentration and distribution is altered in any way. Methods that rely on surrogate substrates, *e.g.* acetylene in place of N_2 for the nitrogenase assay or any isotope used as substrate, are always subject to criticism since it is difficult to ensure in complex matrices like soil that the concentration of the surrogate achieves and maintains the natural concentration at all microsites. Therefore, methods that employ the natural substrate avoid this potential criticism. Thus a major advantage of the acetylene method over the ^{15}N method is that it uses the natural substrate pool and measures a direct result of that process. With the N-isotopes (both ^{15}N and ^{13}N), achieving uniform labeling of natural substrate pools without altering natural concentrations has been particularly difficult if not impossible.

The few studies that have directly compared C_2H_2 and ^{15}N methods are summarized in Table 2. Rolston *et al.* (1982) and Mosier *et al.* (1986) conducted very similar field studies, comparing $^{15}N_2$ production to N_2O production in C_2H_2 treated soils during three irrigation events. Although both studies concluded that there were not important differences between the two methods, estimates of N loss were consistently higher with the C_2H_2 than with the ^{15}N method. This difference may have arisen because multiple treatments of the plots with C_2H_2 caused enrichment of C_2H_2 degraders, which could have increased the rates if available C was limiting denitrification (Terry and Duxbury, 1985; Topp and Germon, 1986; Yeomans and Beauchamp, 1982). This explanation of differences is supported by the fact that in the study by (Mosier *et al.*, 1986), the most marked difference between methods was observed in the last of three irrigation cycles, when the development of C_2H_2 degrading denitrifiers would be most likely.

Parkin *et al.* (1985) also compared the two methods but compared an C_2H_2 based soil core recirculation method with the ^{15}N difference method. Again there were no statistically significant differences between methods, but the N losses were higher when measured by the ^{15}N difference method than by the C_2H_2 method in both soils studied. Higher soil moisture in ^{15}N microplots than in soil outside of the microplots probably enhanced the ^{15}N losses. Furthermore, the difficulty in maintaining uniformly labeled NO_3^- pools, make measurement of total denitrification by ^{15}N imprecise. Parkin *et al.* (1985) observed that rates measured in microplots by ^{15}N were normally distributed while the C_2H_2 core measurements were log-normally distributed (and usually are, Parkin *et al.*, 1988). While normally distributed data are easier to present and synthesize than log-normally distributed data, the advantage in this case is small, since total variance does not appear to be any lower with the ^{15}N method than with the C_2H_2 method.

Since the acetylene and ^{15}N methods gave similar estimates of N loss, we believe that both methods, when used correctly, are equally acceptable quantitative measures of denitrification in nature. Both methods have different advantages and disadvantages that ought to be evaluated for any situation.

Table 2. Summary of field studies in which the acetylene inhibition and ^{15}N methods have been compared for measurement of N losses by denitrification

Study	Condition	Total denitrification: Acetylene Method	Total denitrification: ^{15}N Method	Comments	Authors
I	3 irrig/wk	4.3	4.1	*In situ* cover method: $^{15}NO_3^-$ added	Rolston *et al.*, 1982
	1 irrig/wk	3.4	3.2	Acetylene pumped into soil	
	1 irrig/2wk	2.7	1.9		
II	Clay loam	3.27	10.1	Recirculation cores for acetylene method and ^{15}N balance in *in situ*	Parkin *et al.*, 1985
	Sandy loam	1.71	2.65	cylinder for ^{15}N method	
III	June irrig	2.15	1.86	*In situ* cover method: $^{15}NH_4^+$ added	Mosier *et al.*, 1986
	Early July irrig	1.00	0.75	Acetylene allowed to diffused into soil	
	Late July–Aug. irrig	8.96	6.44[a]		

[a] Significantly different ($P < 0.1$), the rest of the comparisons are not significantly different (statistical comparisons were not done in study I). The single instance of significant difference is caused by one outlier (n = 4), suggesting a log normal distribution. If analyzed by log normal statistics this significant difference may disappear.
irrig = irrigation.

In choosing a method the specific questions to be addressed, the characteristics of the site to be studied, the availability of required equipment and the cost are all important considerations. Comparison studies, while valuable, are frustrating due to the difficulties in demonstrating significant differences due to the high spatial and temporal variability of this process. Since both methods are sound, it is more important to move forward and investigate underlying principles of the denitrification process than to dwell on denitrification methodology. The methods are not now the problem—it is the dynamic nature of the process with its many regulators that cause the problem in budget quantitation. A 'better' method is not the route to understanding the process nor to integrating true variation.

Cores vs in situ *covers*

The need to add C_2H_2 to soil in a controlled atmosphere provided the stimulus for the use of extracted soil cores in denitrification research. The use of cores can be problematic since the coring process may disturb the soil system, and create effects on denitrification rates that are difficult to interpret. This concern led to the development of chamber methods for measuring denitrification. These methods involve placing covers over the soil surface and either measuring the accumulation of N_2O in the air space of the box or sweeping air through the box and analyzing N_2O in the exit air stream (Jury *et al.*, 1982). Chamber methods have been used either with or without C_2H_2, and several approaches have been developed for introducing C_2H_2 into in-field chambers (Burton *et al.*, 1984; Hallmark and Terry, 1986; Ryden *et al.*, 1979). The main advantage of chamber methods is that they allow for in-field measurement of actual fluxes of N gases from soil to the atmosphere.

There are several problems with chamber methods however, that derive from the fact that physical effects inhibit diffusion and cause emission of N gases from the soil surface to be divorced from biological production of those gases. Jury *et al.* (1982) reported that several weeks of monitoring may be required to accurately assess production of N gases associated with a particular rainfall or irrigation event. Soil temperature, which strongly controls N_2O solubility and diffusion, and which varies diurnally, has a strong effect on emissions measured by chamber methods (Blackmer *et al.*, 1982). Diffusion problems can be easily overcome with cores however, either with forced air flow (See: Gas-phase recirculation core below), or by thorough mixing of the air space of the soil core (See: Static core below).

While core methods can disturb the soil environment, chamber methods also affect soil physical conditions. Disturbance effects from driving cylinders into soil can increase rates of gas emission

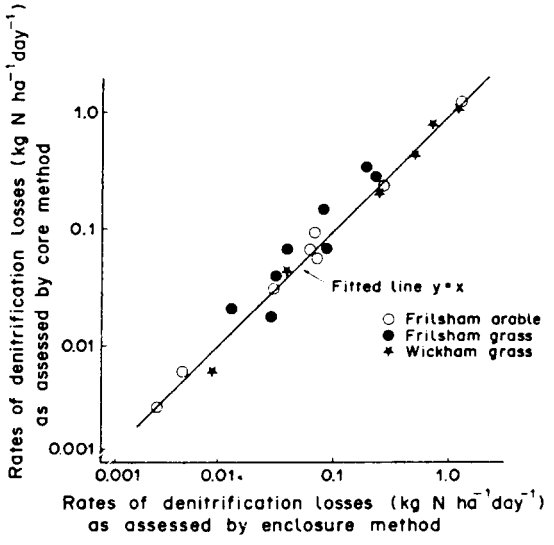

Fig. 1. Comparison of rates of denitrification measured using a cover method with those measured by a static core method on three sites. Reproduced from Ryden *et al.*, Soil Biol. Biochem. 1987, 19, 753–757, by permission.

significantly (Matthias *et al.*, 1980). This effect may be caused by release of gases physically trapped in soil spaces (Goodroad and Keeney, 1984). If so, waiting for some period between insertion of the chamber and sealing should alleviate this problem (Matson and Vitousek, 1987). Chambers can significantly increase soil temperatures (Matthias *et al.*, 1980), especially if long (> 1 hour) incubations are used. A final problem with chambers occurs if gas concentrations become sufficiently high to inhibit diffusion of gases out of the soil. Using short incubation times or flow through chambers minimize this effect.

Burton and Beauchamp (1984) compared two extracted core techniques with two *in situ* chamber methods, but high variability in rates complicated comparisons between the methods. In a comprehensive comparison of cores and chambers, Ryden *et al.* (1987) found a very strong relationship (slope not significantly different from 1) between denitrification rates in cores versus chambers, over a wide range of denitrification rates (Fig. 1). In very wet soils, they found that cores were superior to chambers due to the difficulty of introducing C_2H_2 into, and the slow diffusion of N_2O out of these soils. An additional advantage of cores is that it is possible to run numerous core incubations, cheaply and quickly, while chamber measurements can be expensive and time consuming, limiting the number of replicates and/or sites that can be analyzed.

Core and chamber methods can differ in the method of extrapolating point measurements to larger areas. While chamber measurements must be extrapolated using surface area, core measurements can be extrapolated using either surface area or soil weight. For surface area extrapolation, the flux measurement made from under the area of the chamber is extrapolated to a hectare or square meter basis and represents the activity for that area over the entire soil profile. Values are extrapolated by weight by calculating flux on a per gram of soil basis and extrapolating to an areal basis using bulk density values for the soil under study. Extrapolation by weight allows for evaluation of the contribution of different soil depths and is useful for making comparisons between sites on a per unit weight basis, negating the effects of bulk density.

In summary, when compared directly, cores and chambers provided equivalent measurements of denitrification. While chambers may provide more accurate measurements of instantaneous flux of N gases from the soil to the atmosphere, cores appear to give more direct estimates of N gas production by biological processes. Total N gas production is needed for N budget questions important to ecosystem production and water quality; therefore the core measurements are best for this parameter. Nitrous oxide flux is needed for atmospheric chemistry questions; the chamber methods are most appropriate in this case.

Considerations and protocol for acetylene based methods

Gas phase recirculation core

Background. This method was originally described by Parkin *et al.* (1984) and was developed in our lab from the original concept of Kaspar (1984) and Kaspar and Tiedje (1980). It is based on the principle that acetylene distribution and N_2O recovery from intact soil cores can be more quickly and accurately achieved by introducing mass flow through soil macropores, and that denitrification rates can be more accurately measured in a sealed,

repeatedly sampled system. In this method, a membrane pump recycles soil gas plus acetylene between the soil core and the gas chromatograph sampling loop. The increase in N_2O is continuously measured and the denitrification rate is obtained within 2 hours.

The advantages of the method are: (i) the natural soil structure and thus microsites with their carbon, nitrate and oxygen concentration are preserved, (ii) the assay is the most rapid (less than 2 hours) of any that maintains natural soil structure, (iii) because the production of N_2O is continuously measured, and linearity established, there is less uncertainty about whether the observed rate is influenced by limitations in gas diffusion, (iv) the analytical error is very low (CV < 10%), (v) cores of larger diameter are more accurately assayed than with the static core method (vi) samples with low nitrate concentrations can be analyzed, since a decline in N_2O accumulation rate is readily apparent when the nitrate concentration becomes rate limiting, and (vii) this system is the most convenient for experiments where the same core is reused for determining the effects of other treatments on denitrification. Examples of the latter include addition of water with or without nitrate (Groffman and Tiedje, 1989a; Robertson and Tiedje, 1984), the denitrification hysteresis during wetting versus drying cycles (Groffman and Tiedje, 1988), effect of different oxygen or acetylene concentrations (Parkin and Tiedje, 1984; Robertson and Tiedje, 1987), the effect of air filled porosity (Sexstone *et al.*, 1988), and measurement of the maximum denitrification rate under anaerobic conditions (argon plus acetylene as the recycled gas).

The maximum (anaerobic) denitrification rate is effectively used as the last measurement on a core, so that the previously measured rates on that core can be reference to this standard condition as a means of maximizing the treatment effect and minimizing the influence of variation among replicate cores (Myrold and Tiedje, 1985; Parkin and Tiedje, 1984; Sexstone *et al.*, 1988).

The disadvantages of the recirculation method include the following. Since gas is pumped through the soil, this slight increase in pressure could break water films extending oxygen to more microsites than occurs naturally; however we have not been able to demonstrate this to be more than a theoretical problem (Parkin *et al.*, 1984). Other disadvantages are that the number of cores that can be analyzed is too limited for some purposes (30 cores per day on the system described below), the method will not work for clay soils that are wet, and the equipment required, especially if automated, is complex and moderately expensive and cannot be used at remote sites.

Although not now the most popular acetylene-based method, the recirculation method remains the method of choice when advantages ii through vii (above) are important.

Recommended protocol. The method remains largely as described in Parkin *et al.* (1984). Subsequent improvements have been in automation, valving and plumbing, temperature control of the cores, and in data handling. The diagram of one loop in our current system which has the capacity to simultaneously measure eight cores is shown in Fig. 2. Soil cores of 4.7 cm diameter × 10 to 20 cm length are taken in a plastic liner which fits inside a steel coring device which has a sharpened, tapered, cutting tip. The soil core should fit snuggly in the plastic liner to minimize edge flow of the pumped gas. The metal core is driven into the soil by a hand operated slide hammer. Cores compacted more than 5% are discarded. After collection, the plastic cores are removed from the driver, capped on both ends with butyl rubber stoppers, and placed on ice

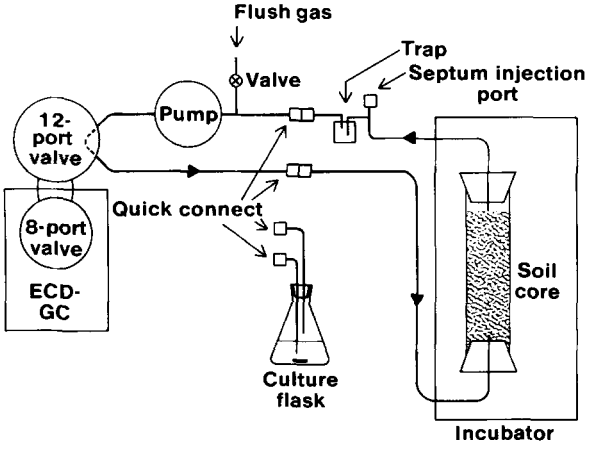

Fig. 2. Diagram of one of the eight parallel loops in our recirculation system to measure denitrification. This system can be easily shifted from core to flask samples by the quick connect fittings. The system can also be easily flushed to remove acetylene or change O_2 content by opening the valve for the flush gas and disconnecting the top quick connect. Acetylene or other gases are added through the septum injection port.

for transport to the laboratory. Cores have been stored at 4°C for up to 19 days without significantly affecting the denitrification rate (Parkin *et al.*, 1984). This feature is often important for large field studies and efficient use of the analytical equipment.

The analytical portion of our system consists of two gas chromatographs each equipped with two ^{63}Ni electron capture detectors and four analytical columns. An 8-port valve (Valco, column switch/backflush to vent) mounted in the GC oven connects two analytical columns to each detector. Thus four analytical columns in each of two gas chromatographs allow eight soil cores to be simultaneously analyzed. At alternating intervals, computer controlled valves switch the two analytical columns between analysis of the sample and backflush. The backflush prevents acetylene and water from reaching the detector. The analytical columns are 1.8 m × 0.32 cm o.d. stainless steel packed with Porapak Q. They are operated at 55°C with a carrier gas flow of 15 ml/min and a backflush of 30 ml/min. The carrier gas is 95% argon and 5% methane and the detector temperature is 300°C. The column conditions are adjusted as needed to optimize the separation of N_2O and CO_2 since the latter can interfere with quantitation of N_2O. We have found that the relative sensitivity of electron capture detectors for N_2O *vs.* CO_2 varies with manufacturer and that it is important to establish whether a particular instrument is adequate for N_2O analysis. Sample peaks are integrated on computing integrators (Hewlett Packard 3390A) with the data accumulated on a microbuffer and then transferred daily to data files in a personal computer. Detection limits on our system are 0.1 ng N_2O-N/ml and 3.0 μg CO_2/ml (Robertson and Tiedje, 1984).

The recirculation system cycles the gas phase between the soil core and a 0.5 ml sampling loop by means of a membrane pump (Neptune Dyna, Scientific Products, McGraw Park, IL) (Fig. 2). The pumps are operated at full speed which recycles the soil atmosphere at 200 to 300 ml/min for most cores. Swagelock quick connects are used to detach the soil core portion if attachment to another type of sample is desired, *e.g.* a culture flask. Serum vials (6 ml) are used as water and soil particle traps prior to the membrane pump. The tubing used throughout is 1/8″ stainless steel, which is important if a reactive gas like NO is to be measured. The sample loop is connected to the gas chromatograph by means of a 12-port valve (Valco, External sample) which connects to two recirculation loops. Both valves are stainless steel with 1/8 inch fittings and are actuated by pneumatic controllers. The valves are controlled by solenoids which are activated from a program stored in a small computer and operated through an external events actuator board. This system allows cores to be analyzed unattended.

To measure the denitrification rate the stored cores are first warmed to the desired temperature and then mounted in the recirculation system (Fig. 2). We currently house the cores in an incubator (with heating and cooling capability) at the *in situ* soil temperature. We have also used room temperature and corrected rates to the *in situ* temperature using a Q_{10} of 2 for denitrification (Rolston *et al.*, 1984; Knowles, 1981). The oxygen concentration of the recirculating gas can be adjusted to that measured *in situ* for the soil macropores (*e.g.* ranged from 14 to 18% in one of our studies), although this is usually not necessary since the sensitivity of denitrification rates to oxygen concentration in this range is low (Parkin and Tiedje, 1984). The acetylene concentration is recommended to be 20% (20 kPa) to insure effective inhibition of N_2O reduction even in soils low in nitrate. At 10 min intervals the computer controlled valves shunt 0.5 ml of the recirculating gas to the analytical columns. N_2O measurements should be made until a linear pattern of N_2O accumulation (constant denitrification rate) is seen. This usually occurs within 15 to 30 min for coarse textured soils and 1 to 2 h for fine textured soils.

Denitrification rates are calculated by multiplying the N_2O concentration in the gas phase by the volume of the gas in the recirculation-core system, correcting for the N_2O dissolved in the aqueous phase, and dividing by the dry weight of the soil. The gas volume is determined by a pressure transducer after injecting a known volume of air into the system (Parkin *et al.*, 1984). The core water content is determined gravimetrically, and the dissolved N_2O is then calculated from the Bunsen relationship (Tiedje, 1982). The detection limit for denitrification rate is 100 ng-N·m^{-2}h^{-1} or 24 mg-N·ha^{-1}day^{-1} (Robertson and Tiedje, 1984).

Table 3. Comparison of variability of N cycle processes and soil parameters in 0.5 ha old-field on sandy loam sampled in fall

	Coefficient of variation[a]
N mineralization	58
Nitrification	70
Denitrification	275
CO_2 production	61
Moisture	52
pH	5

[a] number of samples was 301.
From Robertson *et al.*, 1988.

Static core

Background. The static core is similar to the recirculation core in concept except that the gas phase is static during incubation. This static system has two important advantages over the recirculation system: it offers the capacity to obtain measurements on an even larger number of cores, *e.g.* 200 per day instead of 30 per day on the recirculation system, and the analytical system is less complex and also allows work at remote field sites. The increased sample capacity is particularly important because of the high temporal and spatial variability of denitrification. This is easily illustrated by comparing the coefficients of variation for denitrification to other processes or parameters measured at a study site (Table 3). Given this situation, the preference in field studies at least, is for high sample capacity. The larger sample capacity provided by the static core system has also allowed for the first time, proper statistical evaluation of field denitrification rates (Parkin *et al.*, 1988, and p. 276).

Table 4. Summary of the characteristics and development of the static core method for measurement of field rates of denitrification

Authors	Core size and seal	Incubation conditions	Acetylene concentration	Sampling times	Precautions to achieve gas distribution	No. of replicate cores per site or treatment
Aulakh *et al.*, 1982	6.0 × 15 cm in glass jar	In shade at field temp.	5 kPa	24 h	Al core had slits to foster gas exchange with jar atmosphere	4
Burton and Beauchamp, 1984	5.0 × 10 cm, sealed	*In situ*	1 kPa	0, 2, 4 h	Double wall cylinder with holes to allow acetylene to enter soil from interwall reservoir	4
Groffman, 1985	2.0 × 8 cm, septa on ends	In shade outside	10 kPa	0 and 6 h	Loose fit of soil core in tube	12
Robertson *et al.*, 1987	2.2 × 20 cm septa on ends	Lab, 20–22°C	5 ml (10– 15 kPa)	0 and 24 h[a]	Pumping with 50 ml syringe at 0 time and before each sampling	20
Klemedtsson, 1986 and Svensson *et al.*, 1985	3.2 × 10 cm inside larger sealed core	Lab, 15°C	10 kPa	5 and 15 h	Plastic core had holes punched in sides to foster gas exchange	15
Parkin *et al.*, 1987	1.7–20 cm[c] × 16 cm	Lab, 24–26°C	10 kPa	3, 6 and 18 h	Loose fit of soil core in tube, and automated pumping with syringe[d]	36
Ryden *et al.*, 1987	3.4 × 10 cm inside jar	*In situ*	5 kPa	0 and 24 h	No core support used; texture and roots maintained core integrity	5
Groffman and Tiedje, 1989a and Rice *et al.*, 1988	2.2 × 15 cm, septa on ends	Lab, 22°C[b]	10 kPa	2 and 6 h	Pumping with 30 ml syringe	20
Myrold, 1988	2.5 × 20 cm, septa on ends	*In situ*	10 ml	0 and 24 h	Pumping with 50 ml syringe at 0 time and before each sampling	10

[a] Random subset of 6 cores sampled at 4 h intervals to confirm linearity of response over the 24 h period.
[b] Rates corrected to *in situ* temperature using Q_{10} of 2 (Rolston *et al.*, 1984).
[c] Core diameter of > 4.2 cm recommended from this study to be the optimum for yielding the most reliable estimates of natural denitrification rate (Parkin *et al.*, 1987).
[d] Cores were taken with steel tubes and the intact soil core was then transferred to more loosely fitting plastic tubes for incubation.

A major limitation of the static core system is that the acetylene and N_2O gas distribution is not as efficient. Consequently, other accomodations must be made in the design to improve gas distribution. Table 4 summarizes the features used by various investigators as this static core method has evolved. Two points are important to minimizing the gas distribution problem. First, the core size is usually small both to aid gas distribution and to facilitate driving and handling of larger numbers of cores, and second, all designs (Table 4) have some feature, *e.g.* syringe pumping or exposed core sides, to improve gas distribution. The two other features in the evolution of the static core method are also notable: the number of replicate cores per treatment has increased and the length of assay period has become shorter and more well defined. Both improvements are important to making the static core a more reliable and accurate denitrification assay method.

Recommended protocol. The features of the more recent method (Table 4) are sufficiently similar that any design following these general characteristics is probably acceptable. Development of the high sample capacity static core methods were initiated in the early 1980's by the Swedish group for the 'Ecology of Arable Land Project', and in our laboratory by Phil Robertson and Tim Parkin. Subsequently, Parkin has independently developed a more automated, high capacity protocol (Parkin, 1985; Parkin *et al.*, 1988; Parkin *et al.*, 1987) to support his statistical work.

For use of the static core there are certain features and precautions that warrant discussion. Soil cores have most commonly been taken in a plastic tube housed inside of a steel tube that is driven into the soil. Parkin, however, has recently collected the soil in a steel tube sampler and then transferred the soil core to a slightly larger diameter plastic tube for incubation (Parkin *et al.*, 1987). The loose fit allows acetylene and N_2O to mix more readily along the walls of the core reducing the length of the gas diffusion path into soil. This approach will probably only work in soils that maintain structural integrity through the transfer. While cores of 2 to 4 cm diameter have been used (Table 4), Parkin reports that 10 to 15 kg of soil (> 4.2 cm diameter cores), gives the most reliable results because this sample size was necessary to reasonably sample the 'hot spots' of denitrification (Parkin *et al.*, 1987). However, cores of this size are more difficult to drive into soil and make it heavier to handle large core numbers. Thus, these practical considerations may outweight the slight increase in accuracy afforded by the larger core size.

Cores can be incubated in place, but most commonly are incubated in the laboratory. Both are acceptable methods, but if the core is transported to the laboratory they should be kept stored on ice during transit and held at 4°C. Before incubation, the cores are brought to the desired temperature, degassed if significant N_2O has accumulated to reduce the sensitivity of the measurement, sealed, and 10% (10 kPa) of acetylene added.

Acetylene from a cylinder should be scrubbed through a sulfuric acid train to remove the contaminating acetone (Tiedje, 1982). Acetylene generated from the reaction of carbide rock with water has no acetone and is free of other contaminants that might interfere with the denitrification assay (Hyman and Arp, 1987) (contaminants are produced from the water-carbide reaction but these are not substrates for denitrifiers nor inhibitors of denitrification at the concentrations produced). The most convenient way to foster acetylene distribution throughout soil is to create mass flow by alternately reducing and increasing pressure in the soil pore space, which can be accomplished by pumping with a large syringe. This pumping should be done immediately after the acetylene is added and prior to each gas sampling. Gas samples are taken by disposable plastic syringes and transferred to evacuated gas vials. We use 3-ml, preevacuated Venoject™ vials (Terumo Scientific, N.J.) but any vial used should first be checked for background N_2O and any other contaminants that interfere with the N_2O analysis. Samples of 4 ml are recommended for injection into this vial of ~ 3.3 ml capacity. This volume will not pop the stoppers and the vials can be stored for several months if sealed with silicon. If stored, vials should be surveyed to verify pressurization (*i.e.*, no leakage) prior to analysis. To be certain of the correct quantitation, a series of N_2O standards can be prepared and stored in the same manner. Satisfactory internal standards are not available; we have tried helium but it is too insensitive to detection by electron capture, requiring excessive dilution of the sample.

The N_2O (and CO_2) is analyzed by gas chromatography as described for the recirculation system. Because the static core system can lead to a larger number of samples (thousands), gas vial analysis becomes the rate limiting experimental step. At least three automated systems have been built for this purpose (Klemedtsson, 1986; Parkin, 1985; Robertson and Tiedje, 1985). We are also aware of one report where a commercial autosampler system was used to sample 1 ml vials in a denitrification study (Lowrance and Smittle, 1988).

Particularly important in the static core method is the time of sampling. We now recommend not to sample at 0 time but to use a later time to establish the initial point, after the acetylene is better distributed. If only two sampling times are used, shorter intervals are recommended, *e.g.* 2 and 6 h to insure that nitrate does not become rate limiting. If large sample analysis capacity is available, three sampling points are recommended to insure linearity.

To determine the N_2O produced, the total gas volume of the core and its water content are needed. The pore volume can be estimated from the total known volume of the core and the bulk density of the soil, which is estimated by measuring the length of the soil core and its dry weight. The moisture content is determined gravimetrically. The dissolved N_2O is calculated by using the Bunsen coefficient (Tiedje, 1982). An alternative used by Parkin is to inject a large volume of acetylene into the core prior to incubation and then to measure the pressure increase by means of a pressure transducer (Parkin *et al.*, 1987). This determines if there are leaks as well as allowing calculation of the total pore volume. The gas phase is then mixed by syringe and the excess pressure vented to atmospheric presure prior to the start of incubation. After incubation, the moisture content and soil dry weight are determined gravimetrically.

High spatial and temporal variability in denitrification rates necessitates that careful consideration be given to sampling strategy. Two ways of dealing with the variability problem are; i) taking a large number of samples and ii) accommodating the temporal and spatial variations characteristic of the site in the sampling design.

Taking a large number of samples requires using a static core technique. With this technique up to 200 cores can be dug, incubated, and headspace samples collected in one day. With a gas chromatograph equipped with two detectors and two column switching/backflushing valves, 30 samples an hour (200 per day) can be analyzed by manual injection. With an autosampler, even more analyses can be done. We completed weekly sampling of this type for 10 sites over an 80 km^2 area for several months (Groffman and Tiedje, 1989a).

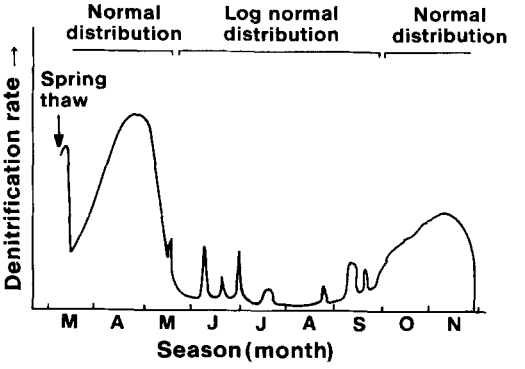

Fig. 3. A generalized diagram of the seasonal pattern of denitrification rates for the northern temperate soils. Peaks of activity in the summer are due to rainfall or to pockets of decaying organic matter. The frequency distribution of rates for these periods is also shown (Groffman and Tiedje, 1989a).

Considering temporal patterns of denitrification can aid in designing sampling strategies. For example, denitrification activity in temperate ecosystems often occurs during brief periods of high soil wetness and low plant activity in early spring and fall (Goodroad and Keeney, 1984; Groffman and Tiedje, 1989a; Myrold, 1988; Schmidt *et al.*, 1988). From our experience, a generalized seasonal pattern of denitrification in our region is illustrated by Fig. 3. Such a pattern may be used as a model for a sampling strategy in which the periods of greater activity are more intensively sampled and less active periods, *e.g.* the summer, are infrequently sampled. The seasonal pattern of variability (*e.g.* Fig. 3) also provides guidance in the number of samples needed.

As a minimum guideline we recommend taking 20 cores per site and 12 to 20 samplings per year planned to encompass periods of higher activity.

Denitrifying enzyme activity (Phase 1)

Background. The denitrifying enzyme assay (DEA), also known as the phase 1 assay, measures the concentration of functional denitrifying enzymes in

a sample at the time of sample collection (Smith and Tiedje, 1979; Tiedje, 1982). This assay does not measure the denitrifying activity of the natural sample, but the denitrifying enzyme concentration of that sample does reflect the environmental history of that site. This assay has been used in a comparative manner to characterize samples and to study experimental treatment effects on denitrification, but it has not been used to provide information on field denitrification rates. Recently, however, two new lines of evidence suggest that this assay may also be useful in field studies of denitrification.

In one study (Groffman and Tiedje, 1989b), found that DEA was strongly correlated with the measured annual denitrification N loss in forest soils of southern Michigan. Furthermore, this assay was found to correlate with soil texture and drainage characteristics of catenas and could be used to predict the denitrification N loss of these sites. Since this assay is more easily done than core assays, a larger number of sites can be sampled, perhaps improving large scale estimates of denitrification. The relationship of DEA to denitrification at this larger temporal and spatial scale may be revealing the effects of selection discussed earlier (p. 262). If so, this provides a rationale for why the DEA may be predictive of natural denitrification losses.

In a second study, Parkin and Robinson (1989) have used the phase 1 assay (DEA) in a stochastic model along with respiration rate to predict denitrification frequency distribution and mean rates. Their approach was based on the fact that a highly variable process, like denitrification, probably cannot be explained by a deterministic model. One of their stochastic models accurately predicted the frequency distribution as well as the mean denitrification rates. This study also illustrates how DEA offers potential for estimation of field denitrification rates.

Recommended protocol. This protocol is based on the phase 1 assay described by Smith and Tiedje (1979) and Tiedje (1982). The method described here includes some further improvements in components and in convenience.

The principle of the method is based on optimizing all requirements for enzymatic activity-saturation with nitrate, an electron donor, no oxygen, and no diffusion limitation—so that the rate of N_2O production is proportional to denitrifying enzyme content. The method can also be used to test whether one of the substrates is limiting by not adding that substrate to the assay.

Soil (25 g) is placed in a 125 ml-Erlenmeyer flask containing 25 ml of a solution of 1 m*M* glucose, 1 m*M* KNO_3, and 1 g/l of chloramphenicol. Chloramphenicol blocks protein synthesis, thus extending the period of linear N_2O accumulation. The flasks are capped with gas impermeable stoppers and made anaerobic by alternately flushing with argon and evacuating 4 times. Purified acetylene is added to the flask to achieve a final concentration of 10% (10 kPa) in the gas phase. Higher concentrations of acetylene should be used if the organic content and biological activity are unusually high (Yeomans and Beauchamp, 1978; Kaspar *et al.*, 1981). The soil slurries are incubated on a rotary shaker. Three replicates are recommended.

The headspace gas is sampled by syringe and the N_2O measured by gas chromatography as described above. At least four determinations should be made during the incubation period to establish linearity. The recommended incubation period is 1 h and should not go beyond 2 h. The dissolved N_2O which is substantial in this case, should be corrected for by using the Bunsen relationship (Tiedje, 1982). Samples can be stored in evacuated glass vials if they cannot be analyzed directly. In mixed, homogeneous, anaerobic soil, the coefficient of variation should be 5 to 15%. Higher variation may indicate incomplete anaerobiosis or natural patchiness of denitrifiers in soil.

In situ *soil cover*

Since *in situ* soil cover techniques are not extensively used in our laboratory, we will not recommend a protocol for their use. We will, however, review the different approaches to adding C_2H_2 to soil and provide some considerations for the development and use of cover methods.

The simplest approach to adding C_2H_2 to a soil cover system is to introduce C_2H_2 into the headspace of the cover. With this approach, the time required for C_2H_2 to diffuse throughout soil can be considerable, and the area of soil that will have

C_2H_2 concentrations sufficient to inhibit N_2O reduction is unknown. As discussed earlier, it is necessary to minimize the time that the cover is in place to avoid temperature and diffusion problems under the covers. Passive diffusion of C_2H_2 into soil can be enhanced by using a hollow 'double-wall' chamber design (Burton and Beauchamp, 1984), or by perforated tubes inserted into the soil which are connected to an above ground manifold through which acetylene flows (McConnaughey and Duxbury, 1986). The advantage of adding acetylene by diffusion is that no aeration changes are induced since mass flow of soil gas is avoided.

Ryden *et al.* (1979) and Ryden and Dawson (1982) developed a procedure where C_2H_2 is introduced into soil by radial diffusion from probes inserted into the soil. With this system, C_2H_2 concentrations required to inhibit N_2O reduction are established within 15 to 30 minutes, and denitrification rates can be measured over a 1 to 2 hour period. Air is continuously swept through the chamber and accumulated N_2O is trapped on molecular sieve, avoiding diffusion problems caused by N_2O buildup in the chamber. The main drawback with this system is the time and expense required to set-up the chambers in the field, which limits the numbers of replicates that can be run.

A third technique for introducing C_2H_2 into soil involves adding C_2H_2 saturated water to field chambers (Hallmark and Terry, 1985; Terry *et al.*, 1986). The major drawback of this technique is that the moisture addition decreases the oxygen status of the soil and thus increases denitrification, but it can be used to approximate irrigated soils or to simulate rainfall events. An additional problem is that C_2H_2 concentrations sufficient to inhibit N_2O reduction may not be maintained as the soil dries. Hallmark and Terry (1985) recommend using both C_2H_2 saturated water and radial diffusion to introduce C_2H_2 into irrigated soils.

At certain locations, most notably sites with acid soils, N_2O may be the natural terminal product of denitrification, eliminating the need for introducing C_2H_2 into soil. Spatial and temporal patterns of denitrification were characterized at an acid soil in Michigan using this approach (Christensen and Tiedje, 1988). The drawback to this approach rests on the uncertainty of whether N_2O reduction is uniformly inhibited and whether respiratory denitrification is the principal source of the N_2O (Robertson and Tiedje, 1987). While bulk soil pH and N_2O reductase activity may be low at a site, microsites of high pH and denitrification activity associated with decomposing plant material may have significant N_2O reduction.

Data analysis

Analysis of skewed data

Many of the difficulties inherent in denitrification research arise after measurements of denitrification rates have been made and appropriate methods to analyze the data are sought. These complexities in the analysis of denitrification data occur principally as a result of the tendency of the frequency distribution of rates to be much better approximated by the lognormal than by the normal distribution. Denitrification rates have been observed to be lognormally distributed when measured using surface chambers (Duxbury and McConnaughey, 1986; Folorunso and Rolston, 1984), in intact soil cores (Parkin *et al.*, 1985), or in anaerobically incubated soil slurries (Parkin *et al.*, 1987). Members of the denitrification group within the project 'The Ecology of Arable Land' have also observed that rates measured in intact soil cores are

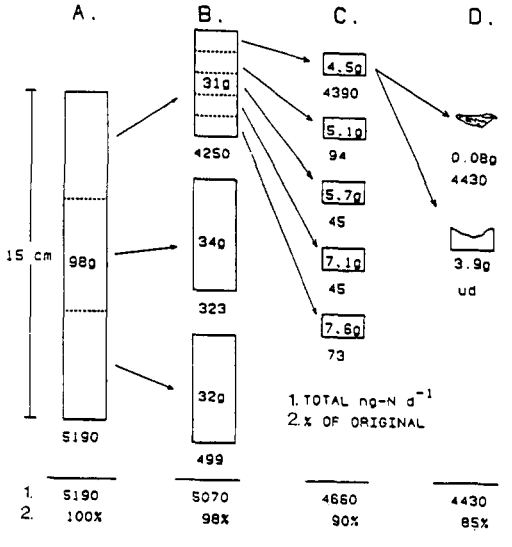

Fig. 4. Results from segmentation of a soil core to localize the active denitrifying sites. Reproduced from Parkin, 1987, Soil Sci. Soc. Am. J. 51, 1194–1199, by permission.

almost invariably better described by the lognormal than the normal distribution.

While the high variability of denitrification has long been recognized, and the skewed frequency distributions are now being realized, the underlying basis for these patterns had not been investigated until Parkin (1987) demonstrated that decaying particulate organic matter created 'hot spots' of denitrification. In one example (Fig. 4), he found that 85% of the denitrification activity of a 98 g soil core was found in a 0.08 g piece of decaying plant leaf. Such 'hot spots' would likely be non homogeneously dispersed, and thus would give rise to the observed lognormal frequency distributions (Parkin, 1987). The generalized seasonal pattern of denitrification (Fig. 3) consists of spring and fall periods when moisture, and probably carbon, are more plentiful (and uniform), and the frequency distribution is observed to be normal (Groffman and Tiedje, 1989a). However, in the summer particularly moisture is much more limited and the organic matter 'hot spots' are the only sites sufficiently depleted in O_2 to allow denitrification, thereby resulting in lognormal distributions for this period (Groffman and Tiedje, 1989a).

The possibility that rates of denitrification may be lognormally distributed requires that the first steps in analyzing denitrification rate data center on the testing of the rates for statistically significant departures from normality. The Kolmogorov-Smirnov test for goodness of fit is one tool for the identification of significant departures from normality. High positive skewness of the distribution of a set of denitrification rates is a strong indication that those rates may be closer to lognormal than normal. The statistical significance of suspiciously high coefficients of skewness can be determined through reference to tabulated values for this coefficient that are statistically significant indications of departures from normality for different sample sizes and at different levels of probability (Pearson and Hartley, 1958; Zar, 1974).

If denitrification data are found not to be normally distributed, the next step appropriate for their analysis would be to apply the same tests for normality to the logarithms of the rates. If the logarithms of the rates are found to be well approximated by the normal distribution, *i.e.*, if the rates are lognormally distributed, then the logarithmically transformed rates can be safely used for popular parametric statistical tests, such as analyses of variance and covariance or regression analyses, that provide accurate results only for normally distributed data. Thus, reliable evaluation of the significance of differences in denitrification rates between experimental treatments or of the significance of trends in denitrification rates is possible using familiar statistical tests even when those rates are lognormally distributed.

Best method to estimate mean and variance of denitrification rates. A surprisingly challenging problem is the accurate estimation of the true mean rate of denitrification from a limited number of rate measurements. Folorunso and Rolston (1984) have calculated that denitrification rates would have to be measured in more than 4000 samples to be able to calculate a value for the mean denitrification rate that was within 10% of the true mean rate for a 3×36 m experimental plot with highly variable, lognormally distributed rates. Parkin *et al.* (1988) have evaluated three different methods for calculating the mean from limited numbers of lognormally distributed measurements. The first of these methods, the arithmetic average, gave relatively efficient and unbiased estimates of the population mean. A maximum likelihood method based on a transformation of the mean and variance of the logarithms of the original measurements gave estimates for the mean that not only were less accurate than the arithmetic average but were also biased overestimates by as much as 73%. The problems of inaccuracy and bias with the maximum likelihood method were exacerbated by decreasing sample size or increasing skewness of the frequency distribution. A third method evaluated by Parkin *et al.* (1988) is a uniformly minimum variance unbiased estimator (UMVUE) that incorporates mathematical expressions that correct for the bias inherent in the maximum likelihood method. The UMVUE method was found to be the most accurate of the three techniques for the estimation of the mean of a lognormal population based on a limited number of samples. The superiority of the UMVUE method was most evident for small sample sizes ($n < 30$) from highly skewed distributions, and its use was recommended for such samples because the accuracy of the UMVUE method is sufficiently greater than that of the arithmetic average to justify the more elaborate calculations that it requires.

Parkin *et al.* (1988) also reviewed three methods for the estimation of population variance analogous to the three methods for mean estimation. They found, again, that the maximum likelihood estimate of population variance was the least accurate of the three methods for small sample sizes. The familiar formula for variance (the sum of the squared departures from the sample mean divided by one less than the sample size) was the least accurate of the three methods for larger sample sizes ($20 < n < 100$). The UMVUE method was the most accurate estimator of population variance for all sample sizes and different degrees of skewness examined by Parkin *et al.* (1988), and they recommended its use for all log-normal samples except for those of small size ($n < 20$) drawn from distributions of low skewness, *e.g.*, with a coefficient of skewness less than 2.

Geostatistical methods

Given the extreme variability of denitrification rates in the field, the integration of rates over space and time becomes a focus of concern. For this purpose, methods of data analysis taken from the body of theory known as geostatistics are gaining in frequency of use for denitrification research (Folorunso and Rolston, 1984; Folorunso and Rolston, 1985; Parkin *et al.*, 1987; Robertson *et al.*, 1988). One geostatistical technique, kriging, offers the ability to predict (with known estimation variance) denitrification rates at unsampled locations through optimal interpolation of rates at sampled locations.

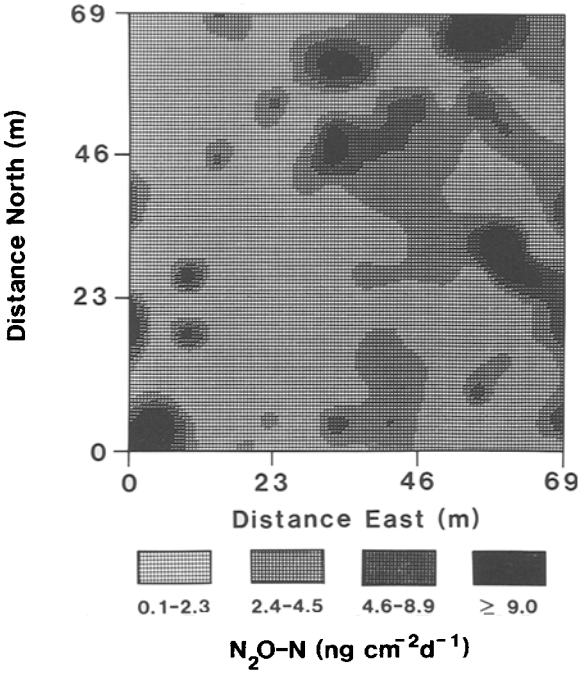

Fig. 5. Isopleth for denitrification across site derived by punctual kriging. The patterns show the variability of this process in this 0.5 ha old field. Reproduced from Robertson *et al.*, 1988, Ecology 69, 1517–1524, by permission.

For rates predicted by kriging to be more accurate estimates than the simple average of all the measured rates, the rates of denitrification must exhibit autocorrelation: a tendency for the variance of the rates at locations close to one another to be less than the variance of rates at widely separated points. Robertson *et al.* (1988) found that denitrification rates in their study exhibited marked spatial autocorrelation, and consequently they were able to use kriging to predict the rates over their 69 × 69 m study site at 1-m intervals as shown in Fig. 5. Denitrification rates do not always appear to be autocorrelated. Folorunso and Rolston (1984) found evidence of spatial autocorrelation of denitrification rates in only one out of twelve transects examined.

Attempts at temporal kriging

Previous efforts to apply geostatistical methods to denitrification research have focused on the use of kriging for the prediction of rates of denitrification at unsampled locations in space. In this section, geostatistical methods such as kriging are applied to the prediction of denitrification rates at unsampled points (dates) in time. For the sake of brevity, the following discussion of temporal kriging assumes some familiarity with the general techniques and terminology of geostatistics. Good general introductions to geostatistics include Journel and Huijbregts, 1978; Vieira *et al.*, 1983; and Webster, 1985.

The massive data base on denitrification rates that has been assembled by members of the project 'The Ecology of Arable Land' is well suited for the evaluation of temporal kriging. The denitrification group measured denitrification rates in three dif-

ferent crops (grass, barley, and lucerne) in two positions relative to the crop plants (within and between rows), and these measurements were carried out at relatively frequent intervals over two entire field seasons (1982 and 1983). We are, thus, in a position of being able to analyze these data to determine whether rates of denitrification are temporally autocorrelated in all of these different field treatments. If evidence of autocorrelation is observed, it then would be possible to examine the stability of the semivariograms from year to year and from crop to crop.

The inhibition of nitrous oxide reductase by acetylene as described by Klemedtsson (1986) was used for the measurement of the rates of denitrification in intact soil cores. For all of the following analyses, common logarithms of the rates of denitrification measured in individual cores were taken, and then the logged rates were averaged. The rates were logged because members of the denitrification group of the project had determined that the rates were almost invariably better described by the log-normal than the normal distribution. These daily logged rates were averaged separately for the three different crops and the two different row-positions.

As an initial evaluation of the extent to which temporal autocorrelation was present in the (averaged logged) rates of denitrification in these studies, semivariograms were constructed for each of the field treatments for both years when full field-season data were collected. No evidence of temporal autocorrelation was evident in the semivariograms for denitrification rates in the barley or lucerne treatments in either year whether between or within crop rows; the semivariograms were flat showing a pure nugget effect (Journel and

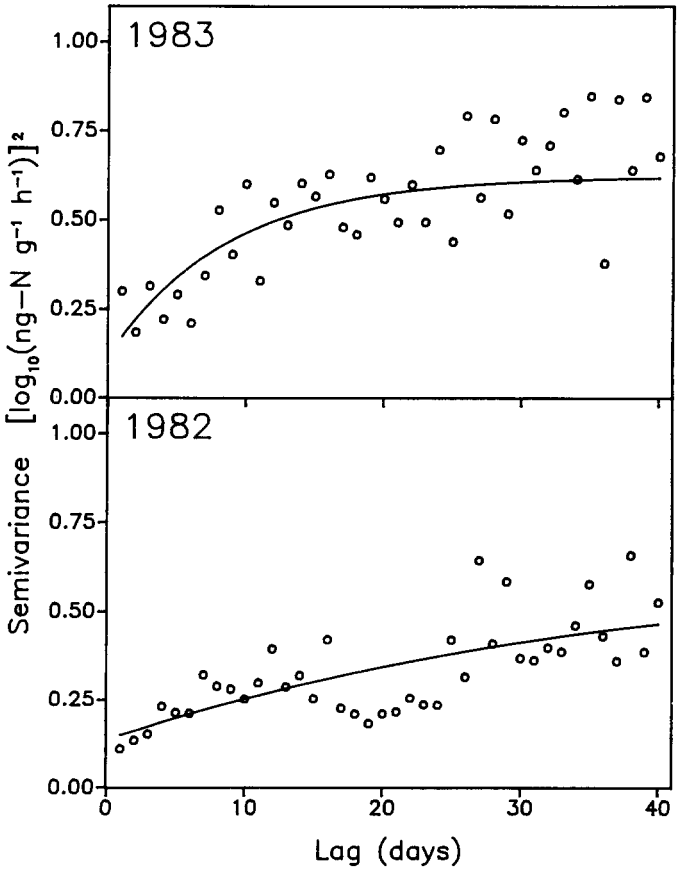

Fig. 6. Semivariograms constructed from logarithmically transformed denitrification rates measured within rows of grass over the course of two field seasons. The smooth curves correspond to the exponential model for semivariograms fit to the experimental values for semivariance by weighted nonlinear regression.

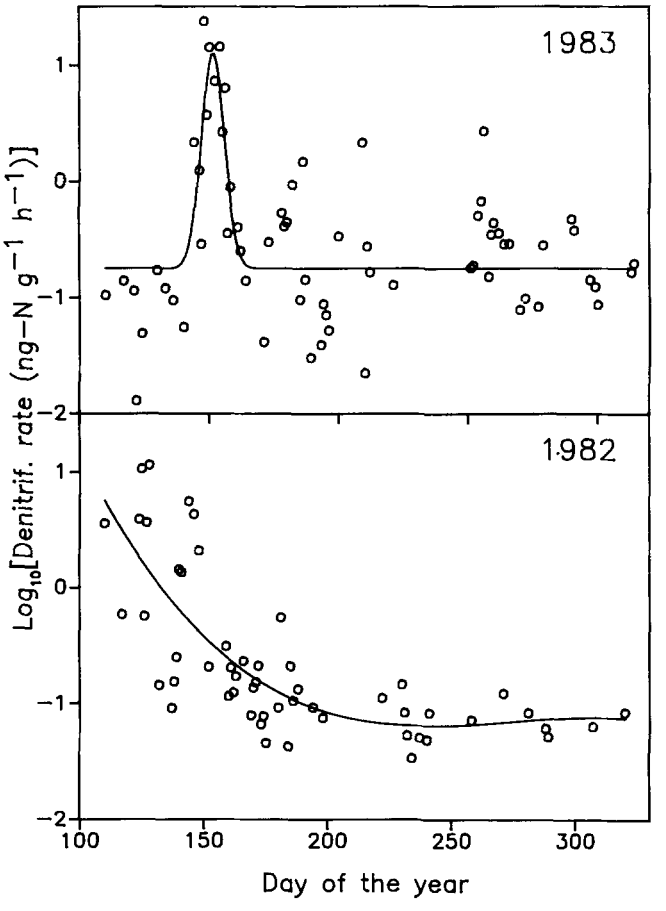

Fig. 7. Trends in logarithmically transformed denitrification rates measured within rows of grass over the course of two field seasons. The trends were removed from the data for subsequent analyses using the curves shown fit to the data.

Huijbregts, 1978; Webster, 1985). Rates of denitrification measured between rows of grass also failed to show evidence of temporal autocorrelation. In contrast, temporal autocorrelation did appear to be present in denitrification rates measured within rows of grass during both 1982 and 1983. Semivariograms calculated from these rates are shown in Fig. 6. The smooth curves shown are fits of the exponential model (Journel and Huijbregts, 1978; Webster, 1985) to the experimental values for semivariance. The models were fit using weighted nonlinear regression in which the weights corresponded to the number of pairs of data used at each value for lag. The observed tendency for semivariance to increase with increasing lag implies that measurements of denitrification made close together in time tended to be more similar to one another than were measurements separated by a long period of time.

To determine whether trends in the denitrification rates within rows of grass were responsible for the observed autocorrelation in the rates, the data were analyzed by curvilinear regression to determine whether denitrification rate varied through either the 1982 or 1983 field season as a simple linear or polynomial function of time. A significant ($P < 0.001$) fit of a cubic polynomial to the logarithms of the rates of denitrification was found for the data collected in 1982 but not for data from 1983. The lower half of Fig. 7 shows the polynomial of best fit and the daily averages of the logarithms of the denitrification rates measured during the 1982 field season. Sixty percent of the variation in the logarthmically transformed rates

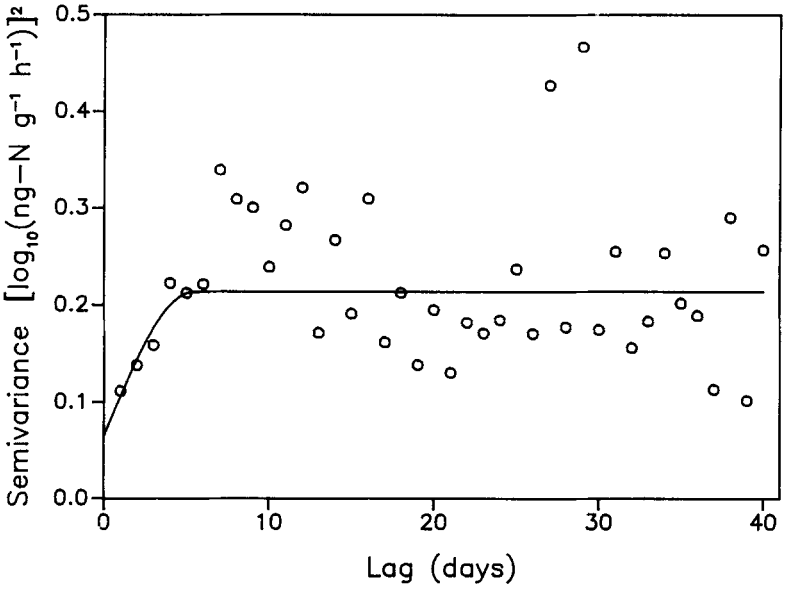

Fig. 8. Semivariogram constructed from the residuals left by a fit of a cubic polynomial function of time to logarithmically transformed denitrification rates measured within rows of grass during 1982. The smooth curve shown corresponds to the spherical model for semivariograms.

measured in 1982 could be attributed to the trend.

Although a simple polynomial failed to provide a statisticaly justifiable fit to the denitrification rates from 1983, a trend can be observed in these data in that a clear peak in activity occurs at about day 150. This peak followed an addition of 120 kg per ha of fertilizer N (Klemedtsson, 1986). Because the peak of denitrification activity could almost be predicted as a consequence of fertilizer addition, it seemed ill-advised to regard the data as stationary, *i.e.*, lacking a trend. Consequently, a model was arbitrarily chosen that had the right general shape to fit the peak, and this model was fit by nonlinear regression to the data. This bell-shaped model (the shape is the same as the normal distribution) is shown fit to the data from 1983 in the upper half of Fig. 7. The model explained a significant ($P < 0.001$) portion (53.9%) of the variability of the logarithmically transformed denitrification rates. Significant trends, thus, appeared to be present in the rates of denitrification measured within rows of grass in both years of the study.

To test for the presence of autocorrelation, semivariograms were constructed for the residuals left by the models used to account for the trends in the rates of denitrification within rows of grass. The semivariogram for the residuals of the model fit to the 1983 data (not shown) was flat offering no indication of temporal autocorrelation. In fact, this semivariogram indicated that rates of denitrification measured one day apart tended to be more dissimilar to one another than rates measured at any more widely separated intervals up to one month. In contrast, the residuals left by the polynomial model used to remove the trend in denitrification rates in grass rows during 1982 appeared to be autocorrelated. A semivariogram constructed from this data appears in Fig. 8 with a fit of the spherical model (Webster, 1985). Two consequences of the removal of the trend in the 1982 data can be seen from a comparison of Fig. 8 with Fig. 6. First, the range over which autocorrelation appears to exist is greatly diminished by the removal of the trend. In addition, detrending has the expected effect of reducing the maximum value for semivariance achieved at the longest lags.

Autocorrelation appeared to be present in the denitrification rates measured between crop rows in grass leys during 1982 even when a trend in those data was removed with a cubic polynomial. The presence of autocorrelation offers an opportunity to use kriging to predict denitrification rates on unsampled dates with more accuracy than would be possible using only the polynomial model. It

seemed of potential value to evaluate the accuracy of those predictions. Accordingly, kriging was used in a jackknifing procedure (Vieira *et al.*, 1983) to predict detrended values of denitrification for all the days when measured values were available. These predicted values were compared to the measured, detrended values, and it was determined that the kriged predictions could account for only 15.2% of the variation in denitrification rates after the polynomial trend had been removed. This reduction in unexplained variation is particularly unimpressive when it is compared to the variation explained (60%) by the polynomial trend in the 1982 data.

The inability of kriging to produce more accurate estimates for the 1982 study in the grass ley can be at least partially attributed to the sampling pattern. Although it is not readily apparent in Fig. 7, denitrification rates were measured within rows of grass during 1982 on many dates that were not within 3 days of another sampling date. Unfortunately, no autocorrelation appears to be present in the detrended rates of denitrification for rates measured 4 or more days apart (Fig. 8). Kriging would not be expected to provide good estimates for rates on dates separated by 4 or more days from the nearest sampling date because the kriging predictions are based on a weighted average of the measured rates in which more weight is given to rates measured on dates close enough to have autocorrelated rates. Thus, for dates separated by 4 or more days from the nearest date of sampling kriging generates a prediction that is nothing more than the unweighted average of all the data except for the rate (temporarily deleted for jackknifing) actually measured on the date for which the prediction is to be made.

The principal conclusion that can be drawn from our analyses of data on denitrification collected through time is that geostatistics does not appear to offer immediately useful tools for the analysis of this kind of data. Supporting this conclusion was our inability to find any evidence of temporal autocorrelation for rates measured in two (barley and lucerne) out of the three crops used by the denitrification group within the project 'Ecology of Arable Land'. Moreover, rates in grass leys that were measured between (rather than within) plant rows also failed to show any evidence of temporal autocorrelation. The rates measured within rows of grass showed superficial autocorrelation. For rates measured during 1983 this superficial autocorrelation was entirely attributable to a nonlinear trend resulting from fertilizer addition. During 1982, denitrification rates within grass rows also showed a strong trend, but there appeared to be evidence of autocorrelation in the rates that was not directly attributable to the trend. This autocorrelation permitted estimates to be generated using kriging that were more accurate than those offered by the detrending regression alone, but the improvement was very small in a quantitative sense. The modest utility of geostatistics in analyzing the data from only one of three crops, in only one of two sampling positions relative to plant rows, and in only one of two years does not encourage one to believe that geostatistical techniques will play a major role in future studies of the variation of denitrification rates measured through time.

Conclusions and recommendations

1. Acetylene and ^{15}N based methods as well as soil core and *in situ* cover sampling methods all give comparable estimates of field denitrification rates. Some of the methods are better suited for particular objectives or sites, but all have been proved to be sound methods for measurement of terrestrial denitrification.

2. The difficulty in quantifying denitrification lies not with the methods, as they accurately measure the process, it is due to the dynamic nature of the process that causes high temporal and spatial variability. Because of this, improvements in quantitation of denitrification are more likely to come from better approaches to analyze, model, and predict the variability than from further work on methodology. It's doubtful, however, that denitrification budget estimates will ever approach the accuracy of most other biogeochemical cycle measurements.

3. In studies of denitrification, perhaps we have too often asked an inappropriate question, namely 'How much nitrogen is lost by denitrification?'. While it is an important question, it can divert too many resources in one direction if that question is too difficult to answer and especially if the same approaches are repeatedly used. In our view, greater opportunities for the future lie in research

at scales other than the traditional field plot and on questions that have been relatively ignored. Particularly important are studies at both larger (landscape, regional) and smaller (microsites, organism, enzyme, gene) scales. We have illustrated examples of the concepts and approaches for work at some of these scales elsewhere (Groffman *et al.*, 1988). Important future opportunities also lie in understanding how denitrification is regulated at the molecular level and how these mechanisms are coupled to the environmental triggers at the microsite. On balance, we believe understanding of denitrification would benefit from more diversity in the questions asked and the scales studied.

Acknowledgements

We particularly thank all those denitrification researchers at Michigan State University who have helped in the evolution of the methods described here: in approximate chronological order they are: Mary Firestone, Scott Smith, Henry Kaspar, Tim Parkin, Alan Sexstone, David Myrold, Phil Robertson, Chuck Rice, and Søren Christensen. We also thank Leif Klemedtsson and Bo Svensson for the Arable Lands denitrification database that was used in the attempt at temporal kriging. The support for this research was provided by the U.S. National Science Foundation.

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