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Denitrification

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Denitrification is the reduction of the nitrogen oxides, nitrate (NO_3^-) and nitrite (NO_2^-), to the gases nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen (N_2). The process is carried out mainly by facultative anaerobes, i.e., organisms that normally use oxygen (O_2) to accept electrons during respiration but in its absence can use nitrogen oxides as electron acceptors. Most denitrifying bacteria are heterotrophs, using organic carbon compounds as a source of energy.

Denitrification is important in ecosystems for several reasons. First, removal of inorganic nitrogen by denitrification can influence the productivity of plants because their growth is frequently limited by nitrogen (Vitousek and Howarth 1991). Second, denitrification is important to water quality. Nitrate is a federally listed drinking water pollutant (Keeney 1987) and is an agent of eutrophication in marine ecosystems (Ryther and Dunstan 1971). Denitrification in soils, wetlands, streams, and groundwater can prevent movement of NO_3^- from intensive upland land uses into aquatic ecosystems. Third, N_2O, one of the gaseous products of denitrification, is a "greenhouse" gas that can influence the earth's radiative budget and plays a role in stratospheric ozone destruction (Prather et al. 1995). Finally, anaerobic metabolism is responsible for a significant portion of energy flow in many soils and wetlands. Denitrification is the most energetically favorable form of anaerobic metabolism, allowing for rates of energy generation close to those in aerobic metabolism (Thauer et al. 1977), and thus is essential to the overall microbial function of anaerobic (i.e., wet) soils.

Denitrification is a difficult process to measure. Methods for measuring denitrification are flawed because they either change substrate concentrations, disturb the soil physical environment, lack sensitivity, or are prohibitively costly in time and

expense (Tiedje et al. 1989). The quantification of denitrification has also been hindered by high spatial and temporal variation in the field. This variation is especially problematic given the lack of methods amenable to the collection of large numbers of samples with reasonable expenditures of time and money.

Available Protocols

The difficulty with measuring denitrification stems from the fact that it is hard to quantify either the production of the terminal end product of denitrification (N_2) or the specific depletion of the substrate (i.e., NO_3^-). It is difficult to measure production of N_2 because of the already high atmospheric concentration of this gas. It is difficult to quantify denitrification by measuring decreases in NO_3^- because this ion is also consumed by plants, heterotrophic microbes, dissimilatory reduction to NH_4^+, leaching, and runoff, and is produced by nitrification.

Like many biological processes, denitrification exhibits high spatial and temporal variability. Rates of denitrification in the field frequently vary over two or three orders of magnitude in both time and space in a wide variety of environments (e.g., Foloronuso and Rolston 1984; Robertson and Tiedje 1985, 1988; Burton and Beauchamp 1985; Robertson et al. 1988; Parkin 1987; Starr et al. 1995). Total soil denitrification is often dominated by very high rates of activity in very small activity centers (hot spots) where O_2 is low and NO_3^- and carbon availability are high (Parkin 1987; Christensen et al. 1990; Murray et al. 1995). High variability hinders quantification of field rates, comparisons of treatments, and evaluation of different methods.

The Acetylene (C₂H₂) Inhibition Method

A major development in denitrification research was the discovery that acetylene inhibits the reduction of N_2O to N_2 (Balderston et al. 1976; Yoshinari and Knowles 1976), making N_2O the terminal product of denitrification. Quantifying denitrification by measuring production of N_2O in the presence of acetylene is relatively easy because of the low atmospheric concentration of N_2O and the availability of sensitive detectors for this gas. Since 1980 acetylene inhibition has been the most common method used to quantify denitrification (Tiedje et al. 1982; Keeney 1986; Tiedje et al. 1989; Nieder et al. 1989; von Rheinbaben 1990; Payne 1991; Aulakh et al. 1992).

Although acetylene-based methods have been widely applied, they have serious drawbacks. Perhaps the most critical problem is that acetylene inhibits the production of NO_3^- via nitrification (Hynes and Knowles 1978; Walter et al. 1979; Mosier 1980). Inhibition of nitrification can lead to underestimation of denitrification rates as NO_3^- pools become depleted during incubations in the presence of acetylene. This problem is especially critical in natural ecosystems, where NO_3^- pools are often inherently low.

Other (less critical) problems with acetylene methods arise from the difficulty of getting acetylene to diffuse to active denitrification sites in soil (Ryden et al. 1979;

Jury et al. 1982; Parkin et al. 1984); from the effects of acetylene on soil carbon metabolism (Yeomans and Beauchamp 1982; Terry and Duxbury 1985; Topp and Germon 1986; Flather and Beauchamp 1992); from the inhibition of chemoautotrophic oxidations (in addition to nitrification) that can provide energy to denitrifiers (Payne 1984); and from the contamination of acetylene with other gases that can affect denitrification (Hyman and Arp 1987; Gross and Bremner 1992). A more critical (but quantifiable) problem is the failure of the inhibition of N_2O reduction at low NO_3^- concentrations (Oremland et al. 1984; Slater and Capone 1989; Seitzinger et al. 1993).

Direct Flux Methods

Although it is difficult to directly measure the fluxes of denitrification substrates or products as discussed earlier, direct flux techniques have application in certain cases. These techniques are particularly useful in situations where the use of acetylene is inappropriate.

Measuring depletion of NO_3^- can be used as a quantification of denitrification when other possible fates of NO_3^- have been either measured or eliminated. These techniques are thus limited to specific laboratory applications.

Production of nitrogen gases has been measured to quantify denitrification in laboratory studies with artificial atmospheres (e.g., without N_2; Seitzinger et al. 1980, 1993; Swerts et al. 1995). The problem inherent in all direct N_2 flux methods is reducing the background level of N_2 gas sufficiently that rates of N_2 production by denitrification are detectable. This can require unacceptably long preincubation times and/or complex laboratory equipment (Aulakh et al. 1991).

Devol (1991) developed a technique for direct field measurement of N_2 production from marine sediments. This technique is based on measuring the accumulation of N_2 gas dissolved in water within a field chamber placed on the sediment surface and may be applicable to flooded soils.

A final approach to direct flux measurement of denitrification is the quantification of changes in $Ar:N_2$ ratios, i.e., a decrease in this ratio is used as evidence of denitrification (Wilson et al. 1990; Martin et al. 1995). These methods are not very sensitive unless a mass spectrometer is used to quantify the $Ar:N_2$ ratio. A mass spectrometer can also be used for very sensitive direct measurement of N_2 production (Thomas and Lloyd 1995).

It is important to note that all direct N_2 flux methods are rather cumbersome, limiting the number of replicate samples that can be run at any one time. This limitation is important given the high spatial and temporal variability of denitrification.

^{15}N Balance Methods

Balance methods are based on tracing the movement of $^{15}NH_4^+$ or $^{15}NO_3^-$ into different ecosystem pools and processes (plants, volatilization, leaching, runoff, soil inorganic and organic pools). In these methods, denitrification is quantified as the ^{15}N unaccounted for at the end of the experiment (Rolston et al. 1979; Parkin et al. 1985; Mosier et al. 1986). This estimate of "unaccounted for N" includes the accu-

mulated errors associated with estimates of the other pools and processes and is thus not very accurate or precise. The precision of this estimate is also limited by how well other loss processes (leaching, runoff, volatilization) are controlled or quantified.

In addition to accumulated error problems, there are also questions about how well added ^{15}N simulates the behavior of soil N. Although added inorganic ^{15}N is likely a good surrogate for fertilizer N, it may not be a good tracer of nitrogen in soil organic matter and microbial biomass. A final concern, common to ^{15}N methods in general, is that addition of ^{15}N can significantly enrich the nitrogen pools under study, leading to artificially high rates of activity.

$^{15}N_2$ Flux Techniques

Techniques have been developed to trace the movement of ^{15}N added to soil into gaseous denitrification products. These techniques generally require high enrichment of soil inorganic nitrogen pools with ^{15}N and thus have primarily been used in situations where soil nitrogen levels are already relatively high (Siegel et al. 1982; Mulvaney 1984; Mosier et al. 1986). However, recent improvements in mass spectrometer techniques have made it possible to make measurements with very low, tracer-level additions of ^{15}N (Brooks et al. 1993). As with ^{15}N balance approaches, although $^{15}N_2$ flux methods can reliably trace fertilizer-derived fluxes, their ability to depict fluxes of nitrogen associated with soil organic matter turnover is less certain (Nielsen 1992).

Perhaps the most important constraint on the use of $^{15}N_2$ flux techniques is that they are expensive and time-consuming. Despite active research in this area (Arah et al. 1993; Avalakki et al. 1995), costs of ^{15}N and for mass spectrometer analysis are high, and sample preparation and collection techniques are time-consuming. These cost and time constraints limit the number of flux measurements that can be made, which is a serious problem given the high spatial and temporal variability of denitrification.

Sample Type—Cores Versus Chambers

The need to add acetylene to soil in a controlled atmosphere motivated the use of extracted soil cores in denitrification research. However, the use of cores creates disturbance effects that are difficult to quantify. The alternative to extracted cores is a chamber method, where chambers are placed over the soil surface and the accumulation of N_2O is measured in the air space under the chamber or in a stream of air circulating through the chamber. A variety of methods have been developed for introducing acetylene to infield chambers (Ryden et al. 1979; Burton and Beauchamp 1984; Hallmark and Terry 1985). The main advantage of chamber methods is that they allow for infield measurement of actual fluxes of nitrogen gases from soil to the atmosphere.

There are several problems with chamber methods for measuring denitrification. Physical processes that inhibit diffusion (e.g., wet and/or fine-textured soils) inhibit the movement of acetylene and N_2O into and out of sites of denitrification activity

in soil. Jury et al. (1982) reported that several weeks of monitoring may be required to accurately assess production of nitrogen gases associated with a particular rainfall or irrigation event. Gas diffusion problems can be easily overcome with core methods, however, either by using forced-air-flow recirculation systems (Parkin et al. 1984) or by thorough mixing of the air space of the soil core, e.g., with a large syringe (Robertson et al. 1987; Groffman and Tiedje 1989). Other problems with chambers relating to pressure, concentration, and temperature changes within the chamber can be accounted for with proper chamber design (Mosier 1989; see Chapter 10, this volume).

Detailed comparisons of core versus chamber approaches have shown that cores produce accurate measurements of soil-atmosphere gas fluxes, except when cores are held for long periods (several days) before incubation (Burton and Beauchamp 1984; Ryden et al. 1987; Aulakh et al. 1991; Dunfield et al. 1995). Ryden et al. (1987) found a very strong relationship between denitrification rates in cores versus chambers, over a wide range of denitrification rates, during 24 hour incubations. In very wet soils, cores were superior to chambers due to the difficulty of introducing acetylene into, and 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, whereas chamber measurements can be expensive and time-consuming, limiting the number of replicates and/or sites that can be analyzed. Dunfield et al. (1995) found that extracted cores produced very similar estimates of soil-atmosphere N_2O and CH_4 fluxes as in-field chamber and soil gas concentration/diffusion flux methods.

Measurement of Denitrification Potentials

The high variability and methodological problems associated with measuring denitrification have led many investigators to resort to measures of denitrification potential. A variety of measures of denitrification potential have been made, where amendments are used, frequently under slurried, laboratory conditions, to increase rates of denitrification above those occurring in nature and to reduce the variability of the process.

Of all measurements of denitrification potential, the assay of denitrification enzyme activity (DEA) developed by Smith and Tiedje (1979) is the most common. In this assay, all limiting factors of denitrification (O_2, NO_3^-, C) are present in excess, growth is inhibited (by addition of chloramphenicol), and the nitrogen gas production measured (usually N_2O in the presence of acetylene) is a function only of the level of enzyme present in the sample.

It was originally hoped that DEA would be strongly related to actual denitrification activity because, in culture at least, the denitrifying enzymes are strictly inducible (Payne 1981). However, DEA has been found to be poorly related to hourly or daily denitrification rates due to the persistence of viable but inactive enzymes in soil (Smith and Parsons 1985; Groffman 1987; Martin et al. 1988; Parsons et al. 1991). However, the DEA assay has proven very useful for comparison of soils, ecosystems, and treatments because it responds well to longer-term variation in

the factors that control denitrification (soil water, NO_3^- availability, carbon availability).

Recommended Protocols

Although many studies have compared different methods of measuring denitrification, there have been few conclusive results (Tiedje et al. 1982; Keeney 1986; Tiedje et al. 1989; Nieder et al. 1989; Payne 1991; Aulakh et al. 1991, 1992; Beauchamp and Bergstrom 1993; Tiedje 1994; Mosier and Klemedtsson 1994). In most cases, high variability has made it difficult to determine differences among techniques. As a result, it is difficult to produce a "consensus" recommended protocol.

We recommend two approaches for assessing denitrification, one for quantification of denitrification potential (DEA) and one for measurement of actual denitrification nitrogen flux (an acetylene-based, static core method). Although we have a high degree of confidence and consensus about the DEA method for quantifying denitrification potential, our recommendation for quantification of actual denitrification nitrogen flux comes with considerable reservations given the problems with acetylene-based methods described earlier. Our recommendation is based on the fact that many studies have used this core method, in a wide range of ecosystems, and several methodological comparisons/validations have been performed (Burton and Beauchamp 1985; Tiedje et al. 1989; Christensen et al. 1991; Aulakh et al. 1991; Groffman et al. 1993b). The method was designed to allow for large numbers of samples to be run simultaneously, and it is thus suitable for ecosystem and landscape-scale studies. However, investigators should be aware of the problems with this method and should be alert for new methodological developments. We did not select a chamber-based method because the problems with chambers (described previously), especially the fact that the number of chamber incubations that can be run at one time is relatively small, outweigh their advantages.

Denitrification Potentials—Denitrification Enzyme Activity

The objective of the denitrification enzyme assay is to measure the maximum activity of the biomass of enzymes present in soil at the time of sampling. In this assay all limiting factors of denitrification (O_2, NO_3^-, C) are removed, growth is inhibited (by the addition of chloramphenicol), and the nitrogen gas produced is measured as the accumulation of N_2O in the presence of acetylene.

Materials

1. Flasks that can be sealed with airtight stoppers, e.g., Corning no. 5020 125 mL Erlenmeyer flasks with an Aldrich no. z12468-0 25.5 mm rubber septa
2. Media capable of providing NO_3^- (100 mg N kg^{-1}), dextrose (40 mg kg^{-1}) and chloramphenicol (10 mg kg^{-1}). The concentration of the media will vary depending on expected activity (see later).

3. Purified acetylene. Commercially available "laboratory" or "welding" grade acetylene can be purified to remove acetone and other contaminants by passing it through two concentrated H_2SO_4 traps and a distilled water trap in sequence. Protocols for this purification are described in detail in Hyman and Arp (1987) and in Gross and Bremner (1992). Relatively clean acetylene can also be produced by adding water to calcium carbide (CaC_2) in an evacuated flask. Water reacts with the CaC_2 to produce acetylene. Caution must be taken to avoid adding too much water to a large amount of CaC_2 because the reaction can be explosive.

4. A gas manifold capable of evacuation (700 mm Hg) and flushing with an O_2-free gas such as N_2

5. A rotary shaker table capable of maintaining 125 rpm

6. Syringes (disposable, 1, 5, or 10 mL) to add acetylene to flasks and to take gas samples from flasks

7. Airtight storage vials for gas samples and standards. Investigators have used a variety of vials to store gas samples, including commercially available blood collection tubes (e.g., Vacutainer or Venoject), headspace autosampler vials, and polypropylene syringes. With any vials, there can be contamination, leakage, or absorption problems that should always be checked for with blanks and spikes. See Chapter 10, this volume, for more detail on these problems.

8. A gas chromatograph equipped with an electron capture detector

Procedure

1. Weigh sieved field moist soil samples (two to three analytical replicates per sample) into flasks and add media (e.g., 25 g soil, 25 mL media). The weight of soil and the amount of media are varied (by trial and error) with the expected activity of the samples. The objective of this variation is to ensure that N_2O concentrations in the headspace of the flask stay within the range of the standard curve used in the gas chromatographic analysis (e.g., 0.3–50 ppm).

2. Seal flasks with stoppers and make soils anaerobic by repeated evacuation and flushing with oxygen-free gas (e.g., N_2 or Ar). We recommend at least three cycles of flushing (1 minute) followed by evacuation to 700 mm Hg vacuum. Flasks should then be brought to atmospheric pressure.

3. Add acetylene to 10% of the volume of the headspace of the flask. Incubating slightly pressurized flasks prevents contamination with laboratory air during sampling and the development of negative pressure in the flasks from sample removal.

4. Incubate the flasks at 125 rpm on a rotary shaker at constant temperature.

5. Take gas samples at 30 and 90 minutes and store them in evacuated, airtight storage vials. A 60 minute sample is recommended but optional.

6. Analyze gas samples for N_2O by gas chromatography. The most common method is to use an electron capture detector at 350 °C with a Porapak Q, 80/100-mesh column (2 m × 0.32 cm), with a carrier gas of 95% Ar/5% CH_4 at a flow rate of between 10 and 40 mL m^{-1}, with an oven temperature of be-

tween 25 and 50 °C. See Chapter 10, this volume, for more details on N_2O analysis.

Calculations

The basic calculation to quantify the amount of N_2O produced by the soil involves multiplying the concentration of N_2O in the headspace of the flask at 30 and 90 minutes by the volume of the headspace and then dividing by the dry weight of soil:

$$DR = [(C_{90} \times H) - (C_{30} \times H)]/(D \times T)$$

where

DR = denitrification rate, expressed as $\mu g\ N \cdot kg\ soil^{-1} \cdot h^{-1}$

C_{30} = N_2O concentration at 30 minutes, expressed as $\mu g\ N_2O–N/L$ headspace (see Chapters 10 or 13, this volume, for formula to convert ppm_v or $\mu L\ N_2O/$ L headspace to $\mu g\ N_2O–N/L$ headspace)

C_{90} = N_2O concentration at 90 minutes, expressed in same way as C_{30}

H = flask headspace volume (it is necessary to account for removal of air by sampling). Volume (L) can be calculated as total flask volume less added media volume less soil volume. Soil volume can be calculated based on bulk density.

D = soil dry weight

T = time (duration) of incubation, expressed as h, e.g., 1 h for samples taken at 30 and 90 minutes

It is necessary to account for N_2O dissolved in solution using Bunsen coefficients that predict the amount of gas dissolved in the liquid phase from the concentration in the gas phase (Moraghan and Buresh 1977; Wilhelm et al. 1977):

$$M = C_g \times (V_g + V_l \times \beta)$$

where:

M = total amount of N_2O in the water plus gas phase

C_g = concentration of N_2O in the gas phase

V_g = volume of the gas phase

V_l = volume of liquid phase

β = Bunsen coefficient (1.06 at 05 °C; 0.882 at 10 °C; 0.743 at 15 °C; 0.632 at 20 °C; 0.544 at 25 °C; 0.472 at 30 °C)

In a shaken assay such as this, it is safe to assume that liquid- and gas-phase N_2O are in equilibrium (i.e., that the Bunsen coefficients are accurate). Total N_2O production values can be converted to an areal basis using bulk density values (see Chapter 4, this volume).

Special Considerations

1. Sampling depth varies with site and experimental objectives. For site comparison work, it is important to sample the soil profile to encompass the most

biologically active zone of the soil (e.g., 0–20 cm). As with all biological activities, activity can be highly stratified in the soil profile, with the 0–2 cm or 0–5 cm depth having much higher activity than lower depths. On the other hand, low, but significant, activity can occur to relatively great depth in the soil profile (e.g., 2 or 3 m in some tropical soils), which in aggregate can be more important than surface soil activity.

2. Recently, there has been concern that chloramphenicol may inhibit the activity of existing denitrification enzymes (Brooks et al. 1992); the effect varies with soil type (Wu and Knowles 1995; Pell et al. 1996). We recommend periodically testing for this effect by running very short term (30 minute) assays with and without chloramphenicol. This testing is especially important for comparisons across different experimental sites.

3. Analytical variability (i.e., variation of samples taken from the same bag of well-mixed soil) for the DEA assay ranges from 10% to 20%. Field variability (i.e., variation of different samples from the same plot) ranges from 25% to 75%.

4. Temporal variability of DEA is much less than for actual denitrification rate. In north temperate forest ecosystems with well-distributed rainfall, six to eight sample dates during the snow-free season are sufficient to characterize this variability. In ecosystems with more marked seasonal changes in moisture (e.g., tropical dry forests), sampling should be stratified by season.

5. Sampling should not be done within 3–5 days of drying and rewetting events if possible (Groffman and Tiedje 1988).

Actual Denitrification Rate

Our recommended "static core" method has been used for ecosystem and landscape-scale denitrification studies for over 10 years (Robertson and Tiedje 1984; Groffman 1985; Robertson et al. 1987, 1988; Myrold 1988; Tiedje et al. 1989; Groffman and Tiedje 1989; Groffman et al. 1993a; Hanson et al. 1994). In this method, 2 cm diameter × 15 cm long intact soil cores are taken in acrylic sleeves and sealed with rubber serum stoppers at both ends. The headspace of the cores is sampled at various time intervals to quantify the accumulation of gases. A pressure transducer is used to quantify headspace volume and to check for leakage of each core. This sampling design allows for highly replicated measurement of denitrification rates and related variables (water content, NO_3^- levels, porosity) on the same samples.

Materials

1. A 2 cm diameter punch auger capable of holding acrylic tube inserts. Several companies manufacture punch augers that hold 2.54 cm diameter acrylic tubing, although the tubing they sell with these samplers is often very thin-walled and not gastight. Gastight, more durable acrylic tubing usually can be purchased from local suppliers. Custom-made samplers, or 2 cm diameter "tube" or "Oakfield" samplers can also be used. Larger-diameter cores may produce

less variable estimates of denitrification rate in some cases (Parkin et al. 1987; Starr et al. 1995). However, sampling with large cores is much more labor-intensive.

2. Rubber stoppers capable of providing an airtight seal in the acrylic tubes. We use Aldrich no. z12468-0 25.5 mm rubber septa.
3. Purified acetylene (as described earlier)
4. Syringes for adding acetylene to core tubes (5 or 10 mL), for mixing acetylene into the soil core (30 or 60 mL), and for removing gas samples from the core tubes (5 or 10 mL).
5. Airtight storage vials for gas samples and standards (as described earlier)
6. A pressure transducer capable of measuring pressure changes induced by a 5 or 10 mL addition of air to the headspace of the core/tubes
7. A gas chromatograph equipped with an electron capture detector

Procedure

1. Intact soil cores (0–15 cm depth) are taken directly into, or are inserted into, the acrylic tubes. We recommend taking 10–20 replicate core samples from each field plot for a maximum of 200 cores per sampling date. Core tubes should be stoppered at the bottom only and stored upright. Incubations should be initiated within 24 hours, although some studies (Breitenbeck and Bremner 1987; Parkin et al. 1984) have shown that intact soil cores can be stored at 4 °C for up to 30 days without a significant effect on denitrification. Such stability cannot be assumed for any given soil, however.
2. To begin the incubation, cores should be sealed with rubber stoppers. Acetylene (to at least 10% of the volume of the headspace) should be added to the headspace of each core and mixed into the soil pores by repeated pumping with a 30 or 60 mL syringe.
3. The cores should be incubated at constant, field soil temperatures and sampled at least twice. For example, cores can be incubated for 6 hours, with duplicate gas samples removed from the headspace after 2 and 6 hours, or single samples removed at different times over the 6 hour incubation period. The headspace of the core should be mixed by repeated pumping with a syringe prior to each sampling. Note that it is important to account for the amount of air removed by each sampling. If the headspace is small relative to the sample, negative pressure develops in the headspace.

   The rate of N_2O production between 2 and 6 hours is taken as the rate of denitrification. The 2 hour lag period before initial sampling ensures that acetylene has diffused into soil pores. It is necessary to run time-course experiments to determine that rates of gas production between the initial and final samples are linear (Fig. 14.1). The final length of the incubation should be chosen based on consideration of depletion of soil O_2 levels or the NO_3^- pool (which motivates a shorter incubation), the detection of low rates of activity (which motivates a longer incubation), and convenience (e.g., time of day). Depletion of the NO_3^- pool results in a decrease in denitrification rate, and depletion of soil O_2 levels can result in an increase in rate (Fig. 14.1).
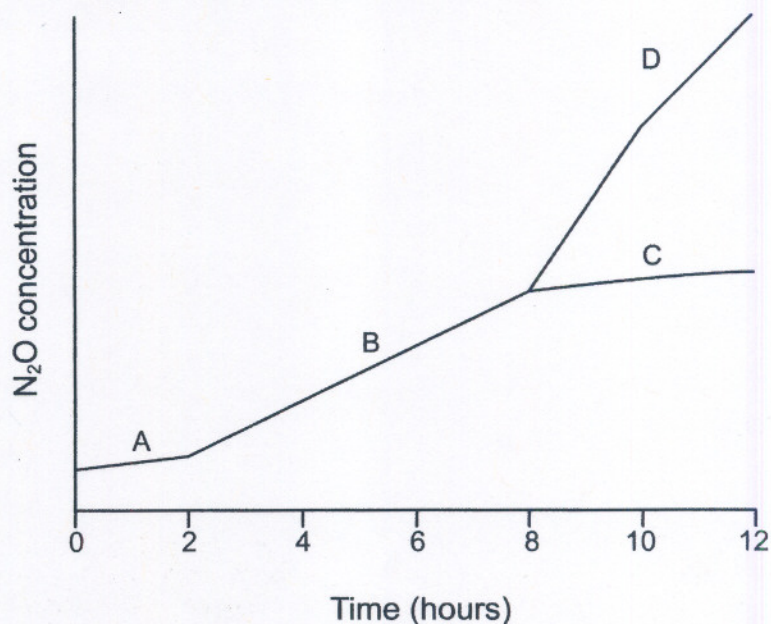
Figure 14.1. Phases of denitrification during intact, static core incubation: A—lag phase while C_2H_2 diffuses into soil pores; B—linear phase; C—NO_3^- depletion phase; D—O_2 depletion phase. Time-course experiments must be performed on subset of samples to ensure that denitrification rates are calculated using data from the linear phase.

4. Store gas samples, blanks, and standards in airtight storage vials and analyze for N_2O as described earlier.
5. Following incubation, cores should be weighed and measured for area and bulk density calculations. The internal headspace volume of each core can be measured with a pressure transducer (Parkin et al. 1984) calibrated to produce volume estimates from pressure changes induced by an addition of 5 or 10 mL of air to the headspace of the core tube. This procedure also facilitates testing for leaks. Alternatively, headspace can be calculated by calculating the volume of the empty tube and subtracting the volume of the soil core (accounting for its pore space and water content).
6. Cores should be processed for soil water content and inorganic nitrogen (see Chapters 3 and 5, this volume).

Calculations

The basic calculation involves quantifying the amount of N_2O produced by the soil by multiplying the concentration of N_2O in the headspace of the core at 2 and 6 hours (or whatever sampling times are used) by the volume of the headspace and then dividing by the dry weight of soil or the surface area of the core. Results are commonly expressed as $\mu g \; N \; kg^{-1} \; d^{-1}$ or as $\mu g \; N \; ha^{-1} \; d^{-1}$:

$$DR = [(C_2 \times H) - (C_1 \times H)]/(D \times T)$$

where

DR = denitrification rate, expressed as $\mu g\ N{\cdot}kg^{-1}{\cdot}d^{-1}$ or $\mu\ N{\cdot}ha^{-1}{\cdot}d^{-1}$

C_1 = N_2O concentration at the first sampling time, expressed as $\mu g\ N_2O{-}N/L$ headspace (see Chapters 10 or 13, this volume, for formula to convert ppm_v or $\mu L\ N_2O/L$ headspace to $\mu g\ N_2O{-}N/L$ headspace)

C_2 = N_2O concentration at the second sampling time

H = core headspace volume (L) (it is necessary to account for removal of air by sampling)

D = soil dry mass equivalent (kg) or core surface area (ha)

T = time between sampling points (d), e.g., 0.17 d for samples removed at 2 and 6 hours

If the headspace is sampled at multiple times during the incubation, the numerator of the equation can be replaced by a regression of N_2O concentration with time ($\mu g\ N_2O{-}N{\cdot}L$ headspace$^{-1}{\cdot}d^{-1}$). It is necessary to account for N_2O dissolved in solution using Bunsen coefficients as described earlier for DEA. In intact cores, it is not always safe to assume that liquid- and gas-phase N_2O are in equilibrium (e.g., N_2O is often supersaturated in soil water), but this is usually a small error unless soils are very wet.

Results can be expressed on an areal basis either by using the bulk density or the surface area of the cores. It is also possible to calculate water or air-filled pore space on each core using bulk density and soil water values (see Chapter 4, this volume).

Estimates of annual or seasonal denitrification nitrogen flux can be produced by extrapolating measured rates over the intervals between sampling dates, i.e., assuming that rates at a sampling date are representative of some period before and/or after that date. The validity of these extrapolations is controlled by sampling frequency and the spatial and temporal variability of the measured rates.

Special Considerations

1. Given the earlier discussion about the depth distribution of activity above (see the section "Special Considerations" for the denitrification potential protocol, above), it may be important to take cores from depths greater than 0–15 cm in some cases. It may also be appropriate to take shallower cores as well.
2. It is impossible to quantify analytical variability of an "intact core" method because cores cannot be subdivided. However, taking multiple samples of the headspace during the incubation allows for evaluation of the analytical variability of the headspace N_2O analysis, which ranges from 5% to 15%. Field variability with this method ranges from 50% to 200%.
3. Knowledge about spatial and temporal dynamics of water, nitrogen, and carbon fluxes in a particular system should be used to design optimal sampling strategies for denitrification. Transitions between cold and warm or between dry and wet seasons are often periods of high denitrification because plants do not dominate water and nitrogen dynamics during these periods. In many

ecosystems, denitrification is most vigorous outside of the plant growing season. Drying and rewetting and freezing and thawing events have also been found to stimulate denitrification. Activity may be significant in unfrozen soils under a snowpack.

Ancillary Data

Ancillary data valuable for interpreting spatial and temporal variation in denitrification include soil temperature, moisture, and NO_3^- content, air-filled pore space, soil respiration, texture, organic matter content, NH_4^+ content and pH, vegetation type and productivity, microbial biomass, and mineralization and nitrification rates. Soil moisture and NO_3^- content are essential ancillary data for the intact core method. Denitrification data are frequently lognormally distributed. Approaches for analyzing such data are described by Parkin and Robinson (1992).

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STANDARD SOIL METHODS

FOR

LONG-TERM ECOLOGICAL RESEARCH

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