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Stable nitrogen isotopes

Study about its use in the assessment of denitrification and N fixation

C. Carratalá Sánchez

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1. Abstract

Losses of N from agriculture, like leaching of nitrate into ground and surface water, volatilisation of ammonia from manure and emission of nitrous oxide produced by denitrification are processes which adversely affect the environment.

Neither of the processes mentioned above is easy to measure, with large uncertainties in quantifications of the nitrogen flows in agriculture as a result. As a possible way of improving quantification in the N cycle, in this paper the possibilities of the use of stable nitrogen isotopes for the measurement of denitrification and biological N_2 fixation are evaluated, because the known research is expensive and not very precise.

It is concluded that the use of stable nitrogen isotopes can result in reliable quantifications of biological N_2 fixation, but is not suitable for quantifications of denitrification in the field.

2. Introduction

Nitrogen occupies a unique position among the essential elements for plant growth because of the rather large amount required by most agricultural crops.

The total amount of N in most soils is appreciable, often exceeding 4000 kg/ha to the depth of ploughing. This N is largely bound to organic forms; in general, only a small amount exists in available mineral forms (as NO_3^- and exchangeable NH_4^+) at any one time. When land is first placed under cultivation, the N content of the soil usually declines and a new equilibrium level that is characteristic for the climate, agricultural practices, and soil type is established. At equilibrium, any N removed by harvested crops or lost to the environment must be compensated for by incorporation of an equivalent amount of N into the soil.

Systems of agriculture that rely heavily on soil reserves to meet the N requirements of plants will show a steadily decrease of N content and cannot long be effective in producing high yields of crops. In the past, manure and biological N_2 fixation were the major means of supplying N for cultivated crops. Currently, N fertilisers have become increasingly important. When used to augment the N supplied by natural processes, N fertilisers can increase yields and improve the quality of the crops. A major concern of present-day farmers is the effective management of N fertilizers for maximum efficiency and minimal pollution of the environment (Stevenson, 1982).

Given that nitrate is highly soluble, excess fertiliser use will lead to excessive concentrations in the soil. Frequent irrigation or rain will leach it to the groundwater, which can be used for drinking water, or to surface waters, in which it can cause eutrophication problems. Furthermore nitrate can accumulate in vegetables for consumption. All of this can cause problems for human health or for environmental quality.

The extent of losses from the N cycle is hard to quantify. Not only the individual processes are hard to measure reliably, but also the extent of the total loss is a problem. The changes in the total amount of N present in the soil is very hard to measure since it concerns small differences in a large pool of spatially uneven distributed N. From the individual processes of N loss, especially denitrification is hard to quantify by the fact that the major product of this process (N_2) is the major constituent of air (Bremner & Hauck, 1982). Other reasons for the uncertainties in the quantities of denitrification are the effects of artefacts in the methodology, operational farm management, seasonal and regional variations in climate, spatial variations in soil conditions, crop residues, soil cultivation and water management. Developing sound methods for measuring denitrification can reduce the uncertainties. In addition, monitoring of denitrification at key-sites, in long-term experiments is needed. These experiments preferable should take into account sub soil denitrification, the effects of crop residues management, the effects of water management and losses of NO_x and N_2O (Kroeze *et al.*, 2000).

Until now common field measurements of denitrification are inaccurate and expensive. More reliable and eventually cheaper methods would be most welcome.

In recent years, the use of N^{15} tracer techniques has resulted in advances in our understanding of the cycling of nitrogen in the soil-plant system. For example, N^{15} tracers have been successfully used to distinguish fertiliser nitrogen from soil derived nitrogen in plant uptake and to quantify mineralization. The objective of this study is to evaluate if N^{15} based methods can also be used to quantify denitrification and biological N_2 fixation under field conditions and to review the advantages and disadvantages of several methods. The methods considered are: natural N^{15} abundance, N^{15} isotope dilution and $N_2{}^{15}$ reduction.

3. Nitrogen cycle in farming soils

The nitrogen cycle is complex and involves many processes (Figure 1), in Chapter 3.1 a general description of the processes is given.

3.1 Nitrogen cycle

Plant uptake and crop extraction

The N that is absorbed by plant roots (plant uptake) is partly removed from the field (crop extraction) and partly returned to the soil as crop residues. Plant uptake must be optimised to get an adequate production and economic benefit. However, the amount of fertiliser needed for optimal crop growth can not be calculated directly from crop extraction. Knowledge on the crop use efficiency and the amount of N mineralized during crop growth is essential.

Mineralization and immobilisation

Mineralization is the transformation of the nitrogen of the organic matter in ammonium (NH_4^+) through enzymatic action of soil micro-organisms. Immobilization is the antagonistic process, this is, incorporation of mineral nitrogen into organic matter by soil micro-organisms. The total amount of NH_4^+ released is the gross mineralization, the difference between the gross mineralization and the immobilisation is defined as the net mineralization.

Nitrification

Nitrification is the complementary phase of mineralization, in which ammonium (NH_4^+) is oxidated to nitrate (NO_3^-). During the processes small proportions of the oxidized NH_4^+ can be emitted as N_2O or NO_x. In soils, this process is mediated primarily by the autotrophic bacteria *Nitrosomonas* and *Nitrobacter*. *Nitrosomonas* oxidizes NH_4^+ to nitrate (NO_2^-), and *Nitrobacter* completes the oxidation to NO_3^-. The *Nitrosomonas* oxidative process may be represented by the following sequence:

$2NH_4^+ + 3O_2 \rightarrow 2HNO_2 + 2H+ + 2H_2O$
$2HNO_2 + O_2 \rightarrow 2\ NO_3^- + 2H^+$

Nitrification is controlled primarily by NH_4^+ and O_2 concentrations.

Rates of nitrification can be measured by mass balance techniques, but considerable nitrate turnover can occur in soils. It requires the use of an isotope technique to measure gross nitrate production (Mosier & Schimel, 1993).

Denitrification

It is generally accepted that denitrification is the major sink for the surplus N in agro-ecosystems on clay and peat soils, though there are only very few measurements that confirm this assumption. Estimated losses via denitrification range from 50 to more than 300 kg ha^{-1} yr^{-1} when based on N balances (Kroeze *et al.*, 2000).

Denitrification is defined as the dissimilatory reduction of oxides of nitrogen to produce N_2O and N_2 by a taxonomically diverse group of aerobic bacteria. The most abundant denitrifiers are heterotrophs, which require sources of electron-reducing equivalents contained in degradable organic matter. The generally recognised reductive sequence is:

$2NO_3^- \rightarrow 2NO_2^- \rightarrow 2(NO) \rightarrow N_2O \rightarrow N_2$

The soil factors that most strongly influence this reductive sequence are O_2, soil water content, NO_3-concentration, pH, temperature, and organic carbon. Reductive enzymes are repressed by O_2 but not by NH_4^+. Nitrous oxide reductase appears more sensitive to O_2 than either NO_3^- or NO_2^- reductase. Therefore, N_2 production predominates in more anoxic sites and N_2O production may be greater under more aerobic conditions. Because of the heterogeneity of the factors controlling denitrification, the spatial and temporal variability of this process in soil is extremely large.

Direct measurements of denitrification in soils are generally centered around quantifying N_2O and N_2 production in and evolution from the soil system. Mostly the production of N_2O and N_2 is measured totally as produced N_2O by blocking the reduction of N_2O to N_2 by the addition of acetylene to the soil atmosphere. Acetylene is usually added to undisturbed soil cores, incubated in jars, but it can also be injected directly in the soil. Also indirect methods based on the disappearance of NO_3^- from the soil have been used in denitrification studies. N^{15} is recently used in both types of studies (Mosier & Schimel, 1993).

Biological N_2 fixation

Reduction of atmospheric N_2 to NH_3, from which it is assimilated into organic N, is carried out by some bacteria, living in symbiosis or in a looser association with plants or free-living in the soil. N_2 fixation is the main natural route of N from the atmosphere into the biosphere and, therefore, into the soils (Hopkins *et al.*, 1998).

The total amount of N returned to the earth each year through biological N_2 fixation has been estimated at 175 Tg, of which about one half (80 Tg) is contributed by nodulated legumes grown for agricultural purposes (Stevenson, 1982).

Deposition

Nitrogen in the form of NH_3 or NO_x can reach the soil or plants by rain (wet deposition) and can be absorbed directly from the atmosphere (dry deposition) In agriculture deposition is mostly low in comparison with fertiliser input. Agriculture contributes appreciably to the deposition of NH_3 through volatilisation from livestock production. Deposition of ammonia contributes largely to acidification of the soil.

Leaching

Excess of rain or irrigation causes leaching, a downward transport of water with soluble salts beyond the rooting zone. Nitrogen is lost by leaching mainly as NO_3^-, although NH_4^+ may be lost from sandy soils.

Surface runoff

Runoff is the flush of water at the soil surface, it is caused as a consequence of excessive rains or irrigation and it is much more common on helling field than on flat fields. Generally nitrogen loss by surface runoff is small, except when runoff takes place shortly after nitrogen fertilization.

Volatilisation

Generally ammoniacal salts react in alkaline medium producing ammonia (NH_3) that is liberated in the atmosphere. Gaseous loss of NH_3 may account for a large part of the turnover of N in grazing systems. Much smaller amount of ammonia can be volatilised from decaying plants.

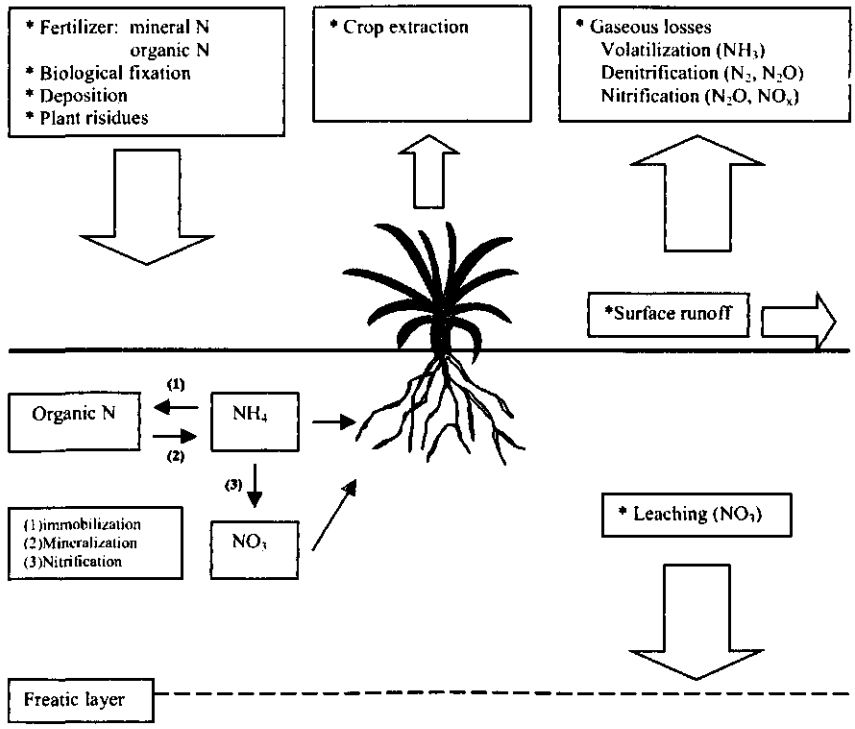

Figure 1. Nitrogen cycle in agriculture.

4. Isotope methods

Since N^{15} enriched N compounds became available at reasonable prices around 1970, the use of stable isotopes in investigations of N transformations in soils has increased dramatically (Bremner & Hauck, 1982).

Stable isotopes of nitrogen N^{14} and N^{15} naturally occur at a ratio of 272:1 or 0.3663 atom% N^{15} in atmospheric N_2. However, there are differences between atmosphere and soil and plants. These differences are caused by the preferential use of the lighter N^{14} isotope in biological processes. So, normally the N transferred through a process is poorer in N^{15} than the nitrogen before the process. For example, NO_3^- after nitrification is poorer in N^{15} than NH_4^+ produced by mineralization, and NH_4^+ still remaining in the soil during nitrification is richer in N^{15} than NH_4^+ produced by mineralization.

Relative enrichment of a nitrogen pool with N^{15} is expressed as δN^{15} (deltaN^{15}):

$$\delta N15 = \frac{\text{atom\% } N^{15} \text{ (sample)} - \text{atom\% } N^{15} \text{ (standard)}}{\text{atom\% } N^{15} \text{ (standard)}} * 1000‰$$

By using these differences in a normal situation (natural N^{15} abundance method) or after artificially increasing the differences through the addition of N^{15}, into the atmosphere (N_2^{15} reduction method) or in the soil (N^{15} isotope dilution method), it is possible to estimate the amount of N that is derived from biological N_2 fixation (Warembourg, 1993). The N^{15} isotope dilution method can also be used to measure denitrification rates (Mosier & Schimel, 1993).

4.1 Denitrification

Denitrification is hard to quantify since its major product (N_2) is the major constituent of air (Bremner & Hauck, 1982). This difficulty can only be overcome by changing the soil atmosphere. Possible changes are replacing all N_2 by helium or the addition of acetylene (C_2H_2). Under an atmosphere free of N_2, the production of N_2 can be measured directly, but the creation of an absolute N_2 free atmosphere in intact soil is very difficult and the risk of leaking remains large (Scholefield *et al.*, 1997). Addition of acetylene blocks the reduction of N_2O to N_2 and the production of N_2O can be used as a measure of denitrification. However, results obtained with this method can be affected by artefacts in the method (Keeney, 1986). Presently, this method is generally supposed to give mostly an underestimation of the actual denitrification rate (Bollman & Conrad, 1997). The N^{15} technique is particularly advantageous because the measurements can be performed under an undisturbed atmosphere, without any addition of other gases.

An example of the use of N^{15} isotopes is the work of Harder Nielsen *et al.* (1997). They tested a probe constructed to detect the denitrification in the subsoil. The probe was designed to label a volume of the subsoil with NO_3^{15} and to subsequently collect gas samples from which the denitrifying activity in the surrounding soil can be estimated by isotopic analysis of N_2. The results were comparable with other methods and it was concluded that this method seemed to be suitable for reliably measuring denitrification in the subsoil.

4.1.1 Calculations

The method involves applying highly N^{15} enriched fertiliser to the soil (>20 atom% N^{15}) and then using a chamber to cover the N^{15} fertilised plot to isolate the atmosphere above the soil for a designated time. This permits determinating the rate of change of N^{15} atoms (in N gases) in the chamber atmosphere over time. Calculations make use of the fact that the soil N gases (principally N_2 under denitrifying conditions) that evolve into the chamber headspace containing normal air do not have the same isotopic distribution as the N gases in the chamber. Utilising this different N^{15} distribution, the method permits calculation of the amount of N gases evolved. Since the N_2^{29} to N_2^{30} ratio is depending on the atom% N^{15} in the total NO_3^- pool, denitrification rates can be calculated not only from the added N^{15}-enriched fertiliser but also from soil N, on the condition that the added N^{15} enriched NO_3^- and the native soil NO_3^- have the same spatial distribution. The atom% N^{15} of the native soil NO_3^- must be known to calculate the total amount of N_2 evolved from the site.

Hereafter, the basic equations used to calculate the total N-gas flux from the soil using mass spectrometry are shown (Mosier & Schimel, 1993). The technique is applicable to laboratory incubation flasks and field studies, and total N gas production is estimated, not only N gas production from the added nitrogen.

1. $dr = (N_2^{29}/N_2^{28})\text{sample} - (N_2^{29}/N_2^{28})\text{reference}$.
2. $dr' = (N_2^{30}/N_2^{28})\text{sample} - (N_2^{30}/N_2^{28})\text{reference}$.
3. Sample = air sample from collection chamber at time, T, after installing the chamber.
4. Reference = air sample from field, i.e., normally air sample taken from the chamber at the moment of closure (t0).
5. 29/28 and 30/28 are isotope ratios determined by the mass spectrometer (N^{29} is N^{14}-N^{15}, N^{30} is N^{15}-N^{15}).
6. X_N^{15} = mole fraction of N^{15} in the soil NO_3^- pool = $2(dr'/dr)/(1+(2(dr'/dr)))$, the soil NO_3^- pool is the total pool of native soil NO_3^- and added NO_3^-.
7. d = fraction of total N gas in the gas collection chamber attributable to denitrification = $dr'/(X_N^{15})^2$.
8. dC = total N_2 gas evolved from the soil into the collection chamber = total N_2 in the chamber volume*d.
9. N_2 flux = $dC/(A*(tT-t0))$ Where A is soil surface area covered by chamber, tT-t0 is the time that the chamber covered the soil, and dC is the change in amount of N_2^{30} and N_2^{29} in the chamber during time T.

The use of these equations requires some assumptions. The first is that the total amount of N_2^{28} inside the gas collection chamber or incubation flask does not change during the sample collection period. The second and major assumption is that the N^{15} label of the NO_3^- in the soil is uniform. One must assume that nitrate from the N^{15}-labeled fertiliser added to the soil mixes uniformly with the unlabeled NO_3^- already in the soil or with unlabeled NO_3^- formed from organic N mineralization. Non-uniform mixing of the N^{15} label will cause underestimation of the total N denitrified (Mosier & Schimel, 1993).

4.1.2 N_2O analysis using N^{15}

The amount of N_2O evolved from a N^{15}-fertilized soil can also be quantified by mass spectrometry, using a simple mass spectrometer with a gas and solid ammonium sampling inlet system. An air sample collected from an incubation flask or soil cover enclosure is introduced into the gas sampling inlet of the mass spectrometer. The inlet sampling loop is immersed in liquid N to freeze out N_2O and the N_2 is analysed. After N_2 analysis, the inlet system is evacuated and a known volume of unlabeled N_2 is added to the sample loop. The sample loop is warmed to gasify the frozen compounds and the N_2O and N_2 are mixed and then passed through the O_2 scrubber. The N_2 produced is then analysed as

described earlier and the same calculation process is used, except that the quantity of N_2O is equal to *d* times the amount of N_2 dilution gas used. The procedure requires that the amount of diluent gas exceeds the amount of N_2O in the sample by 100 times if preceding equations are used (Mosier & Schimel, 1993).

4.1.3 Denitrification experiments

Lab case study 1

This study was performed to observe the effect of acetylene on denitrification. In this study, 10 g samples of mixed soil (a clay loam containing 0.9% C, 0.16% N, pH 7.3) were placed in 125-ml serum bottles and 10 ml aqueous solution containing 2 mg KNO_3-N (99 atom% N^{15}) was added. The bottles were immediately sealed with rubber serum caps and, when desired, acetone-free acetylene was injected to attain 10% (v/v). The bottles were incubated for 3, 6, and 12 hr and 1, 3, and 7 days at 25°C. After these incubation periods the gas headspace was analysed for N_2O and CO_2 by GC (Gas Cromatography) and N_2 + N_2O by IRMS (Isotope Ratio Mass Spectrometry). After gas analysis, the soil from each bottle was extracted with 50 ml 2 M KCl. The KCl extract was analysed for NO_3^- + NO_2^- and NH_4^+ by steam distillation and processed for N^{15} analysis. An aliquot of the KCl extracted soil was Kjeldahl-digested for total N and subsequent N^{15} determination. Samples were analysed for N^{15} with a triple-collector IRMS.

The total N_2 + N_2O production was significantly greater when the soil was exposed to acetylene, at each incubation period. Furthermore, after 1, 3 and 7 days significantly more NO_3^- was reduced in the presence of acetylene (Mosier & Schimel, 1993).

Lab case study 2

One of the potential limitations to the N^{15} technique, particularly in field studies of both nitrification and denitrification, is that the method assumes that the NH_4^+ or NO_3^- in the soil undergoing transformation has a uniform isotopic distribution. The N^{15} added is assumed to mix uniformly with the unlabeled NO_3^- so that the denitrifying microflora encounters a NO_3^- pool that has a uniform N^{15} content. Unless systems are well mixed, it seems unlikely that this uniform N^{15}-NO_3^- distribution can exist. Theoretically, this non-uniform distribution leads to underestimating the total amount of denitrification that occurs in a soil. The following experiment was performed to determine if evolution of N^2 into a common headspace from physically separated NO_3^- pools, containing different N^{15} contents, would give an underestimation of the total denitrification.

One-pint preserving jars (568 ml) were used as incubation chambers. A gas sampling port was made in each rubber-gasketed lid by making a 6-mm-diameter hole and inserting a rubber serum stopper. Silicone caulking was used around the stopper to ensure an airtight seal. Inside each jar three 35-ml liquid scintillation vials were placed. One vial containing 2 ml 1 M NaOH to collect CO_2 and each of the other two vials containing 10 g soil (as described in Lab case study 1). To each vial of soil KNO_3 solution was added, totalling 200 microg N/g soil. The N^{15} enrichment of the NO_3^- varied between 0 and 70 atom% N^{15} in different vials. The individual vials represented separate NO_3^- pools for which the N^{15} enrichment was controlled. After the NO_3^- solution, 1 ml of glucose solution (15 mg glucose-C/ml) was added. Finally, distilled water was added to bring the final moisture content of the soil to 35%. A set of time zero samples was prepared and analyzed immediately to provide a zero sample basis for comparing total and N^{15} values for each experiment. The N^{15}-fertilized soils were then incubated at 25 °C under an air atmosphere for 1 to 7 days. The atmosphere of half of the jars was amended with 5% (v/v) of acetone-free acetylene to block the conversion of N_2O to N_2. Adding acetylene allowed cross-comparison of the amount of N gas produced measured by two different methods. By measuring

N_2O produced in the presence of acetylene by GC and the total N gas produced by IRMS, the two analyses should give the same result for gas produced, unless the N^{15} method underestimated N-gas production.

After designated times, soils were sampled in triplicate and analysed for total N and N^{15}. The remaining soil was extracted with 2M KCl and the extract analysed for NO_3^- + NO_2^- and NH_4^+ and N^{15}.

Samples of the jar's gas phase were removed through the rubber septum in the jar lid using 3-ml polypropylene syringes, fitted with vacuum-tight stopcocks. Two samples were collected each time: 1.5 ml for IRMS analysis and 1.0 ml for N_2O analysis by GC. The total gas volume, accounting for vials, soil and solution, was measured for each jar. Recall that the experiments were set up so that separate vials of soil were amended with NO_3^- having the same or diferent N^{15} enrichments. The gases produced from each vial evolved into the jar atmosphere, where gases mix with the initial jar air atmosphere. Hauck & Bouldin (1961) showed that N_2 molecules formed from different NO_3^- pools physically mix in the gas phase, but they do not mix atomically; the equilibrium reaction N^{15}-N^{15} + N^{14}-N^{14} ← → 2 N^{15}-N^{14} is not of importance. If we compare N-gas production from soils in which two NO_3^- pools are present are compared, one containing no-enrichment and the other containing 70 atom% N^{15} enrichment, using acetylene block and N^{15} methods, the total amount of N evolved was underestimated by the N^{15} method. But when the pools were both either 30 or 50 atom% N^{15} enriched, both methods estimated the same amount of N-gas production. This indicates that this method is indeed only suitable when the added N^{15} enriched N pool is uniformly mixed with the native soil N pool (Mosier & Schimel, 1993).

4.2 N_2 fixation

4.2.1 Natural N^{15} abundance method

This technique, generally known as the dN^{15} method, gives results comparable with other methods but its main value lies in natural ecosystems where these other methods usually cannot be applied.

Successful application of the approach relies on the existence of a genuine, robust and measurable difference in N^{15} abundance between the two N sources (soil N and atmospheric N_2) and in order to satisfy these criteria a number of precautions are required. Determining the dN^{15} of the plant-available soil N is usually achieved by analysis of non-N_2-fixing plants rather than analysis of extractable (mineral) soil N. This is because the former method integrates possible fractionation during plant uptake or during loss of soil mineral N prior to plant uptake. Careful selection of suitable non-fixing reference plants is essential, since they should be ecologically and physiologically comparable with the N_2-fixing plant species in study in all ways apart from receiving a supply of N via fixation. The spatial and temporal variability in N mineralization and plant-available soil concentrations and different isotopic partitioning between roots, shoots, fruits etc. are both relevant considerations in sampling the reference plant. Finally, although the dN^{15} of atmospheric N_2 is usually very close to that of the fixed N, any small differences in isotopic composition arising from fractionation during uptake and assimilation must be accounted for. Given these constraints, the natural abundance of N^{15} should best be regarded as a usually reliable, although not precise, indicator of N_2 fixation (Hopkins *et al.*, 1998).

Basis of the dN^{15} method

Variations in the natural abundance of N^{15} in different ecosystem compartments result from equilibrium and kinetic isotope effects, which, in some cases, have been operating over all of biological time. Equilibrium constants are determined by differences in the structure and energy of two chemical

species at equilibrium, and these differences are affected by the isotopic composition of the two species. Hence, two species at equilibrium (e.g., $H^+ + NH_3 = NH_4^+$) may differ in N^{15} abundance. Kinetic isotope effects almost always result in N^{15} enrichment of substrate and depletion of product because of the tendency of molecules bearing the lighter isotope to react somewhat faster than those which bear the heavier isotope.

Natural N^{15} abundance is expressed as dN15, the per mil N^{15} excess over a standard:

$$d\text{N15} = \frac{\text{atom\% } N^{15} \text{ (sample)} - \text{atom\% } N^{15} \text{ (standard)}}{\text{atom\% } N^{15} \text{ (standard)}} * 1000‰ N^{15}$$

Atmospheric N_2, with 0.3663 atom% N^{15}, is the ultimate reference value, although often a more convenient shelf standard is used for the measurement (Gadish *et al.*, 2000).

The expression for calculating the fractional contribution of biologically fixed N to the total N in N_2-fixing plants, Fndfa, is given by an isotope dilution expression. A convenient form is:

$$\text{Fndfa} = \frac{d\text{N}^{15}_{o} - d\text{N}^{15}_{t}}{d\text{N}^{15}_{o} - d\text{N}^{15}_{a}}$$

where $d\text{N}^{15}_{a}$ is the $d\text{N}^{15}$ value of fixed N in the N_2-fixing plant (as measured in plants forced to depend solely on atmospheric N_2 by growing them hydroponically with N-free nutrient medium), $d\text{N}^{15}_{t}$ is the $d\text{N}^{15}$ value of the total N in the N_2-fixing conditions in which atmospheric N_2 and N from other sources are available, and $d\text{N}^{15}_{o}$ is the $d\text{N}^{15}$ value of N from sources other than atmospheric N_2 (as measured in neighbouring non-fixing plants). Put in words, the fraction of total N derived from atmospheric N_2 is calculated from an interpolation between two pools ($d\text{N}^{15}_{a}$ and $d\text{N}^{15}_{o}$).

Annual or seasonal input of fixed N for a given area may be calculated once an estimate of the fractional contribution of fixed N to the plant has been determined. For this, it is necessary to measure the productivity of the N_2-fixing plant by direct harvest or by applying dimensional analysis techniques, along with data on fractional cover of the ground surface and plant N content (Gadish *et al.*, 2000).

4.2.2 N^{15} isotope dilution method

The isotope dilution method has proved extremely useful in agricultural systems. The method involves labelling the soil available N pool by applying N^{15}enriched fertiliser N at low rates to soil on which N_2-fixing plants are to be grown. By adding N^{15}enriched fertilisers to soil, one ensures that the plant will take up N from soil with a higher N^{15} content than that in the atmosphere. The extent to which this N^{15} enrichment is diluted by atmospheric N in a fixing plant reflects the magnitude of fixation.

Basis of the isotope dilution method

Calculation of the amount of N_2 fixed per unit area or per plant requires measurements of:

- The N^{15} abundance of the N_2-fixing plant.
- The N^{15} abundance of the plant available N in the soil.
- The total amount of N in the N_2-fixing plant.

The isotope dilution method requires that the N^{15} abundance of assimilated soil N in the N_2-fixing plant is known. The basic assumption of the isotope dilution method is that the N^{15} abundance of soil-derived N is the same in N_2-fixing and reference plants. Because of the time and depth dependence of the N^{15} label of the soil plus fertiliser N pool, selection of an appropriate reference plant (one which takes up soil N from the same depth and with the same temporal pattern as the N_2-fixing plant) is crucial for this method. But it is unlikely that, by chance, an ideal reference plant for the N_2-fixing plant of interest would be found growing at the same site. Moreover, there are no practical methods available for determining the suitability of reference plants that can be applied in natural ecosystems. This is a major disadvantage of isotope dilution method for studies in natural ecosystems.

There are two additional serious problems with the isotope dilution method, when applied to natural systems: N fertilisation disturbs the system and in an existing vegetation the N^{15} abundance of labelled soil N taken up by the plants is diluted by N present in the plant at the start of the experiment.

By contrast, in most agricultural studies with annual crops, the experiment starts at the time that seeds are planted and N^{15}-labeled fertiliser is applied. At the end of the experiment, the entire (above ground) plant is harvested. All of the soil N taken up by both N_2-fixing and reference plants is taken from the labelled available soil N pool (Shearer & Kohl, 1998).

Use of N^{15}-enriched materials, generally fertilisers added to the soil, results in access of fixing plants to three nitrogen sources: soil N, fertiliser N, and atmospheric N. But if we assume uniform mixing of the N^{15} fertiliser into the soil, N only two sources of N for the plant remain: soil and atmosphere. In this way the fraction of biologically fixed N to the total N in N_2-fixing plants (Fndfa) can be calculated as follows:

$$\text{Fndfa} = 1 - \frac{N^{15}\text{ atom\% excess (fixing plants)}}{N^{15}\text{ atom\% excess (non-fixing plant)}}$$

This equation is independent of the rate of fertiliser applied and of the yield attained (Warembourg, 1993).

4.2.3 N_2^{15} reduction method

The use of N_2^{15} as a tracer is the most satisfactory method in the field of N_2 fixation. Despite difficulties in its use, the N_2^{15} incubation method remains the absolute method of measurement of N_2 fixation against which others methods should be tested. Moreover, many qualitative and quantitative aspects of translocation and fate of biologically fixed N can be investigated only through the use of N_2^{15}.

The principle of N_2^{15} incorporation is simple. The whole fixing system (soil, plant and root) is exposed to an atmosphere enriched in N_2^{15} for a specified period of time followed by N^{15} determination in the material exposed. The whole system or its separate components may be analysed. The fraction of the total N in the plant that was fixed (Fndfa) during the period of exposure is determined by the equation:

$$\text{Fndfa} = \frac{N^{15}\text{ atom\% excess in sample}}{N^{15}\text{ atom\% excess in atmosphere}}$$

If the total amount of N in the sample is known, it is possible to calculate the amount of N fixed during the exposure period.

The main limitation of the N_2^{15} reduction method is that it is technically difficult. Because the fixing system must be exposed to an N_2^{15} atmosphere of constant enrichment, sophisticated and expensive apparatus are required to prevent leaks and maintain normal environmental conditions. In addition, the method is destructive, which complicates repetitive sampling in long-term experiments.

The N_2^{15} reduction method remains a short-term kinetic measurement and, as such, is not useful for integrated quantification of N_2 fixation. However, it represents a powerful tool in fundamental research because it is the only direct method of estimating N_2 fixation. The isotopic element is incorporated by biological processes and, hence, behaves as a true tracer of fixed N. This allows important applications. It is the only absolute measurement that demonstrates the occurrence of N_2 fixation and can screen bacterial strains and associations between plant cultivars and bacteria for N_2 fixation efficiency (Warembourg, 1993).

However due to technical difficulties and costs of this method, we will limit our study to comparison of the others two methods.

4.2.4 Natural N^{15} abundance vs N^{15} dilution method

The major limitation of the isotope dilution method lies in the main assumption that the N^{15} fertiliser is uniformly mixed with soil N. In fact, the soil N^{15} enrichment changes with time and space, and the use of the method is totally dependent on the comparison between fixing and non-fixing reference plants. Choice of a proper non-fixing plant species is therefore of high importance. It must take up N with identical isotopic composition in the soil and fertiliser pools as does the N_2-fixing plant species. Another requirement of the method is that the amount of N added has to be the same for both plant species. N addition must also be small, because high soil mineral N levels normally inhibit N_2 fixation. In nitrogen-poor soils, the non-fixing plant species may therefore not get enough N to ensure adequate growth. This limits the use of the isotope dilution method.

The inherent simplicity of isotope dilution makes it the method of choice for general purposes. It has been found to be more precise than any other method for estimating N_2 fixation. The larger the proportion of nitrogen in the test crop that is derived from atmosphere, the larger is the difference between N^{15} enrichment of the fixing and non-fixing plants, and the smaller is the error in the estimates (Warembourg, 1993).

In the δN^{15} method, unlike in the isotope dilution method, it is not necessary to apply N for estimating the contribution of N_2 fixation. This is an important advantage in any N_2-fixing system since inorganic N is known to inhibit N_2 fixation and time-consuming field work is avoided. The δN^{15} method has an additional advantage over methods in which N^{15} fertiliser is added. It is especially difficult to apply methods requiring addition of N^{15} fertiliser to study N_2 fixation by established perennials. This disadvantage does not apply to the δN^{15} method because in this method there is no change in N^{15} abundance of plant tissues induced by starting the experiment. Likewise, use of the δN^{15} method avoids the large drop with time during the growing season in N^{15} abundance of N available to the plant that inevitably occurs when N^{15} labelled fertilisers are applied, because of dilution with N at natural abundance that is mineralised from soil organic N.

The major disadvantage of the δN^{15} method is that the difference in N^{15} abundance of N_2-fixing and non-fixing reference plants is very small, usually less than 10‰ N^{15}. In consequence, measurement error and real variation in δN^{15} of N sources other than atmospheric N_2 become important. Given these constraints, it is necessary to establish the magnitude, not only of the measurement errors, but also of the real variation of δN^{15} in each N-pool of interest. Obviously, the δN^{15} method cannot be applied to locations in which the total variation in the value for δN^{15} due to measurement errors and

real variation exceeds the difference between N^{15} abundance in non-fixing reference plants and in N_2-fixing plants.

In the context of the d^{N15} method, issues of isotopic fractionation are important, and experimental design, field sampling, and experimental procedures all must aim at minimising the impact of isotopic fractionation on the N_2 fixation estimate. In contrast, when the isotope dilution method is used with N^{15}-labeled fertilisers, isotopic fractionation needs not be taken into account, because isotopic alteration caused by fractionation is very small compared to the difference in N^{15} abundance being measured (Warembourg, 1993).

Both methods present an additional problem, i.e. they both assume that N^{15} abundance of sources other than N_2 are the same for N_2-fixing and reference plants. This assumption is not necessarily valid if, for example, the two kinds of plants take up N from different soil depths or in different time periods. Hence, care is required in selecting reference plants. The appropriateness of a reference plant may be a less serious problem for the d^{N15} method than for the isotope dilution method, at least with respect to the temporal pattern of uptake of soil available N during the growing season. Since soil N available to the plant is mineralised from a pool of organic N at natural N^{15} abundance, the N^{15} abundance of added N^{15} enriched N is expected to be diluted by this much lower d^{N15} material during the growing season. Such dilution does not occur with the d^{N15} method (Shearer & Kohl, 1998).

5. Discussion and conclusions

Denitrification

There are no major obstacles to using N^{15} measures of denitrification in natural ecosystems where rates of emission are relatively high (tropical ecosystems), and tracer-level (rather than plant fertiliser levels) additions of N^{15} would provide sufficient analytical sensitivity. Indeed, isotope studies of denitrification and N_2O emission from natural ecosystems are badly needed to improve understanding of global trace gas emissions.

Problems exist with isotopic techniques. In natural ecosystems and agricultural ecosystems where emission rates are relatively low, directly measured gas flux emission and isotopic N balance agree well. In systems where N losses are high, the lost N cannot be accounted for by direct gas flux measurements. Because only a few studies have been conducted, we have yet to learn if this is due to methodological problems, or if loss via vectors not simultaneously measured occurred. Problems could also result from interference with gas movement in soils by the chamber employed, non-uniform mixing of isotope in soils, or others factors. The effects of mineralization, immobilisation, and turnover of N and N^{15} enrichment of soil NO_3^- and NH_4^+ and uniformity of isotopic distribution will change over time (Mosier & Schimel, 1993).

It is concluded that the need for an identical spatial distribution of the added N^{15} enriched N source and the soil N source refrains N isotope methods from being suitable for field studies.

N_2 Fixation

The main disadvantage of the isotope dilution method is its uncertainty, but despite this, it is the only easy and reliable method to obtain an integrated estimate of N_2 fixation in the field, providing certain requirements are met.

Because of the difficulties with the δN^{15} method, some scientists have concluded that measurements of the natural abundance of N^{15} are unlikely to have more than qualitative value for research on N_2 fixation. But several tests of the δN_{15} method have indicated that under many conditions, estimates of N_2 fixation based on this method are comparable to those based on more conventional methods. Hence, despite the lack of precision of the δN^{15} method, we can consider it a useful addition to others methods.

It is concluded that three isotopic research methods are principally suitable for the measurement of N_2 fixation. Each method has its own advantages and disadvantages, which makes the methods suitable for different types of research.

As the cost of mass spectrometers falls and they become easier to use, the use of nitrogen isotopes will likely become more common in future routine measurements.

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