Nitrogen Isotope Techniques

Edited by

Roger Knowles **T. Henry Blackburn**

Academic Press, Inc.
Harcourt Brace Jovanovich, Publishers
San Diego New York Boston
London Sydney Tokyo Toronto

This book is printed on acid-free paper. ♾

Academic Press, Inc.
1250 Sixth Avenue, San Diego, California 92101-4311

United Kingdom Edition published by
Academic Press Limited
24–28 Oval Road, London NW1 7DX

Library of Congress Cataloging-in-Publication Data

Nitrogen isotope techniques / edited by Roger Knowles, T. Henry Blackburn.
p. cm. – (Isotopic techniques in plant, soil, and aquatic biology series)
Includes index.
ISBN 0-12-416965-1
1. Stable isotope tracers. 2. Nitrogen cycle–Research–Methodology. 3. Nitrogen–Isotopes–Analysis. 4. Mass spectrometry. 5. Nitrogen–Fixation–Research. I. Knowles, Roger, date. II. Blackburn, T. Henry (Thomas Henry), date. III. Series.
QH324.35.N1N57 1992
581.19'214–dc20 92-13944
CIP

PRINTED IN THE UNITED STATES OF AMERICA
92 93 94 95 96 97 BC 9 8 7 6 5 4 3 2 1

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Preface

Several books dealing with the use of nitrogen (N) isotopes were published during the last 10 years. They covered separately areas such as soil studies, nitrogen fixation, marine systems, and other topics. To our knowledge, this is the first volume to bring together basic aspects of the measurement of the stable and radioactive isotopes of N as well as specific applications in plant, soil, and aquatic biology. This is the second volume in the series *Isotopic Techniques in Plant, Soil, and Aquatic Biology,* published by Academic Press.

Nitrogen Isotope Techniques and other volumes in the series provide reference sources from which detailed methods can be found for isotopic studies, in our case, nitrogen isotopes. The descriptions of methods are sufficiently detailed, so it should be possible to follow procedures without referring to other sources or the primary literature. However, key sources are given to permit such referencing if necessary. The volume should be of particular interest to researchers and students engaged in physiological or ecological studies of natural and agricultural systems. We hope that it may be treated by such persons as a laboratory cookbook.

After an introductory chapter, there follow separate chapters on basic methods for mass spectrometry and emission spectrometry, the two major techniques for the measurement of ^{15}N abundance. Discussion and description of sample preparation, with some introduction to automated methods, is also included. Chapter 4 describes some of the special approaches and techniques necessary when precise determination of ^{15}N natural abundance is desired, and calculation methods are described. Specific calculations are also included when relevant to other chapters.

Nitrogen fixation measurements employing both enriched ^{15}N-dinitrogen and so-called isotope dilution after addition of enriched inorganic-^{15}N are covered in Chapters 5 and 6. Chapter 5 deals with applications in terrestrial (dryland) plant–soil systems, and Chapter 6 deals with aquatic and wetland systems, including the highly important rice paddy. Applications for nitrification and dentrification (the production and reduction, respectively, of oxides of nitrogen) are covered in Chapter 7. Next, Chapters 8 and 9 address terrestrial and aquatic applications in the field of assimila-

tion, mineralization, and turnover of organic nitrogen. Finally, Chapter 10 describes the very specific methods required for the proper use of the radioactive isotope ^{13}N.

We hope that the methods described allow researchers to adapt the procedures to virtually any task encountered in the investigation of nitrogen transformation processes.

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1

Introduction

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The purpose of this introduction is to give an overview of how nitrogen isotope analysis has been used in different ecosystems, how useful this methodology has been, and to give some assessment as to possible future developments and lines of research. The views expressed here are personal and do not necessarily reflect those of the authors of the different chapters, whose forgiveness we beg should they feel that we have undermined their statements. We do not refer to specific chapters and we do not cite specific references.

In general, nitrogen isotope analysis has been useful in the routine measurement of transformation rates of nitrogen-containing compounds in agricultural but less so in natural ecosystems. The employment of these isotopes has, however, been very helpful in elucidating specific pathways and demonstrating that certain reactions are possible. 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, but inherent difficulties remain, and they may be impossible to overcome. Some of these difficulties will be indicated in the context of the application of isotopes to measure specific processes in the nitrogen cycle. The main problem is that of adding trace amounts of label (^{15}N) to very small and reactive pools of target compounds, where reaction rates would be influenced by an increase in substrate concentration. In the future, it is anticipated that increased experimental ingenuity will overcome some of

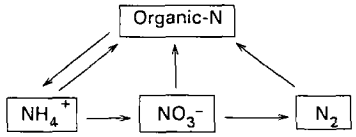

Figure 1 Components and interactions in the nitrogen cycle.

these technical difficulties. We expect to see increased use of high-pressure liquid chromatography and gas chromatography in the preparation of samples for analysis and increased use of the direct analysis of ^{15}N compounds, rather than the conversion of all nitrogen to N_2, before measurement by mass spectrometry. There are very good reasons for using N_2 for mass spectrometry analysis, but its exclusive use may have resulted in a restriction of experimental design.

The most important processes in the nitrogen cycle are outlined in Fig. 1. They have been described in the various chapters in relation to the ecosystems found in water, sediment, and soil. The processes are organic-N mineralization to NH_4^+; organic-N synthesis from inorganic NH_4^+, NO_3^-, and N_2; the oxidation of NH_4^+ to NO_3^- (nitrification); and the reduction of NO_3^- to N_2 (denitrification). We will restrict our observations to the microbiological aspects of the nitrogen cycle; it is the end-products of the microbial reactions that are available for plant uptake and other processes. The mineralization (not remineralization) of organic matter occurs via a number of hydrolytic, fermentative, and oxidative processes to simple C1 compounds and to NH_4^+, irrespective of whether the degradation occurs in oxic or anoxic conditions. Surprisingly little is known about the specific microorganisms involved in the various degradative steps: There are probably as many different types as there are organic compounds. The point of interest is that NH_4^+ is the exclusive end-product of nitrogen mineralization. The synthesis of organic N (biomass) is also carried out by a very diverse group of microbes, probably just as diverse as the degrading microorganisms and may often be the same microorganisms, in many situations. These are heterotrophic microorganisms; they are almost always energy-limited, and, as a result, the metabolic pattern is to utilize the energy in the reduced carbon of an organic molecule, the nitrogen being excreted as NH_4^+. The NH_4^+ will subsequently be taken up and built into biomass. This is the pattern observed in the metabolism of amino acids: deamination, carbon oxidation, and NH_4^+ reincorporation. There is always some direct incorporation of the amino acid directly into biomass, but the proportion is probably small. The order of preference for incorporation is NH_4^+, NO_3^-, and N_2. All but the most fastidious bacteria can utilize NH_4^+ and

many aerobes can use NO_3^-, but only a restricted group of bacteria can utilize N_2. These nitrogen-fixing bacteria may be aerobic or anaerobic and heterotrophic or autotrophic, and the latter may be phototrophic or chemotrophic. All suffer from the same restriction; nitrogen fixation requires the expenditure of much energy and reducing power, and the enzymes are O_2-sensitive. As a result, the process is essentially anaerobic and very often associated directly or indirectly with photosynthesis, where energy is less limiting.

The oxidative step in the nitrogen cycle is the reaction of NH_4^+ with O_2 to give NO_3^- via a number of intermediate molecules. The first step is to NO_2^- by a specialized group of chemoautotrophic bacteria, whose biomass is low but of wide distribution. Nitrite seldom accumulates but is oxidized further to NO_3^-, again by a group of specialized bacteria, most of which are chemoautotrophic. Some heterotrophic nitrification occurs but is thought to be of limited importance. The main significance of nitrification is that it leads to the possibility of leaching losses and of denitrification. This results in the conversion of a readily utilizable compound (NO_3^-) to one that cannot be assimilated by most bacteria or by any eukaryote. Globally, denitrification is balanced by nitrogen fixation, but locally the processes are seldom of equal magnitude. Denitrifying bacteria are generally aerobic heterotrophs, which in an anoxic environment have the capacity to utilize NO_3^- instead of O_2 as an electron sink in the oxidation of reduced carbon or in restricted cases of H_2S.

Ideally, it is desirable to describe an ecosystem's nitrogen cycle in relation to the rates of appearance and disappearance of all the pools, as shown in Fig. 1. In addition, it is helpful to know something about the fate of carbon, its rate of oxidation, and the rate of biomass-C synthesis, thus linking the C and N cycles. In many situations, it is not simply the cycling of C and N between the microbial compartments that is of major interest; it is the availability of N for plant uptake that is of paramount importance. To some extent, the availability of N for assimilation by plant roots may be determined from the net production of inorganic N (NH_4^+ and NO_3^-) in the system, on the assumption that this would be available to the plant. There are some dangers in this approach because the presence of roots will almost certainly affect the O_2 balance in the system and, thus, affect the coupled processes of nitrification and denitrification.

Water, sediment, and soil are the three basic environments, and these have been selected for treatment in the individual chapters. This reflects more the conventions, which are maintained by tradition and scientific institutions (universities and government agencies), than any real basic differences among them, at least in relation to the microbial process involved. Microorganisms can only grow and express their metabolism in

water: The three ecosystems are essentially aqueous. The difference between them lies in the quantity of water present, the quantity of organic input, and the accessibility of O_2. All three factors are interrelated. The amount of water will determine whether O_2 penetration is restricted to diffusion through water or through air, the latter process being significantly more rapid. The quantity of organic input will also determine the depth of O_2 penetration, because O_2 will be consumed by organic-C oxidation. The quantity of organic input will also determine the availability of NH_4^+ to fuel the other N-cycle processes. The only real difference between saltwater and freshwater lies in the high SO_4^{2-} content of the former, which leads to a complete oxidation of organic carbon with the production of H_2S in anoxic marine sediments. In anoxic freshwater sediments, the products of carbon metabolism are CO_2 and CH_4. Oxic systems (soil, mixed waters, organic-deficient sediment, and sediments with rooted plants) are characterized by the presence of NO_3^- as the main inorganic nitrogen compound: Most NH_4^+ is oxidized, the NH_4^+ concentration is low, and the NH_4^+ pool turns over rapidly. In contrast, anoxic systems (water-saturated soils, organic-rich sediments, and stratified waters) contain only the NO_3^- that has been imported; NH_4^+ concentrations are high and this large pool turns over relatively slowly. These facts have considerable significance in the strategy of ^{15}N tracer methodology. Most interest lies, not so much in the completely oxic or the completely anoxic ecosystems, but in the interface between these systems. It is at the interfaces that the most important reactions occur. Ammonium can diffuse or be transported across the interface, from anoxic to oxic, where it may be transformed to NO_3^-. The NO_3^- can then diffuse back to the anoxic and be denitrified. Similarly, O_2 can diffuse into the anoxic regions and lead to similar reactions. Oxic–anoxic interfaces can be extensive and well defined, as in marine and lake sediments and the surface of marshes and bogs. They can also be less well defined and less extensive, as around plant roots and animal burrows in anoxic soils and sediments or in microzones surrounding decomposing organic particles in any of the oxic environments.

The question arises as to why this book is organized in a conventional manner, based on the mutually exclusive water–soil–sediment ecosystems, when it might seem rational to have attempted a more fundamental synthesis, based on oxygen–water–organic relationships. There are several answers to this question, none of which is entirely satisfactory. Most researchers who work with nitrogen isotopes and who will use this book belong to one of the traditional disciplines and might not find a nontraditional format as useful. In other words, they might not buy the book. We cannot complain that the authors have not fulfilled the task assigned to them; they have faithfully restricted themselves to their assigned topic. It is remarkable,

however, how seldom cross-references occur to other ecosystems. It is our hope that the present reader will be less restrictive and will bravely cross the boundaries of tradition and learn that the same processes occur in different systems and that the isotope methods developed for one ecosystem can be profitably used in another. It is for this reason that in this brief survey, we concentrate on the use of labeled substrates, because they may be applied in any environment. First, however, a brief word on the isotopes, their measurement, and their natural abundance.

The N-isotopes are stable ^{15}N and radioactive ^{13}N. Use of the latter has been restricted to very few laboratories. The half-life is very short, the isotope must be produced close to the experimental site, and the methods for analysis of processes and products are very specialized. It must be emphasized that the use of ^{13}N has led to the elucidation of processes that could not otherwise have been investigated. It can be added to label small active pools to determine their turnover; this would be impossible with ^{15}N tracer methodology. We are, however, mostly concerned with ^{15}N, because this is the N isotope that is commonly used. The methodology for measurement of ^{15}N is described in considerable detail in the following chapters. We may be criticized because too many chapters contain descriptions for isotope measurement. It is hoped that this will not be a cause of annoyance to the reader; we thought that it was better to have some overlap rather than to have chapters that were not self-contained. The two main methods for N isotope analysis are emission spectrometry and mass spectrometry. The former, in the past, has not required such large samples as the latter and, for that reason, has been useful in many situations. The $^{15}N/^{14}N$ ratio is determined in N_2, usually prepared by a Dumas oxidation of NH_4^+ or organic N. Newer mass spectrometers require smaller sample sizes for accurate analysis, and better methods allow for the preparation of samples from materials that contain low concentrations of nitrogen. It is usual, if not universal, to measure $^{15}N/^{14}N$ ratios in N_2 in mass spectrometers. Modification of mass spectrometers, particularly in the method of ionization, should allow for more flexibility in the N compounds that are analyzed for ^{15}N content. It is difficult to predict what these compounds might be, but one might anticipate the use of ammonium derivatives, organic compounds resulting from NO_3^- derivatization, and amines. In the past there seems to have been some lack of a spirit of adventure in exploring the analysis of complex organic compounds in favor of the safe, but not always relevant, quest for ever greater precision in isotopic analysis of N_2.

The analysis of natural abundance $^{15}N/^{14}N$ ratios requires considerable precision, because there is not much evidence for large changes in these ratios in natural pools of nitrogen. This is equivalent to deducing that biological processes do not discriminate actively between molecules

containing the light— as opposed to the heavy—isotope. Considerable discrimination occurs during denitrification, probably some during nitrogen fixation, and interesting changes during organic-N mineralization. There is an enormous attraction in being able to use the natural $^{15}N/^{14}N$ ratios to deduce the rate of turnover of, or the source of input to, a particular N pool. The method has great potential, many researchers are working with various aspects of individual processes and food chain transformation, and, hopefully, future developments will fulfill the great expectations of so many laboratories. It is our assessment that results to date have been disappointing.

The analysis of $^{15}N/^{14}N$ ratios after the addition of tracer ^{15}N also requires precision, but often the limiting factor is the low concentration of the particular pool being investigated and the difficulty of obtaining enough sample for analysis. This problem will recur frequently as we consider the use of ^{15}N-labeled NH_4^+, NO_3^-, N_2, and organic N to analyze processes in the N cycle. There are three main experimental approaches: The dilution of the label in the pool may be measured to give the rate of production, or the rate of disappearance from the pool may be measured to give a rate of consumption. The rate of appearance of label in another pool gives the rate of production of that compound.

$^{15}NH_4^+$ *as Tracer*

Three arrows connect NH_4^+ with other pools (Fig. 1), but, in addition, labeled $^{15}NH_4^+$ can be used to see the exchange between dissolved-, exchangeable-, and bound-NH_4^+ pools, in soils and sediments. Possibly the most important use of $^{15}NH_4^+$ is in the measurement of the rate of organic-N mineralization. Ammonium is the final product of organic-N mineralization in all environments, and it is assumed that this NH_4^+ has the normal $^{15}N/^{14}N$ ratio (this remains to be proven). This NH_4^+ dilutes the added $^{15}NH_4^+$ and the rate of dilution reflects the rate of organic-N mineralization. Any disappearance of the $^{15}NH_4^+$, due to uptake or oxidation, and any change in pool size during the experiment can be taken into account in the calculation of the true rate of dilution. It should be remembered that oxic and anoxic ecosystems differ in the size and turnover rate of the NH_4^+ pool. There are real problems in studying tracer dilution when the concentration of NH_4^+ is very low, as in oligotrophic waters. First, it is impossible to add the label at concentrations approaching tracer levels (10%) when the *in situ* concentration may be only 0.5 μM and the rate of NH_4^+ uptake by algal cells is high. Second, processing liters of seawater to get enough sample for analysis is not simple. Some ingenious solutions to the latter problem have been used, and these are described in the relevant chapters. One solution to the adding of label is to make the assumption

that the concentration of added NH_4^+, the end-product of organic-N degradation, does not affect the rate of degradation. There would, therefore, be no disadvantage in adding excess amounts of label. This assumption is very likely true for most situations, where the heterotrophic microorganisms, which are responsible for the mineralization of organic N, are probably energy-limited and not stimulated by the addition of excess NH_4^+. This may not always be true, especially if the substrate(s) for the heterotrophic microbes has a high C : N ratio. This could be the situation in pelagic environments, where the organic substrate used by the heterotrophs might be largely carbohydrate, released by the photosynthetic phytoplankton. Similarly, heterotrophic microorganisms in the oxic rhizosphere may largely depend for energy on organic molecules of low nitrogen content, which have been secreted from the roots. It is unlikely that the addition of large amounts of $^{15}NH_4^+$ to anoxic environments can have any effect on the rate of NH_4^+ production by the heterotrophic population. The *in situ* NH_4^+ pool is usually high in anaerobic sediments, soils, and waters: NH_4^+ is not a limiting nutrient. The problem in these environments is not that of label addition or of obtaining sufficient sample for analysis; it is a problem of a slow rate of NH_4^+ production, which gives a slow rate of $^{15}NH_4^+$ dilution. Usually the gross rate of NH_4^+ production is of little interest, particularly in this type of environment. The net rate of production is of most interest, because this is the rate that determines the amount of NH_4^+ that will be free to be transported to an oxic interface, where it can enter into a more general nitrogen cycle. The measurement of net rates of production does not depend on nitrogen isotope technology.

The second rate that can be measured, following the addition of $^{15}NH_4^+$ to a natural pool, is that of incorporation into biomass. In situations where uptake and incorporation into biomass is the only mechanism for the disappearance of label (taking account of any changes in pool size), the rate of disappearance may be used to calculate the rate of incorporation of NH_4^+ into biomass. Pelagic systems are an example of this type of environment, where the rate of nitrification is insignificant. In this, as in all other systems, there is an incorporation of label into the heterotrophic population, and it is only the surplus NH_4^+ that is available to the algal population. The synthesis of biomass, calculated from $^{15}NH_4^+$ disappearance, may, therefore, be higher than that obtained by measuring directly the amount of ^{15}N that had been incorporated into phytoplankton cells. This latter method is more direct and has been used for many years to measure rates of incorporation into pelagic phytoplankton. The directness of the method is attractive, but there is a penalty to pay. The rate of NH_4^+ uptake is concentration-dependent, and the problem again arises of adding sufficient label to a very small pool so that the uptake of this label may be

detected. This must be achieved without altering uptake rates. It is not surprising that the more indirect method is increasingly used in this type of oxic environment. Measurement of $^{15}NH_4^+$ incorporation into biomass in anoxic environments is complicated by the large NH_4^+ pools, the slow rates of incorporation, and a lack of interest in the rate obtained. The uptake rate can be measured either from $^{15}NH_4^+$ disappearance or from appearance in biomass. The latter measurement is complicated by the very large background of organic N, which is usually present in, for example, anoxic sediments and soils. It is likely that future developments will include methods for the selective extraction of newly synthesized organic N (proteins, polynucleotides), in which the ^{15}N content can be measured. It is not true that there is a total lack of interest in biomass synthesis in anoxic environments. The incorporation of $^{15}NH_4^+$ is one of the few methods for assessing microbial growth in these ecosystems.

Ammonium exists in multiple pools in both oxic soils and anoxic sediments. The NH_4^+ dissolved in pore water is in equilibrium with exchangeable NH_4^+, which is loosely bound to particles. There is a third pool associated with clay mineral lattices, which is in very slow equilibrium with the other two pools. The exchange of NH_4^+ between these pools has been studied by observing the rate of transfer of added $^{15}NH_4^+$ from pore water to the sediment particle pools. It should be noted that these processes have significance in experiments where $^{15}NH_4^+$ is added to soil and sediment pools, to study biological rates. The physical factors that determine NH_4^+ distribution must also be recognized.

The third flux, which involves NH_4^+ (Fig. 1), is the loss to NO_3^-. This process of nitrification is restricted to oxic environments (soils, waters, and the superficial layers of sediments). Although some nitrification occurs in pelagic waters, the rates are low in relation to the other fluxes, which involve NH_4^+ in these waters, and are relatively uninteresting. The rates of nitrification in oxic soils are high: Virtually all the net production of NH_4^+ is rapidly oxidized to NO_3^-. Rates of nitrification are thus equivalent to rates of NH_4^+ production. The most interesting situation in which nitrification occurs is at the oxic–anoxic interface of sediments, roots, worm burrows, etc. It is at these interfaces that coupled nitrification–denitrification occurs, and it is here that the rate of nitrification is most difficult to measure. Nitrification occurs in a very narrow zone at this interface, possibly the zone is of millimeter dimensions, at least in situations where nitrification rates are high. There is thus the technical difficulty of adding $^{15}NH_4^+$ to this layer in tracer amounts, of measuring the specific activity of this NH_4^+ pool, and finally of measuring the amount of ^{15}N label that appears in the very small NO_3^- pool. The concentration of the latter may not exceed 10 μM, ~1 nmol cm^{-2}, or 10 μmol m^{-2}. It might thus require

the extraction of the NO_3^- pool from 1 m^2 of sediment for one ^{15}N determination. While sample size requirement is likely to decrease and extraction methods are expected to improve, there remains the problem of determining the $^{15}N/^{14}N$ ratio in the small and highly dynamic NH_4^+ pool, at the oxic interface. Considerable ingenuity has been demonstrated in the construction of continuous flow chambers, where labeled NH_4^+ can be supplied in the perfusing water. These systems solve some, but not all, of the difficulties involved in measuring rates of nitrification. At present, nitrification can probably be better measured by conventional methods, which do not involve ^{15}N.

$^{15}NO_3^-$ as Tracer

The dilution of added $^{15}NO_3^-$, in all environments, gives the rate of nitrification. The comments in the preceding paragraph are equally relevant, in this context. The situation where it is of most importance to measure the rate of nitrification is also the most difficult. Labeled NO_3^- can be added to sediment or to overlying water in a flow-through system, but in neither situation can the rate of dilution of the sediment pool be measured unambiguously.

The contribution of NO_3^- to the increase in microbial or algal biomass N can be calculated from the appearance of ^{15}N label from NO_3^- in cells. This is of little interest in sediments but is of considerable interest in determining the uptake of NO_3^- by pelagic phytoplankton. It is mostly in this context in which rates of incorporation have been measured. The rate of nitrification is insignificant in this system, so no dilution of added label occurs. It is important that tracer quantities of $^{15}NO_3^-$ be added, so that uptake rates are not stimulated. In many situations it would seem that measurement of NO_3^- disappearance might be a more appropriate methodology. The addition of $^{15}NO_3^-$ to soils has largely been in the context of measuring the rate of incorporation of ^{15}N label into plants. It is important to measure and make allowance for any changes in the $^{15}N/^{14}N$ ratio of the NO_3^- that might occur during the experimental period.

The most significant use for $^{15}NO_3^-$ is in the measurement of rates of denitrification. The appearance of label in $^{15}N_2$ would be related to the rate of reduction of $^{15}NO_3^-$. Flow-through systems with marine sediments, containing $^{15}NO_3^-$ in the perfusing water, allow for equilibrium conditions to be established and for a good measurement to be made of the rate of denitrification from externally supplied NO_3^-. Unfortunately, this experimental procedure does not measure denitrification that depends on internally produced NO_3^-. It seems very likely that future developments will allow for better measurements of coupled nitrification–denitrification rates in sediments. Sadly, this book lacks a chapter on sediment denitrification.

The absence may not be so serious at this time, because it seems that this most important potential use of $^{15}NO_3^-$ has not yet been fully realized. The problems associated with the narrow oxic–anoxic interface in sediment are not relevant in the measurement of denitrification rates in soils. There are usually large NO_3^- pools in soils, which can be labeled with $^{15}NO_3^-$, and the rate of production of $^{15}N_2$ may be relatively easily measured. Denitrification is a slow and quantitatively small component of N cycling in pelagic systems.

$^{15}N_2$ *as Tracer*

Potentially, $^{15}N_2$ dilution could be used to measure the rate of denitrification, but it is never employed in this context. Among other problems would be the difficulty of obtaining a random distribution of label in the N_2 in soils and sediments, the sites of most interest in denitrification studies.

The main use for $^{15}N_2$ has been in the direct measurement of ^{15}N incorporation into nitrogen-fixing populations. This has seldom been used on a routine basis, but it has been very useful in establishing the validity of acetylene reduction measurements, particularly for pure cultures. Nitrogen fixation is inhibited by the presence of fixed nitrogen and is thus of most importance in ecosystems where nitrogen is limiting. This is often true for soils from which plants have depleted the available nitrogen. It is also true for many aqueous, particularly marine, systems, and photosynthetic cyanobacteria are the most significant contributors to nitrogen fixation in these environments.

^{15}N-*Organic Compounds as Tracers*

The use of ^{15}N-organic compounds as tracers has been neglected, partially because of the difficulty of obtaining labeled organic molecules and because the rate of organic-N mineralization can be measured by NH_4^+ production. Labeled urea has been used to determine rates of uptake by phytoplankton and to investigate mutual interference in the uptake of urea, NH_4^+, and NO_3^-. Limited use has been made of ^{15}N-labeled amino acids. It seems unlikely that the use of ^{15}N-labeled organic molecules will become routine, but it is predicted that for specific applications, they will prove to be very useful.

Our final conclusion is that ^{15}N methodology has contributed greatly in process-based studies and that it probably will soon contribute to the advance of our knowledge of N cycling at the ecosystem level.

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Mass Spectrometry

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I. INTRODUCTION

Although other techniques may be employed for N isotope analysis, isotope ratio mass spectrometry is the method of choice for quantitative determinations because it provides unmatched precision. This is no small advantage, in that the sensitivity achieved in detecting tracer N is ultimately determined by analytical precision, and any improvement in precision translates into a gain in sensitivity. As a result, use of mass spectrometry for N isotope analysis has the advantage over other techniques that lower concentrations of tracer N can be detected, which extends the scope of N-tracer research and improves detection of treatment effects. Moreover, ^{15}N-depleted materials may be utilized, and measurements can be made of variations in the natural abundance of isotopic N.

The mass spectrometer is, unfortunately, a complicated and expensive instrument that requires considerable care to operate and maintain, and this has been a major limitation in the use of N isotope techniques. However, the technical difficulties have been reduced substantially by advances in the design of mass spectrometers during the past two decades, due largely to utilization of solid-state electronics. The greatest advance has undoubtedly been the development of the automated mass spectrometer, which can perform isotope ratio analyses on microgram quantities of N, at a rate of up to several hundred samples per day. As a result, many of the analytical problems traditionally associated with N isotope research have been elimi-

nated, particularly for those who submit samples to a laboratory offering N isotope analyses as a service.

Information about the procedures used in N isotope analysis by mass spectrometry can be found in a number of excellent publications, including those by Bremner (1965d), Fiedler and Proksch (1975), Hauck and Bremner (1976), Edwards (1978), Bergersen (1980), Buresh *et al.* (1982), Hauck (1982), Haystead (1983), Fiedler (1984), and Robinson and Smith (1991). In the present treatment, consideration is given to the preparation of samples for analyses by automated mass spectrometers as well as to Kjeldahl–Rittenberg analyses using manually operated instruments. Consideration is also given to basic aspects of mass spectrometry and recent advances in automation, but no attempt is made to evaluate the various instruments that are commercially available.

II. INSTRUMENTATION FOR N ISOTOPE ANALYSIS

A. Principles of Operation

There is considerable variety in the design of mass spectrometers, but the great majority of instruments used for N isotope analysis are single-focusing, magnetic deflection spectrometers with an electron impact ion source, and the analysis is invariably carried out with N in the form of dinitrogen (N_2). Operation of such an instrument during isotope ratio analysis of N_2 is illustrated in Fig. 1, which shows a schematic diagram of the analyzer assembly of a 60°-sector mass spectrometer equipped with double collectors. The analyzer consists essentially of three components: the analyzer tube, the ion source, and the collectors. The analyzer tube is fabricated from stainless steel or an alloy (often Inconel®), with its center flattened laterally and curved through an angle of 60° (other designs use an angle of 90 or 180°). The ion source and collectors are mounted at opposite ends of the analyzer tube via flanges sealed by gold wire O-rings.

From a suitable inlet system (see Section II.D), N_2 is admitted to the ion source through a leak (a constriction in the inlet line that determines the flow rate) for bombardment by electrons from a heated filament. The electron beam is accelerated across the incoming flow of N_2 molecules by a positive potential (typically 70 volts) between the filament and an anode (commonly referred to as a trap), such that the energy of the electrons greatly exceeds the ionization potential of N_2. Ionization results largely from the loss of a single electron to form $^{28}N_2^+$, $^{29}N_2^+$, and $^{30}N_2^+$. To minimize ion–molecule reactions, pressure in the ion source is maintained at <1 mPa by the use of a high vacuum pump (see Section II.C), which

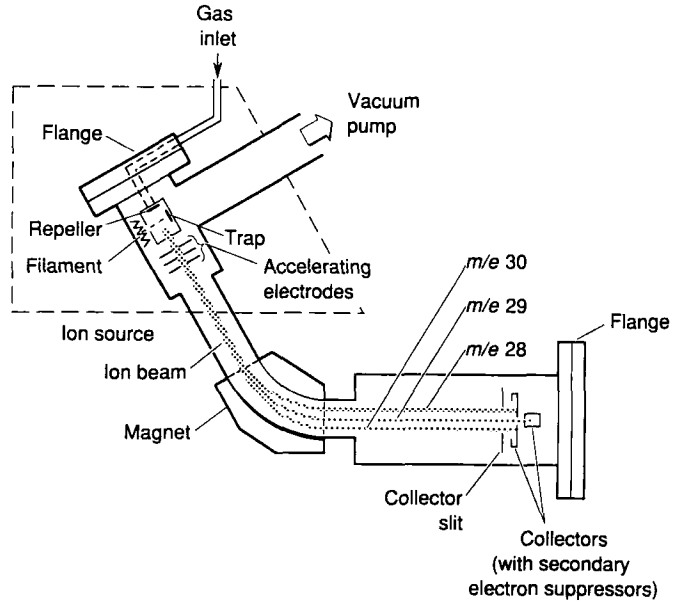

Figure 1 Schematic diagram illustrating operation of a double-collector mass spectrometer during isotope ratio analysis of N_2.

operates continuously. Nevertheless, the efficiency of ionization is quite low (typically of the order of 0.1%), and most of the molecules that enter the ion source are removed by the vacuum pump.

The positive ions are drawn out of the ion source by the combined effects of a small positive potential (e.g., 50 volts) on the repeller and a much larger negative potential across the accelerating electrodes (typically 3000–5000 volts). After passing through an exit slit, the ions enter the magnetic sector, consisting of the curved portion of the analyzer tube positioned between the poles of a permanent magnet or an electromagnet. Their path is curved due to the influence of the magnetic field, the radius of curvature depending on the mass and charge of the ions and their velocity (determined by the accelerating voltage) as well as the field strength. The two latter factors are held constant, so the ions are separated into three beams according to their mass-to-charge (*m/e*) ratios, the radius of curvature decreasing in the order $^{30}N_2^+$ (*m/e* 30) > $^{29}N_2^+$ (*m/e* 29) > $^{28}N_2^+$ (*m/e* 28). Additional beams of lower intensity are also formed (see Table I), due in part to the presence of O_2 and Ar in the N_2 under analysis and to residual gases in the mass spectrometer, including H_2O and CO_2.

Table I
Principal Ions Normally Present during Isotope Ratio Analysis of N_2

m/e	Ion
14	$(^{14}N^{14}N)^{2+}$
14.5	$(^{14}N^{15}N)^{2+}$
15	$(^{15}N^{15}N)^{2+}$
18	$(^{1}H^{1}H^{16}O)^{+}$
28	$(^{14}N^{14}N)^{+}$
29	$(^{14}N^{15}N)^{+}$
30	$(^{15}N^{15}N)^{+}$
32	$(^{16}O^{16}O)^{+}$
40	$(^{40}Ar)^{+}$
44	$(^{12}C^{16}O^{16}O)^{+}$

The intensities of the ion beams corresponding to *m/e* 28, 29, and 30 are directly related to the isotopic composition of the N_2 under analysis, and collection is accomplished using one or more insulated electrodes (anodes) with entrance slits and secondary electron suppression (to reduce noise). With the double-collector arrangement illustrated in Fig. 1, the ion beams corresponding to *m/e* 28 and 30 impinge on one collector (the multicollector), while the beam corresponding to *m/e* 29 impinges on the other (a Faraday cup positioned behind a slit in the multicollector). Focusing of the ion beams is accomplished by regulating the accelerating voltage (for instruments equipped with a permanent magnet) or the magnetic field strength (for instruments equipped with an electromagnet), which changes the *m/e* collected according to the equation

$$m/e = 4.82 \times 10^3 H^2 r^2 / P \qquad (1)$$

where H is the field strength in tesla, r is the radius of curvature in centimeters, and P is the accelerating potential in volts.

The current ($I = 10^{-9}$–10^{-12} A) generated by ions striking a collector is fed to an electrometer with a very high input resistance ($R = 10^{10}$–10^{12} Ω), which produces a proportional voltage ($V = IR$). The voltage is measured using a strip-chart recorder, a digital voltmeter, an integrating ratiometer, or a computer equipped with an analog-to-digital, or A/D, converter.

B. Parameters of Performance

The performance of a mass spectrometer can be measured in several ways. For an isotope ratio instrument, the primary parameters of interest are resolution, precision, accuracy, and sensitivity.

In mass spectrometry, resolution is defined as the ability to separate ion beams that differ in *m/e* ratio. It is most commonly calculated as $m/\Delta m$, where m is the nominal mass (actually *m/e*) for a particular peak in the mass spectrum, and Δm is peak width at 10 or 50% of the peak height. The resolution attainable with most isotope ratio mass spectrometers is <500, which is quite low compared to the resolution afforded by instruments used for exact mass measurements but is more than adequate for isotope analysis of N_2 and other gases of low molecular weight. Some increase in resolution can be achieved by reducing slit widths, but at the expense of sensitivity.

Precision refers to the reproducibility of an isotope abundance measurement. It is commonly expressed in terms of the coefficient of variation (relative standard deviation) for a series of measurements on a single sample (internal precision) or as the minimum detectable difference in isotope ratio between a pair of samples introduced sequentially from a dual-inlet system. Values quoted for commercially available isotope ratio mass spectrometers range from 0.001 to 0.00001 atom% ^{15}N. However, actual precision may be somewhat lower than the quoted value, particularly in analysis of small samples (i.e., those for which the inlet pressure is less than optimal) or samples highly enriched in ^{15}N.

The accuracy of a mass spectrometer for isotope abundance measurements is more difficult to evaluate than precision. Most commonly, evaluation is based on analyses of interlaboratory standards. Of course, the results obtained can be influenced by several factors besides the accuracy of the mass spectrometer, including sample size and purity and, particularly, faulty sample preparation technique, which can lead to isotope fractionation. A loss of accuracy often occurs at high enrichments, due to nonlinearity in the amplification of ion currents by the electrometer(s).

The sensitivity of a mass spectrometer can be defined as the minimum sample size required for optimal accuracy and precision. Conventionally, 0.5–5 mg N are required for N isotope analysis, but recent advances in automation (see Section II.F) have led to a dramatic gain in sensitivity, allowing routine analyses of <50 μg N. Among the factors that contribute to sensitivity are a decrease in the volume of the inlet system (e.g., through use of a smaller sample container) or in the speed of pumping of the ion source and an increase in (1) the size of the leak, (2) the efficiency of ionization, (3) the ion accelerating potential, or (4) the slit widths for source and collector(s).

C. Vacuum Systems

A fundamental requirement of any mass spectrometer is a vacuum system capable of maintaining very low pressure in the analyzer, typically <100

μPa in the magnetic sector and collector region. At such a pressure, the mean free path of ions formed in the source is sufficiently long to preclude collisions with gas molecules that would cause scattering and loss of energy and lead to ion–molecule reactions.

The vacuum system of a mass spectrometer must be designed to ensure rapid removal of un-ionized gas molecules from the analyzer, so pumping is carried out through a high conductance tube connected to the ion source housing. The speed of pumping should be sufficient to minimize memory from a previous sample, but not so high as to seriously limit sensitivity. In some cases, differential pumping is utilized to increase resolution, using a second pump connected to the collector housing.

The low pressures required for operation of a mass spectrometer cannot be attained with a mechanical (rotary) pump. A high vacuum pump must be used. The most common types are the diffusion pump, the ion pump, and the turbomolecular pump.

In the diffusion pump, a fluid having low volatility at room temperature (e.g., polyphenyl ether) is brought to boiling by a heater at the base of the pump. The vapor rises through the chimney assembly and is discharged from several ring-shaped nozzles to form a series of low-pressure vapor jets directed toward the base of the pump. Gas molecules at the inlet to the pump become entrained in the top jet and are carried downward, subsequently becoming entrained in lower jets. This causes the gas to be compressed to a pressure at which it can be removed by a mechanical (backing) pump. The vapor molecules in the diffusion pump condense on the water-cooled walls and return to the boiler.

The major limitation of the diffusion pump for mass spectrometry is backstreaming of the pump fluid, which can affect the background spectrum. To reduce this problem, a baffle or a trap containing liquid N_2 is attached to the inlet of the pump. Alternatively, an ion pump may be used, in which case there are no fluids of any kind. Rather, pumping is accomplished by applying a highly positive potential (3000–7000 volts) to a cylindrical anode placed between two flat cathode plates, usually made of Ti. Gas molecules that enter the pump are ionized by electrons from the anode; the ions impinge upon the cathodes with sufficient energy to be implanted, which causes sputtering of Ti. The sputtered Ti is deposited on a cathode or anode surface, where it acts as a getter film and adsorbs reactive gases such as N_2, O_2, and CO_2. To increase the path of the electrons, and hence the efficiency of ionization, the pump body is mounted between the poles of a permanent magnet. Ion pumps offer considerable convenience, because they operate without cooling water, a backing pump, or liquid N_2, and the flow of current indicates pressure, eliminating the need for a separate pressure gauge. However, ion pumps do not provide the strong pumping of a diffusion or turbomolecular pump, particularly in the case of the noble gases, which can cause serious instability (pressure bursts) with some designs.

Increasingly, the turbomolecular pump is being utilized in mass spectrometry. Pumping is accomplished through momentum transfer from the blades of a high-speed (15,000–100,000 rpm) rotor (turbine) arranged in series with a fixed stator, which compresses the gas molecules for removal by a mechanical backing pump. Compared to the diffusion pump, the turbomolecular pump has the advantage of producing a hydrocarbon-free vacuum without the need for a baffle or liquid N_2, and some models can be mounted in any orientation and operated without cooling water. The major limitations are a higher initial cost and the need for periodic bearing replacement, normally after 20,000–30,000 hr of operation.

D. Inlet Systems

The function of the inlet system is to present a steady stream of sample molecules to the ion source at an extremely low flow rate. Typically, this is accomplished using a vacuum manifold with a variable volume reservoir (a Toepler pump or metal bellows) and pressure gauge for pressure adjustment, connected to the ion source via a critical restriction (leak) to regulate the flow rate.

An important consideration in the design of the inlet system is whether gas flow into the ion source is to be molecular or viscous, which determines the necessary inlet pressure and the type of leak to be used. Molecular inlets find many applications in mass spectrometry, but viscous inlets are generally preferred in measuring isotope ratios, owing to the fact that isotope fractionation occurs during molecular flow. Viscous flow requires an orifice that is large relative to the mean free path of the gas molecules, so the volume of the sample reservoir is minimized to obtain an inlet pressure >1 kPa, and the leak is made by crimping a capillary tube, which provides a larger orifice than the leaks used in molecular inlets.

For isotope analyses requiring the highest accuracy and precision, such as those made to detect variations at the natural abundance level (Chapter 4), ratio difference measurements are made using a double- or triple-collector mass spectrometer equipped with a dual-inlet system. Figure 2 shows a schematic diagram of such a system. Among the components are metal bellows for equilibrating the pressures of sample and reference gases, matched viscous leaks, and a set of four changeover valves to sequentially admit sample and reference gases to the mass spectrometer and to a waste pump. During analyses, the changeover valves are actuated automatically at intervals preset by the operator for multiple comparisons of reference and sample under identical conditions.

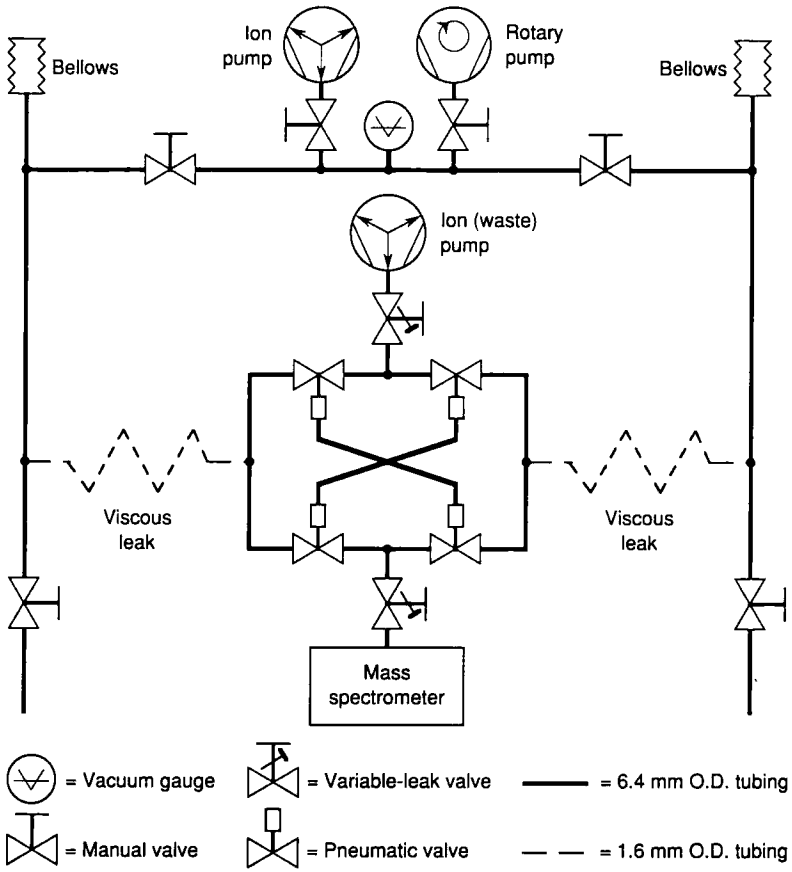

Figure 2 Schematic diagram of a dual-inlet system with matched viscous leaks.

E. Collector Systems

In addition to the double-collector system illustrated by Fig. 1, isotope analyses of N_2 can be performed using a single collector, in which case the ion beams of interest (normally *m/e* 28 and 29) are measured sequentially by regulation of the ion-accelerating potential or the current supplied to the electromagnet (for instruments so equipped). Single-collector measurements are generally considered to be less precise (by about an order of magnitude) than those made using double collectors because of fluctuations during analysis in sample pressure and composition and in the production of ions.

As originally proposed (Nier *et al.*, 1947), double-collector measurements were made by a null balance technique, in which the potentials from the two collectors are balanced against one another using a potentiometer controlled via decade voltage divider switches. This technique has largely been replaced by direct ratio measurements using an integrating digital ratiometer. The ratiometer, introduced in the early 1970s, has proven to be an outstanding advance in isotope ratio mass spectrometry. Not only is a digital display faster and more convenient than the null balance technique, but integration can markedly increase precision, and the output from the ratiometer can be utilized for on-line processing of data by a programmable calculator or computer.

An increasingly common feature of commercially available isotope ratio mass spectrometers is a triple-collector system. Originally developed for C and O isotopic analyses of CO_2 (Morales *et al.*, 1970), a triple collector allows simultaneous measurement of two different ratios, whereas sequential measurements would be required with a double-collector system. The additional capability is considerably less useful for analysis of N_2 than CO_2, because N isotopic abundance can usually be determined from a single ratio, and the determination can be made with equal precision using a double-collector system. However, there is sometimes a need for analysis of two different ratios to determine the relative proportions of all three isotopic species of N_2, as in measuring emission of N_2 into air during denitrification of ^{15}N-labeled NO_3^- (see Chapter 7). In such cases, the necessary ratio measurements can be made more rapidly with a triple-collector mass spectrometer than with a double-collector instrument.

F. Automation

As noted in the Introduction, the practice of mass spectrometry has undergone considerable change during the past two decades, due largely to utilization of solid-state electronics. Of particular significance has been the introduction of low-cost microcomputers, which are now commonly used for controlling the mass spectrometer and monitoring performance as well as for data acquisition. With some instruments, the inlet manifold may be operated under computer control so that unattended analyses can be performed on a series of gaseous samples (typically 20–50 samples per loading); however, in such cases, the preparation of samples (e.g., by hypobromite oxidation of NH_4^+ to N_2) is carried out manually and is off-line, which increases the risk of contamination by atmospheric N_2.

Conventionally, N isotope analyses are performed by the Rittenberg technique, which involves hypobromite oxidation of NH_4^+ to N_2 in the absence of air (see Section III.C). In 1978, a project was initiated by McInteer and Montoya at the Los Alamos National Laboratory to auto-

mate Rittenberg analyses of NH_4^+ salt samples. By 1980, they had developed a working prototype of an automated Rittenberg apparatus (ARA) allowing completely automated N isotope ratio analyses by mass spectrometry (McInteer and Montoya, 1981). With this system, NH_4^+ samples were placed in miniature plastic vials, 137 vials per sample tray. The tray was moved with a modified x–y plotter to sequentially position each vial beneath a pneumatically actuated reaction head designed to make a gas-tight seal with the vial. Air was removed by purging with Freon, hypobromite was added, and the N_2 liberated was allowed to flow through a liquid N_2 trap for removal of Freon before introduction to a manifold connecting a set of custom-fabricated micropneumatic valves. Pressure in the manifold was measured by a pressure transducer, and, if excessive, a valve to vacuum was opened momentarily. The N_2 was then admitted to the mass spectrometer (a double-collector instrument) for isotope ratio analysis. The entire process was under the control of a programmable calculator.

The automated system developed by McInteer and Montoya (1981) had capabilities far beyond those of any conventional isotope ratio mass spectrometer for analysis of ^{15}N; not only could it operate completely unattended, but analyses could be performed with only 25 μg N, at a rate of up to several hundred samples per day. Further improvements were made in the system, including the use of disposable plastic trays to contain samples and the capability for multiple loading of trays (McInteer *et al.*, 1984), and a private business was established to perform automated ^{15}N analyses for the scientific community (Isotope Services, Los Alamos, New Mexico). A commercial version of the ARA designed by McInteer and Montoya (1981), incorporating several refinements, has recently been developed (Mulvaney *et al.*, 1990; Mulvaney and Liu, 1991).

A simpler approach to automation of N isotope analysis involves interfacing an automatic N/C analyzer (ANCA) to a mass spectrometer (Otsuki *et al.*, 1983; Preston and Owens, 1983; Barrie and Workman, 1984; Marshall and Whiteway, 1985; Preston and McMillan, 1988; Barrie and Lemley, 1989; Barrie *et al.*, 1989; Egsgaard *et al.*, 1989; Harris and Paul, 1989; Schepers *et al.*, 1989; Barrie, 1991; Craswell and Eskew, 1991; Jensen, 1991). Operation of such a system (commonly referred to as ANCA-MS) is illustrated schematically in Fig. 3. Samples (normally in the form of a finely ground solid containing 20–150 μg N) are sealed into miniature Sn capsules for loading into the ANCA autosampler (typically a 50- or 66-place unit). From the autosampler, a capsule drops into a combustion chamber (a quartz tube heated to 1020°C through which a He carrier stream flows) containing a catalyst (e.g., Cr_2O_3 granules), finely divided CuO wire (to oxidize hydrocarbons), and Ag wool (to remove S and halogens). Concurrently, a pulse of O_2 is admitted to promote flash combustion of the Sn, which increases the temperature to around 1700°C, ensuring complete oxidation of the sample. The combustion products (CO_2, N_2, NO_x, and H_2O) are swept into a tube containing Cu wire at 600°C, where NO_x species are reduced to N_2, followed by $Mg(ClO_4)_2$ and Carbosorb® traps for removal of H_2O and CO_2. The N_2 is purified by gas chromatography, and a small fraction (≈1%) of the effluent is admitted to the mass spectrometer via capillary tubing connected to a three-way control valve for measurement of *m/e* 28, 29, and 30, from which both total N and ^{15}N may be determined (alternatively, total N may be determined from the ANCA if equipped with a thermal conductivity detector). The bulk of the effluent is exhausted through a coil of capillary tubing to avoid back diffusion of atmospheric N_2.

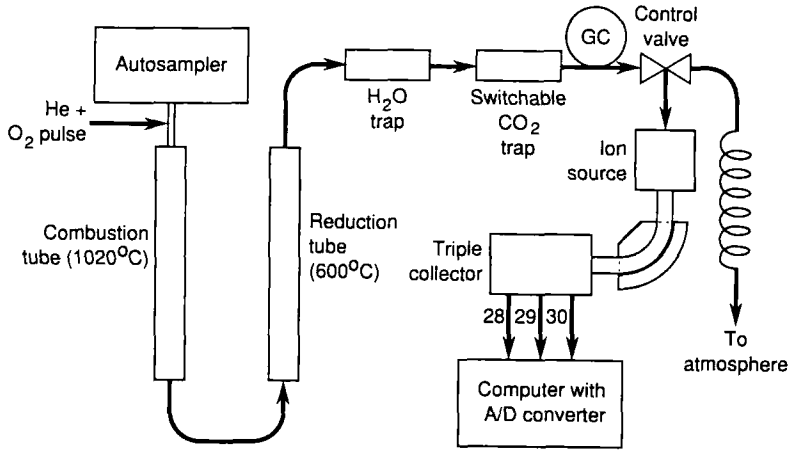

Figure 3 Schematic diagram illustrating ^{15}N analysis by ANCA-MS. (From Europa Scientific Ltd., Cheshire, United Kingdom.)

Use of ANCA-MS for N isotope analysis has expanded rapidly in recent years, and this trend will undoubtedly continue, due in part to the availability of commercial systems consisting of an ANCA, a dedicated magnetic sector mass spectrometer, and a microcomputer. These systems are simpler to operate and maintain than a conventional isotope ratio instrument and are remarkably compact, which makes them portable. They have been successfully used for routine analyses of plant and soil samples and have special value in marine and aquatic biology because analyses can be performed on submicrogram quantities of N (Owens and Rees, 1989), and operation is even possible aboard a ship at sea (Owens, 1988).

III. THE KJELDAHL–RITTENBERG TECHNIQUE FOR N ISOTOPE ANALYSIS

A. General Principles

For N isotope analyses by mass spectrometry, all forms of N in the sample under analysis must be converted into a suitable gas. Dinitrogen is preferred because of its low molecular weight and simple structure (in terms of both molecular and isotopic composition), and because it can be readily generated from a variety of organic and inorganic compounds, is chemically inert, and can be readily pumped from the mass spectrometer.

Although other methods have been used for N isotope analyses of solid or liquid samples by mass spectrometry (Bremner, 1965d; Fiedler and Proksch, 1975; Hauck, 1982; Fiedler, 1984), a three-step procedure is usually employed that involves (1) conversion of labeled N to NH_4^+-N, (2) oxidation of NH_4^+-N to N_2 by alkaline hypobromite in the absence of air, and (3) isotopic analysis of the N_2. This procedure was originally developed by Rittenberg and his colleagues (Rittenberg *et al.*, 1939; Rittenberg, 1948; Sprinson and Rittenberg, 1948, 1949) for use in medical research, but it has been applied more extensively in the agricultural and biological sciences.

Numerous modifications in analytical methodology have been described to improve the speed and convenience of N isotope analysis. Nevertheless, the three-step procedure for this analysis remains a complicated and time-consuming process, and considerable care is required at each step to avoid errors that can arise [for a thorough discussion of the possible errors, see Hauck (1982)]. Moreover, a substantial amount of N is needed (typically 1 mg), which complicates analyses in cases where sample size is limited or the material under study has a low N content (e.g., natural waters), or where the determination must be made on a specific form of N.

B. Conversion of Labeled N to NH_4^+-N

1. Principles

a. Total N Several methods are available for converting labeled N to NH_4^+. The method of choice is determined by the forms of N in the sample under study and by whether or not there is a need to distinguish between them. In most cases, conversion is carried out using the Kjeldahl method or one of its modifications.

The Kjeldahl method is a two-step procedure for determination of total N that involves (1) digestion with concentrated H_2SO_4 to convert organic forms of N to NH_4^+-N and (2) determination of the amount of NH_4^+-N in the digest. To increase the speed and completeness of digestion, additional substances are usually added to the H_2SO_4, most commonly a salt (normally K_2SO_4) to increase the temperature of digestion and a catalyst (Hg, Cu, or Se) to promote oxidation of organic matter. Quantitative determination of NH_4^+-N in the digest is usually accomplished by titration with standard acid of the NH_3 liberated during steam distillation of the digest with alkali.

There are numerous versions of the Kjeldahl method that involve various periods of digestion with different concentrations of salt and different catalysts (see Bremner and Mulvaney, 1982). Sample size also varies. Both macro- and semimicroversions of the Kjeldahl procedure have been employed for conversion of organic N to NH_4^+ for N isotope analysis. Macromethods involve digestion of a sample containing about 10 mg N in a 350- to 800-ml Kjeldahl digestion flask; semimicromethods involve digestion of approximately 1 mg N in a 30- to 100-ml flask. Semimicromethods are generally preferred for the preparation of samples for N isotope analysis, because they provide an appropriate amount of N for the analysis, and there is much less potential for cross-contamination of samples during distillation with a micro-Kjeldahl distillation apparatus than with a macrounit (Hauck, 1982).

During Kjeldahl digestion, H_2SO_4 is consumed in oxidation of organic C to CO_2, which leads to an increase in the salt concentration of the digest and the temperature of digestion. Loss of N occurs at temperatures above about 400°C, which requires a K_2SO_4 concentration of at least 1.3 g ml^{-1} of H_2SO_4 (Bremner and Mulvaney, 1982). This concentration can be attained under some conditions, particularly with Kjeldahl procedures that use high concentrations of K_2SO_4 (>1 g ml^{-1} H_2SO_4), and it is important to estimate the consumption of H_2SO_4 with such procedures so that sufficient acid can be added to compensate for the loss (Fiedler, 1984). This is seldom necessary with the concentrations of K_2SO_4 usually employed in Kjeldahl procedures for total N analysis of soils and plant materials (0.22–0.33 g K_2SO_4 ml^{-1} H_2SO_4), including the procedure described in Section III.B.3.a.

In ^{15}N tracer studies involving Kjeldahl digestion for determination of total ^{15}N, it is essential that all forms of N in the sample under study be quantitatively converted to NH_4^+-N. In some cases, a digestion period of 12–18 hr has been recommended to ensure the absence of volatile amines that interfere in isotope ratio analysis of N_2; however, the period of digestion required for complete conversion of organic N to NH_4^+ depends on the K_2SO_4 concentration, and no interference due to amines has been observed with Kjeldahl procedures for total N analysis of soils and plant materials involving digestion for 5 hr (Bremner, 1965d). In most investigations involving use of ^{15}N-labeled compounds, complete recovery of NO_3^- and NO_2^- is essential, because these forms of N will often have a substan-