Using stable isotopes to follow excreta N dynamics and N$_2$O emissions in animal production systems

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Nitrous oxide (N$_2$O) is a potent greenhouse gas and the leading anthropogenic stratospheric ozone-depleting substance. The tropospheric concentration of N$_2$O continues to increase, with animal production systems constituting the largest anthropogenic source. Stable isotopes of nitrogen (N) provide tools for constraining emission sources and, following the temporal dynamics of N$_2$O, providing additional insight and unequivocal proof of N$_2$O source, production pathways and consumption. The potential for using stable isotopes of N is underutilised. The intent of this article is to provide an overview of what these tools are and demonstrate where and how these tools could be applied to advance the mitigation of N$_2$O emissions from animal production systems. Nitrogen inputs and outputs are dominated by fertiliser and excreta, respectively, both of which are substrates for N$_2$O production. These substrates can be labelled with $^{15}$N to enable the substrate-N to be traced and linked to N$_2$O emissions. Thus, the effects of changes to animal production systems to reduce feed-N wastage by animals and fertiliser wastage, aimed at N$_2$O mitigation and/or improved animal or economic performance, can be traced. Further $^{15}$N-tracer studies are required to fully understand the dynamics and N$_2$O fluxes associated with excreta, and the biological contribution to these fluxes. These data are also essential for the new generation of $^{15}$N models. Recent technique developments in isotopomer science along with stable isotope probing using multiple isotopes also offer exciting capability for addressing the N$_2$O mitigation quest.

Keywords: $^{15}$N, climate change, excreta, nitrogen isotopes, nitrous oxide

Implications

Nitrous oxide (N$_2$O) is a potent greenhouse gas and the leading stratospheric ozone-depleting substance. Its concentration continues to increase, predominantly owing to N$_2$O emissions associated with animal production systems. The number of farmed animals will increase to satisfy growing global population demand. Mitigation is required. Tools to identify mitigation options include the stable isotopes of nitrogen ($^{15}$N). Past and recently developed $^{15}$N methodologies provide tools to better understand the sources of N$_2$O, while tracing the substrates responsible for N$_2$O fluxes over space and time through the environment. Integrating the skill sets and efforts of microbiologists, soil and animal scientists will achieve this.

Introduction

Nitrous oxide (N$_2$O) is a greenhouse gas and the dominant stratospheric ozone-depleting substance emitted by humans in the twenty-first century (Ravishankara et al., 2009). Concentrations of N$_2$O have increased since 1800 and continue to do so at 0.26% per annum (Forster et al., 2007). Anthropogenic emissions (6.7 Tg/year) represent 40% of the total annual (17.7 Tg/year) global emissions of N$_2$O (Forster et al., 2007). The main anthropogenically derived substrates for N$_2$O production include synthetic and organic fertilisers and excreta; thus, agricultural production of N$_2$O (2.8 Tg/year) dominates anthropogenic emission sources (Denman et al., 2007; Schils et al., 2013). Davidson (2009) showed that the observed increase in tropospheric N$_2$O between 1860 and 2005 could be explained by 2.0% of manure nitrogen (N) and 2.5% of fertiliser N being emitted as N$_2$O over this period. Oenema et al. (2005) partitioned N$_2$O emissions from animal production systems (1.5 Tg/year) into five sources: dung and urine from grazing animals deposited in pastures (41%), indirect sources (27%), animal wastes from housing and storage (19%), application of animal wastes to land (10%) and the burning of dung (3%). The majority of these N$_2$O emissions from agriculture occur as a result of excreta being deposited onto soil where microbial reactions, driven by nitrifiers and denitrifiers,
are the major biological pathways for N₂O. The Food and Agriculture Organisation of the United Nations predicts the demand for animal products to increase, and thus animal numbers are projected to increase significantly (OECD_FAO, 2012). Given the current trends in tropospheric N₂O concentrations and the predicted increases as a result of increasing animal-based agricultural production, it is urgent that a fuller understanding of the sources, processes and management factors that contribute to these agriculturally based N₂O emissions is developed. Improved knowledge will lead to mitigation options. It is necessary to differentiate agricultural N inputs from N forms found in the soil and which can also contribute to N₂O fluxes. This permits the tracing of a particular N input through the agroecosystem concerned, over time, enabling a full and detailed understanding of the factors affecting N₂O fluxes, their duration and magnitude. This article aims to increase awareness of the stable isotopes of N and the vital role they can play in distinguishing N₂O sources and fate in animal production systems. Specific reference to methodologies for preparing ¹⁵N-enriched samples for analysis and other practical considerations may be found in the literature (Knowles and Blackburn, 1993; Hauck et al., 1994).

### Stable isotopes of nitrogen

The number of protons in an element (equal to the atomic number ‘Z’) is constant, but the number of neutrons (the neutron number ‘Nn’) may vary. Isotopes of a given element differ from one another owing to the number of neutrons they contain. This variation in neutron number does not affect the gross chemical properties of the element. The mass of an element (the sum of Z + Nn) is the superscripted number to the left of the element designation (Kendall and Caldwell, 1998). Thus, for N with an atomic number of 7, the stable isotopes have mass numbers of 14 (¹⁴N with seven neutrons) and 15 (¹⁵N with eight neutrons). These N isotopes occur naturally in the environment (Sharp, 2007). In air, the natural abundance of ¹⁵N is constant with a ¹⁵N/¹⁴N ratio equal to 1/272 or 0.3676% (Junk and Svec, 1958).

Further terms used when studying the isotopic composition of molecules include the words ‘isotopologue’ and ‘isotopomer’. Isotopologues, as defined by the International Union of Pure and Applied Chemistry, are molecules differing from one another only because of isotopic composition (Sharp, 2007). For example, N₂O with mass 44 (¹⁴N + ¹⁴N + ¹⁶O) and mass 45 (¹⁴N + ¹⁵N + ¹⁶O) are isotopologues of N₂O. However, isotopomers always have the same number of each isotopic atom and as a consequence always have the same mass (Sharp, 2007). For example, the N₂O molecules comprising ¹⁴N + ¹⁵N + ¹⁶O and ¹⁴N + ¹⁴N + ¹⁶O are isotopomers of N₂O (see below).

The stable isotopes of N provide a unique research tool to elucidate the N₂O emission pathways and N₂O fate. Scientists may use N compounds that have been enriched in ¹⁵N. This means that more ¹⁵N has been added to the substrate of interest before its use in the experimental set-up. Such experiments may be described as ‘tracer experiments’ where the extra ¹⁵N added, that is, the enrichment, is far in excess of the natural abundance of ¹⁵N, thus permitting the scientist to ‘trace’ the flow of ¹⁵N through the ecosystem of interest. Alternatively, the scientist may elect to use the ¹⁵N already existing in the ecosystem of concern, at natural levels of abundance, and these are termed ‘natural abundance’ experiments. Initially, the use of ¹⁵N-enriched tracer will be discussed followed by natural abundance. Fractionation causes changes in the natural abundance of ¹⁵N and this is considered below.

### ¹⁵N-enriched tracer studies

As with any experiment, the rationale and aims of a ¹⁵N-tracer study need to be clearly thought out. In a tracer experiment, the information gathered from the measurements made includes not only the concentrations of N in the various N pools (e.g. N₂O μ/l) but also the level of ¹⁵N enrichment (e.g. N₂O atom% ¹⁵N). The term ‘atom% ¹⁵N enrichment’ is an expression that denotes the concentration of ¹⁵N as a percentage of the total mass of stable N atoms (¹⁴N + ¹⁵N) and is calculated numerically as:

\[
\text{atom } %\text{ } ^{15}\text{N} = \frac{\text{No. of } ^{15}\text{N atoms}}{\text{No. of } ^{14}\text{N }+ ^{15}\text{N atoms}} \times \frac{100}{1} \tag{1}
\]

Although several methods may be used to measure the atom% ¹⁵N enrichment of a sample (e.g. Fourier transform infrared (FTIR) determination of ¹⁵N₂O), the most common method to date has been isotope-ratio mass spectrometry (IRMS). Rather than measuring individual atoms to determine atom% ¹⁵N, the mass spectrometer measures the molecules of interest on the basis of their mass, which is a function of their isotopic composition. If the sample is a solid, it must first be combusted to produce N₂, and then it can be carried in a He flow through the mass spectrometer. Similarly, dissolved N forms, such as inorganic-N, also need to be converted either to a solid form, and then to a gas (Stark and Hart, 1996). Alternatively, the sample may already be in a gaseous state (Stevens and Laughlin, 1994; Laughlin et al., 1997). A detailed explanation of mass spectrometry is beyond the scope of this paper and the reader is directed to other sources (e.g. Mulvaney, 1993; Sharp, 2007). In brief, the gas molecule is introduced into the mass spectrometer’s ‘ion source’ where a fraction of the gas molecules are ionised. The positively charged ions are then moved through a magnetic field with the positively charged ions deflected in a circular trajectory, on the basis of their mass to charge ratio (m/z). The charged ions are collected in Faraday cups, thus forming ion currents (I) that are proportional to the quantity of gas. For the N₂O molecule, the masses of interest are 44, 45 and 46, and for N₂ these masses are 28, 29 and 30. For a given molecular species, the ion currents are used to produce ratios (R). Therefore, for N₂, the ratios ²⁹R (²⁹N/²⁸I) and ³⁰R (³⁰N/²⁸I) are derived (Stevens et al., 1993). For solid samples, the ratios ²⁹R and ³⁰R, which are derived from the combusted...
materials, can be used in the appropriate equations to determine the atom% $^{15}$N enrichment (Mulvaney, 1993). Further consideration must also be given to comparing these ratios against standards and determining instrument factors, so that only the true ratio differences between normal and enriched atmospheres are used when deriving gaseous N fluxes (Stevens et al., 1993; Stevens and Laughlin, 1998). A major focus of many $^{15}$N-isotopic studies is to derive N$_2$ and N$_2$O fluxes from the soil nitrate pool. Further detailed explanations of the assumptions, derivations and implementation of the ratios used when determining denitrification of $^{15}$N-enriched NO$_3^-$, and the respective calculation of $^{15}$N-enriched fluxes of N$_2$ and N$_2$O, can be found in the literature (Mulvaney and Boast, 1986; Boast et al., 1988; Mosier and Schimel, 1993; Mulvaney, 1993; Stevens et al., 1993; Stevens and Laughlin, 1998; Bergsma et al., 2001). The ion currents at m/z 44, 45 and 46 are used to calculate the concentration of N$_2$O, in conjunction with a reference gas, whereas its $^{15}$N enrichment is calculated from ratios of $^{45}$R or $^{46}$R (Stevens et al., 1993; Stevens and Laughlin, 1998).

**Using $^{15}$N-enriched substrates in tracer studies**

A potential artefact when using $^{15}$N-tracer studies is that the application of a relatively large rate of N may unnaturally perturb the system under investigation. Fortunately, in agroecosystems, this is less of an issue, as substrates containing $^{15}$N, used in tracer studies to follow N$_2$O and N$_2$ emissions, generally consist of N forms such as fertilisers, excreta (dung and urine) and plant residues, which by their very nature perturb the system. Therefore, these types of N substrates are potentially ideal for use in $^{15}$N-tracer studies, if suitable enrichment of the substrate can be undertaken.

Once the experiment has been conceived, the experimental treatments must be refined. The first step is to consider the rate of N required in any given treatment. The rationale for this is similar to any non-$^{15}$N experimentation where an N substrate is being added. Thought then needs to go into determining which of the various N pools the $^{15}$N tracer will be measured in, how often the N pools will be measured and what will be the period of the experiment. Naturally, the type of N substrate being applied will also have a bearing on the N pools being measured and their frequency of measurement. If the $^{15}$N tracer is to be followed in multiple pools (e.g. soil, plant and gases) over time, dilution of the $^{15}$N tracer may occur immediately in the soil, because of antecedent N, or at a slower rate because of other N inputs resulting from management and/or soil-N mineralisation. The $^{15}$N enrichment in the N pool of interest may also be diluted because of the pool of background $^{14}$N being significantly larger than the $^{15}$N pool evolving (e.g. N$_2$ evolving into ambient air). Alternatively, significant loss of the $^{15}$N tracer may occur early in the experiment. For example, if the experiment aims to follow the contribution of $^{15}$N-enriched urea to an N$_2$O flux, then allowance needs to be made for a significant fraction of the urea fertiliser (~20%), and the $^{15}$N embodied in it, to be potentially lost within hours of the experiment, starting as a result of ammonia volatilisation. Thus, the N pool(s) of interest, dilution and early loss of $^{15}$N from the experimental system need to be considered when deciding on the level of $^{15}$N enrichment to use.

Finally, the last factor to consider when determining what level of $^{15}$N enrichment to use is the sensitivity of the mass spectrometer (Stevens et al., 1993). The more sensitive the mass spectrometer is, the lower the required $^{15}$N enrichment, all things being equal. The experimental set-up and environmental conditions may also determine the level of enrichment to use. When measuring gas fluxes from soils with headspace chambers, the sensitivity increases with high gas fluxes and smaller headspaces (large surface area to volume ratios). Stevens et al. (1993) provide an excellent example of how to determine sensitivity for a mass spectrometer. As $^{15}$N enrichment and gas fluxes decrease, the coefficient of variation will increase and data quality will suffer (Stevens et al., 1993). For example, using an enclosure time of 2 h with an enclosure volume: surface area ratio of 5 : 1, and a NO$_3^-$-$^{15}$N enrichment of 60 atom% Stevens and Laughlin (1998) reported a limit of detection for N$_2$ fluxes of 3.5 g N$_2$-N/ha per day. A point not always recognised by researchers using $^{15}$N enrichment to measure N$_2$ fluxes is the requirement to be able to measure both the $^{29}$R and $^{30}$R ratio. In order to do this, there must be both $^{14}$N and $^{15}$N present. Thus, starting an experiment with a substrate that is 98 atom% $^{15}$N enriched (a commonly available $^{15}$N enrichment), and with little or no potential for $^{15}$N dilution, reduces the chances of measuring robust $^{29}$R and $^{30}$R ratios.

Fractionation results from the differential responses of stable isotopes in either kinetic reactions, where lighter isotopes tend to react faster, or exchange reactions, where heavy isotopes concentrate where bonds are strongest (Fry, 2006). The result is that products have a lighter isotopic composition owing to the fractionation process. The degree of fractionation can be calculated as a fractionation factor. Detailed discussion of fractionation factors is beyond the scope of this article and the reader is directed to other articles (Fry, 2006; Sharp, 2007). It has been experimentally shown that the isotopic fractionation during the production of N$_2$O via denitrification may vary with the $^{15}$N enrichment of the nitrate substrate; however, the effect of such isotopic fractionation was shown to be negligible if the enrichment of the substrate was greater than 0.6 atom% $^{15}$N (Mathieu et al., 2007). It has also been shown that isotopic fractionation does not bias quantifications of gross N transformations in modelling studies when isotopic $^{15}$N enrichment is used (Rütting, 2012). Thus, the use of high levels of $^{15}$N enrichment avoids any bias owing to isotopic fractionation.

**Labelling of plant materials/fertiliser/ruminant excreta with $^{15}$N**

The $^{15}$N content of soil inorganic-N, fertilisers, excreta, plants and gases can all be enriched in order to follow the fate of these compounds in the environment. The degree of enrichment required depends on the material being enriched and the proposed nature of the ensuing experiment. Labelling of dairy cow manure or slurry is generally performed by feeding animals herbage that has been fertilised with
15N-labelled manures have been used to trace manure effects on N cycling in soils and agronomic effects (Bernsten et al., 2007; Bosshard et al., 2009; Bosshard et al., 2011), but the number of studies that have included measures of N2O and its enrichment following manure, slurry or digestate applications are relatively few (Hauck et al., 1994; Dittert et al., 2001; Lampe et al., 2006; Schouten et al., 2012). The study by Dittert et al. (2001) is a good example of a study where 15N tracing was used to demonstrate the potential of a nitrification inhibitor (3,4-dimethylpyrazole phosphate) to reduce N2O emissions from slurry injected into soil. In this instance, the dairy slurry was 15N enriched and the isotopic composition of the soil and N2O pools were monitored. Besides having lower N2O emissions, the 15N enrichment of the N2O emitted was lower in the nitrification inhibitor treatment, indicating that less N2O was derived from the slurry in this treatment. Further such studies are urgently required to assess management and mitigation strategies for reducing N2O emissions. For example, the call to reduce excess N in the diet of the ruminants or the manipulation of the ruminant diet with feeds varying in C:N ratios will have implications for N2O fluxes from faeces that should be ascertained.

In comparison with generating 15N-labelled excreta, the production of 15N-enriched plant residues is relatively straightforward. Plants are grown with appropriate nutrition and water (avoiding leaching events), generally in a sandy matrix to avoid 14N mineralisation from the soil organic-N pool diluting the 15N pool, with N nutrition provided by adding a 15N-enriched fertiliser. Again urea is best avoided so that 15N is not lost unnecessarily owing to NH3 volatilisation. Once the plant is at the required stage of growth, it may be harvested and used in 15N-tracer experiments. Previous results using 15N to apportion N2O sources have shown that emissions from plant residue applications can be short-lived (Frimpong and Bagg, 2010; Frimpong et al., 2011). Ruminant grazing of pasture and forage crops causes fresh litterfall, as animals fail to ingest all harvested herbage (Lodge et al., 2006; Campanella and Bisigato, 2010; Pal et al., 2012). One study, replicating a grazing-induced litterfall event, used 15N tracer to show that fresh litter deposition contributed to the N2O flux (1% of N applied) from the soil surface and enriched the soil inorganic-N pool (Pal et al., 2013). Experiments have also been conducted using 15N-enriched N2 to study the fate of biologically fixed N2. For example, Carter and Ambus (2006) showed that easily degradable clover residues (Trifolium sp.) made a minor contribution to N2O fluxes. Other studies have shown that the dynamics of N2O emissions derived from 15N-labelled residues are impacted upon by earthworms (Giannopoulos et al., 2011). Modelling of 15N studies conducted by Delgado et al. (2010) suggest that residues should not be treated in the same way as fertilisers in terms of N2O emissions, and they call for more residue studies to examine N2O losses from agroecosystems. This will be done best by using 15N tracing.

The study of fertiliser applications using 15N tracer is perhaps the easiest of the substrates to deal with, as it requires little preparation other than perhaps diluting the
acquired $^{15}$N-enriched isotope fertiliser to a level of enrichment suitable for the experimental objectives. One consideration is the form to apply the fertiliser in. The easiest but perhaps the least conventional way to do this is to water a fertiliser solution onto trial plots. But again the experimental design and objectives need to be considered.

**A role for $^{15}$N-enriched $N_2O$**

It is well recognised that $N_2O$ may be consumed in the soil profile by denitrifiers (Chapuis-Lardy et al., 2007). The use of $^{15}$N-enriched $N_2O$ is an underutilised tool that can increase our understanding of $N_2O$ production and fate in agroecosystems. Again, owing to its very nature, the $^{15}$N-stable isotope acts as a tracer for the $N_2O$ molecule when it is added to the soil. If $N_2O$ is sufficiently enriched in $^{15}$N and of high enough concentration, it is theoretically possible to observe $N_2$ production, but the large $N_2$ background makes it impractical. However, using soil columns, Clough et al. (2006) demonstrated that the addition of $^{15}$N-enriched $N_2O$, along with an inert tracer gas SF6, could be used to calculate an $N_2O$ sink (consumption plus absorption by water), whereas the corresponding decrease in the $^{15}$N enrichment between successive soil depths enabled $N_2O$ production in the soil profile to be calculated simultaneously, as the $N_2O$ diffused through the soil. One reason for the low uptake of $^{15}$N-tracer studies directly using $N_2O$ is the cost of commercially available $^{15}$N-enriched $N_2O$. However, $^{15}$N-enriched $N_2O$ can be made and collected on a small scale in the laboratory by gently and carefully heating small quantities of ammonium nitrate using an oil bath or muffle furnace (Friedman and Bigeleisen, 1950). Further studies with $^{15}$N-enriched $N_2O$ are required to increase our understanding of the factors affecting $N_2O$ : $N_2$ ratios in soils (e.g. carbon supply and soil pH) to enable the design of $N_2O$ flux mitigation strategies focused on soil and manure management.

**Modelling $N$ transformations, $N_2O$ and $N_2$ emissions using $^{15}$N-tracer studies**

The first models developed using $^{15}$N-tracer data focused on determining gross production and consumption of mineral N, on the basis of the exchange between organic and mineral N (Kirkham and Bartholomew, 1954). These early models were simple enough to allow the development of straightforward analytical solutions. However, process-specific gross N rates including production of an N species (e.g. $NO_2^-$) from various sources can only be quantified with models that are based on more realistic N-transformation concepts, such as those developed by Myrold and Tiedje (1986) and Barradough and Puri (1995). The set of simultaneous equations developed is solved using numerical integration with parameters in these models determined by suitable parameter optimisation routines (Mary et al., 1998). Further developments of more realistic and arguably more complex analysis models utilise parameter optimisation routines that can handle large numbers of parameters, such as Markov Chain Monte Carlo techniques (Mueller et al., 2007; Ruetting and Mueller, 2007). Currently, $N_2O$ fluxes can be described by such models so that source partitioning and rates of $N_2O$ consumption/production can be derived (Abbasi and Muller, 2011), and the microbial processes responsible for the observed $^{15}$N dynamics (e.g. autotrophic v. heterotrophic nitrification or denitrification) can also be determined (Stange and Dohling, 2005). Useful future developments in these models would be the use of longer time periods and the use of $^{15}$N-labelled substrate pools to realistically mimic excreta or slurry deposition. To advance our understanding of N-transformation processes related to various soil organic N pools, there needs to be more utilisation of $^{15}$N labelling in experiments, where various organic N and mineral N pools are $^{15}$N labelled. Although the costs of these experiments ($^{15}$N label and the analysis costs) are relatively high, the data from such studies are essential, if we are to fully understand the role of soil N-transformation processes that produce $N_2O$ from animal excreta. Studies with $^{15}$N-enriched substrates focusing on inputs other than inorganic N are beginning to appear and provide insights into the effects of substrate additions on gross N dynamics. For example, Nelissen et al. (2012) modelled soil mineral N dynamics following the application of $^{15}$N-labelled biochar to a soil. With the advances in analytical techniques and analysis models ($^{15}$N-tracing models), it is now possible to analyse complex system dynamics. It is mainly the costs associated with $^{15}$N-tracing studies that may prevent further large-scale experimental work. Furthermore, analytical challenges are still to be solved, such as the development of reliable field methods to quantify for instance the $N_2/N_2O$ ratios.

**Utilising $^{15}$N-enriched tracers to understand microbial contributions to $N_2O$ dynamics**

The predominant biological processes in soils forming $N_2O$ predominantly include nitrification, nitrifier denitrification, nitrification-coupled denitrification and denitrification (Wrage et al., 2001; Kool et al., 2011b). The use of $^{15}$N on its own cannot differentiate between the inorganic-N sources contributing to $N_2O$ fluxes or determine the significance of individual processes to soil-derived $N_2O$ emissions. Thus, Wrage et al. (2005) devised a novel dual isotope method ($^{15}$N and $^{18}$O (oxygen)) to assign $N_2O$ production to these processes. The method assumed (a) no preferential removal of $^{18}$O or $^{16}$O during nitrifier denitrification or denitrification, (b) the $^{18}$O signature of the applied $^{18}$O-labelled water would remain constant over the experimental period and (c) exchange of O between $H_2^{18}O$ and $NO_3^-$ would be negligible. Following the application of N to a silt loam soil at 50% water-filled pore space, the assumptions were validated. Wrage et al. (2005) showed that nitrifier denitrification is a significant source of $N_2O$ in soil. However, the assumption that there was negligible exchange of O between $H_2^{18}O$ and $NO_3^-$ was subsequently proven to be violated (Kool et al., 2007; Kool et al., 2009a and 2009b). Thus, the dual isotope method was revised by introducing an additional $^{18}$O-labelled $NO_3^-$ treatment so that O exchange during denitrification could be accounted for (Kool et al., 2010 and 2011a), and it was subsequently shown that
nitrifier denitrification made a significant contribution to the 
N₂O fluxes in a number of soils examined. This method holds 
great promise for furthering our understanding of the role of 
biological processes in producing N₂O and needs to be 
applied across a wider range of soils and agroecosystems, in 
particular.

One of the most intriguing and exciting isotopic develop-
ments in recent years has been that of stable isotope probing 
(SIP) of nucleic acids. The method relies on microorganisms 
assimilating significant quantities of the isotope concerned. 
This has been used successfully to trace uptake of 13C-
labelled compounds into the DNA or RNA of soil micro-
organism (Radajewski et al., 2003). Previously uncultivated 
N₂ fixers, which assimilate N, have been identified using 
this method with 15N (Buckley et al., 2007). However, the 
direct use of SIP to identify dissimilatory organisms and/or 
conditions that promote N₂O production/consumption has 
generally been limited to conditions where denitrification is 
optimal and where 13C compounds are dosed to identify 
organisms operating in the denitrifying conditions. For 
example, a study by Ishii et al. (2011) supplied 13C-labelled 
succinate with and without N₂O to determine what microbes 
were undertaking N₂O consumption in rice paddies soils. 
This examination of denitrifiers by 13C-proxy, under denitrifying 
conditions, in the absence or presence of substrates has 
merit, but it should also utilise 15N-labelled N substrate to 
further strengthen the findings (fate or change in 15N sub-
strate would provide information on denitrification activity), 
and with emphasis placed on RNA–SIP under such condi-
tions, as RNA provides information on active microorgan-
isms. It is not yet understood how individual nitrification 
or denitrification genotypes affect N₂O production (Braker 
and Conrad, 2011). This methodology holds much promise 
and needs to be applied widely to excretal and fertiliser 
inputs in agroecosystems, so we can determine the key 
microbes and their function as it relates to nitrification and 
nitrification processes.

Natural abundance studies

Natural abundance studies utilise the naturally occurring 
isotopic composition of the molecule in question and report 
The abundance of the atom concerned in delta notation (δ) in 
units of ‰:

\[ \delta x = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000 \tag{2} \]

where \( \delta x \) is the value of the heavy isotope in the sample 
\( (R_{\text{sample}}) \) relative to a standard \( (R_{\text{standard}}) \), where the inter-
national standard for \( \delta ^{15} \text{N} \) is N₂ in air. The measurement of 
molecules at natural abundance may also be carried out 
using IRMS (see above), infrared or laser spectroscopy (as 
noted below).

An area where natural abundance of N isotopes has come 
into play with respect to N₂O is in the field of isotopomers. 
The N₂O molecule is linear and when 16O is the oxygen 
isotope in the molecule it takes the form \( ^{14} \text{N}^{15} \text{N}^{16} \text{O}, \)
\( ^{15} \text{N}^{14} \text{N}^{16} \text{O} \) or \( ^{14} \text{N}^{14} \text{N}^{16} \text{O} \). The intramolecular distribution of 
\( ^{15} \text{N} \) at the central (\( \alpha \)) or end (\( \beta \)) positions of the molecule are 
assessed by studying the abundance of \( ^{15} \text{N} \) in the molecule. Using \( \delta \)-notation, \( \delta ^{15} \text{N} \) denotes the difference in the \( ^{15} \text{N}^{14} \text{N} / \text{^{15}N}^{15} \text{N} \) ratio with respect to a standard, usually atmospheric N₂ (Mohn et al., 2012). The relative difference in the ratios of 
\( ^{14} \text{N}^{15} \text{N}^{16} \text{O} / ^{14} \text{N}^{14} \text{N}^{16} \text{O} \) and \( ^{15} \text{N}^{14} \text{N}^{16} \text{O} / ^{14} \text{N}^{15} \text{N}^{16} \text{O} \) are denoted \( \delta ^{15} \text{N}^\alpha \) and \( \delta ^{15} \text{N}^\beta \), respectively, whereas bulk value, 
\( \delta ^{15} \text{N}_{\text{bulk}} = (\delta ^{15} \text{N}^\alpha + \delta ^{15} \text{N}^\beta )/2 \) (Mohn et al., 2012). The value for \( \delta ^{15} \text{N}_{\text{bulk}} \) in the troposphere is reported to range from 6.3 
and 6.7‰ depending on location and time of sampling (Mohn 
et al., 2012). Another important piece of data able to be 
derived from the isotopomer measurement is the site pre-
ference (SP = \( \delta ^{15} \text{N}^\alpha - \delta ^{15} \text{N}^\beta \)) of the N₂O molecule. This is 
deemed independent of the isotopic composition of the 
substrate the N₂O molecule derives from and supplies pro-
scess information (Mohn et al., 2012).

Before development of instrumentation for measuring 
\( \delta ^{15} \text{N}^\alpha \) and \( \delta ^{15} \text{N}^\beta \), only the average \( ^{15} \text{N} \) value of the N₂O molecule was determined \( \delta ^{15} \text{N}_{\text{bulk}} \). Isotopomers of N₂O can 
now be measured using FTIR spectroscopy (Griffith 
et al., 2009), tuneable diode laser absorption spectroscopy 
(Pathey et al., 2006), gas chromatography–IRMS (Toyoda and 
Yoshida, 1999; Rockmann et al., 2003; Kaiser et al., 2004) 
and more recently quantum cascade laser absorption spec-
troscopy cavity-ringdown (QCLAS) spectroscopy utilising 
mid-infrared lasers (Mohn et al., 2012).

It is well recognised that N₂O isotopomers can be used to 
constrain the atmospheric N₂O budget and they confirm that 
the increase in atmospheric N₂O is a result of anthropogenic 
perturbation of the N cycle (Yoshida and Toyoda, 2000; 
Park et al., 2012). Initial results examining nitrification and 
denitrification processes showed that different groups of 
organisms produced differing isotopomer signatures, and that 
SP values of 33‰ and ~0‰ were characteristic of 
nitrification and denitrification, respectively (Sutka 
et al., 2003; Sutka et al., 2006).

Isotopomer science is in its infancy in the context of 
examining N₂O fluxes and sources from agroecosystems. The 
isotopomer analyses of N₂O have been applied to examine 
the effects of various treatments on N₂O production and 
consumption including: biogas residue application to soil 
(Koster et al., 2011), cropping soils receiving organic 
and synthetic fertilisers (Toyoda et al., 2011), comparisons of 
tropical forest and cropping soils (Park et al., 2011), soil 
moisture conditions (Well et al., 2006; Jinuntuya-Nortman 
et al., 2008; Bergsterrann et al., 2011), composting (Maeda 
et al., 2010), stimulated soil denitrification using glucose (Meijide 
et al., 2010), cultivation of temperate grassland (Ostrom et al., 2010), 
microbial processes (Bol et al., 2003; Toyoda et al., 2005; Perez 
et al., 2006; Sutka et al., 2006; Well et al., 2008), the effect of 
ruminant diet on subsequent slurry N₂O fluxes (Cardenas et al., 
2007) and ruminant urine (Yamulki et al., 2001). Most of these 
early studies were of short duration and had limited temporal 
sampling. The study by Park’s et al. (2011) suggested that 
the \( \delta ^{15} \text{N}_{\text{bulk}} \) data could be used for distinguishing N₂O fluxes.
from fertilised and natural ‘background’ fluxes, and that the SP $\delta^{15}$N results could be used to differentiate between consumption and production of N$_2$O by microbial pathways. Enticingly, the use of QCLAS has been shown to be capable of continuous analysis of N$_2$O isotopomers with identification of N$_2$O source processes possible (Mohn et al., 2012). This technology also needs to be deployed across agroecosystems to help understand not only excreta sources of N$_2$O, and temporal dynamics, but also to explore and demonstrate the success of mitigation options.

Conclusion

The increasing trend in tropospheric N$_2$O emissions as a result of existing and projected increases in anthropogenic animal production systems demands mitigation options. These can only be implemented if N$_2$O emission sources and their temporal dynamics can be traced in conjunction with N$_2$O fate. The stable isotopes of N and associated methodologies provide the tools to achieve this tracing. More $^{15}$N-tracer studies are needed to ascertain soil and excreta contributions to N$_2$O dynamics. Recently new research fronts using SIP and isotopomers of N$_2$O offer exciting potential as diagnostic tools to evaluate effects and mitigation success. Collaborations between microbiologists, animal production specialists and soil scientists will bring much needed synergies to address the N$_2$O issue.

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