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Using stable isotopes to follow excreta N dynamics and N_2O emissions in animal production systems

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Nitrous oxide (N_2O) is a potent greenhouse gas and the dominant anthropogenic stratospheric ozone-depleting emission. The tropospheric concentration of N_2O 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_2O, providing additional insight and unequivocal proof of N_2O 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_2O emissions from animal production systems. Nitrogen inputs and outputs are dominated by fertiliser and excreta, respectively, both of which are substrates for N_2O production. These substrates can be labelled with ^{15}N to enable the substrate-N to be traced and linked to N_2O emissions. Thus, the effects of changes to animal production systems to reduce feed-N wastage by animals and fertiliser wastage, aimed at N_2O 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_2O 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_2O mitigation quest.

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

Implications

Nitrous oxide (N_2O) is a potent greenhouse gas and the leading stratospheric ozone-depleting emission. Its concentration continues to increase, predominantly owing to N_2O 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_2O, while tracing the substrates responsible for N_2O 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_2O) 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_2O 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_2O (Forster *et al.*, 2007). The main anthropogenically derived substrates for N_2O production include synthetic and organic fertilisers and excreta; thus, agricultural production of N_2O (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_2O between 1860 and 2005 could be explained by 2.0% of manure nitrogen (N) and 2.5% of fertiliser N being emitted as N_2O over this period. Oenema *et al.* (2005) partitioned N_2O 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_2O emissions from agriculture occur as a result of excreta being deposited onto soil where microbial reactions, driven by nitrifiers and denitrifiers,

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are the major biological pathways for N_2O. 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 N_2O 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 N_2O 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 N_2O 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 N_2O 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 N_2O sources and fate in animal production systems. Specific reference to methodologies for preparing ^{15}N-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 (^{14}N with seven neutrons) and 15 (^{15}N with eight neutrons). These N isotopes occur naturally in the environment (Sharp, 2007). In air, the natural abundance of ^{15}N is constant with a ^{15}N/^{14}N 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, N_2O with mass 44 ($^{14}N + ^{14}N + ^{16}O$) and mass 45 ($^{14}N + ^{15}N + ^{16}O$) are isotopologues of N_2O. 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 N_2O molecules comprising $^{14}N + ^{15}N + ^{16}O$ and $^{15}N + ^{14}N + ^{16}O$ are isotopomers of N_2O (see below).

The stable isotopes of N provide a unique research tool to elucidate the N_2O emission pathways and N_2O fate. Scientists may use N compounds that have been enriched in ^{15}N. This means that more ^{15}N 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 ^{15}N added, that is, the enrichment, is far in excess of the natural abundance of ^{15}N, thus permitting the scientist to 'trace' the flow of ^{15}N through the ecosystem of interest. Alternatively, the scientist may elect to use the ^{15}N already existing in the ecosystem of concern, at natural levels of abundance, and these are termed 'natural abundance' experiments. Initially, the use of ^{15}N-enriched tracer will be discussed followed by natural abundance. Fractionation causes changes in the natural abundance of ^{15}N and this is considered below.

^{15}N-enriched tracer studies

As with any experiment, the rationale and aims of a ^{15}N-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. N_2O μl/l) but also the level of ^{15}N enrichment (e.g. N_2O atom% ^{15}N). The term 'atom% ^{15}N enrichment' is an expression that denotes the concentration of ^{15}N as a percentage of the total mass of stable N atoms ($^{14}N + ^{15}N$) and is calculated numerically as:

$$\text{atom}\,\%\,^{15}\text{N} = \frac{\text{No. of}\,^{15}\text{N atoms}}{\text{No. of}\,^{14}\text{N} + ^{15}\text{N atoms}} \times \frac{100}{1} \qquad (1)$$

Although several methods may be used to measure the atom% ^{15}N enrichment of a sample (e.g. Fourier transform infrared (FTIR) determination of $^{15}N_2O$), the most common method to date has been isotope-ratio mass spectrometry (IRMS). Rather than measuring individual atoms to determine atom% ^{15}N, 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 N_2, 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 N_2O molecule, the masses of interest are 44, 45 and 46, and for N_2 these masses are 28, 29 and 30. For a given molecular species, the ion currents are used to produce ratios (R). Therefore, for N_2, the ratios ^{29}R (^{29}I/^{28}I) and ^{30}R (^{30}I/^{28}I) are derived (Stevens *et al.*, 1993). For solid samples, the ratios ^{29}R and ^{30}R, 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_2O 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_2O, 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_2O, in conjunction with a reference gas, whereas its ^{15}N enrichment is calculated from ratios ^{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_2O 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. $^{15}N_2$ 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_2O 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_2O 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

^{15}N-enriched fertilisers or by feeding ^{15}N-enriched urea. Powell *et al*. (2004) describe in detail a *forage* method where feeding of ^{15}N-enriched forage resulted in an increase in the ^{15}N enrichment of the urine, the endogenous N (microbes and microbial products from the rumen, intestine and hind gut plus digestive tract N) and the undigested feed N. Silage components had ^{15}N enrichments of 1.17 to 6.44 and the resulting manure was ~2.5 atom% ^{15}N. They also describe a *urea* method where ^{15}N-enriched urea was fed to ruminally fistulated cows that resulted in ^{15}N enrichment of the urine and the endogenous N but not the undigested feed N, as no ^{15}N-enriched feed was provided (Powell *et al*., 2004). Single dosing with 5 atom% ^{15}N urea produced urine ~1.25 atom% ^{15}N, whereas repeated 50 g doses at regular intervals produced urine ~1.25 to 2.15 atom% ^{15}N. Of particular interest in a study by Powell *et al*. (2004) are the results showing the time that elapses between administering the ^{15}N-enriched forage or urea and the observed peak in ^{15}N enrichment of the excreta. Lampe *et al*. (2006) produced ^{15}N-enriched (0.72 atom%) slurry by feeding steers ^{15}N-enriched hay and maize silage. Yue *et al*. (2012) generated a more highly ^{15}N-enriched manure by letting the animal's gut empty out for 2 days and then feeding mixed silage (26.6 atom% ^{15}N), resulting in cattle manure with 8.0 atom% ^{15}N. Manures of other animal species (sheep and pigs) have been similarly labelled (Sorensen and Thomsen, 2005; Bosshard *et al*., 2011).

The choice of method for ^{15}N-labelling excreta depends on the intention of the experiment. Obviously, ^{15}N labelling of animal excreta is expensive and labour intensive, with due attention needed to be given to animal ethics. However, it provides a genuine urine or faeces matrix that is ^{15}N labelled. In the case of urine, it avoids the researcher having to choose between synthetic urine mixtures where potential bias in N_2O fluxes may occur as a result (Kool *et al*., 2006), or having to further dope collected unenriched urine with ^{15}N urea to generate ^{15}N enrichment in the collected urine. As seen from these results (Powell *et al*., 2004), the degree of ^{15}N enrichment is too low to follow evolution of N_2 fluxes, which is ideally 40 to 60 atom% ^{15}N, but it is sufficient to allow the detection of ^{15}N-enriched N_2O to partition the N_2O sources. An example of a study that used fresh urine labelled with ^{15}N urea is that of Taghizadeh-Toosi *et al*. (2011) who, upon applying the ^{15}N-enriched urine to pasture, were able to partition N_2O sources and show the presence of biochar-mitigated urine-derived N_2O emissions, with the contribution of urine to N_2O emissions lower in the presence of biochar.

The recent work by Jost *et al*. (2013) is highly relevant when choosing a method to look at manure contributions to N_2O emissions. Jost *et al*. (2013) found that total N_2O emissions were correlated with faecal microbial biomass N, showing the significance of including the endogenous N component. This implies that the *forage* method would be best, if the rationale for the experiment was to determine N fluxes from faeces (e.g. N_2O). Others have collected manure and only labelled inorganic-N pools of the manure by adding ^{15}N-enriched inorganic-N salts, for example, (Paul and Beauchamp, 1995) to trace N cycling of the inorganic-N.

Studies using ^{15}N-labelled manures have been used to trace manure effects on N cycling in soils and agronomic effects (Berntsen *et al*., 2007; Bosshard *et al*., 2009; Bosshard *et al*., 2011), but the number of studies that have included measures of N_2O 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 ^{15}N tracing was used to demonstrate the potential of a nitrification inhibitor (3,4-dimethylpyrazole phosphate) to reduce N_2O emissions from slurry injected into soil. In this instance, the dairy slurry was ^{15}N enriched and the isotopic composition of the soil and N_2O pools were monitored. Besides having lower N_2O emissions, the ^{15}N enrichment of the N_2O emitted was lower in the nitrification inhibitor treatment, indicating that less N_2O was derived from the slurry in this treatment. Further such studies are urgently required to assess management and mitigation strategies for reducing N_2O 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 N_2O fluxes from faeces that should be ascertained.

In comparison with generating ^{15}N-labelled excreta, the production of ^{15}N-enriched plant residues is relatively straightforward. Plants are grown with appropriate nutrition and water (avoiding leaching events), generally in a sandy matrix to avoid ^{14}N mineralisation from the soil organic-N pool diluting the ^{15}N pool, with N nutrition provided by adding a ^{15}N-enriched fertiliser. Again urea is best avoided so that ^{15}N is not lost unnecessarily owing to NH_3 volatilisation. Once the plant is at the required stage of growth, it may be harvested and used in ^{15}N-tracer experiments. Previous results using ^{15}N to apportion N_2O sources have shown that emissions from plant residue applications can be short-lived (Frimpong and Baggs, 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 ^{15}N tracer to show that fresh litter deposition contributed to the N_2O 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 ^{15}N-enriched N_2 to study the fate of biologically fixed N_2. For example, Carter and Ambus (2006) showed that easily degradable clover residues (*Trifolium sp*.) made a minor contribution to N_2O fluxes. Other studies have shown that the dynamics of N_2O emissions derived from ^{15}N-labelled residues are impacted upon by earthworms (Giannopoulos *et al*., 2011). Modelling of ^{15}N studies conducted by Delgado *et al*. (2010) suggest that residues should not be treated in the same way as fertilisers in terms of N_2O emissions, and they call for more residue studies to examine N_2O losses from agroecosystems. This will be done best by using ^{15}N tracing.

The study of fertiliser applications using ^{15}N 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 SF_6, 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_3^-) 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 Barraclough 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 (Muller *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 N_2O fluxes in a number of soils examined. This method holds great promise for furthering our understanding of the role of biological processes in producing N_2O and needs to be applied across a wider range of soils and agroecosystems, in particular.

One of the most intriguing and exciting isotopic developments 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 ^{13}C-labelled compounds into the DNA or RNA of soil microorganism (Radajewski *et al.*, 2003). Previously uncultivated N_2 fixers, which assimilate N, have been identified using this method with ^{15}N (Buckley *et al.*, 2007). However, the direct use of SIP to identify dissimilatory organisms and/or conditions that promote N_2O production/consumption has generally been limited to conditions where denitrification is optimal and where ^{13}C compounds are dosed to identify organisms operating in the denitrifying conditions. For example, a study by Ishii *et al.* (2011) supplied ^{13}C-labelled succinate with and without N_2O to determine what microbes were undertaking N_2O consumption in rice paddy soils. This examination of denitrifiers by ^{13}C-proxy, under denitrifying conditions, in the absence or presence of substrates has merit, but it should also utilise ^{15}N-labelled N substrate to further strengthen the findings (fate or change in ^{15}N substrate would provide information on denitrification activity), and with emphasis placed on RNA–SIP under such conditions, as RNA provides information on active microorganisms. It is not yet understood how individual nitrification or denitrification genotypes affect N_2O 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 ‰:

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

where δx is the value of the heavy isotope in the sample (R_{sample}) relative to a standard (R_{standard}), where the international standard for δ^{15}N is N_2 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 N_2O is in the field of isotopomers. The N_2O molecule is linear and when ^{16}O is the oxygen isotope in the molecule it takes the form $^{14}N^{15}N^{16}O$, $^{15}N^{14}N^{16}O$ or $^{14}N^{14}N^{16}O$. The intramolecular distribution of ^{15}N at the central (α) or end (β) positions of the molecule are assessed by studying the abundance of ^{15}N in the molecule. Using δ-notation, δ^{15}N denotes the difference in the $^{15}N/^{14}N$ ratio with respect to a standard, usually atmospheric N_2 (Mohn *et al.*, 2012). The relative difference in the ratios of $^{14}N^{15}N^{16}O$ to $^{14}N^{14}N^{16}O$ and $^{15}N^{14}N^{16}O$ to $^{14}N^{14}N^{16}O$ are denoted $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$, respectively, whereas bulk value, $\delta^{15}N^{\text{bulk}} = (\delta^{15}N^{\alpha} + \delta^{15}N^{\beta})/2$ (Mohn *et al.*, 2012). The value for $\delta^{15}N^{\text{bulk}}$ in the troposphere is reported to range from 6.3 to 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 preference ($SP = \delta^{15}N^{\alpha} - \delta^{15}N^{\beta}$) of the N_2O molecule. This is deemed independent of the isotopic composition of the substrate the N_2O molecule derives from and supplies process information (Mohn *et al.*, 2012).

Before development of instrumentation for measuring $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$, only the average ^{15}N value of the N_2O molecule was determined ($\delta^{15}N^{\text{bulk}}$). Isotopomers of N_2O can now be measured using FTIR spectroscopy (Griffith *et al.*, 2009), tuneable diode laser absorption spectroscopy (Pattey *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 spectroscopy cavity-ringdown (QCLAS) spectroscopy utilising mid-infrared lasers (Mohn *et al.*, 2012).

It is well recognised that N_2O isotopomers can be used to constrain the atmospheric N_2O budget and they confirm that the increase in atmospheric N_2O 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 N_2O fluxes and sources from agroecosystems. The isotopomer analyses of N_2O have been applied to examine the effects of various treatments on N_2O 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; Bergstermann *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 N_2O 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 $\delta^{15}N^{\text{bulk}}$ data could be used for distinguishing N_2O 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_2O by microbial pathways. Enticingly, the use of QCLAS has been shown to be capable of continuous analysis of N_2O isotopomers with identification of N_2O 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_2O, and temporal dynamics, but also to explore and demonstrate the success of mitigation options.

Conclusion

The impending increase in tropospheric N_2O emissions as a result of existing and projected increases in anthropogenic animal production systems demands mitigation options. These can only be implemented if N_2O emission sources and their temporal dynamics can be traced in conjunction with N_2O 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_2O dynamics. Relatively new research fronts using SIP and isotopomers of N_2O 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_2O issue.

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