AUTOMATED N_2O/N_2 ANALYSIS – A NEW TOOL FOR STUDYING DENITRIFICATION DYNAMICS AND TESTING MITIGATION STRATEGIES

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Abstract:

The bulk of nitrous oxide (N_2O) from New Zealand agriculture is produced from denitrification – the four step process by which NO_3^- is progressively reduced to atmospheric N_2 ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). N_2O can be emitted from the soil when denitrification is incomplete and the last step of this process [$N_2O \rightarrow N_2$ (known as N_2O consumption)] does not occur. To properly understand the dynamics of N_2O emission from soil denitrification, we must not only know the rate of N_2O emission but also the rate of N_2O conversion to N_2. However, quantification of this final step is challenging due to the high background level of atmospheric N_2. We present an analytical system (unique in New Zealand and based on a Norwegian design), which quantifies N_2O consumption by directly measuring microbial production of both N_2O and N_2 using soil incubated in an initially N_2-free atmosphere. We simultaneously quantify the overall rate of NO_3^- removal together with rates of N_2 and N_2O production to identify factors influencing the shift from N_2O production to N_2O consumption. We describe the system, which we call the Denitrification Dynamics Gas Chromatograph (DDGC), present initial results and discuss how the tool can be applied to test mitigation strategies.

Introduction:

Denitrification is an important component of the nitrogen cycle, and provides a key ecosystem service by removing excess NO_3^- from soils, sediment and groundwater, but also is the major source of agricultural nitrous oxide (N_2O) in New Zealand (Ministry for the Environment, 2012). Globally, it accounts for about 60% of total N_2O emissions (Kroeze et al., 1999). In temperate grasslands the annual loss of N through denitrification has been estimated to be 5.6 Tg (Saggar et al., 2013).

Microbial denitrification is a four-step process that starts with NO_3^- and tends to end with production of both N_2O and N_2 ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). Although denitrification is a well-studied process, a comprehensive understanding of factors influencing denitrification all the way to N_2 has been impeded by measurement difficulties. The large atmospheric background N_2 concentrations in air (789,000 ppm) and water make it difficult to analytically measure small amounts of N_2 produced from denitrification (Davidson and Seitzinger, 2006).

In the absence of a comprehensive process-based understanding of denitrification, there are several questions relevant to nitrogen cycling in New Zealand pastures where knowledge gaps remain. In terms of NO_3^- removal from soil, critical questions include "How much NO_3^- is denitrified to N_2O and how much NO_3^- is completely denitrified to N_2?" A key metric is the denitrification product ratio, which is defined as the amount of N_2O produced relative to total amount of nitrogen gas end-products, i.e., $N_2O/(N_2O + N_2)$. The product ratio depends on the nitrous oxide reductase (N_2OR) activity, which catalyses consumption of N_2O (Zumft and Matsubara, 1982).

A growing body of evidence suggests that the gene encoding for N_2OR is not ubiquitous (Philippot et al., 2011), and gene expression depends on soil conditions. Low pH, for example, inhibits N_2OR activity (Simek and Cooper, 2002). Consequently, transformation of N_2O to N_2 is considered the rate limiting step for denitrification in acidic soils (Herold et al., 2012; Knowles, 1982). Measurement of both N_2O and N_2 is critical for understanding the dynamics of denitrification in agricultural soils. Investigating ways to enhance the activity of N_2OR may reduce the $N_2O/(N_2O + N_2)$ product ratio, which is one avenue toward mitigation of agricultural N_2O emissions.

A common but indirect approach to measuring the product ratio is the acetylene inhibition technique (Yoshinari and Knowles, 1976). This involves anaerobic incubation of soils and injection of acetylene to block the last step in denitrification ($N_2O \rightarrow N_2$). The acetylene deactivates N_2OR, so accumulation of N_2O in the headspace represents the sum of N_2O and N_2 production that would occur in the absence of acetylene. The product ratio is determined by the ratio of N_2O accumulation under uninhibited conditions to N_2O accumulation under acetylene-inhibition conditions. However, there is now an abundance of literature identifying artefacts associated with the acetylene inhibition technique (Groffman et al., 2006). For example, acetylene inhibits nitrification (as well as N_2OR) and provides a carbon source for denitrification. Inhibition effectiveness is also less than 100% due to diffusion limitations in soil.

^{15}N-tracer techniques offer an alternative means for measuring denitrification in soils but they are expensive in terms of the need for laborious sample preparation and expensive instrumentation (Groffman et al., 2006).

The scientific community has called for direct and cost-effective measurement of N_2 and N_2O produced via denitrification for decades, and recent advances now make this possible. Here, we present a technique based on a previous instrument design and incubation method (Molstad et al., 2007), where rates of denitrification and the denitrification product ratio were determined in an N_2-free atmosphere. We refer to this technique as Denitrification Dynamics Gas Chromatography (DDGC). We expect the technique to provide novel insights into the regulation of N_2O and N_2 production.

Methodology:

The DDGC Technique

Briefly, the method comprises a technique for incubating soils in a N_2-free atmosphere and an automated technique for sampling the headspace contents of the vials. Headspace samples are analysed using gas chromatography to quantify N_2 and N_2O, the end products of denitrification, as well as CO_2, CH_4 and O_2.

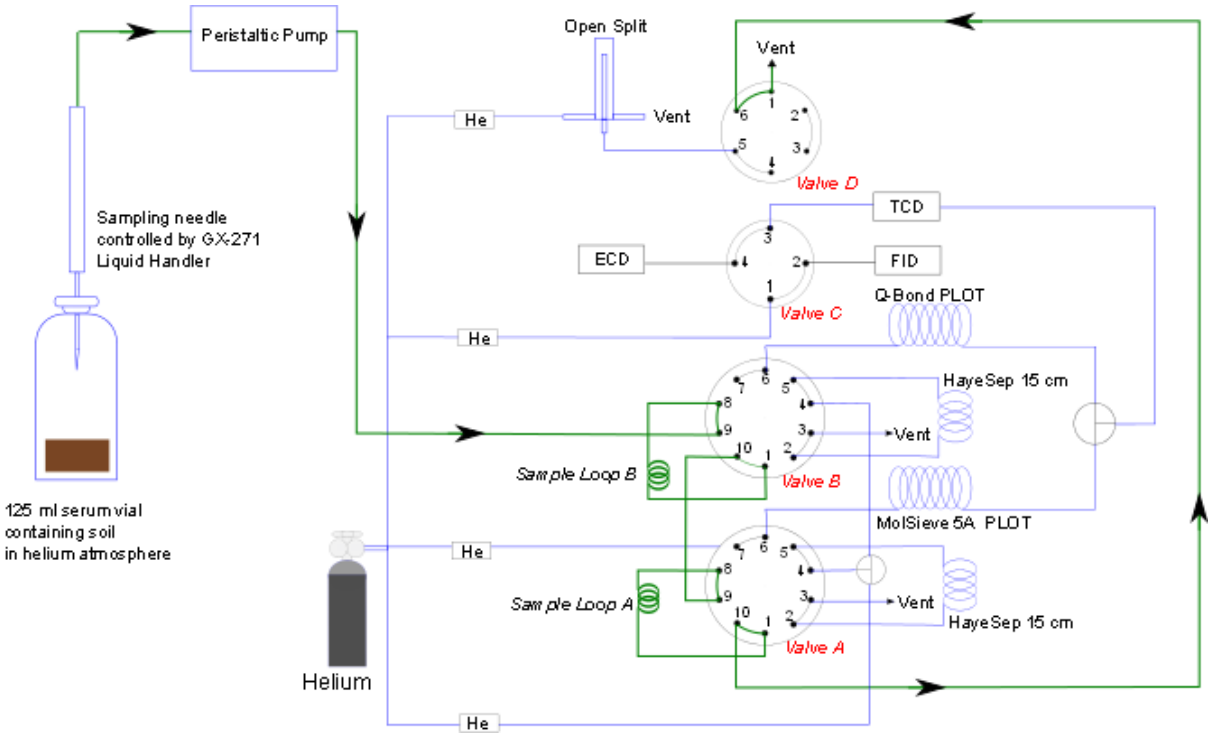

Figure 1 Schematic of DDGC. The sample is drawn from the vial and pumped through a series of two 0.25 ml sampling loops at a rate of 9.4 ml/min. The contents of the loops are injected onto two packed pre-columns (packed with HayeSep Q material) and subsequently onto two capillary columns: a Q-Bond PLOT column for separation of CO_2 and N_2O; and a MolSieve 5A PLOT column for the separation of CH_4, N_2 and O_2. The eluent then passes through the TCD for detection of (N_2O, N_2, O_2) and upon exit can either be directed to the ECD (for detection of N_2O, CO_2) or to the FID (detection of CH_4) using Valve C. Following sample injection, Valve D is switched and the peristaltic pump operated in reverse mode to allow helium to replace the head space of the vials that was lost during sampling. Ultra-high purity helium is used as the carrier gas throughout the entire system.

(a) Soil Incubation.

About 20 g (dry weight equivalent) of moist soil is sieved (2 mm), homogenised and placed in a 125 mL serum vial (Sigma Aldrich, Part No. 98334, Milwaukee, WI, USA) sealed with a Butyl rubber/PTFE lined septum and an aluminium crimp seal (Grace Discovery, Part No. 95584). The vials are evacuated to ~2 mbar then backfilled with 99.999% pure Helium. This evacuation/helium rinsing procedure is repeated a further four times. The vials are placed on rack that is immersed in a temperature-controlled water bath that lies beneath a Gilson GX-271 Liquid Handler (Gilson, UK).

The evacuation procedure is capable of removing 99.96% of the N_2 headspace leaving a typical residual N_2 concentration of 330 ppm. This low level of N_2 concentration can be measured with a typical precision of 18% (59.6 ppm). At higher concentrations of N_2 in the vial, the relative precision of the measurement improves, for example, at concentrations of 1484 ppm, the precision is 4% (54.1 ppm).

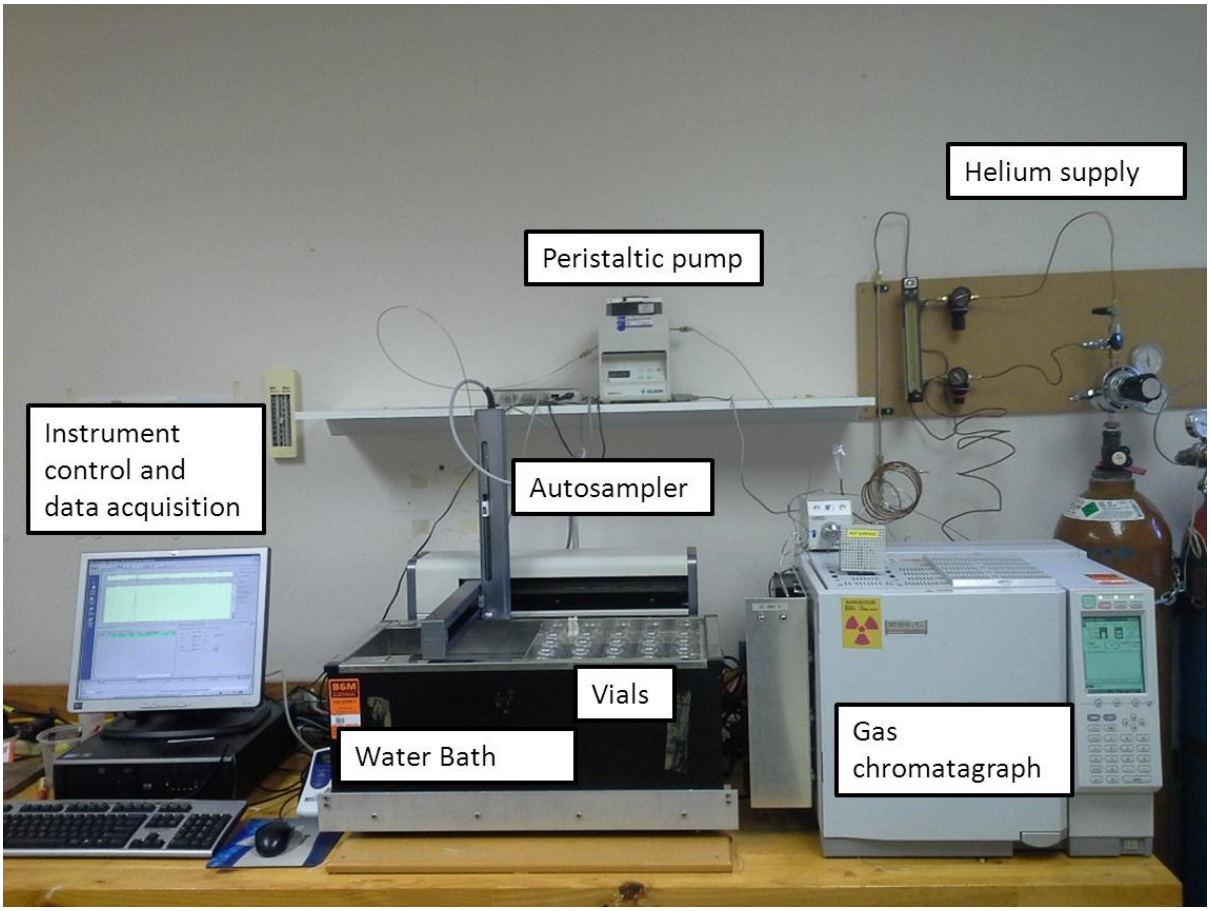

Figure 2 Photograph of the DDGC. The system comprises a Shimadzu 2010-Plus Gas Chromatograph (GC) with an ECD, FID and TCD detectors (right), a Gilson GX271 Liquid Handler to Automatically Sample the vials from the sample rack, which is immersed in a temperature-controlled water bath.

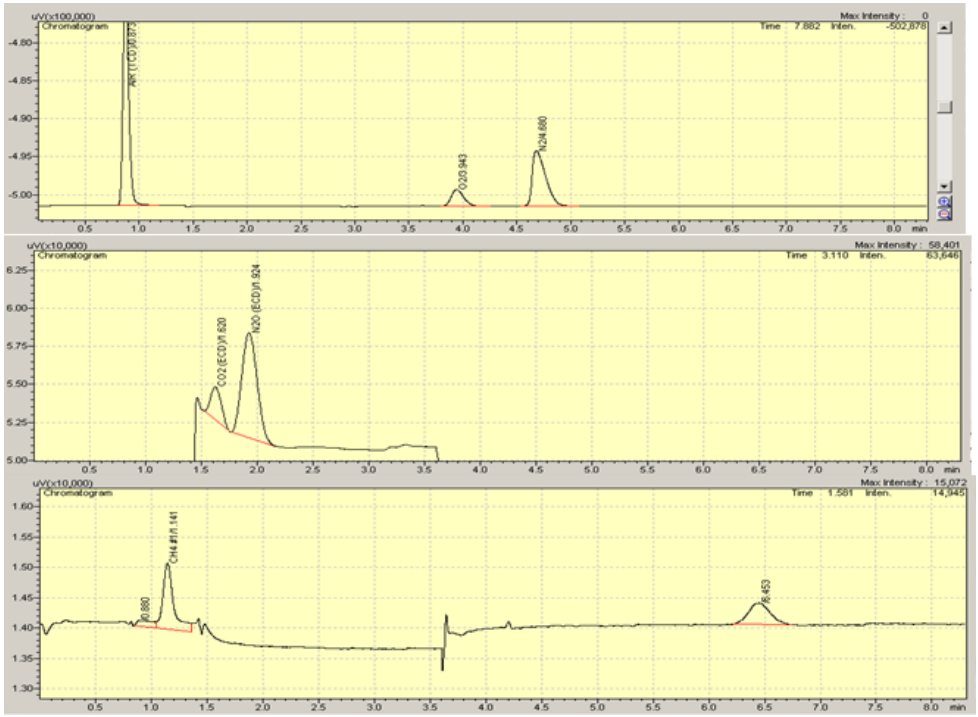

Figure 3 Chromatograms from the DDGC showing (a) detection of O_2 and N_2 by the TCD, (b) detection of CO_2 and N_2O by the ECD and (c) detection of CH_4 by the (FID, eluting first from the Q-Bond column at 1.1 minutes and then from the Molecular Sieve 5A column at 6.5 minutes.

(a) Automated Sampling and Analysis

The automated sampling and analysis is under control of the Trilution Software (Trilution, Gilson, UK). The software controls the GX-271 Liquid Handler, the Gilson MP3 Peristaltic pump and also sends a signal to the gas chromatograph to initiate analysis. The gas chromatograph is a Shimadzu 2010-Plus (Shimadzu Corp, Kyoto, Japan).

One minute before the desired time of sampling, helium is directed from the open split to flush the sample line. The sample needle is then injected into the vial and ~5 ml of the vial contents is directed through the sample line (green lines in Fig. 1). Following injection, Valve

D is switched and the peristaltic pump is operated in reverse mode so that helium can be drawn from the open split through the sample loops and back into the sample vial to replace the volume removed be sampling. The sample loops are allowed to equilibrate with atmospheric pressure and then injected onto the Hayesep Q packed pre-columns. After the gases of interest have eluted from the packed pre-columns (1.6 minutes), they pass onto the capillary columns, and the pre-columns are then back-flushed with helium carrier gas to remove water vapour and excess oxygen.

A photograph of the sampling and analytical components of the DDGC is shown in Figure 2. Chromatograms from the TCD, ECD and FID are shown in Figure 3.

Experiment 1: Testing of the DDGC System

A trial was conducted to test the entire procedure of soil incubation, vial evacuation, sample injection and analysis. The soil used for the test was a stored soil that had previously been used for denitrification studies (Jha et al., 2013). The soil was the upper-most layer (0-100 mm) of a Manawatu Fine Silt Loam that had been treated with effluent, collected in October 2010 and stored at 4°C. We acknowledge that the long storage time of the soil created microbial conditions that were not representative of field conditions. However, the primary purpose of Experiment 1 was to test methodology.

20 g dry weight equivalent of field-moist soil was sieved (<2 mm) and placed in 20 125-ml serum bottles. The soil was wetted to 75% water holding capacity either with deionised water (10 vials) or with a solution of D-glucose and KNO_3 that provided an amendment of 22 μg NO_3^- and 250 μg Glucose-C per g dry weight soil (10 vials), similar to substrates used by Jha et al. (2013).

Each vial was evacuated to a pressure of 5 mbar and backfilled with Ultra-High Purity (99.999%) helium (Gas Code 220, BOC, Auckland, New Zealand). Five additional vials were also evacuated and backfilled with helium to act as sample blanks and allow quantification of N_2 contamination. The 25 vials were then placed in the sampling rack and analysed 6 times each over the period of a week.

Experiment 2: Determining the effect of added NO_3^- and Glucose

A second experiment was conducted to determine the separate and combined effects of added NO_3^- and glucose. Here, a freshly sampled soil was used (Manawatu Fine Silt Loam collected 14 January, 2014 and incubated within 24h) and amendments were increased slightly to reflect amendment ratios common in the literature (100 μg NO_3^- and 500 μg glucose-C per g dry weight soil). We compared four treatments (with 5 replicates): (A) Control (only water added), (B) added NO_3^- (water + 100 μg-NO_3^- g $soil^{-1}$), (C) added glucose (water + 500 μg-C g $soil^{-1}$), and (D) added NO_3^- plus added glucose (water + 100 μg-NO_3^- g $soil^{-1}$ + 500 μg-C g $soil^{-1}$). The water was added to achieve a final moisture content of 100% water holding capacity. The vials were closed and sampled 11 times for gas analysis over a one week period. The first 8 samplings were conducted as 4 pairs of sampling 3-4 hours apart so that we could determine short term flux rates at various stages of the incubation.

Mineral N concentrations were measured in a set of parallel incubations using the same proportions of soil, water and amendments as described above. These incubations were conducted in 12.5 ml sealed Exetainers (LabCo, Lampeter, UK), and the concentration of dissolved NO_3^-, NO_2^- and NH_4^+ was measured on six occasions during. At each of the 6 samplings 3 replicates were removed and extracted with 2M KCl for NO_3^-, nitrite and ammonium determination (Blakemore et al., 1987).

Results:

Experiment 1.

N_2 production

The DDGC was easily capable of measuring the rates of N_2 production that occurred in both the amended and unamended soil incubations (Figure 4). In the unamended soil, rates were low initially but increased gradually over the incubation. In the amended soil, much greater rates occurred, particularly between 31and 67 hours. Over this interval the unamended soil produced N_2 at a rate of 0.12 μg N_2-N g(dry weight soil)$^{-1}$ h^{-1} while the amended soil produced N_2 at a rate that was 20 times higher (2.7 μg N_2-N g(dry weight soil)$^{-1}$ h^{-1} (Table 1, Figure 4)). The amended rate of denitrification was similar to that measured on the same soil by Jha et al. (2013) who found rates of 2.5 μg N_2-N g(dry weight soil)$^{-1}$ h^{-1}.

After 67 h, rates of N_2 production continued increased in the unamended soil, while the amended soils produced very little further N_2.

Given the worst case precision of 18% (relative standard deviation) at 330 ppm, we can calculate that the smallest difference in N_2 concentrations we can reliably measure is 98 ppm. This figure was calculated by finding what the minimum concentration difference between two subsequent measurements would need to be in order that their respective 95% confidence intervals do not overlap. If the interval between these measurements was one hour and 20 g (dry weight equivalent) of soil was used, this would allow a denitrification rate of 1 μg N_2-N g-dwt-soil^{-1} h^{-1} to be detected.

Ability to measure N_2O concentration

The DDGC was capable of measuring N_2O concentrations over a wide range of concentrations. The ECD was able to measure N_2O concentrations ranging between 2 ppm to 3000 ppm, whereas the less sensitive TCD detector could measure N_2O concentrations ~750 ppm and above. In the unamended treatment headspace concentrations rose linearly to a maximum of 7000 ppm at 100 hours and then declined to almost initial concentrations in the following 65 hours (Figure 5). The decrease in N_2O concentrations corresponded both in time and, roughly, in magnitude with the increase in N_2 concentration shown in Figure 4, and indicated that N_2O consumption was occurring.

The N_2O concentration of the amended vials followed a markedly different pattern, peaking after 41 hours and then decreasing sharply until all N_2O had disappeared by 89 hours. Interestingly, for both treatments, the N_2O concentration peaked at the same value before consumption occurred.

The N_2 production rates observed for the amended soils was consistent with a sigmoidal ("S"-shaped) growth, whereby the microbes responsible for the production first underwent a slow lag phase due to a shortage of individuals and/or low rates of enzyme synthesis (in this case, the N_2OR enzyme). This was followed by an exponential growth phase, where the microbial process was not limited by substrates or other resources. The final phase was a plateau phase, where the organisms are subject to resource limitations. In this case, it seems most likely that the precursor for N_2 production, N_2O, was the limiting resource as it became almost entirely

Table 1 Rates of N_2 and N_2O production during different phases of the incubation

Incubation phase	N_2 μg N_2-N g(dwt soil)$^{-1}$ h^{-1}		N_2O μg N_2-N g(dwt soil)$^{-1}$ h^{-1}	
	Control	Added C + N	Control	Added C + N
20 – 31 h	0.03	0.20	0.55	1.59
31 – 67 h	0.12	2.65	0.51	-1.16
89 – 164 h	0.64	-0.03	-0.32	0.00

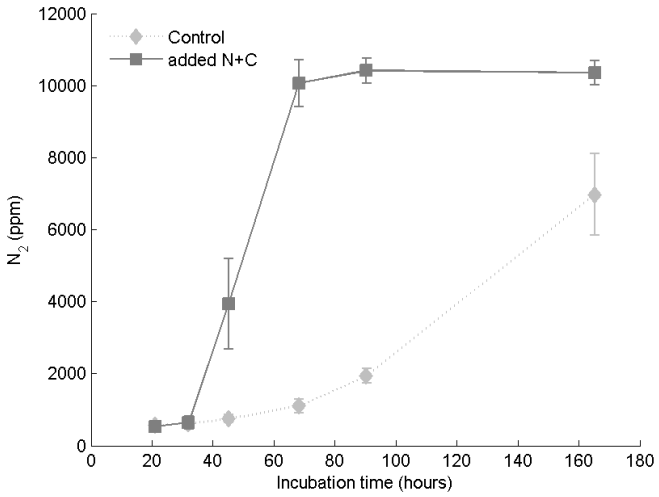

Figure 4 Headspace N_2 Production of incubated Manawatu Fine Silt Loam either amended with NO_3^- and glucose (Added C + N) or unamended (Control). The N_2O concentrations shown here were measured by TCD. Error bars represent 1 standard deviation (n=10).

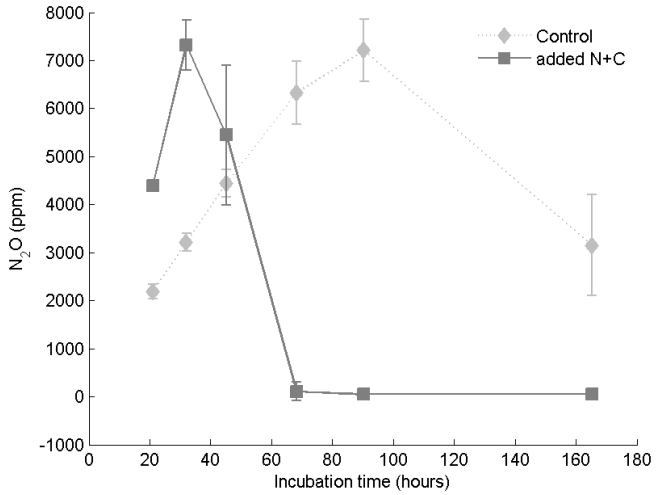

Figure 5. Headspace N_2O Concentration of incubated soil amended with NO_3^- and glucose (Added C + N) or unamended (Control).

depleted after 67 hours. Molecular measurements of gene abundance would provide valuable information to confirm the existence of such growth patterns.

The N_2 production rates in the unamended soil appeared to be entering an exponential phase at the late stages of the incubation, and this corresponded with the sharp decline in N_2O concentration after 100 hours.

Experiment 2: Effect of NO_3^- and glucose on denitrification rates and the product ratio

This experiment was designed to investigate the separate and combined effect of glucose and NO_3^- addition on the rates of N_2O and N_2 production.

In the NO_3^--Only treatments, soil NO_3^- concentrations decreased sharply. Although the amount of NO_3^- addition was the same for both the NO_3^--only and the NO_3^-+Glucose treatment, the initial measurement of NO_3^- in the NO_3^-+Glucose treatment was less than 50% of that in the NO_3^- -only treatment. We attribute this difference to much greater rates of NO_3^- reduction in the NO_3^-+Glucose treatment, which led to a substantial portion of the NO_3^- being consumed in these vials before the sampling for mineral N extract had been sampled. Accordingly, NO_3^- was completely depleted in the NO_3^-+Glucose treatment by 48 h whereas NO_3^- was not depleted in the NO_3^--Only treatment until 116 h.

The highest rate of N_2 production occurred in the NO_3^-+Glucose treatment where the cumulative N_2-N exceeded 50 μg/g after 170 h (Figure 6). This response indicated that NO_3^- and glucose interacted strongly to promote the conversion of N_2O into N_2. N_2 production was higher in the Control treatment compared to the NO_3^--Only treatment, supporting previous work that NO_3^-addition favours the production of N_2O relative to N_2, thereby increasing the product ratio (Firestone et al., 1979). The lowest rates of N_2 production occurred in the Glucose-Only treatment, indicating that glucose addition alone had an inhibitory effect on denitrification

The N_2O concentration in the vials represents a balance between N_2O production and N_2O consumption. In the Control treatment, N_2 rose without accumulation of N_2O in the headspace. In the NO_3^--Only treatment, N_2 rose with a large accumulation of N_2O in the headspace. In the Glucose-Only treatment, neither N_2 nor N_2O increased. However in the NO_3^-+Glucose treatment, N_2O rose to 24 μgN/g (~4200 ppm) by 51 h and decreased rapidly thereafter, while the N_2 concentration increased rapidly after 51 h and then levelled off.

The decrease in N_2 concentration that was apparent in the later phases of the incubation is hard to explain biologically, and is thought to be an artefact caused by uncertainties in amount of N_2 leakage into the vial that occurs after several injections. This quantity is well constrained in the early incubation stages but becomes increasingly variable at the late stages due to variations among replicates in individual leakage rates.

The headspace concentration of N_2O and N_2 at the end of different stages of the incubation reflects the net cumulative production of these gases and is shown in Figure 7. The product ratio (N_2O / N_2O+N_2) is calculated directly from these quantities. Overall, rates of denitrification were greatest in treatments amended with NO_3^--N. In the added N treatment, the product ratio increased sharply from 0 at the beginning to ~0.67 at the end of the incubation. In the added N+C treatment, the product ratio was high (0.5 to 0.6) over the first

51 hours but had dropped to 0 by the end of the incubation as all the N_2O had been consumed.

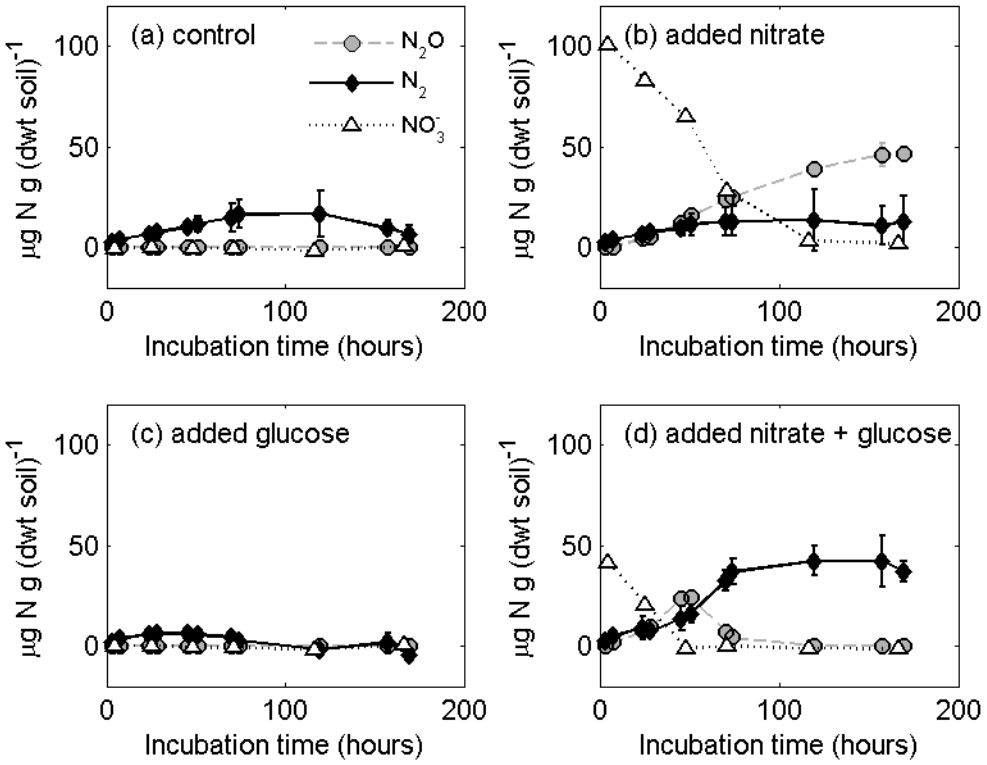

Figure 6 N_2O and N_2 in vial head space and NO_3-N in soil as a function of incubation time. Note the units of the vertical axis are different to those used in Figure 4 and 5 to allow NO_3^- to be plotted on a similar scale. Error bars represent 1 SD. The N_2O and N_2 values can be converted to ppm by multiplying by 176.3.

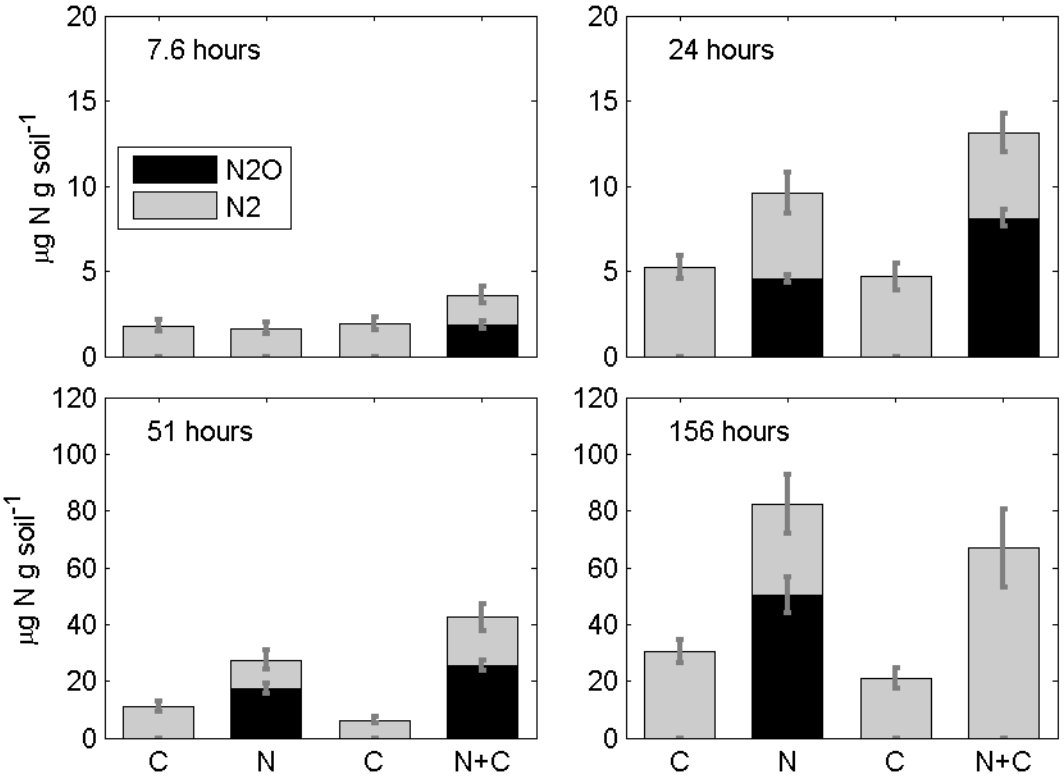

Figure 7. Accumulated denitrification end-products at different stages of the incubation. The total amount of gaseous N i(N_2 + N_2O) s depicted by the combined height of bars, and the shaded regions indicate the relative contributions from each gas. Error bars denote 1 SD (n=4). Note the larger vertical scale in the lower panels.

Discussion

1. What new information does this method reveal about denitrification?

This methodology has the ability to provide better understanding of the last step of denitrification (the reduction of N_2O to N_2) and valuable insights into the activity of the N2OR enzyme. There are two aspects to this system that improve upon previous methodology: first, the direct measurement of N_2 production in a helium atmosphere avoids the artefacts associated with using acetylene inhibition; second, the automated nature of the analyses provides a high level of quality control and substantially reduced labour requirements, which provides for reliable, high frequency measurements of gas production rates from incubated soil.

The method is best suited to experiments in which production rates of N_2 are relatively high since the direct detection of N_2 production using this technique is less sensitive than estimating N_2 production by measuring N_2O production under acetylene inhibition. In these experiments, N_2 production was well in excess of the detection limit under both amended and control soil treatments.

This technique therefore provides an improved means to measure the potential activity of the N_2OR enzyme in the soil. It should be kept in mind that this technique takes place under controlled lab-based conditions. While it would be more desirable to measure in situ rates of potential N_2OR activity, there are no simple methods to make these measurements in undisturbed soil. Therefore, to maximise the applicability of the results from this assay to the field, it is crucial to develop a consistent methodology so that results from different soils and different incubation conditions can be easily compared. Qin et al. (2014) has recommended protocols to achieve this, with a particular emphasis on short incubation times, thereby minimising the incubation effect.

2. What do the amendment effects tell us about the regulation of denitrification and the product ratio?

Experiment 1 revealed that the stored soil responded dramatically to the addition of $NO_3^-$and glucose by first undergoing a stage of rapid N_2O production and subsequently undergoing a rapid stage of N_2O consumption. In the unamended soil, N_2O consumption began to accelerate only towards the end of the experiment. The soil was relatively high in NO_3^-at the beginning of the experiment suggesting that it was the addition of glucose-C that was most important for the high rates of production and consumption in the second experiment. The decrease in the product ratio that occurs with the addition of labile C has been noted previously (Weier et al., 1993a).

Under both treatments, the product ratio was highly dynamic, changing continuously throughout the incubation, and indicating that the metric is most useful when expressed together with the incubation time so that the context of the incubation stage is acknowledged. Importantly, the product ratio increased over the later stages of the incubation, indicating that there is a lag between N_2O production and N_2O consumption.

The acceleration in N_2O consumption in the latter stages of the incubation is of interest because it indicates the staggered nature of the denitrification sequence, and suggests that there are certain optimal conditions for maximal N_2O consumption that occur only at the later stages of incubation.

The high product ratio found with the NO_3^--Only treatment is consistent with findings from several studies that have found high product ratios associated with high soil NO_3^- concentrations (Senbayram et al., 2011; Weier et al., 1993b).

Since Experiment 2 used freshly collected soil, it is not directly comparable to Experiment 1, which used soil stored at 4°C. However Experiment 2 allowed the individual effects, as well as the combined effect, of added $NO_3^-$and added glucose to be determined. Glucose alone seemed to promote pathways other than denitrification, while NO_3^-alone created the largest accumulation of N_2 and N_2O, and the largest product ratio. The combination of both $NO_3^-$and glucose had a strong interactive effect on the product ratio, reducing it to zero after 100 hours.

3. *How can the methodology be applied to enhancing NO_3^- removal and mitigating N_2O emissions in grazed pastures?*

There is keen interest in developing strategies to increase the rate of NO_3^- removal in high nitrogen environments. Enhancing denitrification rates is an obvious tactic but carries the risk of increased N_2O emission rates, effectively leading to "pollution swapping". Likewise, strategies aimed at reducing the N_2O from denitrification would ideally prevent excess NO_3^- from being available for loss via leaching or run-off. By providing the means to quantify both the rate of denitrification and the rate of N_2O consumption, the DDGC is an ideal tool to test such strategies under highly controlled and reproducible conditions.

The development of strategies to enhance N_2O consumption in the soil will require us to understand what factors are limiting the enzyme N_2OR, and in turn, the expression of the gene that codes for this enzyme, nos-Z. The initial experiments described here underscore the importance of particular substrates in determining the product ratio. More detailed experiments are underway to investigate other factors important for the regulation of this enzyme, including temperature, the presence of oxygen and the availability of metallic co-factors known to be important for denitrification enzymes.

Conclusion

We have developed a methodology that measures both the rate of denitrification and the relative amounts of the end product gases. This system measures N_2 directly, avoiding the artefacts associated with previous techniques. The system will allow testing of strategies aimed at reducing the N_2O emitted from denitrifying environments and/or enhancing the rate of NO_3^- removal from high nitrogen environments.

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