

PRELIMINARY STUDIES TO MEASURE DENITRIFICATION ENZYME ACTIVITY AND DENITRIFICATION RATE IN NEW ZEALAND PASTURE SOILS

Neha Jha¹, Surinder Saggar², Russ Tillman¹ and Donna Giltrap²

¹*Soil & Earth Sciences Group, Institute of Natural Resources, Massey University,
Palmerston North 4442, New Zealand*

²*Landcare Research, Palmerston North 4442, New Zealand*

Abstract

Denitrification is a microbially mediated process in which nitrate is reduced to a number of gaseous products, including N₂O and N₂. It contributes about 60% of the total N₂O emissions globally and is the primary process of N₂O production in New Zealand pasture soils. In soils actual and potential denitrification rates are determined by acetylene (C₂H₂) inhibition (AI) of N₂O reduction and by measuring denitrification enzyme activity (DEA) respectively. In this paper the term denitrification refers to emission of mixture of gases (N₂O and N₂) as the product of denitrification. A review of published papers on these methods raised some concerns about the effect on measured denitrification rate of nitrification inhibition caused by C₂H₂, and also the effect of high concentrations of chloramphenicol (added during the DEA assay) on the activity of the various reductase enzymes involved in denitrification. Therefore, preliminary studies were conducted to investigate these effects and to standardise these methods for future use. The experiments involved incubations in N₂ atmosphere (O₂ free) with and without C₂H₂, and DEA measurements with varying chloramphenicol concentrations (0-100 ppm). The AI technique was also used to assess denitrification in samples of surface and sub-surface of this pasture soil from control and urine applied plots, in order to observe the change in denitrification rate with incubation time, urine application and soil depth.

The results of the preliminary experiment showed denitrification rate and DEA were higher for the soils incubated with C₂H₂ than without C₂H₂ and suggested C₂H₂ had little effect on N₂O production (denitrification) during the measurement period. There was no significant effect of <10 ppm chloramphenicol concentration on DEA, however higher concentrations caused a reduction in measured DEA. Application of urine-N increased denitrification rate up to 24 hours of incubation followed by slower or no increase, in the surface and sub-surface soil. The findings of these preliminary experiments were used to develop the protocols for these methods for future use in research.

Introduction

Denitrification, an anaerobic microbial process that converts NO₃⁻ and NO₂⁻ to inert dinitrogen (N₂), maintains the sustainability of an agricultural system. If incomplete, it contributes to global warming by emitting NO and N₂O as intermediate products. There are numerous approaches to quantify denitrification, and all the existing approaches have some limitations (Groffman et al. 2006). These difficulties in measuring denitrification arise from its spatial and temporal variability, methodological restrictions and difficulty in quantifying the dominant end product N₂ against its high ambient concentration in the atmosphere.

The most commonly used approach for estimating denitrification involves measuring the production of N_2O from the soils in the presence of acetylene (C_2H_2) which inhibits N_2O reduction to N_2 (Balderston et al. 1976; Yoshinari and Knowles 1976). This makes the easily detected N_2O the sole product of denitrification. Soil samples are incubated in the presence and absence of C_2H_2 , and the difference in N_2O produced during the incubation is used to calculate the amount of N_2 produced during denitrification. The acetylene inhibition (AI) technique has been applied successfully in studies which have led to an understanding of the spatial and temporal variability of denitrification (Groffman et al. 1999).

A slight modification of the AI method is used to estimate denitrification enzyme activity (DEA). Soil samples supplied with excess moisture, C and NO_3^- together with chloramphenicol to inhibit the *de novo* synthesis of reductase enzyme are incubated in the presence of C_2H_2 in anaerobic conditions (Tidje et al. 1989). The N_2O emitted gives an estimate of potential denitrification of soil and the ambient reductase enzyme activity.

Although it is the most common method to measure denitrification, there have been concerns raised about the AI method. These include inhibition of nitrification by C_2H_2 , slow diffusion of C_2H_2 into the soil cores and contamination of C_2H_2 with other gases (Seitzinger et al. 1993). There have also been concerns raised about the use of high concentrations of chloramphenicol in the DEA assay, which inhibits the actively participating reductase enzymes along with the *de novo* enzymes and denitrifiers hence leads to underestimation of potential denitrification and enzyme activity of soils. A large number of reviews have been published describing improvements to the method itself and identifying its limitations (Tiedje et al. 1982; Tiedje et al. 1989; Aulakh et al. 1992; Barton et al. 1999; Groffman et al. 1999, 2006).

The preliminary experiments conducted during the start of the current study were focussed on the issues related to AI and DEA methods of quantifying denitrification, and the objective was to develop a set of protocols to be followed in future research.

Methodology

Collection of soil samples

The soil used for current study was Tokomaru silt loam, classified as Argillic-fragic Perchley Pallic soil (Hewitt 1992) collected from Massey University Dairy Farm 4 (40° 22' S, 175° 36' E). Soil samples for denitrification enzyme activity (DEA) experiments were collected in July 2010. Soil cores 2.5 cm diameter and 10 cm long were obtained randomly from the farm, taken to lab, sieved through 2mm sieve and stored in cold storage until used for analyses. For denitrification rate experiment the soil cores 2.5 cm diameter were collected from Massey University Dairy Farm 4 from the plots already under a field trial in which different treatments of N with and without DCD were applied to study their effect on N_2O emissions in April 2010. Intact cores were collected from control and cow urine (700 kg N ha^{-1}) applied plots where cow urine was applied 3 months before sampling at 0-10 and 10-20 cm depths, stored in polythene bags and immediately transferred to incubation jars when taken to lab.

Determination of denitrification enzyme activity (DEA)

There were two sets of experiments run to study the DEA for pasture soils. In Experiment 1 the acetylene and chloramphenicol concentrations were used separately and together to assess their effect on N_2O emission during DEA. In Experiment 2 the effect of various concentrations of chloramphenicol was assessed on N_2O emissions during DEA.

Gas Sampling for experiment 1

A 10g of field moist sieved (2mm) soil was placed in 125ml conical flasks and 20ml of deionised water and 8ml of DEA solution was added to make a slurry. The solution was prepared by dissolving 0.45g KNO₃, 0.77g D-glucose and 2mg chloramphenicol in 1L deionised water which provided 50µg NO₃⁻ and 250µg C g soil⁻¹. The flasks were sealed air tight using suba seals. Air was scrubbed from flasks by flushing with N₂ gas to create anaerobic conditions. Ten percent of the headspace volume of the flask was replaced by purified C₂H₂, and the syringe was flushed 2-3 times to ensure proper mixing of C₂H₂ into the soil sample.

Air samples for time 0 were taken immediately and transferred to 11ml evacuated vials. Then all the flasks were placed on a shaker set at 125 rpm and incubated at 25°C in a constant temperature room. Thereafter, gas samples were taken at 1hr, 2hr, 4hr, and 6hr of incubation. A 5ml gas sample was collected from each flask each time and the same amount of N₂ gas was replaced into the flasks. The collected samples were diluted to 25ml by adding 20ml of N₂ gas and then were transferred to 11ml evacuated vials. Gas samples were analysed by gas chromatography (GC) for N₂O production in each flask. Shimadzu GC 17 A (Japan) equipped with back flush system was used which has a ⁶³Ni- electron capture detector operating at column, injector and detector temperature of 55, 75 and 330°C respectively.

Gas Sampling for experiment 2

Similar methodology as described above for gas sampling was used in experiment 2 where various concentrations of chloramphenicol 0, 2, 10, 20, 50 and 100 ppm were used to assess effect of chloramphenicol concentration on DEA.

Calculations for DEA

From the measured volumetric N₂O concentrations the amount of N₂O contained in the water and gas phases is given by:

$$N_2O \text{ (}\eta\text{L-N}_2\text{O-N gsoil}^{-1}\text{hr}^{-1}\text{)} = (\text{ppm N}_2\text{O}) \times (V_g + V_l \alpha) \times \text{Dilution factor} / (\text{weight of soil (g)} \times \text{incubation time (hr)})$$

where, ppm N₂O is the concentration of N₂O in diluted gas sample from flasks analysed by GC

V_g is the volume of gas phase in flasks

V_l is the volume of liquid phase (soil + water) in flasks

α denotes Bunsen absorption coefficient = 0.544

Weight of soil used is 10g

Denitrification Enzyme Activity = N₂O concentration (nL-N₂O-N gsoil⁻¹hr⁻¹) × density of N₂O (1.8264 × 10⁻⁶ g µL⁻¹ at normal temperature (25°C) and pressure (1 atm))

Derived from the above calculations the DEA is measured as µg N₂O-N kgsoil⁻¹ hr⁻¹

Determination of Denitrification Rate

Gas Sampling: Five of the collected cores were put in 1L Agee jars, and the lids screwed on tightly. In half of the jars 10% of the headspace volume was filled with purified C₂H₂ (to stop the conversion of N₂O to N₂). All the jars were kept in a constant temperature room maintained at 25°C.

The first set of gas samples were collected immediately after adding C₂H₂ to the jars. Subsequent samples were taken after 3hr, 6hr, 9hr, 12hr, 24 and 48hr hours of incubation. A 10 ml sample was taken from each jar and at the same time an equal amount of air was replaced to maintain the same air pressure inside the jars. The collected gas samples were diluted by adding 15ml of N₂ gas to make the volume to 25ml and then were transferred to evacuated glass vials. Gas samples were analysed by gas chromatography (GC) for N₂O production in each flask. Shimadzu GC 17 A (Japan) equipped with back flush system was used which has a ⁶³Ni- electron capture detector operating at column, injector and detector temperature of 55, 75 and 330°C respectively.

Calculations for Denitrification Rate

From the measured amount of volumetric N₂O concentrations, the amount of N₂O contained in the sampled gas from the headspace of each of the gas jar is estimated as

$$\text{N}_2\text{O-N mass } (\mu\text{g}) = \text{N}_2\text{O-N Concentration} \times \text{Density of N}_2\text{O} \times \text{Volume of the jar}$$

N₂O-N Concentration is the amount of N₂O present in the gas sample analysed by GC in $\mu\text{g L}^{-1}$.

Density of N₂O is $1.8264 \times 10^{-6} \text{ g } \mu\text{L}^{-1}$ at normal temperature (25°C) and pressure (1 atm)

N₂O-N emitted per gram soil per hour from each jar is measured as:

$$\text{N}_2\text{O-N } (\mu\text{gh}^{-1} \text{ kg soil wt}^{-1}) = \text{N}_2\text{O-N } (\mu\text{g}) / (\text{Incubation time (hr)} \times \text{wt of soil (kg)})$$

Statistical Analysis

The analysis of variance using General Linear Model (GLM) procedure for N₂O emitted during denitrification from various treatments was performed using SAS 9.2 software. Tukey's studentized Range (HSD) test at 5% significance level was used to study the mean comparisons of the treatments and paired t-test to compare the differences among the treatments.

Results and Discussion

Effect of C₂H₂ and chloramphenicol on N₂O emissions during denitrification

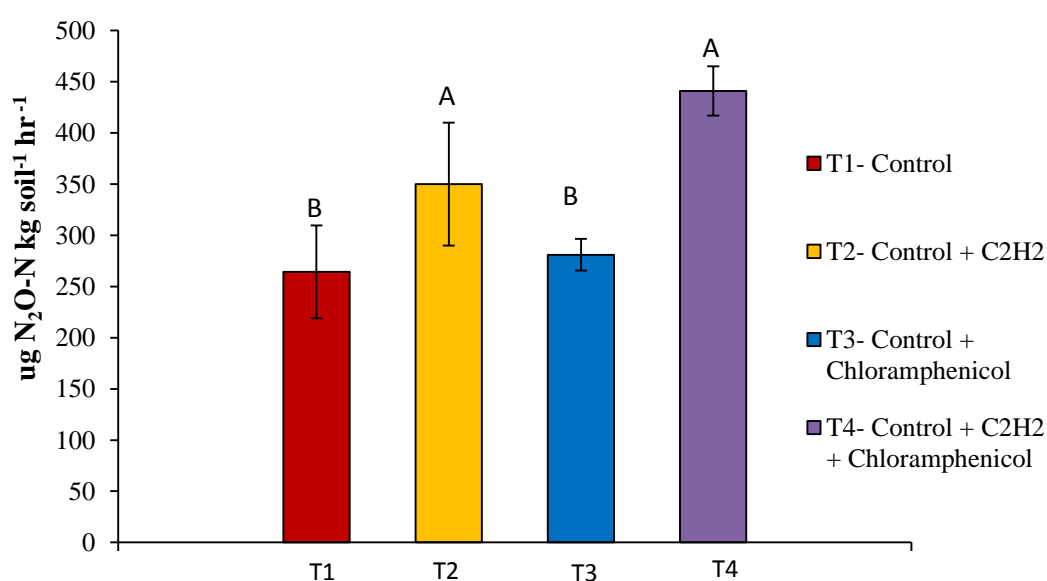


Fig. 1: Effect of C₂H₂ and chloramphenicol on N₂O emissions during denitrification, error bars denote standard error

Total N₂O emissions ranged between 264 and 441 µg N₂O-N kg soil⁻¹hr⁻¹ (Fig. 1) with the control (T1) treatment having the lowest emissions. Statistical analysis shows when the soil is incubated with C₂H₂ (10%) (T2 and T4) there is a slight increase in N₂O emissions. At the same time there is no effect of chloramphenicol (2 ppm) addition on the N₂O emissions, and it is similar for treatments T1 and T3 or T2 and T4. Incubations carried out in the presence of C₂H₂ (T2, T4) inhibited the conversion of N₂O to N₂ producing N₂O as the major product of denitrification and thus produced higher N₂O emissions than in incubations without C₂H₂. When chloramphenicol was added to the soil (T3 and T4), it did not pose any inhibitory effect on the enzyme activity and thus did not affect the N₂O production. These results are in accordance with a study by Dendooven and Anderson (1994) who studied the activity of reduction enzymes involved in denitrification in pasture soil, and found higher N₂O production from soils incubated with C₂H₂ and no inhibitory effect of chloramphenicol on N₂O emissions during denitrification. From the above results, it can be concluded that C₂H₂ and chloramphenicol doesn't have inhibitory effect on denitrifier's activity to produce N₂O during denitrification and it is safe to use both C₂H₂ and chloramphenicol in control rates to assess denitrification and DEA of soil samples.

Effect of chloramphenicol concentrations on DEA in soils

DEA values varied from 125 to 245 µg N₂O-N kg soil⁻¹hr⁻¹ depending on the concentration of chloramphenicol (Fig. 2) The DEA values decreased with increasing concentration of chloramphenicol with the lowest DEA (125 µg N₂O-N kg soil⁻¹ hr⁻¹) measured for T6 (100 ppm). Chloramphenicol is used in DEA assays to inhibit the activity of *de novo* reductase enzymes and growth of novel denitrifiers, without interfering with the functioning of the existing enzymes and denitrifiers (Frankling and Snow 1989). The amount of chloramphenicol should be kept at an optimum level during the incubations so that it does not interfere with the denitrifier activity. Murray and Knowles (1999) reported that use of chloramphenicol in higher concentrations (>100 ppm i.e. 0.1g l⁻¹) has some detrimental effect on denitrifier activity.

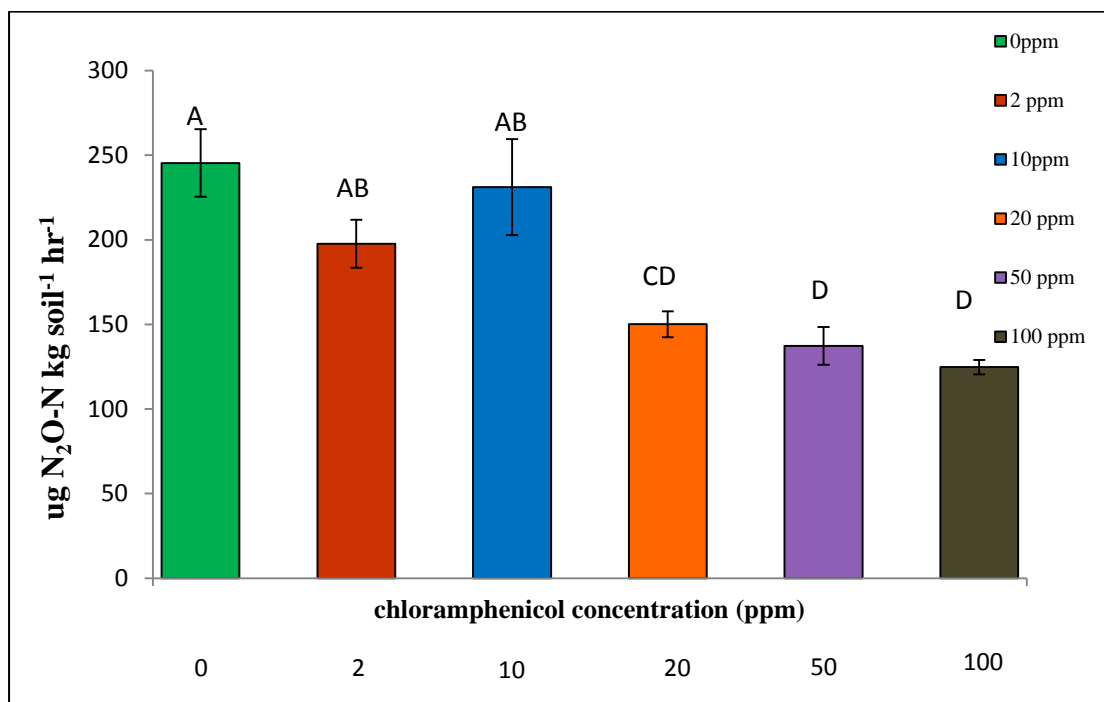


Fig. 2: Effect of Chloramphenicol concentrations on DEA in soils

Chloramphenicol not only depressed *de novo* synthesis of enzymes, but it also deactivated the enzymes already actively participating in denitrification, hence leading to underestimation of denitrification. Results from this study suggest that during DEA analysis chloramphenicol concentration should be kept low to avoid the negative effect of chloramphenicol on DEA. Use of 10ppm chloramphenicol is recommended to avoid the inhibition of denitrifiers and their enzyme activity.

Effect of urine-N application and duration of incubation on denitrification rate of soils

Denitrification rates ranged from 3.20 to 7.12 $\mu\text{g N}_2\text{O-N kg soil}^{-1} \text{hr}^{-1}$ in surface soils and from 2.43 to 5.35 $\mu\text{g N}_2\text{O-N kg soil}^{-1} \text{hr}^{-1}$ for sub-surface soils (Fig.3). The denitrification rate was higher for soils that had cow urine applied, than the control soils and it is also higher for surface soils (0-10cm) than sub-surface soils. The difference in denitrification rate among the both treatments is significant only in surface soils, in subsurface soils it is almost similar for both control and urine treated soils. In all of these soils the denitrification rate was higher during the first 24 hours of incubation than during the next 24 hrs.

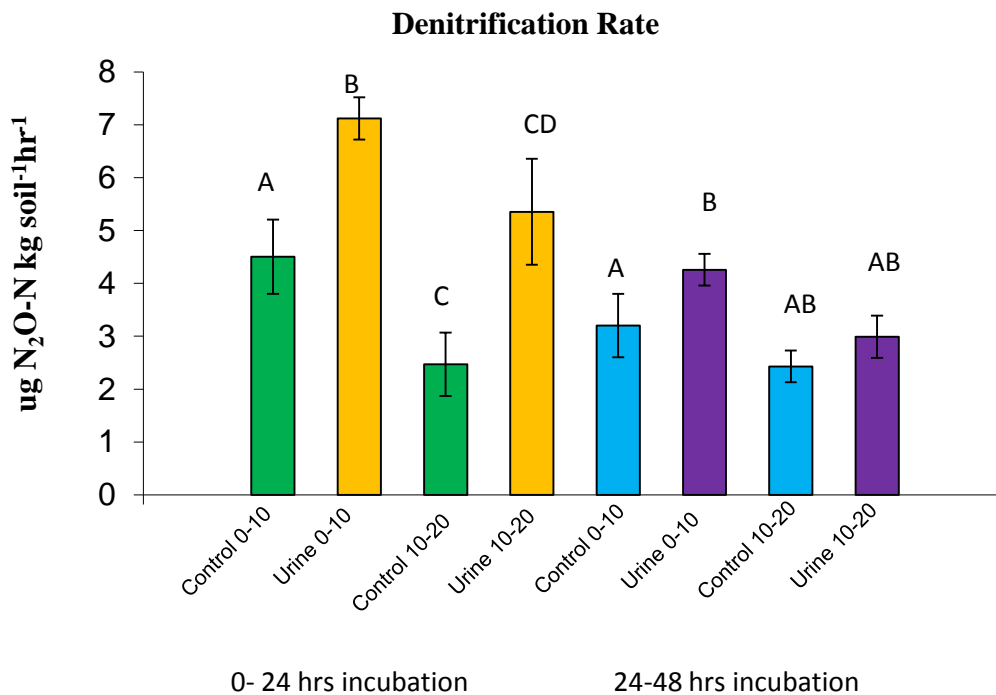


Fig. 3: Effect of Urine-N application and incubation time on denitrification rate in soils.

Urine application to soil supplements the soil with added nutrients in the form of N and C. It also creates an alkaline pH in the soil. Thus conditions are favourable for denitrifiers to actively participate in denitrification. The results from the current experiment are in accordance with the work done by Chatskikh et al. (2005) and Luo et al. (1999) which show increased denitrification rate with the application of animal excreta in the form of urine and dung to the soil. In a similar study de Klein et al. (2003) found increased N_2O emissions in NZ grassland soils up to 5 months after urine application to soils. Higher substrate availability in the form of C and N influences the bacterial and enzymatic activity in the surface soils and hence higher denitrification rate is observed in these treatments.

With increasing incubation time, microbes utilize the substrates that are present and their availability starts decreasing with time. After 24 hours, when C sources decrease in the soil, denitrifiers start decomposing the added C₂H₂ by using it as a source for C, making less availability of C₂H₂ to block the conversion of N₂O to N₂ thus interfering with the denitrification activity, showing decreased denitrification rate between 24-48 hours of incubation (Zaman et al. 2008).

Standard protocols to be followed for the AI and DEA techniques

The objective of conducting these preliminary experiments was to standardise the AI technique to measure both denitrification and DEA. Based on the results of the preliminary experiments following protocols will be followed in further experiments:

- Use 10 % of Headspace of C₂H₂ to inhibit the conversion of N₂O to N₂.
- Concentration of chloramphenicol should kept low (10 ppm) during the DEA assay to measure potential denitrification.
- The duration of incubation should be 24 hrs for denitrification rate.
- Conduct the denitrification measurements under controlled temperature, specifically at 25°C.
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