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# Plant canopy nitrous oxide emissions

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## Report prepared for MAF

AgResearch contract to FRST SLMACC project C10X0827

February 2010

2020 SCIENCE

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**February 2010**

Saman Bowatte, Paul Newton, Mark Lieffering, Phil Theobald and Shona Brock

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# 1. Executive summary

This project investigated whether leaves of grazed pasture plants contribute to atmospheric $N_2O$ emissions and whether this could provide an alternative target for mitigation technologies beyond those directed at soil microorganisms.

Previous studies have shown that plants can release $N_2O$ by two mechanisms: a) $N_2O$ produced by soil microorganisms is transported to the atmosphere through the plant, or b) $N_2O$ produced by the plant during photoassimilation of plant N. In this project we investigated a third possible mechanism viz. $N_2O$ production by ammonia oxidising bacteria (AOB) colonising plant leaves exposed to high $NH_3$.

Our experiments demonstrated that leaves of plant species in New Zealand grazed pastures can contribute to $N_2O$ emissions and leaves exposed to high $NH_3$ release greater $N_2O$ compared to those exposed to ambient air. Using molecular techniques we have demonstrated that AOB are present on pasture leaves.

We measured detached leaves and excluded mechanisms a) and b) above; the resulting emission of 0.15 µg $N_2O$-N g leaf $DM^{-1}d^{-1}$ can therefore be ascribed to the by-product losses when AOB carry out nitrification. Scaling this to an area basis gives 0.015 -0.03 mg $N_2O$-N $m^{-2}d^{-1}$ when herbage mass of 100 – 200 g $m^{-2}$ (equivalent to the average standing biomass of N.Z. pastures). By comparison, Di and Cameron (2008) found emissions from the plant and soil of 0.06 mg $N_2O$-N $m^{-2}$ $d^{-1}$ after urea application, 60% of which could be ascribed to nitrification. We also measured emissions from leaves on plants growing in soil of 0.6-1.2 kg $N_2O$-N $ha^{-1}yr^{-1}$; this compares to average emissions for New Zealand sheep grazed pastures being 4-6 kg $N_2O$-N $ha^{-1}yr^{-1}$ (Saggar et al 2008). Comprehensive future experimentation is required to validate these estimates.

# 2. Introduction

Emissions from New Zealand grazed pastures represent a significant source of atmospheric $N_2O$ (De Klein and Eckard 2008); the loss of N has serious consequences for the environment and farm profitability as well as contributing significantly to New Zealand's greenhouse gas emissions. New solutions to reduce agricultural $N_2O$ emissions are urgently required.

Current $N_2O$ mitigation technologies target two soil microbial processes: nitrification and denitrification. Nitrification is an aerobic process that oxidises ammonium ($NH_4^+$) to nitrate ($NO_3^-$) with $N_2O$ as a by-product; denitrification is an anaerobic process that reduces $NO_3^-$ to $N_2$, with $N_2O$ an obligatory intermediate.

In this project we investigated the potential of pasture leaves to contribute to atmospheric $N_2O$ emissions and the scope this might provide for an alternative mitigation technology directed at plants rather than the soil. Emissions of $N_2O$ from plant leaves have been observed previously from wheat (Smart and Bloom 2001, Zou et al 2005), canola (Chang et al 1998), barley (Chang et al 1998), beech (Pihlatie et al 2005) and ryegrass (Chen et al 1999). These studies proposed two mechanisms. First, plants serve as conduits for $N_2O$ between the soil and atmosphere; the $N_2O$ dissolved in the soil solution is absorbed by plant roots along with water and then conveyed to the leaves in the transpiration stream and, along with evaporation of the water at the leaf surface, the $N_2O$ is released into the atmosphere (Chang et al 1998, Ferch and Römheld 2001). Second, plants produce $N_2O$ themselves through photoassimilation of nitrite ($NO_2^-$) (Smart and Bloom 2001). Smart and Bloom (2001) suggested that about 5-6% of total soil-plant ecosystem (i.e. on an area basis) $N_2O$ emissions might be due to this plant production mechanism.

In this project we tested a possible further mechanism – $N_2O$ emissions from ammonia oxidising bacteria (AOB) that colonise plant leaves, particularly in high $NH_3$ environments. AOB are largely responsible for nitrifcation in soils but have also been identified on plant leaves. Our evidence for a canopy source of $N_2O$ from AOB is 1) the presence of AOB on leaves determined by molecular detection of AOB genes from the DNA extracted from pasture leaves exposed to high $NH_3$ environments, 2) direct measurement of emissions from leaves when exposed to a high $NH_3$ environment, and 3) inference from field chamber measurements (see section 3.5).

In this report we discuss laboratory and field experiments conducted to test our hypothesis that leaf-based AOB are a source of $N_2O$ emissions from pastures and, if so, determine the significance of this mechanism to total $N_2O$ emissions from New Zealand pastoral lands. The specific objectives of each of the experiments presented in this report were:

Experiment 1: investigate AOB presence in leaves collected from grazed pastures of different locations. This initial experiment was to confirm previous research that AOB can actually be found in/on leaves.

Experiment 2: test whether detached leaves collected from grazed pasture produce $NO_3^-$-N when exposed to a $NH_4^+$-N solution. This would give an indication of whether the leaves had the capacity to convert $NH_4^+$ to $NO_3^-$ and hence, if this was an AOB mediated reaction; have the potential to produce $N_2O$ during $NH_3$ oxidation.

Experiment 3: following on from Experiment 2, the objective of this experiment was to determine levels of $N_2O$ production when pasture leaves were exposed to high atmospheric $NH_3$. The results of this experiment will help to test our hypothesise that the AOB in leaves used $NH_3$ and produce $N_2O$ during $NH_3$ oxidation.

Experiment 4: the emissions from detached leaves may have potential artefacts and preliminary tests were conducted to develop an assay to monitor $N_2O$ emissions from non-detached leaves.

Experiment 5: emissions of $N_2O$ from pasture soil and plants are frequently observed almost immediately after urine is applied. No satisfactory explanation has been made for these emissions. We hypothesised that the emissions might come from AOB colonising leaves exposed to the high $NH_3$ environment created by urine application. In order to test our hypothesis a field experiment was carried out.

# 3. Experimental Methods

## 3.1 Investigation of AOB presence in pasture leaves

Leaves of different pasture species were collected from various locations in the North Island. In March 2009, leaves of ryegrass (*Lolium perenne*), cocksfoot (*Dactylis glomerata*) and white clover (*Trifolium repens* ) were collected from a paddock at

Massey University Dairy Unit No. 1. These leaves were collected from observable urine patches. In May 2009 leaves of ryegrass, white clover and prairie grass (*Bromus wildenowii*) were collected from a paddock within 200 m of the urea production plant at Kapuni; the area surrounding this plant has been shown to have elevated atmospheric $NH_3$ concentrations. During spring 2009 several samplings were made of ryegrass leaves from sheep grazed paddocks at AgResearch Grasslands and from the AgResearch FACE experimental site at Flock House.

Total DNA was extracted from the plant leaves using a MO BIO plant DNA extraction kit according to the manufacturer's instructions. A Polymerase chain reaction (PCR) targeting the amoA gene of AOB (Bowatte et al 2008) was carried out to check whether the DNA extracted from pasture leaves contained DNA of AOB. Some successful PCR products were subjected to denaturing gradient gel electrophoresis (DGGE) and DNA sequenced to identify leaf AOB species using the procedure described in Bowatte et al 2008.

## 3.2 Laboratory test to measure the nitrification capacity of detached leaves

In this experiment we wanted to test whether detached leaves produce $NO_3^-$-N when exposed to a $NH_4^+$-N solution. This would give an indication of whether the leaves had the capacity to convert $NH_4^+$ to $NO_3^-$ and hence, if this was an AOB mediated reaction, have the potential to produce $N_2O$. Ryegrass leaves were collected from the experimental plots where urine was added (see section 3.5). Two grams of fresh leaves were added to 50 ml of 0.005 M sterilised $NH_4SO_4$ (Bowatte et al 2008) or 50 ml sterilised $H_2O$ in 250 ml sterilised flasks and continuously shaken for 4 days at 25°C in the dark. The $NO_3^-$ and $NO_2^-$ levels in the solutions were then measured after 18 h and 4 d.

## 3.3 Laboratory test to measure $N_2O$ emissions from detached leaves

A series of preliminary laboratory tests were conducted (not presented in this report) to develop an assay to determine $N_2O$ emissions from detached leaves both in the presence and absence of ammonia ($NH_3$); the assay was designed to exclude emissions from the soil and from endogenous plant production (by incubation in the dark to prevent photoassimilation). The final assay was set up as in Figure 1 used

ryegrass leaves collected from five urine patches in a sheep grazed pasture (AgResearch Grasslands). Fresh leaves (1 g) were loosely contained in a plastic mesh pouch and kept in sterilised 100 ml volumetric flasks with 40 ml of 2M $NH_4SO_4$ ( buffered to pH=7.5 to produce $NH_3$). Flasks were sealed and a gas sample was taken to determine initial $N_2O$ levels of the ambient air. Ryegrass leaves were also kept in a empty flask as a control. Flasks were incubated in the dark at 25 $^oC$ to minimise photorespiration derived $N_2O$ and after 24 h gas samples were taken and $N_2O$ levels were determined using gas chromatography.

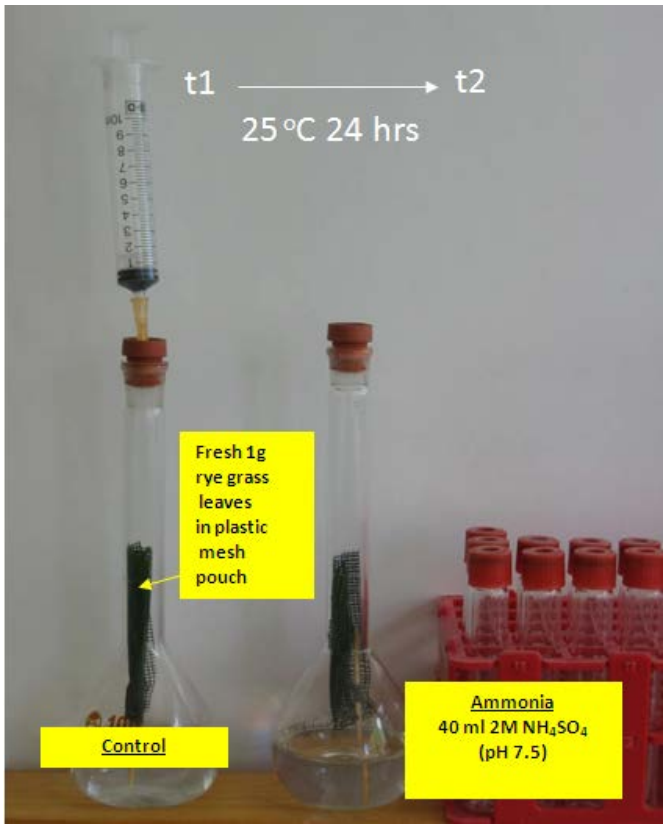


Figure 1: Experimental set up to detect $N_2O$ emissions from detached leaves and detached leaves exposed to high ammonia ($NH_3$) environments.

This assay was then used to screen leaves of four different pasture species collected from the AgResearch (FACE) experimental site. The pasture species tested were 1. Ryegrass 2. Sweet vernal (*Anthoxanthum odoratum*) 3. Kentucky bluegrass (*Poa pratensis*) 4. Yorkshire fog (*Holcus lanatus*)

## 3.4 Laboratory test to measure $N_2O$ emissions from leaves on plants growing in soil

The emissions from the experiment described in section 3.3 may have potential artefacts from using detached leaves and preliminary tests were conducted to develop an assay to monitor $N_2O$ emissions from non-detached leaves that will identify whether these artefacts are substantive.

A soil block (28 cm x 28 cm x 15 cm) with a clump of intact ryegrass plants was removed from an observable urine patch in a sheep grazed paddock at AgResearch Grasslands. Three groups of leaves were inserted into separate 2L Büchner flasks (see Fig. 2). The mouth of each flask was sealed from the ambient atmosphere using cotton wool and blue tack. A plastic tube with a rubber septum at one end was fitted to the glass tube at the neck of Büchner flask. This was used to sample air immediately after the flask was sealed and after 2 and 4 h. The gas measurements from three flasks at two time intervals were regarded as replicates in this experiment.
Shorter 2h periods as opposed to the 24 h periods used in detached leaves experiment was chosen to prevent emitted $N_2O$ dissolving in condensation water from transpiration. Also, during this period the laboratory temperature was maintained at 30°C to decrease condensation on the flask walls. This experiment was done under natural light levels so the plants could actively photosynthesise. At the conclusion of the gas sampling the leaves inside the flasks were cut and dried at 60°C for dry matter determination

Figure 2: Laboratory set up to monitor $N_2O$ emissions from intact leaves of pasture plants.

### 3.5 Field experiment to test whether the initial $N_2O$ emissions from urine patches are from the plant canopy

When urine is applied to a pasture, $N_2O$ emissions are stimulated as a consequence of increased nitrification (i.e. the transformation of $NH_4$ to $NO_3$); this nitrification takes perhaps 4-7 days to occur. However, emissions of $N_2O$ are frequently observed almost immediately after the urine is applied (Sherlock and Goh 1983, Li and Kelliher 2005). No satisfactory explanation has been made for these emissions. We hypothesised that they might come from AOB colonising leaves exposed to the high $NH_3$ environment created by urine application.

In order to test our hypothesis a field experiment was carried out in late September 2009 at a sheep grazed block of mainly ryegrass pasture at AgResearch Grasslands campus. The herbage was trimmed to 2 cm and two weeks later, when herbage mass was the equivalent of 1280 kg DM $ha^{-1}$, urine containing 1.5%N was collected from sheep. The urine was applied at a rate of 150 ml to a circular area of 0.03 $m^2$; this is a similar concentration to a typical sheep urination event (Haynes and Williams 1993). Three treatments with four replicates were tested giving a total of 12 plots. The treatments were: 1. urine applied to plots with herbage (i.e. the regrowth from the previous trimming). 2. urine applied to plots without herbage (trimmed to ground level prior to urine application with minimal pasture regrowth) 3. plots without herbage and without urine (to test any cutting effects); no solution was added to these plots.

The $N_2O$ emissions from each plot at 1, 3, 6 hours and 2, 3, 4 and 9 days after urine application were measured using a static chamber technique (De Klein et al 2003). Ammonia volatilisation was measured using passive $NH_3$ samplers (Carran and Theobald 2000) at ground and canopy height (13 cm above the soil surface). Changes in soil mineral N levels were monitored using ion exchange resin membranes (Bowatte et al 2008a).

## 4. Results

### 4.1 Investigation of AOB presence in pasture leaves

In contrast to our preliminary results, none of the leaf samples collected from urine patches in a cattle paddock at Massey University No. 1 Dairy showed positive AOB

PCR results indicating that AOB were not colonising the tested leaf samples or at least not in detectable numbers for PCR testing.

However, most leaf samples collected from a paddock near to the urea production plant at Kapuni showed positive AOB-PCR which is consistent with our findings prior to the work conducted here.

In the preliminary experiments we observed that some leaf samples collected from sheep urine patches at the AgResearch FACE site showed positive AOB-PCR. However, in this project we found no positive AOB-PCR for leaves collected from this site.

On various other occasions we have collected leaf samples from sheep grazed paddocks at AgResearch Grasslands for other experiments in this project and have found positive AOB-PCR in leaf samples.

Where AOB were present the AOB PCR were subjected to DGGE analysis (Fig. 3) and DNA sequencing of PCR products to identify the AOB species.


Figure 3: The denaturing gradient gel electrophoresis (DGGE) banding patterns of partial amoA gene of AOB species colonising ryegrass, clover and browntop leaves.

The DGGE bands in same position (Y axis) have the same DNA sequence and thus represent the same AOB species. The DGGE banding pattern demonstrated that different pasture species harbour different AOB species community.

The identified AOB DNA sequences were confirmed by searching the international sequence data base with the BLAST program (www.ncbi.nlm.nih.gov/BLAST). The DNA sequencing revealed that the identified pasture leaf AOB species were closely related to *Nitrosospira* species. Some of the leaf AOB DNA sequences were a close match to some soil AOB sequences identified from New Zealand soils.

## 4.2 Laboratory test to measure the nitrification capacity of detached leaves

The results of the laboratory experiment to test whether the detached pasture leaves produce $NO_3^-$-N when mixed with a $NH_4^+$-N solution are shown in Table 1.

Table 1: Amounts of nitrate ($NO_3^-$-N) and nitrite ($NO_2^-$-N ) detected when 2 g of ryegrass leaves were mixed with 50 ml 0.005 M $NH_4^+$ solution or 50 ml sterilised water. Values within brackets are standard error of mean (n = 3).

| Substrate | After 18 hours | | After 4 days | |
|---|---|---|---|---|
| | $NO_3^-$-N (µg N/ml) | $NO_2^-$-N (µg N/ml) | $NO_3^-$-N (µg N/ml) | $NO_2^-$-N (µg N/ml) |
| $H_2O$ | 0 | 0 | 0.10 (0.019) | 0 |
| $NH_4^+$ solution | 0.01 (0.003) | 0 | 0.37 (0.047) | 0.03 (0.02) |

After 18 hours $NO_3^-$-N was detected only in the $NH_4^+$ solution. After 4 days $NO_3^-$-N was detected in both the $NH_4^+$ solution and water. However, higher $NO_3^-$-N was detected in leaves mixed with the $NH_4^+$ solution compared to leaves mixed in water. This indicates that nitrification occurred when leaves were mixed with $NH_4^+$ solution and that some $NO_3^-$-N was transferred from leaves to water.

## 4.3 Laboratory test to measure $N_2O$ emissions from detached leaves

The average $N_2O$ emissions from detached ryegrass leaves collected from five urine patches of sheep grazed paddocks are illustrated in Fig. 4. Nitrous oxide was emitted

by pasture leaves and the extent of emission was greater when leaves were exposed to ammonia levels higher than the ambient air.

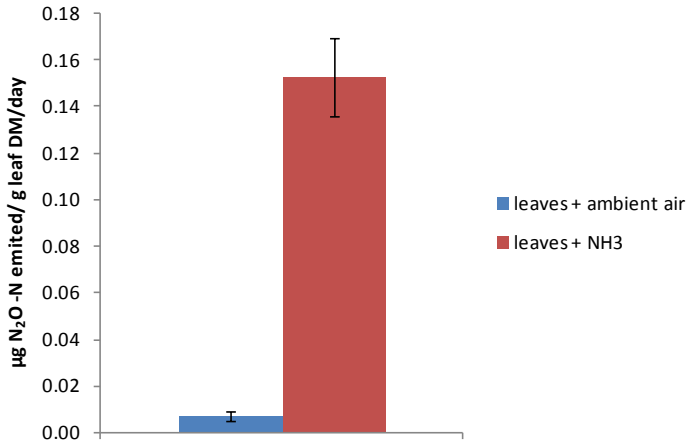


Figure 4: Nitrous oxide emissions from detached ryegrass leaves collected from urine patches of sheep grazed paddocks exposed in ambient air and detached leaves exposed to high ammonia environments. Error bars are standard error of mean (n=5).

There were no $N_2O$ emissions from the leaves of four different grass species collected from the AgResearch FACE site when exposed to ambient air (data not presented). However all four species emitted $N_2O$ in the presence of $NH_3$ with ryegrass tending to have the lowest emission rate (Fig. 5).

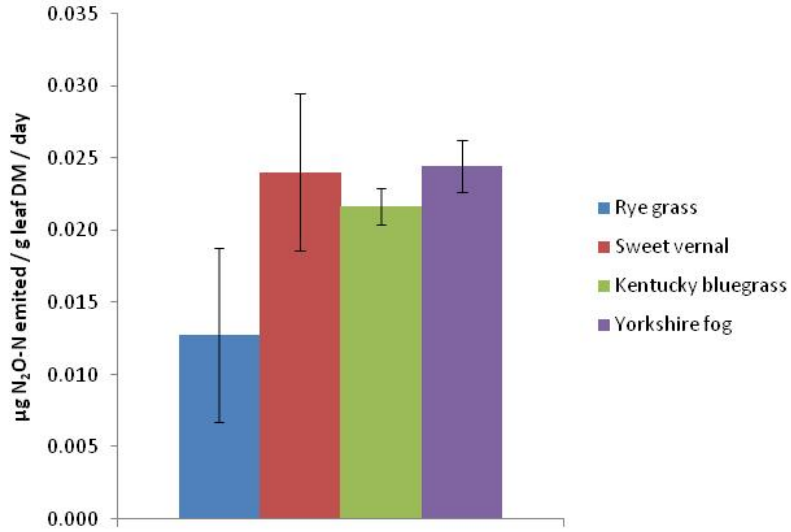


Figure 5: Nitrous oxide emissions from detached leaves of different pasture species exposed to high $NH_3$ levels Error bars are standard error of mean (n= 3).

## 4.4 Laboratory test to measure $N_2O$ emissions from leaves on plants growing in soil

$N_2O$ emissions were also measured from leaves that were still attached to the plant in the flask experiment (see section 3.4). There were three replicate flasks with intact leaves and from each flask two measurements were taken at 2 hour intervals (Table 2). The detected emission from flask 1 was reasonably similar in both measurements and higher than the second measurement from flask 2.

Table 2: Amounts of $N_2O$ emissions from intact pasture plants 2 and 4 h after leaves were sealed in flasks.

| Flask | µg $N_2O$-N emitted/ g leaf DM/day | |
|---|---|---|
| | After 2h | After 4h |
| 1 | 1.7 | 1.6 |
| 2 | nd | 0.6 |
| 3 | nd | nd |

nd: not detected

## 4.5 Field experiment to test whether the initial $N_2O$ emissions from urine patches are from the plant canopy

The results of the field experiment to test the relationship of pasture canopy and initial $N_2O$ emissions from urine patches are illustrated in Figures 6, 7 and 8.

Ion exchange resin membrane adsorbed $NO_3^-$-N and $NH_4^+$-N indicated there was no soil $NO_3^-$-N build up (Fig. 6) up to 9 days after urine application. The $NH_3$ passive samplers adsorbed significant amount of $NH_3$ during the first 3 days indicating high $NH_3$ volatilisation occurred from the urine patches (Fig. 7). A significant amount of $N_2O$ was emitted during the first few days after urine application and there was a considerable amount of $N_2O$ emitted in the first few hours after urine application (Fig. 8).

In contrast to our hypothesis, the initial $N_2O$ emission from urine patches of this experiment was greater in the plots without herbage (see Fig. 8).

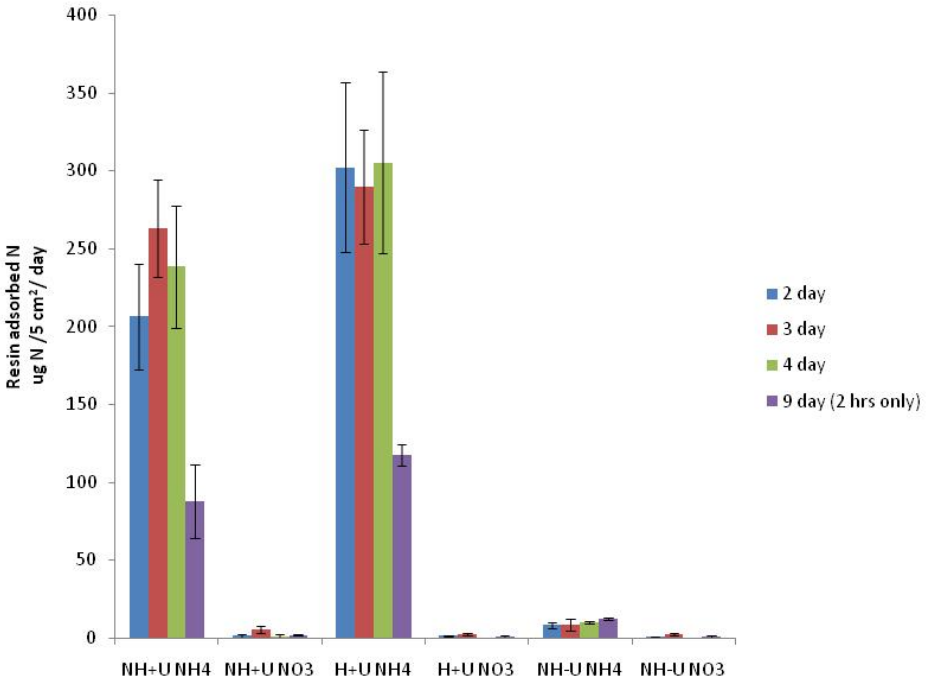


Figure 6: Mineral N changes after urine application with time indicated by resin adsorbed N. NH4 = ammonium N, NO3= nitrate N, NH+U = urine added plots without herbage, H+U = urine added plots with herbage, NH-U = plots without herbage and without urine added. Note, on the 9 day resin adsorbed N is only for 2 hours. Error bars are standard error of mean (n=4).

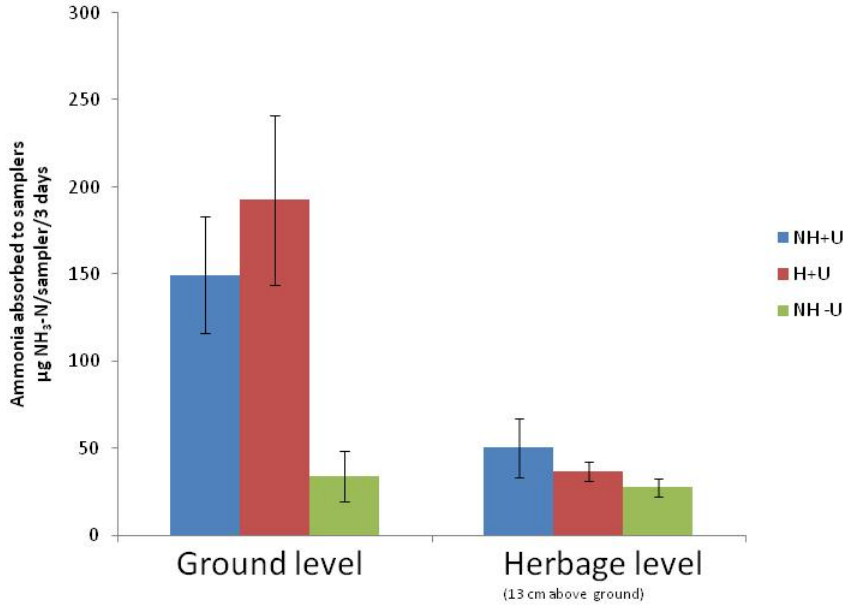


Figure 7: Ammonia volatilisation indicated by $NH_3$ absorbed to passive samplers for 3 days. Passive samplers were placed at ground level and 13 cm above ground level. NH+U = urine added plots without herbage, H+U = urine added plots with herbage, NH-U = plots without herbage and without urine added. Error bars are standard error of mean (n=4).

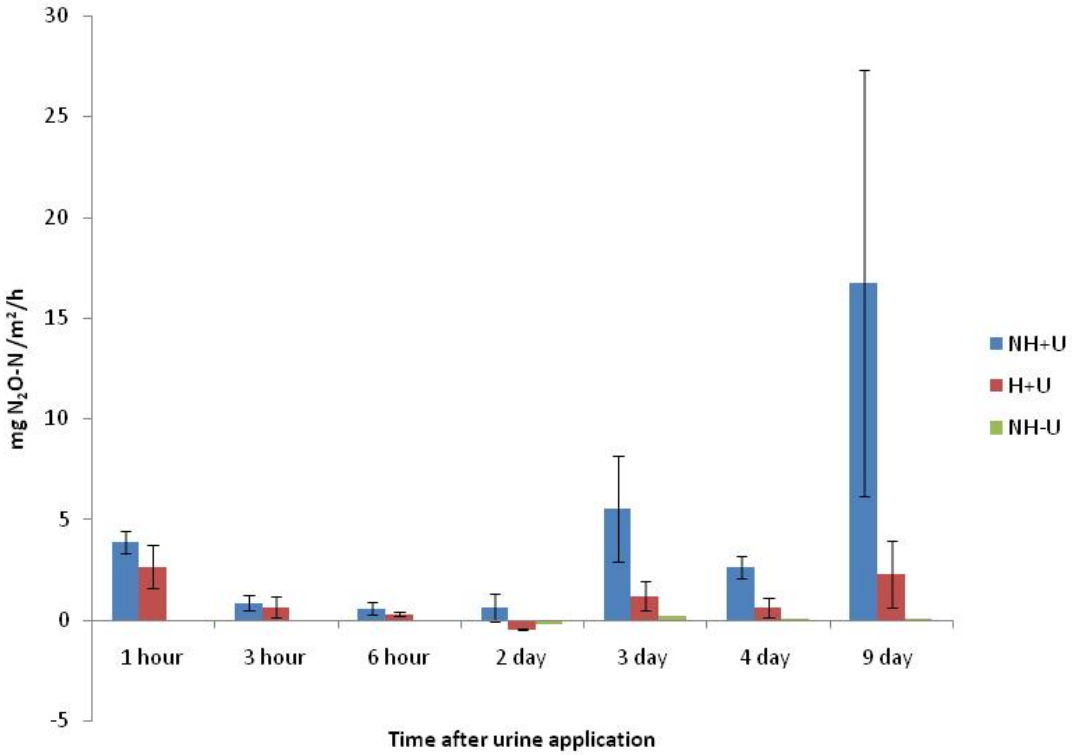


Figure 8: Nitrous oxide emissions after urine application. NH+U = urine added plots without herbage, H+U = urine added plots with herbage, NH-U = plots without herbage and without urine added. Error bars are standard error of mean (n=4).

## 5. Discussion

The experimental results from this project and the data available from the literature (Chang et al 1998, Chen et al 1999, Smart and Bloom 2001, Pihlatie et al 2005, Du et al 2008) demonstrate that plant leaves emit $N_2O$. Our detached leaf assay, which excluded both soil and endogenous plant source of $N_2O$ demonstrates that emissions from bacteria associated with plant leaves is highly plausible.

We found AOB on leaves, particularly when the leaves were from high $NH_3$ environments (urine patches or close to the Kapuni urea plant) but their presence was variable. Using a known quantity of partial amoA gene ligated plasmids as a template DNA we found that our PCR assay for AOB presence has a minimum detection limit of $10^6$ AOB cells; this gives a population size of the same order as found in 1 g soil ( Di et al 2009) and this is in line with the only other measurement we could find in the literature which recorded $10^7$ AOB cells on 1 g dry weight spruce needles (Papen et al 2002). Further investigation is required to understand what causes the variability in

leaf AOB detection using the PCR technique. However, the presence of AOB is consistent with other results that have shown AOB on spruce needles (Papen et al 2002) and weeds of paddy fields (Bowatte et al 2006). There is also evidence from the literature that leaves take up $NH_3$ partly due to the leaf AOB (Papen et al 2002) and that ryegrass and clover leaves can completely absorb $NH_3$ produced near the ground surface (Denmead et al 1976). Our finding of $NO_2^-$-N and $NO_3^-$-N production when ryegrass leaves were mixed with a $NH_4^+$ solution is strongly indicative of AOB activity; we assume that $NO_2^-$-N was produced by $NH_3$ oxidised by AOB on the leaves and then converted to $NO_3^-$-N by leaf $NO_2^-$ oxidising bacteria. However, further testing is required to confirm this hypothesis. AOB are known to produce $N_2O$ during $NH_3$ oxidation (Shaw et al 2006). We observed $N_2O$ production when pasture leaves were exposed to high $NH_3$ (see section 4.3) and hypothesise that the AOB in leaves used $NH_3$ and produce $N_2O$ during $NH_3$ oxidation. We are confident that in this experiment $N_2O$ emissions from leaves were not produced by the other two potential mechanisms for canopy emissions; detaching the leaves discounted the possibility of $N_2O$ transportation from the soil and the incubation under dark conditions removed the possibility of plant $N_2O$ production during photoassimilation.

The detached ryegrass leaf material from urine patches (Fig. 4) produced 0.15 µg $N_2O$-N g leaf $DM^{-1}d^{-1}$. If we assume a herbage mass of 100 g $m^{-2}$ (equivalent to the average standing biomass of N.Z. pastures) this scales to 0.015 mg $N_2O$-N $m^{-2}$ $d^{-1}$; if we assume 200 g leaf DM $m^{-2}$ then this is 0.03 mg $N_2O$-N $m^{-2}$ $d^{-1}$. Emission rates from pasture treated with urea have been found to be 0.06 mg $N_2O$-N $m^{-2}$ $d^{-1}$ with 60% being ascribed to nitrification (see Table 1 Di and Cameron 2008). The number of AOB per g soil and per g of plant DM appears to be similar. We assume this from the literature ($10^6$ from a Canterbury soil (Di et al 2009) and $10^7$ on spruce needles (Papen et al 2002) together with our assay which we calculate can only detect AOB presence when the copy number is greater than $10^6$. This means that on an area basis the number in the soil will be substantially larger (c. $10^{15}$ cells $ha^{-1}$ vs. $10^{12}$ cells $ha^{-1}$). However, it is not only the number of bacteria that is important – it is also their activity. It seems likely that the function of soil AOB may be frequently negatively affected by changes in soil moisture (Stark and Firestone 1995, Avrahami and Bohannan 2007) and we suggest that the environment of the leaf may be more optimal for AOB activity over a much wider span than that of soil moisture levels. It is our hypothesis that the plant moisture status remains similar and hence optimal for bacteria even when soil moisture levels are low. In addition, Papen et al (2002) argued that conditions found in phyllosphere are favourable for the occurrence and activity AOB, namely: 1. availability of $O_2$ and $CO_2$ outside and inside leaves, 2. well protected from UV-A and

UV-B light, 3. optimal pH for AOB in stomata cavity and 4. $NH_3$ availability outside and inside leaves.

There was some evidence (Fig. 5) to suggest that AOB activity differed among grass species but clearly more work is required to confirm this. If this is the case however, it could represent a possible mitigation strategy by altering pasture species.

We also measured emissions from intact leaves which we assume combined emissions from potentially the soil (taken up and emitted through the plant), from endogenous plant production and from leaf AOB. The maximum rate we measured was 1.7 µg $N_2O$-N g leaf $DM^{-1}d^{-1}$. Assuming a herbage mass of 100 g $m^{-2}$ this gives 0.17 mg $N_2O$-N $m^{-2}d^{-1}$ or 0.34 $N_2O$-N $m^{-2}d^{-1}$ if the herbage mass is 200 g $m^{-2}$. Extrapolating to a year gives 0.6-1.2 kg $N_2O$-N $ha^{-1}yr^{-1}$; conventionally it is assumed that for New Zealand, the emissions from a sheep grazed pasture are in the range 4-6 kg $N_2O$-N $ha^{-1}yr^{-1}$ (Saggar et al 2008). This indicates that the canopy can potentially play a significant role in total emissions particularly if the leaf environment is more consistently conducive to nitrification than the soil environment where soil moisture limitation can frequently occur.

We now need to proceed to refine our assays with detached and non-detached leaves to absolutely confirm that AOB are the source of emissions; to determine maximum rates of emission from AOB and endogenous plant production; and to determine how widely these canopy sources are expressed in field situations. Our attempt to identify canopy sources in the field (section 4.5) were not successful, we are not sure why this was the case but the disturbance associated with cutting the herbage may introduce artefacts and we need a more controlled experiment if we are to reliably partition emissions to different sources.

Current techniques for measuring $N_2O$ emissions from pasture, including the methodology used to determine emissions factors for greenhouse gas reporting, involve chambers that cover both the soil and plants (De Klein et al 2003, Luo et al 2007). If there is a significant flux of $N_2O$ from AOB colonising plant leaves this will be reported as part of the total emission (as it should be). What this pathway opens up is the possibility of new mitigation strategies that targets the leaf rather than just the soil. Although the results from this project are not conclusive enough to confirm this mechanism, it has provided evidence to support our hypothesis and encouragement to proceed with further research.

# 6. Conclusion

We found that:

1. Leaves of pasture species produce $N_2O$ both in the presence and absence of $NH_3$.
2. Emissions may vary between grass species.
3. Detached leaves can produce emissions (assumed to be from nitrification by AOB) at a rate similar to the contribution from nitrification by soil AOB (in the study of Di and Cameroon 2008 this was 60% of total emissions)
4. The plant canopy is involved in emissions (by transport of soil emissions, by endogenous production and by leaf AOB) that may be a substantial fraction of the total emissions recorded in pasture.

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