# Ammonia-oxidizing bacteria dynamics affected by plantain under synthetic cattle urine patches

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## Abstract
Plantain has been suggested as a nitrous oxide ($N_2O$) and nitrate ($NO_3^-$) leaching mitigation option as it may induce biological nitrification inhibition (BNI) via plantain root exudation, which affects the activity of ammonia-oxidizing bacteria. This preliminary study compared the abundance of the ammonia monooxygenase gene (*amoA*) in soils containing either plantain and white clover, or ryegrass and white clover. The plants were sown in pots and grown in a greenhouse. Two months after sowing, synthetic cattle urine was applied to half the pots, and rhizosphere and bulk soil samples were collected 30 and 90 days after urine application. The *amoA* gene abundance was measured using real-time quantitative PCR. The abundance of *amoA* genes in rhizosphere soil around ryegrass plants and in bulk soil under ryegrass/white clover were higher in pots treated with urine than the no-urine controls. *AmoA* gene abundance in bulk soil under plantain/white clover was higher in pots treated with urine ($P<0.05$) but not in rhizosphere soil around plantain plants ($P>0.05$) compared with the control. Furthermore, *amoA* gene copy numbers in the rhizosphere soil around plantain plants were lower than for ryegrass plants ($P<0.05$). However, there was no difference in the abundance of *amoA* genes in bulk soil of either combination of plant species evaluated ($P>0.05$). The results suggest that, in the time frame of our experiment, plantain could induce a BNI effect in the rhizosphere soil but not in the bulk soil.

**Keywords:** *Plantago lanceolata*, BNI, rhizosphere, nitrous oxide

## Introduction
Plantain (*Plantago lanceolata*) has been suggested as an option to reduce nitrous oxide ($N_2O$) emissions and nitrate ($NO_3^-$) leaching from New Zealand farm systems. Luo et al. (2018) found that pure plantain swards reduced $N_2O$ emissions from cow urine patches by on average 28%, compared with a ryegrass (*Lolium* spp.) monoculture. Similarly, Simon et al. (2019) showed that $N_2O$ emissions in both no-urine and cow-urine treatments during a field trial were inversely related to the proportion of plantain in the sward. Emissions from urine of cows fed ryegrass/white clover (*Trifolium repens*) applied to a 100% plantain/white clover sward were 44% lower than that in a 100% ryegrass/white clover sward, while emissions from no-urine treatments were 78% lower. Gardiner et al. (2017) showed that applying an extract of plantain leaf with cattle urine to a field experiment reduced soil $N_2O$ emissions by 50%. Three potential explanations for these earlier findings on the effect of plantain on $N_2O$ emissions are: (1) a reduction in the nitrogen (N) concentration in urine (and total urinary N output) from cows fed on plantain (Luo et al. 2018; Simon et al. 2019); (2) changes in the soil microclimate (e.g. pH, porosity, moisture) under plantain affecting soil microbial processes (de Klein et al. 2020); and (3) plantain root exudates inducing biological nitrification inhibition (BNI) (Gardiner et al. 2017; Li et al. 2018).

Biological nitrification inhibition refers to the ability of root exudates to inhibit the soil ammonia-oxidizing bacteria (AOB) responsible for the first step (ammonia oxidation) of the nitrification process (Li et al. 2018). Ammonia oxidation is catalysed by the enzyme ammonia monooxygenase (AMO) and encoded by the AMO gene (*amoA*), so AOB activity can be assessed by quantifying the *amoA* gene abundance in soil (Rotthauwe et al. 1997).

Finding ways to reduce the abundance, and more specifically the activity, of *amoA* is vital to slow down the nitrification process and reduce the risk of N losses through $N_2O$ emissions and $NO_3^-$ leaching from soils (Podolyan et al. 2014; Sarr et al. 2020). We used the two most contrasting species combinations from our earlier work (S0 and S100; Simon et al. 2019) in a pot-trial to undertake a preliminary exploration of the BNI capacity of plantain in soil. Pots were sown with 20/4/0 kg seed $ha^{-1}$ (S0) or 0/4/12 kg seed $ha^{-1}$ (S100) of perennial ryegrass/white clover/plantain, respectively. We quantified bacterial *amoA* gene abundance to test our hypothesis that soils supporting plantain/white clover would have lower *amoA* gene abundance than ryegrass/white clover soils, as a result of the capacity of plantain to biologically inhibit the nitrification process in the soil. The rhizosphere, which is the narrow zone of soils around the roots, is the hotspot for plant-soil-microbial interaction. Therefore, we further hypothesise

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that BNI activity would occur much more quickly in the rhizosphere of plantain than in the bulk soil, and/or at much stronger level.

## Materials and Methods

### Plant establishment and urine application

An experiment was carried out at AgResearch Invermay, in Mosgiel, Otago, NZ between February and July 2018. The study was conducted in a greenhouse under semi-controlled conditions. The roof of the greenhouse consisted of fine mesh nylon cloth that allowed some rainfall to enter. Light conditions were not impaired by the cloth roof, and temperatures were monitored throughout the experiment (averaged 20°C). We used 12-cm deep pots filled with soil, and randomly distributed them within the greenhouse. The soil used was a moderately well-drained Wingatui silt loam (Hewitt 2010), collected at 0–20 cm soil depth from a field experimental area of AgResearch in Mosgiel (45.8° S and 170.3° E). The soil had been cultivated for many years with ryegrass/white clover pastures, and had the following chemical characteristics when collected: pH = 5.9, Olsen P = 23 mg $L^{-1}$, K extractable = 0.5 $cmol_c$ $dm^{-3}$, Total base saturation = 75.7%. Before use, soils were well mixed and passed through a 5-mm sieve to remove large soil particles. Original water content (47% w/w) and percent moisture at field capacity (60% w/w) were measured immediately after collection, and 5 kg of fresh soil (3.4 kg dry weight equivalent) was packed into each pot.

Plantain (*Plantago lanceolata* cv. Agritonic) and white clover (*Trifolium repens* cv. Tribute) seeds (at sowing rates equivalent to 12 and 4 kg $ha^{-1}$, respectively) or ryegrass (*Lolium perenne* cv. One50 AR37) and white clover seeds (at sowing rates equivalent to 20 and 4 kg $ha^{-1}$, respectively) were distributed homogeneously on top of the pots and covered with a 3-cm soil layer. Fifteen pots were planted for each seed combination. After sowing, the pots were watered to maintain soil moisture at field capacity (60% w/w). Following germination, pots were watered approximately twice a week to maintain soil moisture at field capacity. The objective of this study was to understand the effect of plantain on *amoA* gene abundance and relate this to a potential BNI effect so the soil moisture content was control maintained and not variable tested between treatments.

Plantain was established at 100% sowing rate, compared with 100% ryegrass. The number of seeds were standardised per pot and a pricking process was utilised prior starting measurements to standardise the distribution of plants in each pot. Plants were trimmed to 5 cm height three times prior to urine application to maintain the homogeneity of the stand and stimulate growth. At 60 days after sowing (DAS), a mixture of chemicals was combined to generate synthetic cattle urine with composition based on a pasture diet (Fraser et al. 1994), and 10 L $m^{-2}$ was added to all the treatments, except for the no-urine control pots, which received the equivalent volume of water. The experimental layout is presented in Table 1.

### Plant harvesting and soil sampling

Five replicates per treatment were destructively sampled at 60 DAS (before urine application), and the remaining pots (five replicates per treatment) were harvested at 90 and 150 DAS (30 and 90 DAUA, respectively). Cores containing plant roots were shaken by hand to release the loose soil, and the rhizosphere soil corresponding to the soil firmly attached around the roots of plantain and ryegrass was collected on clean trays using tweezers. Bulk soil samples where no roots were visible were also collected from each treatment. The soil samples were stored in 15-mL containers and transferred to the laboratory in cold conditions (-4°C).

### DNA extraction and qPCR of bacterial *amoA* genes

Soil microbial DNA was extracted from 0.25 g of rhizosphere or bulk soil samples using the DNeasy® PowerSoil® Kit (QIAGEN Group). The abundance of bacterial amoA gene was quantified by qPCR with primer set amoA1F (Rotthauwe et al. 1997) and amoAR1 (Avrahami et al. 2003) on a Lightcycler 480 qPCR instrument (Roche Molecular Systems Inc.). Each PCR reaction was prepared in a final volume

**Table 1** Overview of treatments and soil sampling dates in relation to days after sowing and days after urine application in a pot experiment to evaluate the effect of plant species on *amoA* gene abundance in soil. Synthetic urine was applied 60 days after sowing. On each sampling day, both rhizosphere and bulk soil were sampled.

| Plant species | Days after sowing (DAS) | Days after urine application (DAUA) |
|---|---|---|
| | 60 | No urine |
| | 90 | No urine |
| Ryegrass/white clover | 90 | 30 |
| | 150 | No urine |
| | 150 | 90 |
| | 60 | No urine |
| | 90 | No urine |
| Plantain/white clover | 90 | 30 |
| | 150 | No urine |
| | 150 | 90 |

of 15 µL including 11 µL of SensiFAST SYBR (no-ROX) supermix (Bioline), 1 µL of forward primer and reverse primers (10 µM) (Sigma-Aldrich), 2 µL of soil microbial DNA at 5 ηg µL$^{-1}$ or standard curve DNA, or water (no template control). All samples were run in triplicate with Taq Hotstart activation at 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 57°C for 20 s, 72°C for 20 s, and a final extension step at 82°C for 10 s. Fluorescence was measured at 82°C for amplicon quantification. After amplification, an amplicon melting curve was recorded in 1°C steps between 50°C and 95°C. The final step comprised of cooling to 40°C for 30 s.

The standard curve for the bacterial *amoA* gene was created using the approach given in Wakelin et al. (2013), where PCR product was cloned into the pGEM®-T Easy Vector (Promega Corporation). The plasmid DNA was quantified in a stock solution of the standards using a NanoDrop ™ 2000 Spectrophotometer (Thermo Scientific), and from this gene copies per ng template were calculated (Wakelin et al. 2013). Absolute quantification of gene copies was calculated against a standard curve generated by serial dilution of the stock plasmids. Specific amplification of the *amoA* gene was checked by a melting curve analysis to ensure that a single product was generated. qPCR reaction products were also run by agarose gel electrophoresis to confirm the specificity of a single qPCR product of the correct size (491 bp). Briefly, 5 µL of qPCR products were separated by electrophoresis in a 1% w/v agarose gel containing RedSafe DNA stain (Chembio Ltd, UK) in 0.5 × Tris-Borate-EDTA buffer for 30 min at 100V. The bands were visualised under UV light in a UVIdoc transilluminator (UVITEC, UK) and the correct product size verified by comparison to a molecular weight marker EasyLaddder I (Bioline, UK) which was run on the same agarose gel.

### Statistical analyses

The data of *amoA* gene abundance extracted from rhizosphere and bulk soils were subjected to a normality test (Shapiro Wilk) and log transformed before analysis. Two-way ANOVA was applied and a Tukey's test using the statistical software SISVAR® (Ferreira 2011) was performed when a significant effect was detected ($P<0.05$); this identified the differences between treatments.

## Results

The abundance of bacterial *amoA* gene in soil was highly influenced by the input of N via synthetic urine application for most of the treatments (Figures 1 and 2). However, the urine effect on AOB activity in soil differed between rhizosphere and bulk area when applied to plantain/white clover pots. In the rhizosphere soil of the ryegrass/white clover pots, synthetic urine application significantly increased *amoA* gene abundance on both sampling days as compared to no-urine controls (from 6.21 to 7.40 $\log_{10}$ gene copies g$^{-1}$ soil and from 5.26 to 7.92 $\log_{10}$ gene copies g$^{-1}$soil, at 30 and 90 DAUA, respectively ($P<0.05$) (Figure 1)). The abundance of the *amoA* gene increased significantly in the bulk soil in pots of ryegrass/white clover treated with synthetic urine on both sampling dates when compared to no-urine controls (from 7.08 to 7.50 $\log_{10}$ gene copies g$^{-1}$ soil and from 7.01 to 8.02 $\log_{10}$ gene copies g$^{-1}$ soil, at 30 and 90 DAUA, respectively ($P<0.05$) (Figure 2)). *AmoA* gene abundance also increased significantly in the bulk soil of plantain/white clover pots treated with synthetic urine compared with no-urine controls (from 6.84 to 7.26 $\log_{10}$ gene copies g$^{-1}$ soil and from 6.97 to 7.98 $\log_{10}$ gene copies g$^{-1}$ soil, at 30 and 90 DAUA, respectively, ($P<0.05$) (Figure 2)). However, there was

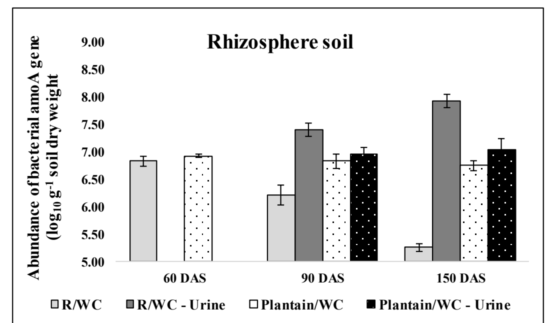


**Figure 1** Abundance of bacterial *amoA* gene in samples taken at 60, 90 and 150 days after sowing (DAS) in the rhizosphere area of ryegrass/white clover (R/WC) and plantain/white clover for urine and no-urine treatments. Bars represent mean abundances of five replicate samples with respective standard deviations of the log values.

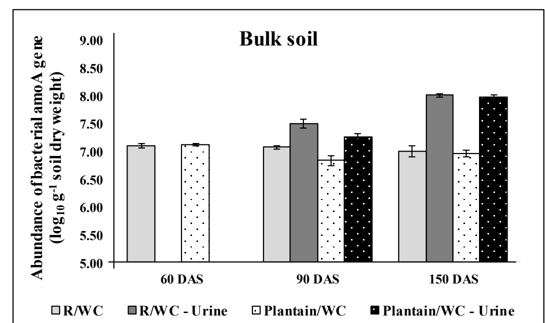


**Figure 2** Abundance of bacterial *amoA* gene in samples taken at 60, 90 and 150 days after sowing (DAS) in bulk soil of ryegrass/white clover (R/WC) and plantain/white clover for urine and no-urine treatments. Bars represent mean abundances of five replicate samples with respective standard deviations.

no significant difference in the *amoA* gene abundance of the rhizosphere soil around plantain plants grown with white clover in pots treated with urine compared with no-urine controls (P>0.05).

The *amoA* gene abundance in the bulk soil of plantain/white clover were similar to those in the ryegrass/white clover bulk soil in both urine or no-urine treatments, indicating no effect of plant species on *amoA* gene abundancy in the bulk soil area (P>0.05) (Table 2). However, at 90 DAUA, rhizosphere soil around plantain plants in pots with white clover treated with urine had significantly lower *amoA* gene abundance than rhizosphere soil around ryegrass plants in pots with white clover (7.04 vs 7.92 $log_{10}$ gene copies $g^{-1}$soil (P<0.05) (Table 2)). In contrast, *amoA* gene abundance was significantly greater in rhizosphere soil surrounding plantain plants in non-urine treated pots with white clover, than that for ryegrass plants grown with white clover at the second and third sampling dates (6.83 vs 6.21 $log_{10}$ gene copies $g^{-1}$ soil and 6.75 vs 5.26 $log_{10}$ gene copies $g^{-1}$ soil at 90 and 150 DAS, respectively (P<0.05) (Table 2)).

## Discussion

In our previous trial we found that $N_2O$ emissions from urine patches were lower in plantain/white clover sward compared with the ryegrass/white clover sward (Simon et al. 2019). In this trial we isolated one of the potential mechanisms for this reduction; biological nitrification inhibition as expressed by amoA genes. Our results showed an increase of bacterial *amoA* gene abundance in soil supporting ryegrass/white clover after synthetic urine application, which is indicative of a response by the AOB community to the N input in soil. A similar effect was reported by Podolyan et al. (2014). The lack of a urine-application effect on the *amoA* gene abundance in plantain/white clover rhizosphere soil in our study may be due to the fact that plantain can inhibit soil nitrification rates through the paralysation of ammonia-oxidizing bacterial activity. Sorghum (*Sorghum bicolor*) is a tropical grass with BNI capacity and Sarr et al. (2020) showed that exudates were released once N reached the rhizosphere area of sorghum roots. They also found that these exudates inhibited ammonia oxidisation activity, which consequently inhibited the nitrification process.

Our study showed that the abundance of *amoA* genes was significantly lower in the rhizosphere of plantain plants grown with white clover than that in the rhizosphere of ryegrass plants grown with white clover following application of synthetic urine, indicating the

**Table 2** Abundance of bacterial *amoA* gene ($log_{10}$ copies $g^{-1}$ soil dry weight) in rhizosphere and bulk soils of ryegrass/white clover and plantain/white clover after cattle urine application.

| **Rhizosphere soil** | ***amo*A gene ($log_{10}$ copies $g^{-1}$ soil dry weight)** | | |
|---|---|---|---|
| *No urine* | **60 DAS** | **90 DAS** | **150 DAS** |
| Ryegrass/white clover | 6.83 Aa* | 6.21 Bb | 5.26 Bc |
| Plantain/white clover | 6.92 Aa | 6.83 Aa | 6.75 Aa |
| *Urine* | | **90 DAS/30 DAUA** | **150 DAS/90 DAUA** |
| Ryegrass/white clover | | 7.40 Ab | 7.92 Aa |
| Plantain/white clover | | 6.95 Aa | 7.04 Ba |
| **Bulk soil** | | | |
| *No urine* | **60 DAS** | **90 DAS** | **150 DAS** |
| Ryegrass/white clover | 7.11 Aa | 7.08 Aa | 7.01 Aa |
| Plantain/white clover | 7.12 Aa | 6.84 Aa | 6.97 Aa |
| *Urine* | | **90 DAS/30 DAUA** | **150 DAS/90 DAUA** |
| Ryegrass/white clover | | 7.50 Ab | 8.02 Aa |
| Plantain/white clover | | 7.26 Ab | 7.98 Aa |

* Uppercase letter compares plant species at the same sampling time and lowercase letter compares sampling dates in each plant species treatment, Different letters represent (P<0.05) according to Tukey's test.

potential BNI capacity of plantain. Carlton et al. (2019) compared the effect of plantain on the AOB community in soil and found a lower AOB community abundance in soil from a pasture mixture of ryegrass, white clover and plantain compared with just ryegrass/white clover pasture after application of cattle urine during summer. In contrast, Podolyan et al. (2020) did not find differences in soil AOB abundance between plantain and ryegrass/white clover, which might be related to the sampling method utilised, as these authors did not collect rhizosphere soil.

The results obtained for bulk soils were different to those for rhizosphere soils. The *amoA* gene abundance increased significantly in the bulk soil of plantain/white clover when synthetic urine was added, as compared to no-urine control at both sampling dates. This may suggest that, if present, a BNI effect of plantain via root exudation might be restricted or mainly attributed to the rhizosphere area. A previous meta-analysis study showed that interferences in the N cycle and bacterial activity in soil attached to plant roots (rhizosphere soil) were significantly higher than those in bulk soil (Finzi et al. 2015). The phenomenon that some plant root effects occur only in the narrow rhizosphere zone but not in bulk soil is very common, and is known as the rhizosphere effect (Cheng 2009). Microbial communities can differ significantly between the rhizosphere and the bulk soil, with some groups of microbes stimulated in the rhizosphere soil by root exudates while other groups are suppressed (Shi et al. 2015; Shi et al. 2016). As a result, enzyme activities are also likely to differ between rhizosphere and bulk soil (Kuzyakov & Blagodatskaya 2015). We did not observe any potential BNI effect in the bulk soil of the plantain/white clover pots, but this could have been due to our experimental time frame of five months. As the specific BNI compounds could gradually diffuse out from the rhizosphere zone, it is possible that a BNI effect in the bulk soil could have occurred at a later date.

Our results also showed that *amoA* gene abundance in the rhizosphere area of ryegrass/white clover under the no-urine treatment decreased over time, possibly due to the depletion of N available as substrate. In contrast, *amoA* gene abundance in the rhizosphere of plantain/white clover under the no-urine treatment remained stable, which might be related to the capacity of plantain to maintain *amoA* activity in soil even under potentially low N concentrations. This effect is described as the capacity of certain plants to survive under severe scarcity of N, as found in previous studies with tropical forage grasses that are well adapted to the low-N environments of South American Savannas (Ishikawa et al. 2003; Subbarao et al. 2007).

## Conclusions/Practical implications

The results support our hypothesis that plantain can inhibit a*moA* gene activity in rhizosphere soil. This suggests that plantain has a potential BNI capacity and a potential to reduce N losses from cattle urine. Further work is needed to confirm this potential BNI mechanism by linking the *amoA* gene with soil $NH_4^+$ and $NO_3^-$ levels and $N_2O$ emissions from plantain and assessing the levels of this gene from the rhizosphere soil of clover roots. Plantain is a promising forage species for reducing $N_2O$ emission; future work should also focus on how to best incorporate and manage plantain in pastoral systems.

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