The effect of Doxycycline laden slurry application on *Lolium Perenne* growth, greenhouse gas emissions and ammonia volatilisation

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Abstract

The objective of this study was to explore greenhouse gas and ammonia emission changes with regard to different slurry Doxycycline residue concentrations with and without vegetation, in turn relating this to the underlying microbial processes related to their production. Under greenhouse conditions, *Lolium Perenne* single variety was sown in four replicates for each Doxycycline slurry treatment: i) 0mg/L ii) 10mg/L iii) 200mg/L and iv) no slurry (plant control) which was then applied to each of the following pot and soil types: i) sand-grass, ii) sand-Bare soil, iii) silt loam-grass and iv) silt loam-bare soil. The gaseous emissions of CO_2, N_2O, CH_4 and NH_3 were sampled in a reverse exponential fashion using a static flux chamber connected to a photo-acoustic gas monitor for up to 27 days. Grass was cut at intervals and then fully harvested after treatment for C:N and Dry weight measurements. Nitrogen Use Efficiency and greenhouse gas equivalents were additionally calculated.

No consistent significant effects of the slurry Doxycycline treatment were observed in the four different soil and Pot conditions. However, in silt loam bare soil pots, 200mg/L Doxycycline CH_4 emissions were <44% higher than the other slurry treatments, with 15% increase across all treatments directly after application. This was contrary to Sanz et al. (1996), displaying no effects of Doxycycline inhibiting methanogenisis and similar results have not been found in the literature. A significant 55% increase in N_2O emissions from 0mg/L silt loam grass pots and 200mg/L silt loam bare soil pots suggest a possible effect of Doxycycline on reducing denitrification processes as seen in other experiments (Halling-Sorensen 2001). Additionally, CH_4, and CO_2 emissions were significantly higher in grass pots compared to the bare soil pots (24% and 81% respectively. However this may have been due to variation in emissions between the bare soil and grass pot types, associated changes in slurry surface application and plant respiration and soil aeration. N_2O emission rates were significantly higher in sand soil after 7 and 8 days, potentially due to greater moisture retention and anaerobic conditions forming. Though other effects of pot and soil type were observed, there were no other significant effects of the slurry Doxycycline treatment on the other gases or *L. perenne* yields. However, in 0mg/L pots, root Nitrogen % was 40% higher (p=. 06) compared to the No slurry treatment, suggesting an effect of the Doxycycline on root N uptake. Although there was insufficient slurry exposure time for this to become significant given the high amount of Nitrogen unaccounted for from the slurry application. A significant 65% increase in Biomass Yields was seen in silt loam soil compared to the sand soil.

Large variations in gaseous emission rates within groups were observed, and a number of factors that could minimise this in future experiments were identified and extrapolated**.** This experiment has presented a practical method for observing the effects of antibiotics on the soil and plant biome. In addition, no other experiment using this antibiotic, procedure or magnitude has been found in the literature. However no firm conclusions regarding the effect of Doxycycline on greenhouse gas and ammonia emissions or *L. perenne* could be made due to certain limitations of the experiment. However, recording no negative effects from the slurry application on plant growth or significant changes in gaseous emission is in itself a result.

Introduction

In order to satisfy the growing global demand for animal protein through intensive management, antibiotic usage is predicted to increase 67% between 2010-30 (Van Boeckel et al. 2015). However, studies have shown excreted Livestock antibiotics decrease a soil potentials for degrading contaminants (Boxall et al. 2003); disturb normal soil processes such nitrification, denitrification and anammox reactions (Halling-Sorensen 2001; Arikan et al. 2006); with various negative effects on soil invertebrates such as springtails, earthworms and enchytraeids (Di Nica et al. 2015; Thiele-Bruhn 2003; Hammer et al. 2016); and plants, specifically Doxyxycycline on arabidopsis growth (Moullan et al. 2015). Therefore, it is imperative to develop a more thorough understanding of the effect of high concentrations of residual antibiotics on crop growth and the soil biome under more field-like conditions.

Bacteriostatic antibiotics, such as Tetracyclines, function through prohibiting protein synthesis at the cell ribosomal level, suppressing ATP production, in turn decreasing cellular respiration and inhibiting bacterial growth (Lobritz et al. 2015). Soil microbes are tightly linked with Global Carbon (C) and Nitrogen (N) cycles, further influenced by a number of environmental factors(Oenema et al. 2001). Nitrous oxide (N_2O), Carbon dioxide (CO_2) and Methane (CH_4) are the three most important greenhouse gases (GHGs) that cause radiative forcing of climate change (Smithson 2002). Ammonia (NH_3) emissions cause the acidification and eutrophication of nitrogen-limited ecosystems (Sutton et al. 2008). Microorganisms are involved in the production (and possible consumption) of all four gases (Oenema et al. 2001). Hence, observing the affect of antibiotic residues on these gases also represents the opportunity to indirectly observe the effect on the underlying soil microbial processes. Whilst most CO_2 GHG production is due to fossil fuel emissions and land use change; 40% of NH_3, 65% of N_2O and 37% of CH_4 (Steinfeld and Wassenaar 2007) of global emissions are directly associated with livestock (9% for CO_2). Therefore, understanding which soil processes they affect is integral to minimizing current and future GHG, NH_3 emissions and other environmental or social impacts from agriculture.

Over 60% of antibiotic resistance has been associated with the use of livestock antibiotics (Smith et al. 2002). Though, Dutch farmers have made an overall 56% reduction in antibiotic use in between 2005-12(Speksnijder et al. 2015), this does not follow the global trend (Van Boeckel et al. 2015). Tetracyclines have a half-life of 54 days and a high propensity to persist in the system and manure (Thiele-Bruhn 2003). In turn, livestock use of antibiotics remains a considerable contributor to the widespread antibiotic resistance (Thiele-Bruhn 2003). Therefore, it is imperative that the potential environmental effects of antibiotics need to be further understood. Not only for current human health and antibiotic resistance, but also more importantly for stricter stewardship of their use for future generations(Bengtsson and Greko 2014).

Tetracyclines remain the antibiotic class with the highest use in livestock production in the Netherlands (MARAN 2016). Doxycycline represents 42% of the Tetracycline sales, with over 11 daily doses per animal per year within the veal calf industry (MARAN 2016). Doxycycline has been shown to inhibit mitochondrial function and cause mild to severe growth retardation in Arabidopsis seedlings from watering with a concentration of 25 μg/L for 7 days (Moullan et al. 2015). Given slurry is used to fertilize crops and improve yields, a reduction in plant growth due to high antibiotic residues could have significant economic and ecological importance for farmers. Hence investigating further effects of this antibiotic is extremely relevant. Furthermore, below in Table 1 is a brief description of the microbial production or alteration of CO_2, NH_3, N_2O and CH_4 and observed effects of Tetracyclines under lab conditions on their production or emissions.

Table 1. The microbial formation of CO_2, CH_4, N_2O and NH_3 and known laboratory effect on these processes by Tetracyclines.

	CO_2	CH_4	N_2O	NH_3
Microbial production pathway	Root & microbial respiration. Soil mineralization exceeds assimilation (Oenema et al. 2001).	Anaerobic (or lack of suitable electron receptor) methanogenisis of easily degradable organic matter (typically Volatile fatty Acids (VFA) in slurry) (Oenema et al. 2001).	Intermediary and by product of nitrification and anaerobic denitrification (Kowalchuk and Stephen 2001).	Hydrolysis of Urea ($CO(NH_2)_2$) to CO_2 + NH_3 and subsequent volatilization (Burgos et al. 2010).
Effect of Tetracycline	Induced SOS response in *Vibrio cholera* and decreased microbial respiration and CO_2 production (Bernier and Surette 2013). Selects for a higher fungi:bacteria ratio, and in turn a higher microbial growth efficiency and lower CO_2 emissions (Jastrow et al. 2007)	Prevent full reduction of butyric acid (VFC) decreasing batch Methane production by 25-45% (Sanz et al. 1996).	Inhibit denitrification and nitrification, decreasing N_2O emissions (Halling-Sorensen 2001).	Prevent the growth of *Nitrosomonas e.,* thereby preventing nitrification of ammonia, increasing NH_3 emissions (Halling-Sorensen 2001).

However, though there are laboratory studies investigating the effect of Teratracyclines, there have been few intensively sampled field trials, further yet on Doxycycline specifically, regardless of its high use. Under greenhouse conditions, this represents a controlled yet more realistic environment to explore the effect of Doxycycline on the soil C and N cycles and crop yields. 1 gram of rhizosphere soil can contain over 10 billion bacterial cells, of thousands of species (de Vrieze 2015). In addition, antibiotic adsorption is also strongly influenced by the presence of clay mineral, soil organic matter, manure and sludge (Golet et al. 2003). Therefore experimenting with differing soils and vegetation (possible photorespiration and reabsorption) (Sommer et al. 1997) is vital as different microbial communities may respond differently to Doxycycline.

With this, the following questions were formed with respect to the underlying biological processes:

1) How do antibiotics (specifically Doxycycline) affect the GHG (CO_2, CH_4 and N_2O) and NH_3 emissions?

2) Does Doxycycline affect the relative yield or Nitrogen assimilation of the crop?

3) Does this effect differ with the soil type or vegetation?

Materials and methods

Experimental setup

In order to explore the effect of antibiotics on the soil and plant biome and in turn GHG and NH_3 emissions, the following experimental design was made (Table 2). Where 10mg/L represents known observed levels of Tetracyclines in the field (Arikan et al. 2006), 200mg represent concentrations when used as a bactericide, eliminating 20% of soil bacteria (Colinas et al. 1994). 0mg/L is the Doxycycline gas emission control, and a No slurry grass treatment represents the grass growth control in response to the slurry. Carbon dioxide and Methane emissions have been shown to be affected by vegetation due to photorespiration from plants and in turn Carbon assimilation for methanogenisis (Joabsson et al. 1999). Separating the root and microbial contributions to CO_2 emissions also remains difficult (Inselsbacher et al. 2011). Hence a Bare soil treatment was also included. Antibiotic adsorption is also strongly influenced by the presence of clay mineral, soil organic matter, manure and sludge (Golet et al. 2003). Therefore experimenting with differing soils and clay contents is extremely interesting given for potential comparison and future applicability of the results. The sorption of antibiotics in soil is also strongly related to pH of the soil (Thiele-Bruhn 2003), hence this was also monitored (Table 3).

Table 2. The Experimental Design with the different slurry treatments, soils and pot type s.

	Sand soil		**Silt loam**	
	Bare soil	Grass	Bare soil	Grass
No antibiotic slurry (0mg/L)	4	4	4	4
Antibiotic added slurry at level observed in the field (10mg/L Doxycycline in slurry)	4	4	4	4
Antibiotic added slurry at maximum known natural levels (200mg/L Doxycycline in slurry)	4	4	4	4
No slurry Control (NS)	-	4	-	4
Total Pots	28		28	

Greenhouse preparation

26kg (+-100g) of the two soil types with vastly different clay % (Table 3) were measured out into each of the 56 30x35cm pots. Saucers were put underneath the pots to prevent soil loss from the drainage holes, before the different soils (Four for each soil type) where evenly allotted onto the 'block' greenhouse tables (Appendix 1). The dry soil was then thoroughly soaked and left overnight. Dry samples were taken of the two soils for basic soil characteristic analysis and profiling as seen in Table 3 below.

Table 3. The different soil characteristics of the silt loam and sand soil used in the experiment.

Soil type	Clay %	Silt %	pH	Sand %	C:N ratio	Deliverable N (kg/Ha)	Deliverable S (kg/Ha)	P Stock P-AL (mg P_2O_5/100g)	K Stock (mmol+/kg)	Organic matter %	CEC mmol+/kg	Soil microbial N (mg/kg)
Silt loam clay (fluvial)	31	47	7.3	17	10	95	13	31	75	3.5	227 (99% full)	33
1 part black sand, 3 parts sand	<1	11	6	81	17	45	15	48	505	4	69	34

From the Initial Experiment, temperatures of 10 degrees or less made it difficult to record a saturated ammonia emission curves. Therefore, the greenhouse temperature was set to 24 degrees during the day and 18°c at night with 16 hours of sunlight.

Plant preparation and harvest

The following day (27th of January), Perennial Ryegrass (*Lolium Perenne*) single variety was sowed into the grass treatment pots at a rate of 15kg/ha, avoiding the edges of the pot. For reference to the timeline of events, see Table 4. For a 30cm diameter cylinder this corresponds to .0706m^2 (area of tested pot) *1.5g=~.11g of seed per cylinder. (Seeding rate 15 000g/10 000m^2=1.5g/m^2). This was measured on a Mettler Toledo AE to .01g. When counted, this equated to approximately 50 seeds. As per Hoogsteen et al. (2015), *L. perenne* has negligible root growth below 30cm. Therefore; pots with a depth of 30cm were deemed sufficient for the experiment.

For the first month to encourage seedling growth and keep bare topsoil moist in 16-hour daylight, plants were watered every day. However after initial development this was reduced to every second day. A moisture sensor meter was used to ensure even watering of pots**.** Both soils had a relatively large residual weed seed banks, therefore weed stems were removed as soon as they sprouted without disturbing the grass seedlings (Appendix 2, Picture 3). However, this was not possible for other grass weed seeds as they were unable to be identified at such an early stage. The silt loam clay top surface soil caked between watering (Appendix 2, Picture 1 & 2), hence the seedling density and seed emergence was lower and slower than that of the sand soil however after a month of growth there was no significant difference between pots in the number of tillers.

On the 15th of March, after a sunny weekend, four of the silt loam pots started flowering. Upon closer inspection, it was seen to be *Poa annua* (Appendix 2, Picture 4). All silt loam pots had flowered before the first cut, however this was not the case for the sand soil pots. As unlike *L. perenne*, *P. annua* can flower at any time of the year. The 6th of April, all grass was cut. A ruler was used to cut and measure out 5cm on all the grass tillers in each pot this. All the fresh material was then placed in a pre-weighed aluminium tray and then weighed again for the fresh weight of the grass. Subsequently the grass was dried for 24 hours at 70°c. Samples were then weighed again for dry weight measurements before being separately ground up to 1mm length for C:N analysis. This was done on three (four for the sand soil) separate occasions as seen in the Table 4 below.

After a final round of gas emission measurements for all pots, the silt loam pots were harvested on the 31st of May. This involved cutting the growth to 5 cm as per previous procedures. Thereafter, the remaining stems were cut to the soil level. Due to some slurry residues, the stems were thoroughly

washed before being dried at 70°c for 24 hours. The dry weight was then recorded before the sample was ground to 1mm and stored in separate dry airtight containers for C:N analysis.

The roots proved harder to extract given the 20% clay content of the silt loam soil (Appendix 2, Picture 10). Two medium pressure hoses were used to wash away the soil leaving the root system intact with a .5cm sieve underneath to catch any root particles detached. The roots were then dried for 24 hours at 70°celcius. The dry weight was then recorded before the sample was ground to 1mm and stored in a dry container fro C:N analysis. A Shimadzu DK6200 scale was used to weigh all samples to 2 decimal places in grams.

Table 4. The experimental timeline.

C:N analysis

A Retsch mm200 was used to ball mill the 1mm dried samples to a fine powder. Thereafter, total N and total C contents were determined using the Dumas Method with a CHN1110 Element Analyser (CE instruments, Milan, Italy). Values were recorded to 4 decimal places and then Analysed with the rest of the data in R.

Slurry collection and application

Slurry was collected from de Hooilanden Blaarkoop Organic Farm to avoid presence of antibiotic residues. The slurry composition (Total Annomical Nitrogen, TAN), moisture content (DM %) and type of soil all influence the NH_3 emissions (Sommer and Hutchings 2001). In table below, the general characteristics of the slurry can be observed.

Table 5. The General slurry characteristics.

Slurry collection (2/5/2017)	Grams NH_4-N/kg	Grams NH_3-N/kg	Grams N/kg fresh manure	% Dry matter	pH	C:N ratio
Sample 1	1.03	0	3.65	11.48	7.51	18.92

The slurry was then thoroughly mixed in the bucket with any roughage or other large bedding materials removed. When weighed with a Shimadzu DK6200 scale, .192L of slurry equated to approximately 220 grams (corresponding to typical slurry application of $20m^3\ ha^{-1}$). This was then measured out for all of the 48 slurry treatment pots; they were then put in cool storage (3˚c) until further use. The slurry samples that were needed the next day were taken out and left in the greenhouse overnight. This minimized any potential bias in slurry temperature when applied to the pots, potentially affecting gas emissions (Sawamoto et al. 2016; Uchida et al. 2011; Sommer and Olesen 1991). Doxycycline Hyclate powder was provided by the Wageningen Animal Sciences Department. For the pots that had antibiotics added to the slurry, the Doxycycline Hyclate was measured out with a Mettler Toledo AE, to the first decimal mg above that which was calculated below. As Doxycycline Hyclate is equivalent to 437mg Doxycycline potency, this was accounted for in the calculations for 200mg and 10mg Doxycycline/L below.

Calculations for relative concentration of Doxycycline in the slurry application:

200mg Doxycycline/L slurry
=200mg/ (1L/.19L)
=200mg/5.21
=38.4mg Doxycycline per pot

To account for Doxycycline Hyclate
= (1000mg/432mg)*38.4mg
=2.3*38.4mg
=88.9mg Doxycycline Hyclate per pot.

10mg Doxycycline/L slurry
=10mg/ (1L/.19L)
=10mg/5.21
=1.9mg Doxycycline per pot

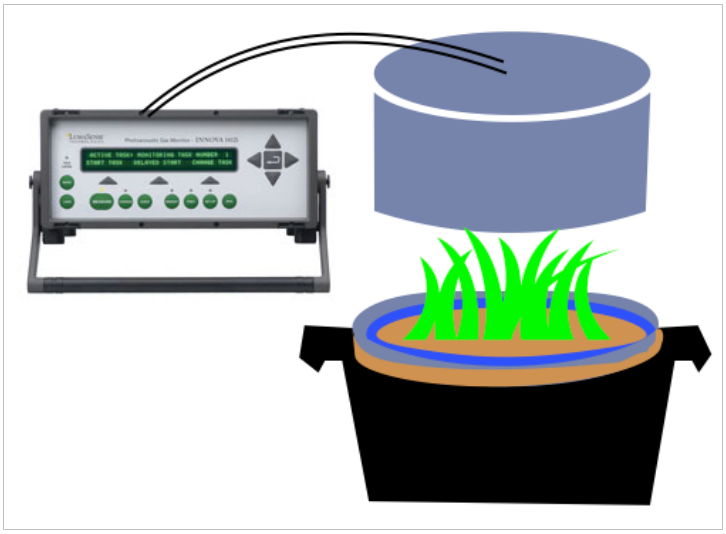
To account for Doxycycline Hyclate
= (1000mg/432mg)*1.9mg
=2.3*1.9mg
=4.4mg Doxycycline Hyclate per pot.

The Doxycycline Hyclate was then dissolved and shaken in 40 mL of water before being added to the slurry container. This was then also rinsed with an additional 10ml of water to ensure the entire measured dose of antibiotic went into the slurry. Slurry samples were then shaken for 20 seconds. To account for the added water in the antibiotic samples, 50mL of water was also measured out and incorporated through shaking for those slurry samples without antibiotics. NH_3 volatilization occurs through the connective mass transfer from the boundary layer of the slurry to the air above the surface (Burgos et al. 2010). Therefore, the surface area of the slurry application was kept inside the cylinder and was evenly poured out in a replicable circular inwards motion to minimize changes in surface area (Appendix 2, Picture 5 &6).

Air monitoring and flux measurements

INNOVA 1412 photoacoustic filed gas analyser
An INNOVA 1412 photoacoustic filed gas analyser was used to monitor the ammonia, nitrous oxide, methane and carbon dioxide emissions as well as water vapour from the pots (Table 6). The air filter was changed prior to the commencement of the experiment and the machine had not been used since the previous calibration. The detection thresholds of the gases as per manufacturer are 0.2 ppm for NH_3, 0.03 ppm for N_2O, 0.4 ppm for CH_4 and 1.5 ppm for CO_2. The measuring accuracy for the multi-gas analyser according to data sheets from the manufacturer is ±2–3%. Hard plastic hollow cylinders, 30cm in diameter and 10 cm meters deep were with a double edged top were used to hold the flux chamber in place. Cylinders were pushed at least 6cm deep into the soil with the help of a cushioned hardwood slab and soft edged hammer. A plumb rule was then used to level out the cylinder before water was carefully poured into the double lining to create and air-watertight environment for the flux measurement chamber as it was put on top. The measurements were set to 20 minutes each in order to achieve NH_3 saturation curves.

Table 6. The INNOVA photo-acoustic Field Monitor Setup in the greenhouse with a watertight lining.



Two 1-meter long polytetrafluoroethylene (Teflon) tubes were attached to the closed flux chamber. Before and after the start of each measurement, ambient air was sampled for 2-4 minutes (depending on gas concentrations) to clear the tubes of the previous cylinder's residual gas levels. These tubes were changed as soon as there was a cinch or fold in the tubing, otherwise at least once a week to prevent ammonia build up (ammonia has high affinity for water from moisture buildup from the previous measurement in the tubes) and ensure good airflow and measurements. The inside of the flux chamber was wiped with a clean towel after each measurement to prevent moisture build-up. Additionally, the tube and the closed flux chamber were kept in a dry environment each night till they were used again the next morning.

Gas measurements

Grass leaves were rearranged so as not to come in between the double edged cylinder and the flux chamber, potentially affecting the measurements. However in the case of the silt loam soil, moving all the grass was not possible alone as there was too much regrowth so quickly after the cutting (Appendix 2, Picture 8). However, carbon dioxide emissions followed a linear increase for these cases ($R^2 > .9$) hence the data was kept for analysis.

Sampling schedule

As there was only one INNOVA machine and 56 pots, the application of the slurry and gas sampling was spread out. The sampling schedule for the silt loam and sand can be seen in Appendix 3. Due to visible effects of the slurry on the grass, the sampling schedule was changed for the sand pots with all grass sand pots sampled over two days then followed by the bare soil pots. In addition, to prevent possible problems with the grass getting stuck in the double lining, the sand grass was cut the day of the measurements. This cutting was not enough for C:N analysis; hence it was dried at 70 degrees and then weighed for dry weight measurement. This was also done with the control pots (no slurry) that were not receiving slurry treatment.

From the initial monitoring with the silt loam, it was also seen that bare soil pots return to normal CO_2 flux levels after 5 days (Figure 1). Therefore, bare soil Pots weren't monitored again till the end of the experiment. Bare soil sand pots were only measured for this time period as well.

Data Analysis

The gas emission measurements from each pot were compiled with that of the other cylinders into a larger datasheet for statistical transformation in R. The concentrations were taken in parts per million (ppm) this was then converted into milligrams per cubic metre (mg/m^3) through the Equation 1 below.

Equation 1.

$$\text{Concentration in } mg/m^3 = ppm\ of\ gas * \frac{Molar\ mass\ of\ gas}{Volume}$$

The measurements were plotted in R, with decreasing concentrations (due to remnant gas in the tubing from the previous measurement) removed from the data vector. A slope was then fitted to the using the least squares method. This was then used to calculate the gaseous emission rates of CO_2, N_2O and CH_4 (R) in units mg/m^2/h from the pots using the Equation 2 below:

Equation 2.

$$R = 60 * Bi\frac{Vt}{Ac}$$

Within this equation, B_i is the fitted (initial) linear slope of the separate gases, V_t is the total volume of air during the measurement ($1.6548*10^{-2}$ m^3) and A_c is the surface area of the pot within the cylinder ($6.8349*10^{-2}$m^3) (Shah et al. 2016). The V_t was calculated summing the cylinder volume 4cm above the soil line after insertion ($2.734*10^{-3}$m^3), the cap volume of the internal chamber ($1.367*10^{-2}$m^3), the volume inside the gas monitor ($1.4*10^{-4}$) and the volume inside the tubes ($4.7*10^{-6}$ m^3). For NH_3, a Non-rectangular Hyperbola was fitted with the following equation:

Equation 3.

$$[NH_3] = D + \frac{1}{2A}\{B_i \times t + C - \sqrt{(B_i \times t + C)^2 - 4A \times B_i \times C}\ \ \}$$

Where A is the curvature parameter (0>1), B_i is the initial slope, C is the equilibrium and D is the concentration at time (Nienhuis and Lantinga 2013). From this equation the initial slope (B_i) is used to calculate the NH_3 emission rate (mg/m^2/h) from Equation 2. This was done using SAS software. However when fitting the Non-hyperbolic Parabola to the measurements, in certain cases the data did not fit with the model. The volatilization of NH_3 from liquid is dependent on the partial pressure of the NH_3 in the air and the relative concentration in the liquid slurry (Petersen et al. 2014). Therefore, when measurements are airtight the NH_3 concentration reaches equilibrium. Measurements that exhibited no curvature or levelled off but then continued to increase were then removed from the data set as they may have been representative of INNOVA malfunction, moisture build-up or an air leak. This represented 43 out of 119 measurements for NH_3 on the first day of which 27 were from grass pots and 16 were from bare soil Pots (18 from sand grass, 10 from sand bare soil, 9 from silt loam grass and 6 from silt loam Bare soil).

The gaseous emission rate was upgraded from mg/m^2/minute to mg/m^2/ hour through a conversion factor of 60 (Equation 2). This facilitated the calculation of long-term total emissions through the use of area under the curve (AUC) calculations in R. The rollmean function was used, giving multiple means of the emission rate and time between each consecutive measurement point and multiplying this together to get an AUC value for each of the 48 pots with slurry treatments.

Nitrogen Use Efficiency (NUE) (Table 9) was calculated using the difference method:

Equation 4.

$$NUE\ (\%) = \left(\frac{N\ crop\ uptake\ (Slurry) - N\ crop\ uptake\ (Control)}{Added\ N\ \ (Slurry)}\right) \times 100$$

Where the N crop uptake (slurry) is the N g/m^2 taken up by the Pots that had had a slurry treatment, N crop uptake (Control) is the amount of N g/m^2 taken up by the No slurry treatments and Added N (slurry)

is the amount of N added in the slurry treatment, also upgraded to g/m^2. The Nitrogen unaccounted for within the experiment was calculated with the following equation:

Equation 5.

$$Unaccounted\ N\ (\%) = 100 - \left(\left(\frac{Measured\ gaseous\ N\ loses + (N\ crop\ uptake(Slurry) - N\ crop\ uptake(Control))}{Total\ N\ applied}\right) \times 100\right)$$

Where the measured gas losses are the sum of NH_3-N and N_2O-N emissions in g/m^2, and the rest of the input is explained from the description of Equation 4.

Statistical analysis

Given the slight differences in the time between the emission measurements, comparison of means was done between the actual measurement numbers instead of relative time. For NH_3 and CH_4, emissions were only significant (Slope $R^2 \geq .9$) in the first 24 hours. Therefore, the analysis was kept to the first two (silt loam) or three (sand) measurements that were taken during the first day, for these gases. Analysis of Variance (ANOVA) was used in R studio to statistically analyse the hourly emissions and the Area under the curve (also total emissions, AUC) of the different gases between different treatments. Mean values of the emissions between groups were used to further explore significant results. For CO_2 and N_2O, where emissions remained significant during the whole experiment, the emissions from the first 24 hours, as well as all other measurements where evaluated. A two-way ANOVA was used for AUC and emission rate, also allowing for possible interaction of the slurry (0, 10 or 200mg Doxycycline/L), soil (silt loam or sand) or pot type (Bare soil or grass). After each ANOVA, a Levene test was performed to make sure that the data for variances were similar (a non significant Pr (>F) value). Residuals were also plotted, and data was log transformed to conform to Normality assumptions if plots were skewed. In addition to all ANOVAs, Levene tests for Interaction with different contrasts for the slurry treatments were also performed. Unless mentioned, all data analysis passed these tests. The data sheet was also separated into four separate parts (sand-Bare soil, sand-grass, silt loam-Bare soil, silt loam-grass) for trend analysis and post hoc pairwise t-tests.

ANOVA was also used to explore the effect of slurry, soil and pot type on: Total dry matter, N%, C%, Total plant N, Total plant C, Total plant C:N, daily dry matter growth.

Results

Carbon dioxide

The mean values within treatments of the hourly CO_2 emissions were plotted for visual comparison in Figure 1. The data was split into 4 different datasets to allow for proper comparison given the different number of measurements depending on the soil and pot type. There were no significant effects of slurry on hourly CO_2 emissions over any time period or the full course of the experiment (Table 7).

When the total CO_2 emissions were calculated (AUC) from the first 14 days (all gas measurement were for at least this time period), there was a significant effect of pot type [$F_{(1,36)} = 807.193$, $p = 2*10^{-16}$] with an 81% increase in CO_2 emissions from grass pots (Table 7). This effect was also significant for hourly emission rates at all time periods of the experiment.

Table 7. The accumulated averaged gas emission from the various measured GHG and ammonia over the course of the experiment for the different soil and slurry Types.

Soil type	Pot type	Slurry treatment (Doxycycline mg/L)	Accumulated Gas emissions over Course of experiment (AUC)				Greenhouse gas CO_2 Equivalent[6] (g/m2)	
			CO_2 (kg/m²)	CH_4 (mg/m²	N_2O (mg/m²)	NH_3 (mg/m²)[2]	CH_4 (g/m²)	N_2O (g/m²)
Sand	**Bare soil**	0	10.83[a1)2)]	471[a3)4)]	255[ab4)5)]	554[7)]	11.8	76
		10	9.71[a]	411[a]	240[ab]	508	10.3	71.5
		200	9.96[a]	439[a]	248[ab]	585	11	73.9
	Grass	0	47.17[b]	440[a]	244[ab]	759	11	72.7
		10	49.90[b]	454[a]	255[ab]	317	11.4	76
		200	45.35[b]	476[a]	239[ab]	758	11.9	71.2
Silt loam	**Bare soil**	0	4.82[a]	160[b]	183[zb]	100	4	54.5
		10	11.89[a]	217[bc]	166[ab]	214	5.4	49.5
		200	5.86[a]	328[d]	115[a]	165	8.2	34.3
	Grass	0	51.86[b]	256[bd]	253[b]	149	6.4	75.4
		10	42.38[b]	297[bd]	240[ab]	147	7.4	71.5
		200	40.06[b]	336[cd]	198[ab]	108	8.4	59

[1)] Different Letters within a column indicate a significant ($P<0.05$) difference between treatments

[2)] The CO_2 values were calculated over 14 days (the minimum treatment time between scenarios for better comparison of total emission rates)

[3)] The CH_4 and NH_3 values were calculated over the first ~4 hours for silt loam and ~5 Hours for sand.

[4)] The first replicate of silt loam 200mg/L Doxycycline was removed as there was only one measurement taken on the first day, hence not AUC could be fitted.

[4)] The N_2O values were calculated over the first 3 days so that all emission slope measurement had an $R^2>.9$.

[5)] N_2O and CH_4 have global warming potential 298 and 25 times higher than CO_2 respectively (Landman 2010).

[6)] NH_3 total emissions were not statistically analyzed as too many measurements were removed preventing proper comparison (Figure 4).

The greenhouse gas equivalents of the gases were calculated in Table 7. Even though CH_4 total emissions were on average higher than N_2O regardless of the shorter sampling period, how the slurry treatment affects N_2O gases will impact the global warming potential more regardless of this given the higher global warming potential of N_2O compared to CH_4. Especially since only 3 days of data was used to model N_2O whilst emissions were still recorded during the full course of the experiment. However these measurements did not have a slope R^2 of >.9, therefore were excluded from this Analysis. Overall leading to an underestimation of N_2O emissions in Table 7. Additionally, for CO_2, the greenhouse gas potential does not include the CO_2 fixation and Carbon storage in the Plants compared to the bare soil pots for instance.

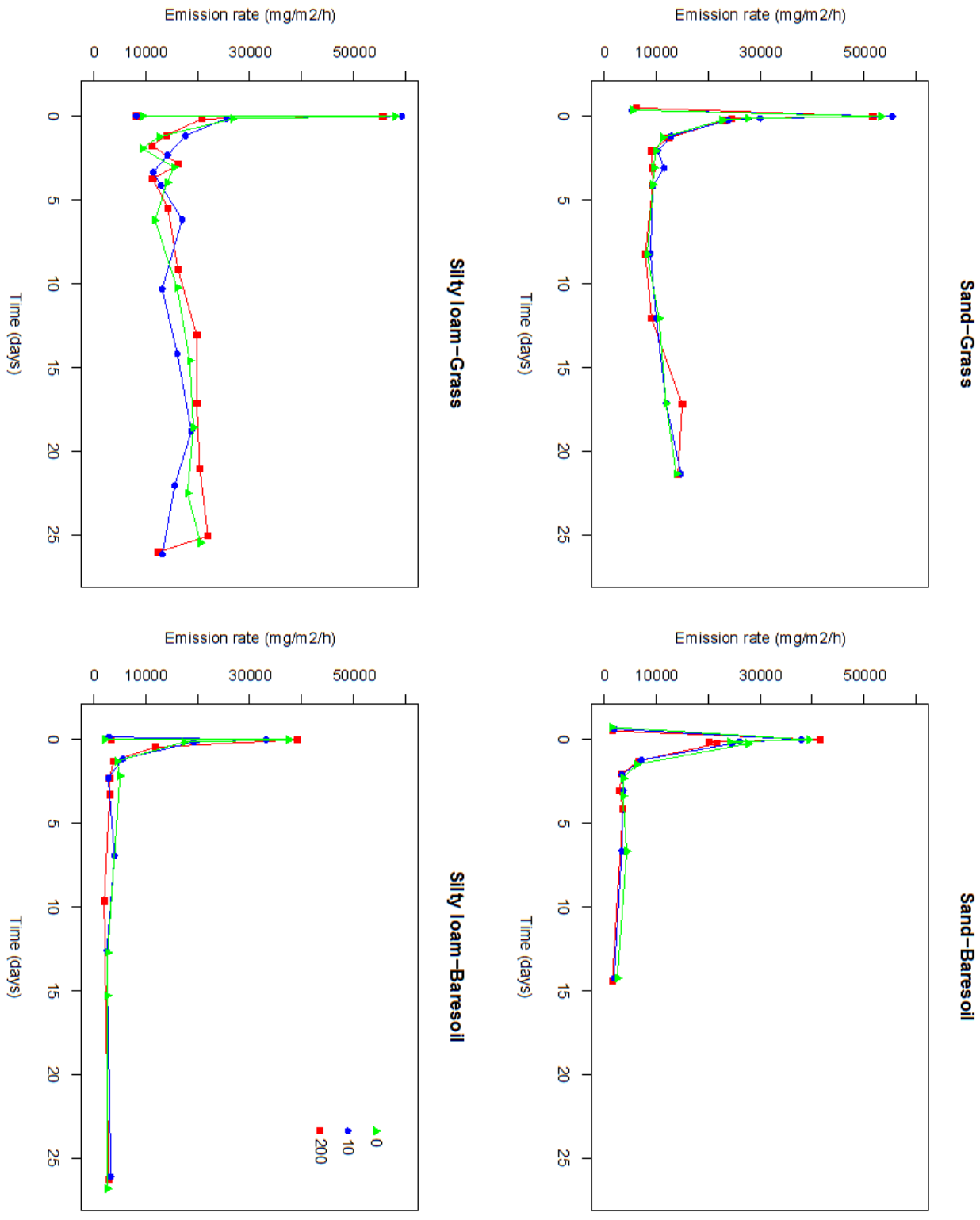


Figure 1 The Carbon dioxide emission rates over time with regards to slurry treatment, soil type and pot type. **All emission rates have an R^2>=.9. 0, 10 and 200 represent the relative mg/L concentration of Doxycycline in the slurry application.**

Nitrous oxide

The mean hourly N_2O emission rates with respect to treatment over the course of the experiment were plotted for visual comparison in Figure 2. Plotted N_2O emission slopes with an R^2<=.9 were not used for the analysis, this represented 93 of the 493 slopes, mostly excluding initial (control) measurements and later (after 3 to 4 days) Bare soil measurements. There were no significant effects of the slurry treatment on the N_2O emission rate over the full course of the treatment even with different time periods selected.

However, when the first 7 and 8 days were selected for comparison, there was a significant effect of the soil type on N_2O emissions [$F_{(1,283)}$ =4.57, p=.033]. Tukey post hoc tests revealed N_2O emissions were 18% higher in the sand soil compared to the silt loam.

The total emissions (AUC) where then calculated for N_2O (Table 7). In order to retain all replicates whilst also using only emission rate slopes with an R^2 => .9, the analysis was then done only with the first three days of data. As seen in Figure 2, N_2O levels return to original levels after 2 days in bare soil pots, suggesting that any effect of the antibiotics on the slurry treatment can also be seen in this time. Data did not conform to Homogeneity of Variance assumption (Levene test) [[$F_{(11,36)}$ =2.54, p=.017]. Therefore the data was split into soil types. There was a significant effect of pot type in silt loam soil [[$F_{(1,18)}$ =8.742, p=.00844]. Post hoc analysis showed that there was a significant difference in N_2O emissions between the 200mg/L Bare soil compared to 0mg/L grass with a 55% increase in emissions from the 0mg/L grass (Table 7).

Methane

The mean values of the CH_4 emissions were plotted for visual comparison in Figure 3. CH_4 emission rates returned to pre-treatment levels within 24 hours. When the hourly emission rate data was explored with R, there was only a significant effect of pot type on the CH_4 emissions [$F_{(1,31)}$ =11.761, p=.00173] for the first measurement (directly after application, also the max CH_4 emission rate). Post hoc analysis revealed significantly higher CH_4 emissions from grass pots (24%).

The AUC total emission CH_4 measurements were only available for the first 6 hours for the sand and 4 hours for the silt loam treatments (Figure 3). This automatically significantly increased the CH_4 total emissions from sand pots [$F_{(1,35)}$ =74.658, p=3.35 $*10^{-10}$]. Hence, the data was split into soil and pot types for further analysis. There were no significant effects of the slurry treatment on the sand CH_4 total emissions. However a significant effect of slurry on the silt loam Bare soil Pots [$F_{(2,8)}$ =10.1, p=.0065]. Post hoc showed a significant increase in CH_4 emissions from 200mg/L compared to 10mg/L and 0mg/L with 44% and 52% more emissions respectively. There was also a significantly increased (53%) CH_4 emissions from the 200mg/L grass silt loam compared to 0mg/L bare soil silt loam (Table 7). It can be observed that in all soil and pot types, the highest recorded CH_4 emissions are from, the 200mg/L slurry treatment. Though this does not continue and is not significant, it is on average 15% higher than the other slurry treatments directly after application (Figure 3).

Ammonia

The mean values of the NH_3 emission were plotted for visual comparison in Figure 4. There were no significant effects of the slurry treatment on the NH_3 hourly gaseous emission rates over the first day. There was a significant effect of the soil on hourly emission rates [$F_{(1,69)}$ =9.508, p=.00294]. Post hoc Analysis revealed this was due to a significant difference between sand 200mg/L and Silt 200mg/L. Data was split into separate soil and pot types for further separate analysis. Given A large number of measurements had been removed, all datasets also needed to be log transformed in order to balance residuals and conform to Normality Assumptions.

When only hourly emissions from the first measurements were selected for, Post hoc analysis of sand bare soil revealed a decreasing trend (p=.09) of 200mg/L Doxycycline treatment compared to 10mg/L. This was also the case for the silt loam bare soil pots (p=.09) though there was no significant effect of the slurry treatment in the ANOVA analysis. No other significant effects were observed at any other time periods. AUC analysis was not conducted on NH_3 as there were too many measurements missing. However an Average of the remaining measurements was used to calculate the amount of Nitrogen lost through volatilisation for Nitrogen Use Efficiency calculations in Table 9. Though not significant, in the silt loam pots, the hourly emissions were 28% less compared to the other slurry treatments at time of slurry application, and 35% less compared to 0mg/L and 45% less than 10mg/L after 3 hours. There was also a tendency for the NH_3 emission rate to increase with time in the sand pots (Figure 4).

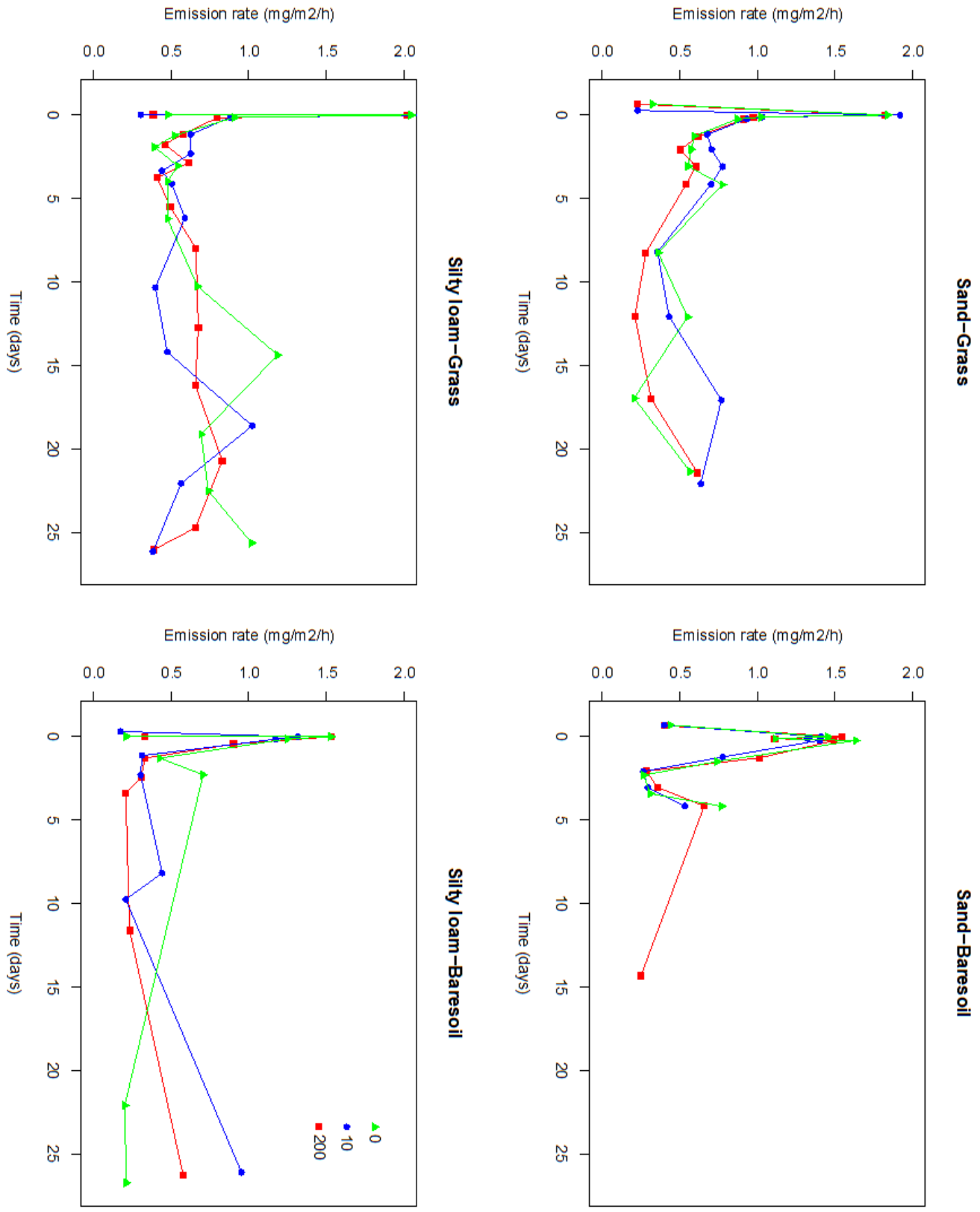


Figure 2. The nitrous oxide emission rates over time with regards to slurry treatment, soil type and pot type. All emission rates have an R^2>=.9. 0, 10 and 200 represent the relative mg/L concentration of Doxycycline in the slurry application.

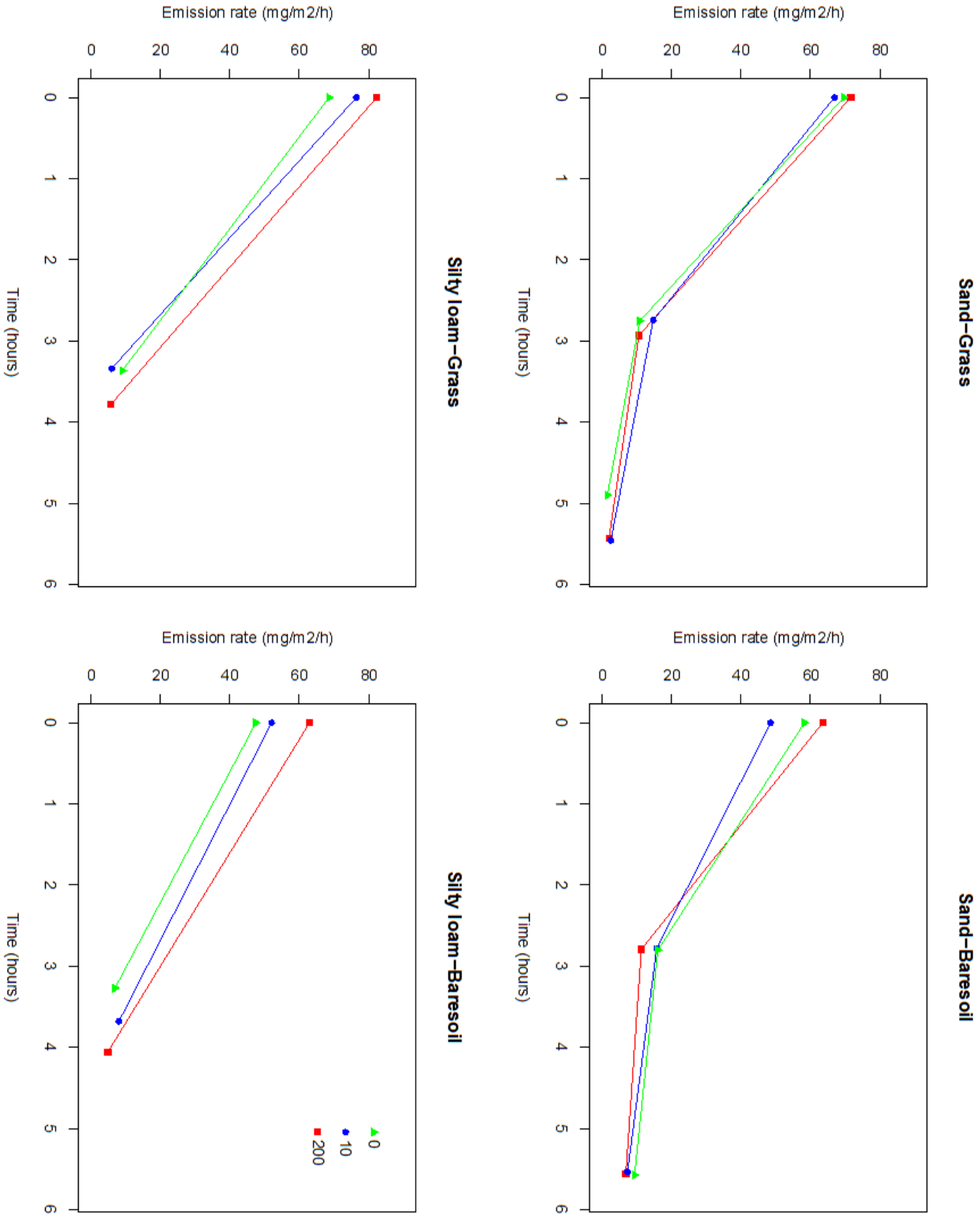


Figure 3. The methane emission rates over time with regards to slurry treatment, soil type and pot type. All emission rates have an R^2>=.9. 0, 10 and 200 represent the relative mg/L concentration of Doxycycline in the slurry application.

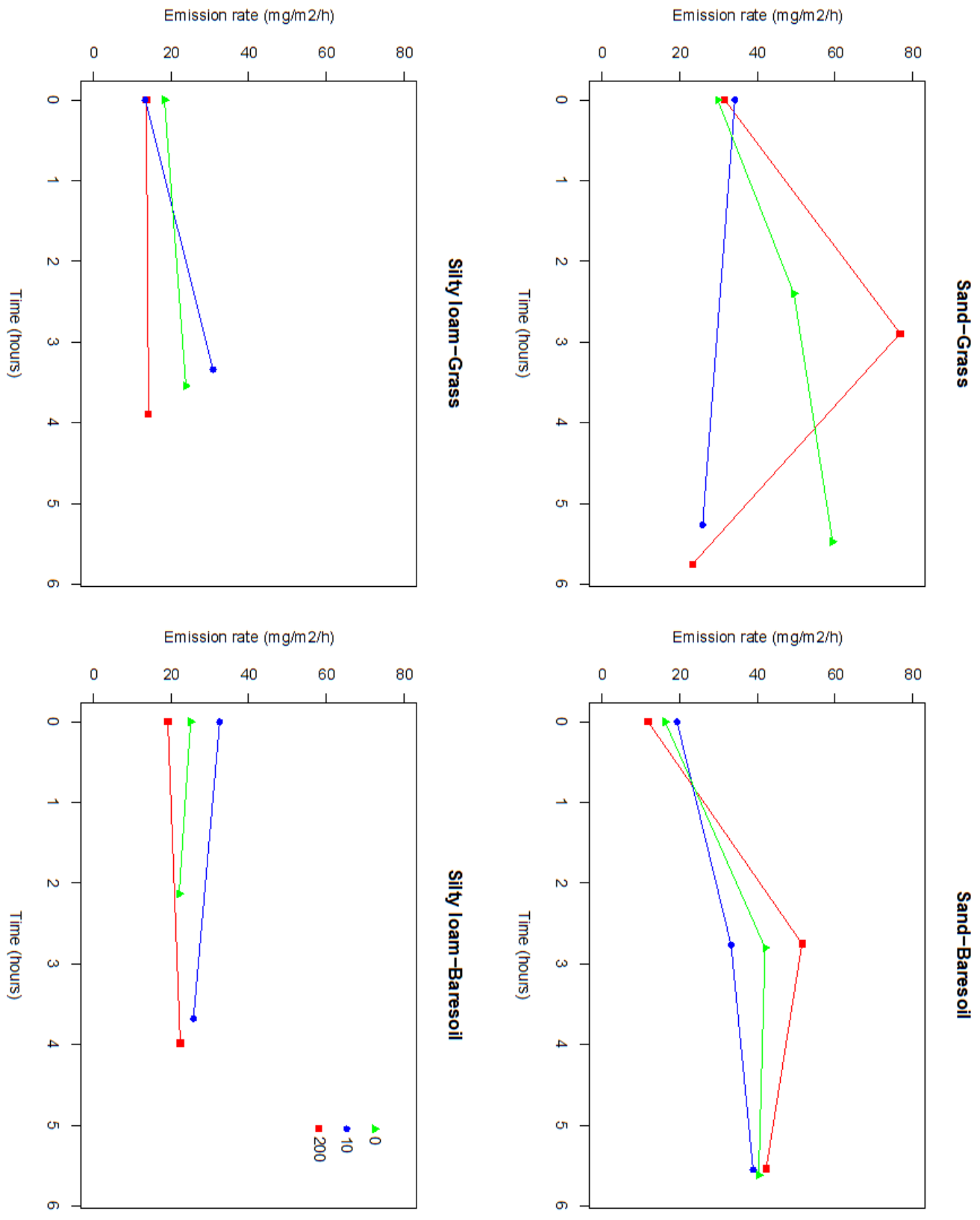


Figure 4 .The ammonia emission rates over time with regards to slurry treatment, soil type and pot type. 43 of the original 119 measurements were removed from analysis. As seen in the sand grass, no emission curves form the second measurement of 10mg/L Doxycycline conformed to the Non-hyperbolic Parabola emission model preventing AUC analysis. 0, 10 and 200 represent the relative mg/L concentration of Doxycycline in the slurry application.

Plant analysis

A two-way ANOVA of the average total biomass production between the soils and the slurry treatments showed no effect of the slurry treatment (when combined), however as seen in Figure 5, a significant increase (65%) in biomass production due to soil type [$F_{(1,24)}$ =671.053, p=$2*10^{-16}$]. Post hoc analysis showed a significant difference between all sand biomass (Dry weight) samples compared to silt loam soil (Figure 6), regardless of the slurry treatment. The plant data was then split into two separate sand and silt loam parts in order to conform to Levene Test of equal variances. The relative Nitrogen % difference between the two soils can also be observed in Figure 7. There was no significant difference between Cut 1 and Cut 4 in the sand, however a significant 50% reduction in N compared to Cut 2. This was not the case in the silt loam soil where all cuts were overall significantly different, and decrease after the first cut.

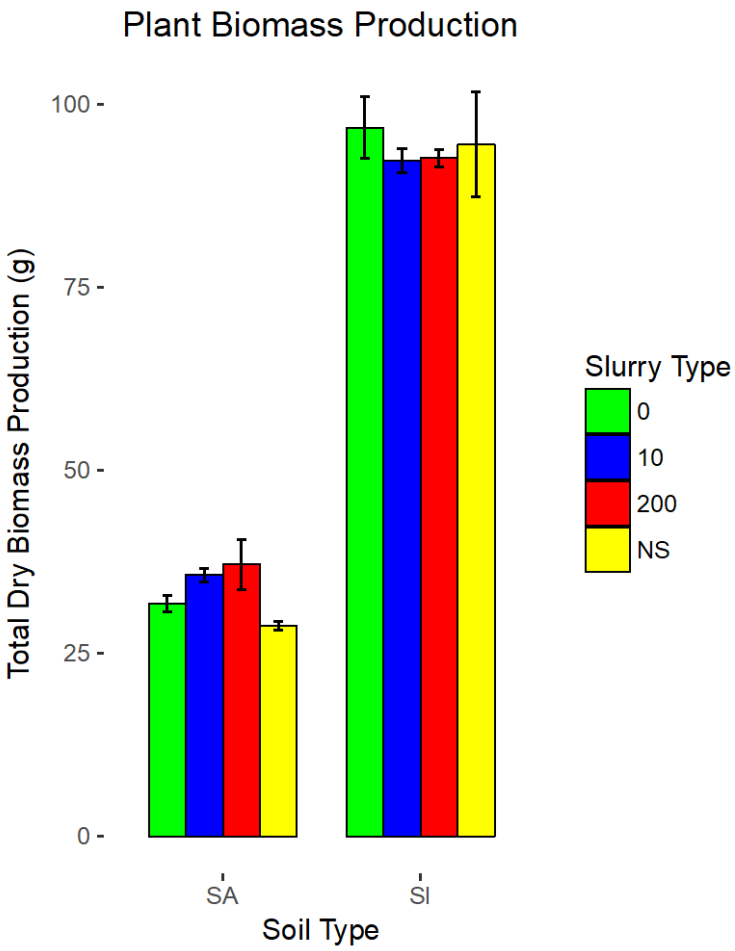


Figure 5. The Total Biomass (all cuts, stems and roots) of the *L. perenne* depending n the soil type (SA=sand, SI=silt loam) and Doxycycline mg/L slurry treatment (NS=No slurry). Error bars show standard error of the mean.

Sand soil

There was no significant effect of the slurry treatment on the Total Nitrogen % (N % of each part multiplied by the dry weight), daily growth or C:N ratio on the combined cut or plant part data. However there was a significant effect of the slurry on the total Carbon %[$F_{(3,12)}$ =5.464, p=.0133] and in turn the Total Biomass [$F_{(3,12)}$ =4.015, p=.0342]. Further post hoc analysis showed this was due to a significantly lower biomass and in turn Carbon content of the No slurry (NS) treatment compared to that of the 200mg/L (23% decrease) and a trend (p=.09) for the 10mg/L (20%) slurry treatment. However, there was no significant difference between 0mg/L and no slurry with just a 10% decrease in Biomass in the No slurry treatment. This can also be seen in Figure 5 and Table 8 below.

A two-way ANOVA with the only daily growth (Dry weight/number of days growth) data from Cut 2 and the Cut 4 indicated a significant effect of the slurry treatment [$F_{(3,24)}$ =8.502, p=.000504] and a significant interaction between the Cut and slurry [$F_{(3,24)}$ =12.628, p=$3.74*10^{-5}$]. An interaction plot and post hoc analysis revealed this was due to 74% decrease in the Dry weight production from the No slurry treatment in the Cut 4 compared to all other slurry treatments, whilst there were no significant differences from the slurry treatment in Cut 2. This is also evident in Figure 6, of the Dry weight production.

There was a significant effect of the slurry treatment on the stem Dry weight [$F_{(3,12)}$ =5.336, p=.0144] and

plant Carbon (C % *Dry weight) [$F_{(3,12)}$ =5.004, p=.0177]. Post hoc analysis revealed this was due to a significant increase (~10%) in Dry weight between 0,10 and 200 mg/l Doxycycline treatments and the No slurry treatments (Figure 6). The 0mg/l Doxycycline stem dry weight or Carbon production was not significantly different to any other treatment

This procedure was repeated with the root data, no effect of the slurry treatment on Dry weight however a significant effect of the treatment on root Nitrogen (Dry weight * N%) [$F_{(3,12)}$ =3.499, p=.0497]. Post hoc analysis showed that this was due to a trend (p=.06) 40% increase in 0mg/L slurry treatment Nitrogen content compared to the No slurry control treatment. Though not statistically significant, there was also a 38% increase in root Nitrogen % between the 0mg/L and the no slurry treatment (Figure 7).

Silt loam

There was no significant effect of the slurry treatment on any of the measured grass factors regardless of clear differences in the standard error bars. However, the dry weight, N, C and other factors were significantly different depending on the part of the grass as seen in Figure 6, Figure 7, Table 8. However this is well represented in the graph for theoretical purposes.

Table 8. Herbage yield (kg DM ha^{-1}) of *L. perenne* under greenhouse conditions under different slurry treatments and soil types.

Soil type	Slurry treatment (Doxycycline mg/L)	Cut/part[3)]						Total
		1	2	3[1)]	4	Stems	Roots	
Sand	**0**	1255[a2)]	512[a]	50[a]	515[a]	769[a]	1364[ab]	4495[a]
	10	1484[a]	462[a]	42[a]	516[a]	859[a]	1687[ab]	5052[a]
	200	1375[a]	552[a]	66[a]	608[a]	839[a]	1816[ab]	5258[a]
	No slurry	1468[a]	567[a]	63[a]	135[b]	658[a]	1175[a]	4068[b]
Silt loam	**0**	3546[b]	3789[b]	-	1989[c]	2256[b]	2126[ab]	13710[c]
	10	3722[b]	3196[b]	-	2178[c]	2038[b]	2033[ab]	13067[c]
	200	3424[b]	3369[b]	-	2065[c]	2237[b]	2025[ab]	13122[c]
	No Surry	3748[b]	3294[b]	-	1502[c]	2150[b]	2690[b]	13387[c]

[1)] Only sand pots had an additional third cut after just one week of growth before slurry application

[2)] Different Letters within a column indicate a significant (P<0.05) difference between treatments

[3)] The separate cuttings, stem and root harvests were completed on the same day for all slurry treatments within that soil type.

NUE

Nitrogen losses form NH_3-N ranged form >1% (200mg silt loam grass) to <5% for 0mg/L and 200mg/L sand grass treatments (Table 9). Losses from N_2O were more constant all around 1%, with no significant differences. The high amount of N unaccounted for does not include potential N leaching, lost N through N_2 emissions or any other pathways. Unreliable NH_3 emissions prevented further Statistical analysis of the NUE and N losses.

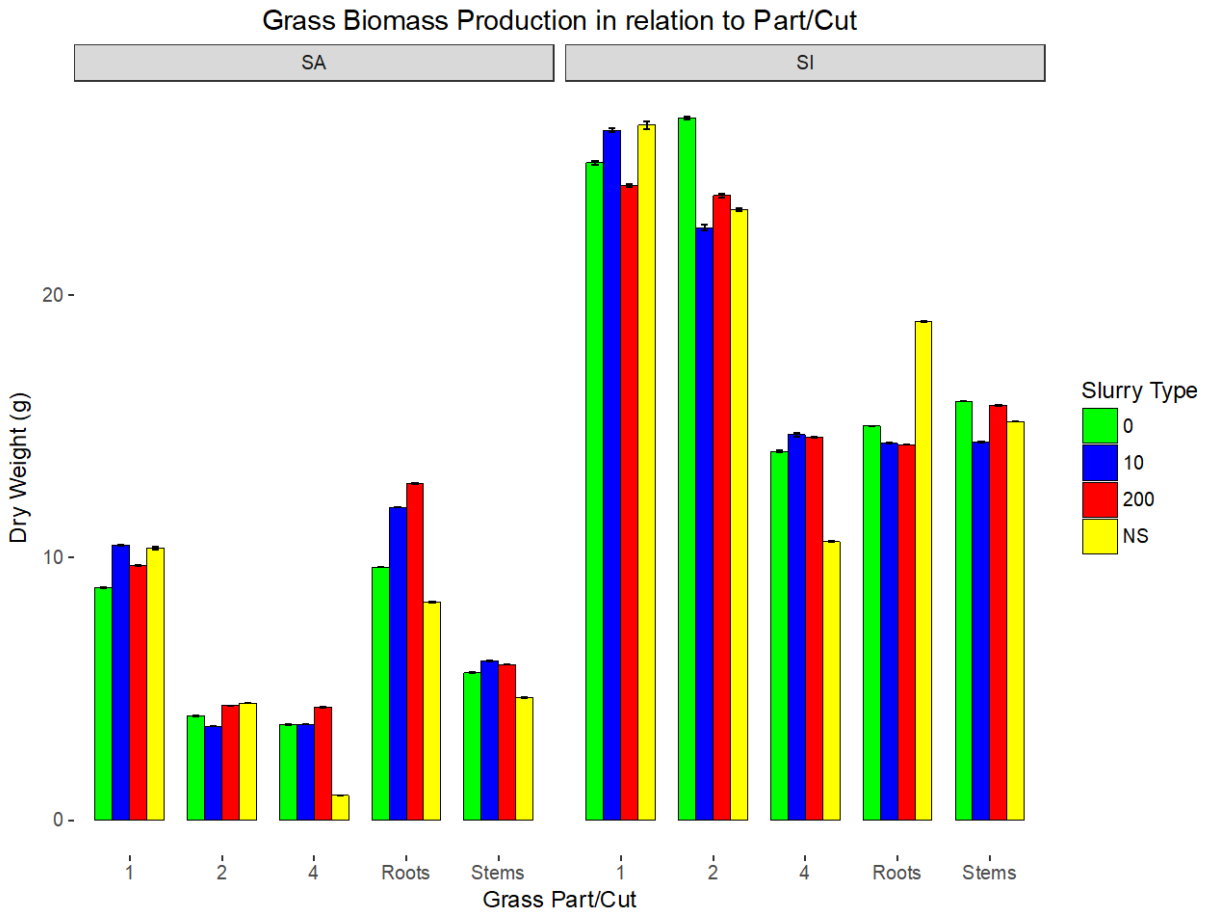


Figure 6. The grass Dry weight production in relation to slurry treatment and the Cut/Part of the grass for the two soils (SA= Sand and SI-Silt Loam). Cut 3 of the sand soil was not included. Error bars show standard error of the mean.

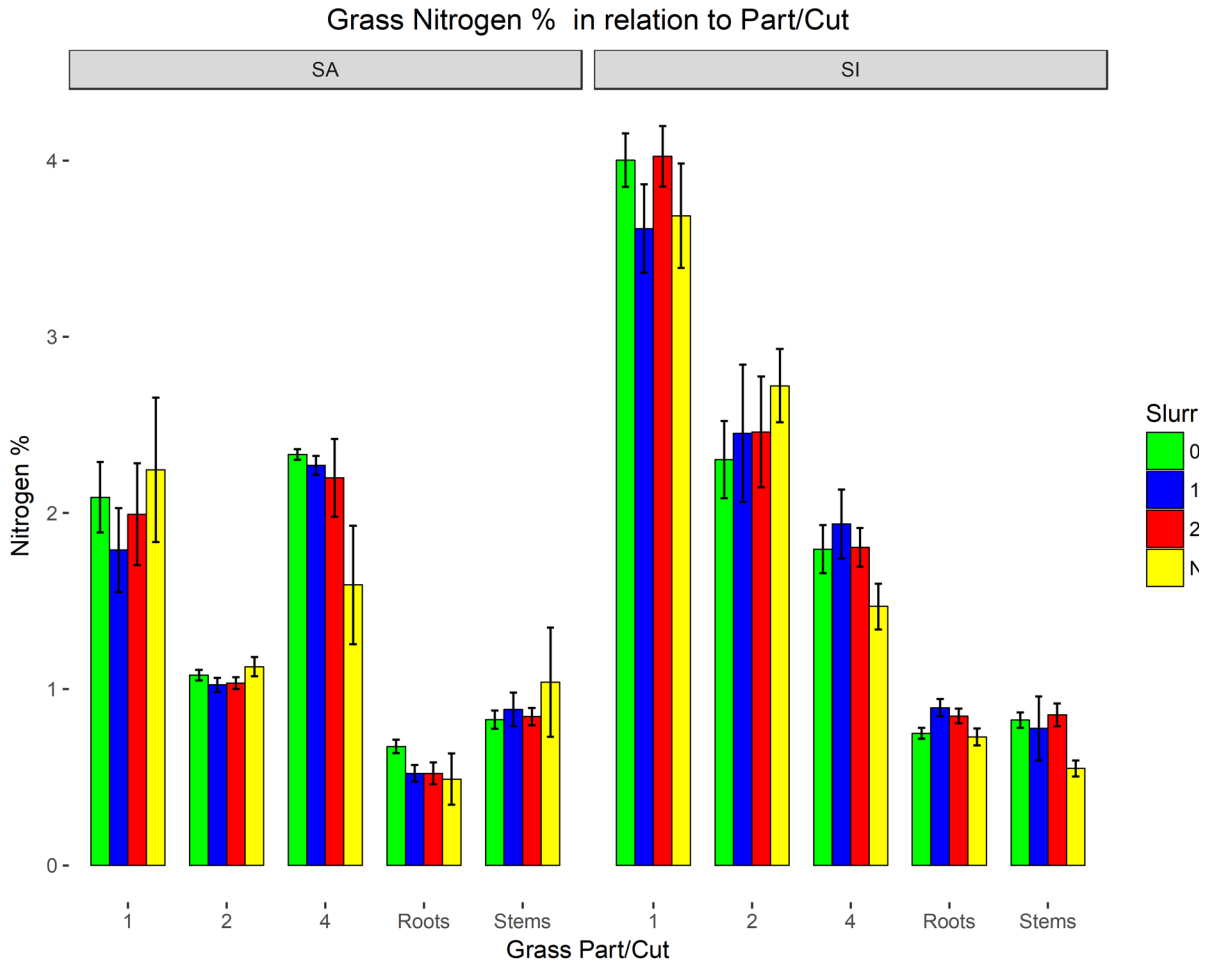


Figure 7. The Nitrogen percentage (%) in the different grass Parts and Cuts for the two soils (SA= Sand and SI-Silt Loam) and different slurry treatments. Cut 3 of the sand soil was not included as insufficient dry matter was harvested for C:N analysis. Error bars show standard error of the mean.

Table 9. The Nitrogen Use Efficiency of the different slurry treatments with respect to soil types.

Soil type	Slurry treatment (Doxycycline mg/L)	Total N uptake in grass (g/m^2)	Slurry Nitrogen Losses (g/m^2)		Total Measured N losses[1] (g/m^2)	Total N Applied from slurry (g/m^2)	Nitrogen Use Efficiency[2] (%)	Nitrogen Unaccounted for[3] (%)
			N_2O-N	NH_3-N				
Sand	0	6.16	.155	.624	.780	11.72	3.79	88.6
	10	6.08	.162	.261	.423	11.72	3.21	92.4
	200	6.39	.152	.623	.776	11.72	5.37	86.7
	No slurry	5.61	-	-	-	11.72	-	-
Silt loam	0	30.76	.161	.123	.284	11.72	11.86	82.8
	10	29.84	.153	.121	.274	11.72	5.59	90.7
	200	30.27	.126	.089	.215	11.72	8.51	87.6
	No slurry	29.03	-	-	-	11.72	-	-

[1] Measured N losses (g/m^2) is the sum of NH_3-N + N_2O-N and does not account for N lost as N_2 or other pathways such as leeching.

[2] Nitrogen Use Efficiency was calculated using the difference method as explained in Equation 4.

[3] The nitrogen unaccounted for was calculated using the described Equation 5.

Discussion

Carbon dioxide

Though the type of slurry treatment did not effect the CO_2 emissions, there was a significant increase due to vegetation (pot type). Grassland has been shown to have over 25% higher soil respiration rates compared to fallow soil (Raich and Tufekcioglu 2000). In addition, the grass stems increased the surface area of the slurry application, known to increase gas emissions (Sommer and Hutchings 2001).

The high peak of CO_2 following the application of the slurry may be due to a number of processes. A strong CO_2 flush often follows application of anaerobically stored slurry as Carbonates in the slurry are dissociated in contact with an acidic soil (Rochette et al. 2004; Sommer and Sherlock 1996). This is also seen following urine application, with urea hydrolysis forming Carbonate ions, that are further hydrolyzed to form CO_2 by urease enzymes (Sherlock & Goh, 1983). Over the more long-term period of the experiment, other processes may contribute to the elevated CO_2 levels. Slurry application results in higher CO_2 emissions due to increased soil microbial Carbon pool (Aarons et al. 2009), plant growth and in turn root respiration(Chu et al. 2007) (~increase by 10mg/m2/h). Combined with microbial respiration in the rhizosphere itself, this can account for 12-38% of respiration in agricultural soils (Raich and Tufekcioglu 2000). This is evident in Appendix 2 and Table 7 with overall significantly higher CO_2 emissions form the grass pots compared to bare soil. However, separating the root and microbial contributions to CO_2 emissions remains difficult even with the bare soil control, as root exudation and microbial activity is a symbiotic relationship (Inselsbacher et al. 2011).

In addition to this, given the gas chamber was dark, this would have prevented photosynthesis. With no light available, CO_2 levels would increase comparatively as the plant leaves continue to respire, using oxygen and glucose in order to produce energy, resulting in CO_2 and water production. This respiration is partitioned into two components of construction and maintenance respiration. Maintenance respiration, representing protein synthesis and replacement, has been shown to increase with increasing temperature (Ryan 1991), whilst construction maintenance is represents new tissue synthesis from glucose and minerals. Due to the pausing of the photosynthesis, the overall CO_2 emissions would have been overestimated within the closed flux chamber. However, together with the aforementioned factors, the other forms of plant respiration help explain the overall higher CO_2 emissions from grass pots, and especially from the silt loam soil, compared to the bare soil pots.

Nitrous oxide

Though Tetracyclines have been shown to inhibit denitrification (Halling-Sorensen 2001), no effect of the antibiotic treatment significantly affected N_2O emissions within the same pot type (grass/bare soil). However there was a significant 55% decrease in N_2O emissions between the 200mg/L bare soil compared to 0mg/L grass from silt loam AUC from the first 3 days (Table 7), suggesting a decrease denitrification due to the slurry treatment. Root exudates can also serve as an additional Carbon source for denitrifer activity, increasing N_2O emissions(Philippot et al. 2009). Additionally, root penetration (decreasing compaction) in soil creates channels for faster N_2O diffusion (Philippot et al. 2009) further increasing grass pot N_2O emission compared to bare soil Pots. However, given there were no other significant effects of pot type on the hourly emission rate or total emission rate, there may have been an effect of the Doxycycline on the N_2O emissions.

Though plants take up Nitrogen, reducing the substrate for nitrification and denitrification (Inselsbacher et al. 2011), a study from Velthof et al. (2003) saw no significant difference in N_2O fluxes after application of pig manure. This was regardless of technique used, which may increase or decrease the surface area of the slurry rather; fluxes were more affected by rain. Suggesting that moisture content would have more of an effect on N_2O measurements due to the anaerobic conditions created and typically needed for its production. This may also explain why N_2O hourly emission rates were 18% higher (after 7 days) in sand pots compared to silt loam. The silt loam soil was extremely compact, with the surface layer drying out, caking and causing water to run down the sides of the pot rather than be absorbed through the top layer of the soil. In turn, decreasing the chances of anaerobic conditions forming.

Contrary to Gavrichkova and Kuzyakov (2008), though slurry application increased N_2O emissions (35 ug/m2/h in the study), it did not return to baseline levels within 8-14 days, rather continued to increase over time after the initial peak (Appendix 3). Given the experiment was in the greenhouse, there was minimal Air movement, NH_3 deposition can also contribute to N_2O emissions by increasing N cycling in the system (Davidson 2009). Initially temperatures in the greenhouse were set to 18°c at night and 24°c during the day, the bad spring weather kept the greenhouse at this temperature for the first two weeks. However, over the course of the experiment, the temperature increased significantly above this to over 35°c at times, and potentially even warmer in the dark Flux Measurement Chamber. Temperature and aeration are seen as the two main environmental factors affecting denitrification and nitrification and in turn N_2O production. Both reactions increase with an increase in soil temperature(Rochette et al. 2004; Firestone and Davidson 1989), possibly explaining the steady increase over time in N_2O emission, as well as potentially why sand pots had higher hourly emission rates after the 7-8 day period as mentioned above.

Ammonia

The confounding effects of the Doxycycline (200mg/L) slurry treatment on NH_3 gaseous emission rates

between soil types (increased in sand, decreased in silt loam) weaken the weight of the observed trends (p=.09) of the sand and silt loam Bare soil (200mg/L) pots decreasing hourly emission rates compared to 10mg/L. However, they do raise the question if slurry application and temperature had been better controlled or if the INNOVA had been recalibrated, if this would have significantly affected the NH_3 gaseous emission rates for future experimentation? Especially since on average in the silt loam pots, the hourly emissions of the 200mg/L pots were 28% less compared to the other slurry treatments at time of slurry application, and 35% less compared to 0mg/L and 45% less than 10mg/L after 3 hours. However it was predicated that due to the observed effects of Tetracyclines on preventing the growth of *Nitrosomonas e*., thereby preventing nitrification of ammonia (Halling-Sorensen 2001), NH_3 gaseous emission rates would increase. Which is the opposite of the observed in this case. However there are a number of different *Nitrosomonas* species that also oxidise ammonia into Nitrate, making significant differences in NH_3 emissions more difficult to observe.

In addition, the non-conformation of 36% of the measurements to a Non-Rectangular Hyperbola made NH_3 data analysis more problematical with less or no replicates and uneven sample sizes in certain treatments. This was mostly due to measurement either exhibiting no curvature, or levelling off as in equilibrium, only to continue to increase. This issue was not aided by the fact that not all-initial concentrations of the NH_3 were 0 at the start of the measurement (D in Equation 3). Though tubes were flushed for at least two minutes between measurements, the humid conditions in the greenhouse enhanced by the stickiness of NH_3molecules (Shah et al. 2006) made initial concentrations higher than they were in certain pots (especially those towards the end of the sampling for the day). This in turn, may have led to an underestimation of the initial increase (B_i) of the gas in certain Pots, then used to calculate the hourly emissions. Moreover, there was no significant difference between pot types (bare soil or grass) as observed in the other gases, even though the grass pots had the slurry unintentionally spread over a larger area. However NH_3 can also be taken up by plant through stomatal and cutical pathways (Sutton et al. 1995) with plant canopy absorption reducing NH_3 losses by up to 25% in spray application of slurry (Sommer et al. 1997).

Following the hydrolysis of Urea ($CO(NH_2)_2$) $\rightarrow$ $CO_2 + NH_3$, the ammonia is then in equilibrium by a dissociation reaction between nonvolatile ammonium (NH_4^+) and volatile NH_3(Burgos et al. 2010). There was also a tendency for the NH_3 emission rate to increase with time in the sand pots (Figure 4). The volatilization of NH_3 from liquid is dependent on the partial pressure of the NH_3 in the air and the relative concentration in the liquid slurry (Petersen et al. 2014). CO_2 emissions affect NH_3 emission rates as both the total inorganic carbon (TIC= $CO_2 + HCO_3^- + CO_3^{-2}$) and TAN acting as buffers to NH_3 volatilization. CO_2 has a lower solubility and is therefore initially emitted more rapidly than NH3 (Petersen et al. 2014). This may help explain why CO_2 peaks after the first application whilst the NH_3 emission rate continued to increase with time. In the greenhouse, there was no wind and a build up of NH_3 emissions, high temperature and moisture. This is temperature and pH dependent, with increased pH and temperature also increasing NH_3 emissions (Génermont and Cellier 1997). This may partially explain why NH_3 emission rates did not decrease as previously observed in field studies of Nienhuis and Lantinga (2013), however does not explain the unreliable Non-rectangular Hyperbolic emission curves recorded by the INNOVA. The INNOVA Photo-acoustic sensor had not been used for a number of years. Though it was recording linear CO_2, N_2O and CH_4 measurements, 20% of the N_2O data could not be used due to unacceptable R^2 values, decreasing certain parts of the dataset and most probably also underestimating total AUC emissions from the experiment. It is unknown if the sometimes uncharacteristic measurements were due to the machine malfunctioning or other circumstances caused by the greenhouse conditions. Together the accumulation of the aforementioned factors, the confounding and insignificant results depending on the soil type, no clear conclusions regarding the effect of Doxycycline in slurry on NH_3 emissions can be made.

Methane

A significant effect was seen on the AUC total CH_4 emissions between the different slurry treatments on silt loam soils [$F_{(2,8)}$ =10.1, p=.0065]. This was also the case with a 53% increase in total CH_4 emissions for 200mg/L grass compared to bare soil. However, as seen in Appendix 5, the timing of the second silt loam 200mg/L measurements was marginally later than for the other treatments, in turn increasing the AUC value even if emission rates had already returned to control levels. This is further supported by the fact that there were no significant effects of the slurry on the sand soil. This was unexpected as actual CH_4 formation from Methane oxidation is negligible on mineral soils (Rodhe et al. 2015) that in principle it can be ignored for this experiment. Possibly a reason for not seeing more significant effects of the Doxycycline slurry treatment compared to the experiment of Sanz et al. (1996) is due to the unrealistic concentration of Butyric acid, as in the experiment it comprises of 33.33% of the VFA, however normally accounts for 8% or less of VFAs in normal cow slurry(Patni and Jui 1985). With this said, an increase in ruminal pH occurs when cattle change from fermenting starch (concentrates) to fiber (more grass based) as in the Hooilanden farm, which in turn produces less propionic acid, and more acetic and butyric acid(Dijkstra et al. 2011). Additionally, CH_4 formation through methanogenisis is a strictly anaerobic process (Amon et al. 2006), the Doxycycline was only added 24 hours prior to the slurry treatment, and then aerated as to thoroughly mix the antibiotic into the slurry. Hence this was perhaps not enough time for anaerobic conditions to reform and the Doxycycline treatment to take proper effect.

There was a significant effect of the pot type on the hourly Methane emissions [$F_{(1,31)}$ =11.761, p=.00173] on the first measurement resulting in 24% reduction in emission rates from bare soil Pots. CH_4 production has been correlated with vascular plant production due to the linkage between recently fixed Carbon and organic material needed for methanogenisis (Joabsson et al. 1999). However in the short term, grass pots had significantly higher vegetation, allowing the slurry to spread over a larger surface area and intern, increased CH_4 emissions through initial surface exposure and diffusion as with the other gases.

Plant

The Doxycycline slurry treatment did not affect dry matter plant yields in sand pots. This may have been due to the short exposure of the grass to the treatment (22 days of slurry compared to a total time period of 126/127 days from sowing to final harvest seen in Table 3). Especially since during the first week after a cutting, majority of the Nitrogen for regrowth comes from remobilized organic N (proteins) stored in the stubble and roots, and not from the applied N (de Boer et al. 2016). Nevertheless, a 74% decrease in the dry weight production from the No slurry treatment in the Cut 4 in sand pots compared to all other slurry treatments suggests that initial effects of the slurry addition on *L. perenne* (Table 8, Figure 6). However time constraints prevented the continuation of the treatment, potentially dulling effects of the treatment.

Though there was a 36% more root biomass of sand 200mg/L treatment compared to the corresponding no slurry treatment, there was no significant difference between treatments (Figure 6, Table 8). However, root biomass is a general indicator of root activity as it includes old, new and dead roots. A higher N content indicates an increased root uptake of Nitrogen; in turn a higher percentage of active roots. *L. perenne* root life span typically lasts 14 weeks (fine roots and nitrogen rich habitats) (Van der Krift and Berendse 2002). Hence, roots from the first few weeks of the experiment may have already been included in the root biomass even though they were not active. Therefore, the N% of the roots is potentially a better indicator of the effect of the slurry treatment on *L. perenne* as both uptake and remobilisation of root nitrogen are used for post-defoliation regrowth (Thornton et al. 1993). There, was a trend 40% increase in the Nitrogen content in the root of the 0mg/L sand treatment compared to the no slurry control slurry exposure, however not with the other two slurry treatments. The inverse significant

effect however was seen on increased stem dry weight from 10mg/L and 200mg/L treatments [$F_{(3,12)}$ =5.336, p=.0144] whilst no significant difference in the C:N ratio was observed. However though this one factor is not sufficient to say the Doxycycline affected grass growth, it does urge the repetition of such an experiment for a longer period of slurry exposure. This is especially the case for the silt loam where no significant effect of the slurry treatment on any factor was observed.

On sand soil and established *L. perenne* pasture 2867kg DM/ha after 100kg N/ha fertilisation after 34 days of growth (de Boer et al. 2016). This value is in between the Cut 1 values of the two soils in Table 8. Potentially suggesting that a reason for no statistical effect of the slurry treatment on yields in the silt loam is due to the soil not being sufficiently nutrient limited, as it was a lot higher than the experiment of de Boer et al. (2016). There was a significant increase in biomass production from the silt loam compared to the sand soil; this is most probably due to the higher amount of available N in the soil (Table 3). However, all cut yields decreased after the after Cut 1 (Table 8), even after the slurry application. Given the slurry was quite thick, this suggests a higher manure compared to urine fraction compared to mixed slurry. This signifies also a higher ratio of inorganic Nitrogen compared to inorganic nitrogen. 40 to 90% of the total N content in slurries may be in organic form, only available to the plant after mineralisation(Chadwick et al. 2000). As seen in Table 5, the C:N ratio of the slurry is over 15, suggesting that after initial application, the high C:N ratio would result in the immobilisation of the Nitrogen added rather than mineralisation (Chadwick et al. 2000). In an experiment from Chadwick et al. (2000), even after 199 days, only 20% of the organic N from dairy slurry had mineralised, with this fraction highest after 45 days. This may have resulted in insufficient slurry treatment exposure time before grass harvest, possibly explaining the lack of significant biomass production increase from the silty loam soil as not enough of the organic Nitrogen fraction of the slurry had yet been mineralised.

After one month of growth, grass tillers in the pots were counted. Though there was no significant difference in number of tillers, in the sand pots, the 200mg/L pots had 17% more tillers than the 0mg/L treatment. This is also reflected in the dry weight of the Cut 1 in Figure 2. This difference in tillers was even more pronounced in the silt loam soil with 45% more tillers in 10/mg compared to 200mg/L. This large variation may have been due to the normal-low seeding rate (25kg seed/ha) and bad germination especially prominent in the silt loam soil due to the drying out and caking of the topsoil. This was exacerbated with the 16-hour days selected for the greenhouse conditions and insufficient watering in the initial plant developmental stage. Ideally, a higher seeding rate and initially watering the pots two to three times per day could have avoided this possible flaw in the experiment. However, recording no effects of Doxycycline on gas emissions or *L. perenne* growth within this exposure period is also in itself a result. However, getting these results this under more homogenous plant and slurry conditions would strengthen the power of this experiment.

NUE

The Nitrogen Use Efficiency of the silt loam pots was higher than that of the sand pots, however the amount of available N from the soil is not taken into account with these calculations (Table 9). There are noticeably around 80% or more of the added Nitrogen unaccounted for, again expressing the time constraints for the experiment. In the case of this experiment, leaching is not accounted for, as pots were never watered to the extent that water would overflow in the saucer, in turn allowing for nutrient leaching. As seen in Appendix 2, Picture 9, fine new roots were forming in slurry residue on the surface. It takes approximately three months for dung pats to visually disappear from pasture (Lantinga et al. 1987). Therefore, the short exposure (22 days for sand pots) of the grass to the treatment may have hindered the exposure of the Doxycycline on the grass. With the hard silt loam clay surface encourage water runoff down the side of the pot, it is possible that some of the Doxycycline was not thoroughly exposed to the

grass. Especially since Doxycycline has a high solubility of 50mg/mL (Bogardus and Blackwood 1979) and had been mixed into the slurry. *L. perenne* typically only has root growth to 30cm(Hoogsteen et al. 2015)(pot depth was 30cm), however there was not a lot of root growth at this depth, potentially suggesting less exposure of the antibiotic compared other experiments when the Antibiotic was manually mixed into the soil such as Jastrow et al. (2007). This technique ensures better exposure of the antibiotic to the soil organisms, and potentially the effect of the antibiotic on gas emissions. Decreasing NH_3 volatilization, as well as leaching and other pathways where vital N sources are lost, increases the NUE of the crop. This is beneficial to farmers whilst also minimizing possible environmental damage, though certain preventative measures may also lead to an increase in other GHG emissions(Hou et al. 2015). However given the unreliable NH_3 results, a full statistical analysis of the effect of the slurry treatment on NUE was not possible.

Experimental Design and Future Recommendations

Given the Experiment was novel experimental research regard to the affect of Doxycycline on GHG and NH_3 emission under different soils and vegetative conditions. A number of procedures could have been done differently to ensure more reliable results. When sampling the bare soil Pots and the grass pots simultaneously, the application of the manure from the first grass pots to the last took over five days (due to limited greenhouse opening hours on the weekend). Even after two days the effects of the manure treatment was visual on the grass growth response (color) (Appendix 2, Picture 4 compared to Picture 8). Hence it was decided that for the sand soil, all the grass samples would be sampled first (over only two days), and then the Bare soil plots would be sampled to prevent experimental bias from earlier slurry treatment. This spread the slurry treatment application out over two weeks total, potentially changing the slurry. In a study by Sawamoto et al. (2016), CH_4 formation continued in stored slurry even at 5°c. However, at a rate 94% lower than at 35°c, suggesting increased storage could potentially alter the total emissions regardless of slurry treatment. In addition, with the Night temperature in the greenhouse at 18°c, and the daytime temperature at 24°c, slurry applications in the morning may have had slurry that was colder in temperature than samples later in the day. This could have been prevented through having an additional INNOVA machine for monitoring the Bare soil pots for instance, and storing the slurry a heated controlled temperature chamber however this was not possible for the experiment.

However, a large possible factor that may have affected the results was the lack of soil and air temperature measurements. This was initially not a factor giving it was winter and the greenhouse was heated to controlled temperatures, however due to unforeseen delays in the experiment, it became a problem especially from the 17th of May when temperatures in the greenhouse exceeded 30 degrees (ACCU weather), with large variations in temperature from measurements done first early in the morning, compared to those in the middle of the day. This could be avoided in the future with a simple temperature probe inside the cylinder during the measurement and in turn being able to use Water Vapor measurements from the INNOVA readings. Warmer than predicted temperatures, does mean that some gas emissions might have been underestimated given the relationship between partial pressure and temperature.

The large differences in gaseous emission observed within treatments further stresses the need for a more controlled slurry application. This also involves stricter time intervals between measurements, as see in Figure 3, the CH_4 emissions decrease rapidly, and small differences in time, resulted in larger differences between emission rates and then also the AUC calculations (time * emission rate) as certain measurements were taken earlier or later than others due to the greenhouse closing or tardiness in previous measurements increasing the time intervals. Though this was not such a problem for CO_2 and N_2O as they are monitored over a long time period, it makes a large difference for NH_3 and CH_4 emissions

rates and AUC (Figure 3 & 4). The slurry had not been well mixed prior to collection so it was quite solid, requiring the addition of water for mixing in the Doxycycline and applying the slurry to the soil (Appendix 2). This could also be avoided if slurry collection had included more liquid fraction, or urine. Additionally, aerating and diluting the slurry has also been shown to reduce CH_4 emissions regardless of an increase in temperature (Amon et al. 2006). Hence, gas emission rates may have been underestimated due to the watering down of the slurry to spread it more easily, this also may have changed the pH and affected potential NH_3 emissions (Génermont and Cellier 1997).

The caking of silt loam soil and the subsequent effect on seed emergence, in turn grass establishment was detrimental to the power of the experiment. Uneven sward establishment within the pot also indirectly changed the surface area application of the slurry. This effect could potentially be removed by having a central shallow cylinder keeping the slurry application to a certain area, with a high concentration of grass around the outside. Unforeseen delays in the experiment due to the INNOVA machine and then complications in procuring control dosed antibiotic slurry delayed the time-plan of the experiment. Together with the time limits in the greenhouse and rising temperature, the grass exposure to the slurry treatment was too short, and also at a more advanced growing stage rather than directly after the First Cut. Though single variety *L. perenne* was sown, in the silt loam pots, there were up to 50% *P. annua* grass plants (Appendix 2, Picture 4). This made grass comparison between the two soil types and in turn slurry treatments not reliable as it was another species with different growth characteristics and flowering times. In turn changing C:N ratios, the above and belowground yields of the grass with *P. annua* plants more competitive above ground and L*. perenne* below ground (Brede and Duich 1986). This can in turn change yields and C:N results, especially since the *P. annua* was flowering. Though it was assumed that there were an equal % of *P. annua* in all pots, in future experimentation, this situation can be avoided through using a different soil type, pre-soaking grass seeds (faster emergence) and sowing the chosen grass species at a higher density to hinder weed seed establishment.

The soil was watered in the evenings after all the measurements had taken place to minimise experimental bias. However (De Klein and Van Logtestijn 1994) reported that a wet soil could increase denitrification losses by 6-9% of the applied N. Though pots were spatially random, in order to keep times between measurements as even as possible they were sampled in the same order as slurry was applied. This may also have led to the first pots in the morning being sampled having a higher moisture content in the top layer of the soil. In Wageningen soil Quality chair group experiments, the pots are smaller (15x15 cm) and then easily weighed in between measurements or straight after the last measurement for that day to ensure equal moisture values in the experiment. This minimises both potential N_2O production and plant growth bias.

Unfortunately there weren't enough cylinders for all 56 pots when taking gas measurements. Rather there were only 12, resulting in a lot of soil disturbance to the Pots between measurements (Appendix 2, Picture 7). Especially as extracting the cylinder also moved the soil around, and inserting them may have damaged root growth. Near the end of the experiment, it also made it difficult to keeps the cylinders at the same height in the soil, as larger cavity and compressed soil ring had formed. This made it extremely difficult to insert the cylinder, especially in the silt loam, which was already quite compact and hard on the surface due to the high clay content. For more long term monitoring such as in this is experiment, it would be recommended to have enough cylinders for all pots. This will minimise changes in air volume, ensure the measurement is air tight, whilst reducing potential soil and plant disturbance.

Contrary to Initial experimental Design, the Doxycycline was added to the excreted slurry rather than given to the animal. This enabled greater control over the amount of antibiotic added to the different

treatment Pots. However also meant that the antibiotics were only in the slurry for ~24 hours before it was applied, whilst realistically, it would be in the slurry for longer periods of time. Though the processes of gas formation are known (Table 1), the relative time period of this is still not well known for this experiment and may have altered gaseous emissions more if they had been added earlier. Antibiotics have been used to improve the livestock feed efficiency, in turn decreasing excreted N content by 5-25% (Han et al. 2001). There is a linear relationship between the Crude Protein (CP) in the diet and manure pH, manure N content and Total Annomical Nitrogen (TAN = NH_3 + NH_4^+), which will in turn affects possible N_2O production. Decreasing dietary CP content can lower NH_3 emissions from slurry by up to 65% (Hou et al. 2015). Therefore, it remains difficult to keep an experiment on plants controlled, as you will need to control either the amount of slurry applied, or the amount of N through the slurry applied, which may also lead to difference in other essential nutrients. In addition, previous studies concerning swine biogas production reveled a tendency of insignificant effects of the Tetracyclines on production when it was added to the slurry as in Lallai et al. (2002); Beneragama et al. (2015), whilst up to a 30% reduction in CH_4 production was observed in Arikan et al. (2006); Masse et al. (2000) when the antibiotic was dosed to the animal and slurry was collection before and after this treatment. Sandkvist et al. (1984) suggested additional antibiotic metabolites from the gastrointestinal tract of pig might be inhibiting to bacterial activity in the manure. This may especially be the case with Doxycycline as 57% was found to be excreted through manure and urine (Zhao et al. 2010), though up to 90% of certain antibiotics have been found to be excreted in unaltered form (Kemper 2008).

Doxycycline represent just one of many antibiotics used in the livestock industry. It was chosen for the experiment given its high use within the Veal Industry, however also Swine (MARAN 2016). Actually collecting slurry from dosed animals would have been preferred to make the experiment more realistic. If time had not been limiting, applying the slurry an additional time would have been preferred. Also experimenting with different antibiotics is also extremely relevant. Overall, there were a number of factors that can be improved upon and changed in order to make future experiments more reliable and observe the true effect of the slurry Doxycycline treatment on *L. perenne* growth, GHG and NH_3 emissions. This includes and is not limited to: cylinders for all pots, higher seeding rates and better control of weed species, monitoring of Temperature 24/7, keeping slurry application to a defined area to control surface exposure, stricter time intervals between measurements (AUC), more measurements in the first 24 hours to monitor CH_4 and NH_3 better, recalibration of the INOVA photo-acoustic machine, adding the antibiotics to the slurry earlier, greater control of slurry application temperature, potentially having two INOVA machines to reduce bias between grass growth and slurry application, naturally a more liquid fraction of the slurry so minimal water addition is needed, applying the manure earlier in the grass developmental stage and then leaving the treatment for a longer period before full harvest.

Conclusions

This study presents a novel yet viable method for simultaneously observing the effect of antibiotic residues in slurry on the underlying soil microbial N and C cycles as well as plant yields under more realistic conditions. No consistent effects of the slurry Doxycycline treatment were observed in the four different soil and pot conditions. However, in silt loam bare soil pots, 200mg/L Doxycycline CH_4 emissions <44% higher than the other slurry treatments, with 15% increase across all treatments directly after application. Additionally, a significant 55% increase in N_2O emissions from 0mg/L silt loam grass pots and 200mg/L silt loam Bare soil pots suggest a possible effect of Doxycycline on denitrification processes. Though other effects of Pot and soil type were observed, there were no other significant effects of the slurry treatment on the other gases or *L. perenne* yields. Though not significant, there was trend ($p=.06$) of higher root N% in 0mg/L compared to the No slurry treatment. 0mg/L root N% was also higher than the other treatments suggesting an effect of the Doxycycline on root N uptake though insufficient treatment

exposure time may have numbed this effect. A significant 65% increase in biomass yield was seen in silt loam soil compared to the sand soil. Whilst N_2O emission rates were significantly higher in sand soil after 7 and 8 days, potentially due to greater moisture retention and anaerobic conditions forming. CH_4 emissions were significantly higher (24%) in grass pots compared to the Bare soil Pots. Though a number of factors decreased the reliability of results, especially NH_3, a comprehensive representation of GHG emissions following slurry application was made. However, in identifying the weaknesses that need to be addressed for future studies within GHG and NH_3 emission management and mitigation, this experiment has presented a practical method for observing the effects of antibiotics on the soil and Plant biome.

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References

Aarons, S. R., C. R. O'Connor, H. M. Hosseini, and C. J. P. Gourley. 2009. Dung pads increase pasture production, soil nutrients and microbial biomass carbon in grazed dairy systems. *Nutrient Cycling in Agroecosystems* 84 (1): 81-92. http://dx.doi.org/10.1007/s10705-008-9228-5.

Amon, B., V. Kryvoruchko, T. Amon, and S. Zechmeister-Boltenstern. 2006. Methane, nitrous oxide and ammonia emissions during storage and after application of dairy cattle slurry and influence of slurry treatment. *Agriculture, Ecosystems & Environment* 112 (2): 153-162. http://dx.doi.org/https://doi.org/10.1016/j.agee.2005.08.030.

Arikan, O. A., L. J. Sikora, W. Mulbry, S. U. Khan, C. Rice, and G. D. Foster. 2006. The fate and effect of oxytetracycline during the anaerobic digestion of manure from therapeutically treated calves. *Process Biochemistry* 41 (7): 1637-1643. http://dx.doi.org/10.1016/j.procbio.2006.03.010.

Beneragama, N., M. Iwasaki, S. A. Lateef, and K. Umetsu. 2015. The effect of cefazolin on biogas production from thermophilic and mesophilic anaerobic co-digestion of dairy manure and waste milk. *Journal of the National Science Foundation of Sri Lanka* 43 (4): 369-376. http://dx.doi.org/10.4038/jnsfsr.v43i4.7971.

Bengtsson, B., and C. Greko. 2014. Antibiotic resistance-consequences for animal health, welfare, and food production. *Upsala Journal of Medical Sciences* 119 (2): 96-102. http://dx.doi.org/10.3109/03009734.2014.901445.

Bernier, S. P., and M. G. Surette. 2013. Concentration-dependent activity of antibiotics in natural environments. *Frontiers in Microbiology* 4: 14. http://dx.doi.org/10.3389/fmicb.2013.00020.

Bogardus, J. B., and R. K. Blackwood. 1979. Solubility of doxycycline in aqueous solution. *Journal of pharmaceutical sciences* 68 (2): 188-194.

Boxall, A. B. A., D. W. Kolpin, B. Halling-Sorensen, and J. Tolls. 2003. Are veterinary medicines causing environmental risks? *Environmental Science & Technology* 37 (15): 286A-294A. http://dx.doi.org/10.1021/es032519b.

Brede, A., and J. Duich. 1986. Plant interaction among Poa annua, Poa pratensis, and Lolium perenne turfgrasses. *Agronomy Journal* 78 (1): 179-184.

Burgos, S. A., N. M. Embertson, Y. Zhao, F. M. Mitloehner, E. J. DePeters, and J. G. Fadel. 2010. Prediction of ammonia emission from dairy cattle manure based on milk urea nitrogen: Relation of milk urea nitrogen to ammonia emissions. *Journal of Dairy Science* 93 (6): 2377-2386. http://dx.doi.org/https://doi.org/10.3168/jds.2009-2415.

Chadwick, D., F. John, B. Pain, B. Chambers, and J. Williams. 2000. Plant uptake of nitrogen from the organic nitrogen fraction of animal manures: a laboratory experiment. *The Journal of Agricultural Science* 134 (2): 159-168.

Chu, H. Y., Y. Hosen, and K. Yagi. 2007. NO, N2O, CH4 and fluxes in winter barley field of Japanese Andisol as affected by N fertilizer management. *Soil Biology & Biochemistry* 39 (1): 330-339. http://dx.doi.org/10.1016/j.soilbio.2006.08.003.

Colinas, C., E. Ingham, and R. Molina. 1994. POPULATION RESPONSES OF TARGET AND NONTARGET FOREST SOIL ORGANISMS TO SELECTED BIOCIDES. *Soil Biology & Biochemistry* 26 (1): 41-47. http://dx.doi.org/10.1016/0038-0717(94)90193-7.

Davidson, E. A. 2009. The contribution of manure and fertilizer nitrogen to atmospheric nitrous oxide since 1860. *Nature Geoscience* 2 (9): 659-662.

de Boer, H. C., J. G. C. Deru, N. J. Hoekstra, and N. van Eekeren. 2016. Strategic timing of nitrogen fertilization to increase root biomass and nitrogen-use efficiency of Lolium perenne L. *Plant and Soil* 407 (1): 81-90. http://dx.doi.org/10.1007/s11104-016-2917-y.

De Klein, C. A. M., and R. S. P. Van Logtestijn. 1994. Denitrification in the top soil of managed grasslands in The Netherlands in relation to soil type and fertilizer level. *Plant and Soil* 163 (1): 33-44. http://dx.doi.org/10.1007/bf00033938.

de Vrieze, J. 2015. THE LITTLEST FARMHANDS. *Science* 349 (6249): 680-683.

Di Nica, V., L. Menaballi, G. Azimonti, and A. Finizio. 2015. RANKVET: A new ranking method for comparing and prioritizing the environmental risk of veterinary pharmaceuticals. *Ecological Indicators* 52: 270-276. http://dx.doi.org/10.1016/j.ecolind.2014.12.021.

Dijkstra, J., O. Oenema, and A. Bannink. 2011. Dietary strategies to reducing N excretion from cattle: implications for methane emissions. *Current Opinion in Environmental Sustainability* 3 (5): 414-422. http://dx.doi.org/https://doi.org/10.1016/j.cosust.2011.07.008.

Firestone, M. K., and E. A. Davidson. 1989. Microbiological basis of NO and N2O production and consumption in soil. *Exchange of trace gases between terrestrial ecosystems and the atmosphere* 47: 7-21.

Gavrichkova, O., and Y. Kuzyakov. 2008. Ammonium versus nitrate nutrition of Zea mays and Lupinus albus: Effect on root-derived CO2 efflux. *Soil Biology & Biochemistry* 40 (11): 2835-2842. http://dx.doi.org/10.1016/j.soilbio.2008.08.003.

Génermont, S., and P. Cellier. 1997. A mechanistic model for estimating ammonia volatilization from slurry applied to bare soil. *Agricultural and Forest Meteorology* 88 (1): 145-167.

Golet, E. M., I. Xifra, H. Siegrist, A. C. Alder, and W. Giger. 2003. Environmental exposure assessment of fluoroquinolone antibacterial agents from sewage to soil. *Environmental Science & Technology* 37 (15): 3243-3249. http://dx.doi.org/10.1021/es0264448.

Halling-Sorensen, B. 2001. Inhibition of aerobic growth and nitrification of bacteria in sewage sludge by antibacterial agents. *Archives of Environmental Contamination and Toxicology* 40 (4): 451-460.

Hammer, T. J., N. Fierer, B. Hardwick, A. Simojoki, E. Slade, J. Taponen, H. Viljanen, and T. Roslin. 2016. Treating cattle with antibiotics affects greenhouse gas emissions, and microbiota in dung and dung beetles. Paper read at Proc. R. Soc. B.

Han, I. K., J. Lee, X. Piao, and D. Li. 2001. Feeding and management system to reduce environmental pollution in swine production. *Asian Australasian Journal of Animal Sciences* 14 (3): 432-444.

Hoogsteen, M. J. J., E. A. Lantinga, E. J. Bakker, J. C. J. Groot, and P. A. Tittonell. 2015. Estimating soil organic carbon through loss on ignition: effects of ignition conditions and structural water loss. *European Journal of Soil Science* 66 (2): 320-328. http://dx.doi.org/10.1111/ejss.12224.

Hou, Y., G. L. Velthof, and O. Oenema. 2015. Mitigation of ammonia, nitrous oxide and methane emissions from manure management chains: a meta-analysis and integrated assessment. *Global Change Biology* 21 (3): 1293-1312. http://dx.doi.org/10.1111/gcb.12767.

Inselsbacher, E., W. Wanek, K. Ripka, E. Hackl, A. Sessitsch, J. Strauss, and S. Zechmeister-Boltenstern. 2011. Greenhouse gas fluxes respond to different N fertilizer types due to altered plant-soil-microbe interactions. *Plant and Soil* 343 (1-2): 17-35. http://dx.doi.org/10.1007/s11104-010-0597-6.

Jastrow, J. D., J. E. Amonette, and V. L. Bailey. 2007. Mechanisms controlling soil carbon turnover and their potential application for enhancing carbon sequestration. *Climatic Change* 80 (1-2): 5-23. http://dx.doi.org/10.1007/s10584-006-9178-3.

Joabsson, A., T. R. Christensen, and B. Wallén. 1999. Vascular plant controls on methane emissions from northern peatforming wetlands. *Trends in Ecology & Evolution* 14 (10): 385-388. http://dx.doi.org/https://doi.org/10.1016/S0169-5347(99)01649-3.

Kemper, N. 2008. Veterinary antibiotics in the aquatic and terrestrial environment. *Ecological Indicators* 8 (1): 1-13. http://dx.doi.org/10.1016/j.ecolind.2007.06.002.

Lallai, A., G. Mura, and N. Onnis. 2002. The effects of certain antibiotics on biogas production in the anaerobic digestion of pig waste slurry. *Bioresource Technology* 82 (2): 205-208. http://dx.doi.org/10.1016/s0960-8524(01)00162-6.

Landman, W. 2010. Climate change 2007: the physical science basis. *South African Geographical Journal* 92 (1): 86-87. http://dx.doi.org/10.1080/03736245.2010.480842.

Lantinga, E., J. Keuning, J. Groenwold, and P. Deenen. 1987. "Distribution of excreted nitrogen by grazing cattle and its effects on sward quality, herbage production and utilization." In *Animal Manure on Grassland and Fodder Crops. Fertilizer or Waste?*, 103-117. Springer.

Lobritz, M. A., P. Belenky, C. B. M. Porter, A. Gutierrez, J. H. Yang, E. G. Schwarz, D. J. Dwyer, A. S. Khalil, and J. J. Collins. 2015. Antibiotic efficacy is linked to bacterial cellular respiration. *Proceedings of the National Academy of Sciences of the United States of America* 112 (27): 8173-8180. http://dx.doi.org/10.1073/pnas.1509743112.

Masse, D. I., D. Lu, L. Masse, and R. L. Droste. 2000. Effect of antibiotics on psychrophilic anaerobic digestion of swine manure slurry in sequencing batch reactors. *Bioresource Technology* 75 (3): 205-211. http://dx.doi.org/10.1016/s0960-8524(00)00046-8.

Moullan, N., L. Mouchiroud, X. Wang, D. Ryu, E. G. Williams, A. Mottis, V. Jovaisaite, et al. 2015. Tetracyclines Disturb Mitochondrial Function across Eukaryotic Models: A Call for Caution in Biomedical Research. *Cell Reports* 10 (10): 1681-1691. http://dx.doi.org/10.1016/j.celrep.2015.02.034.

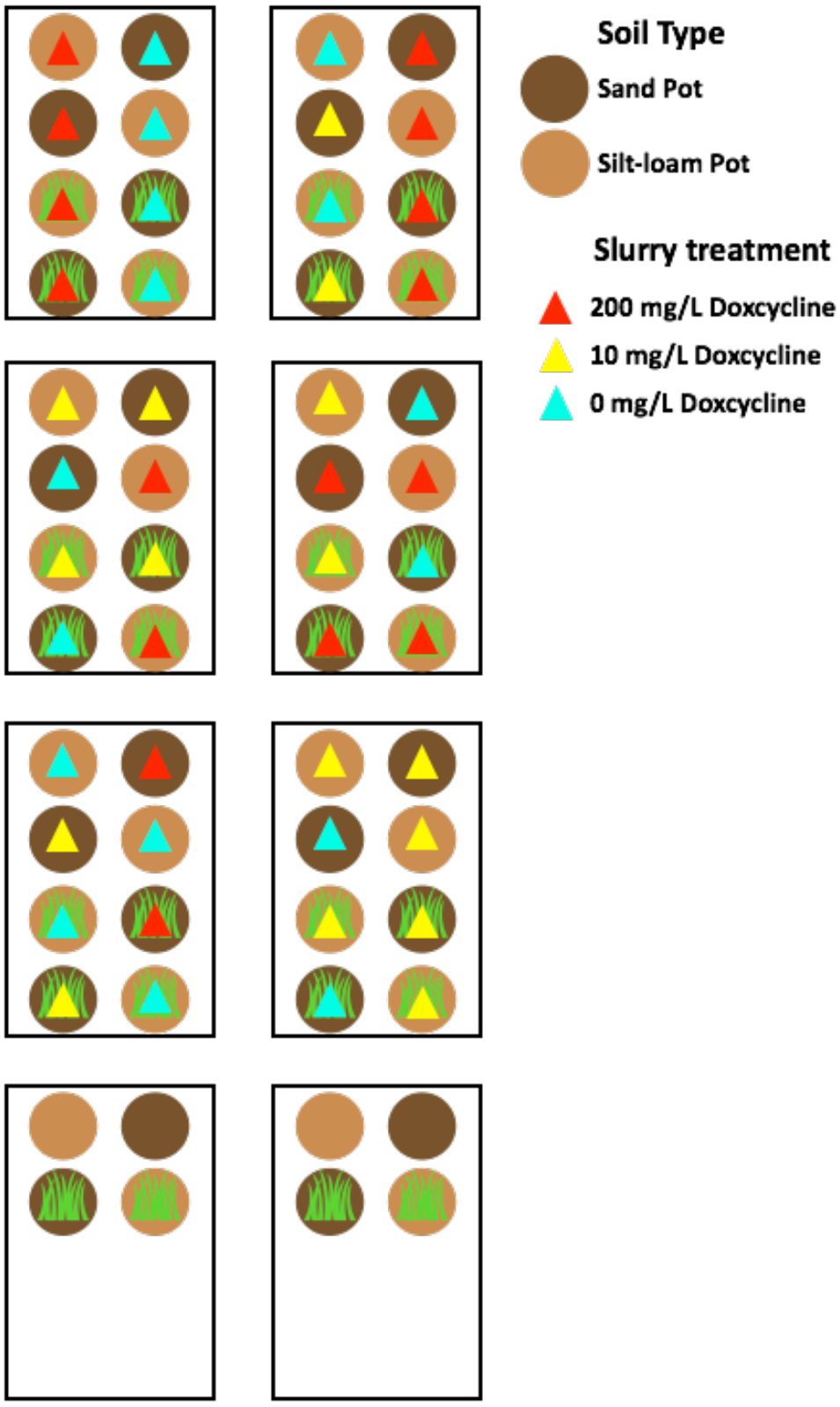
Nienhuis, C., and E. Lantinga. 2013. Thesis report.

Oenema, O., G. Velthof, and P. Kuikman. 2001. Technical and policy aspects of strategies to decrease greenhouse gas emissions from agriculture. *Nutrient Cycling in Agroecosystems* 60 (1): 301-315. http://dx.doi.org/10.1023/a:1012601113751.
Patni, N. K., and P. Y. Jui. 1985. Volatile fatty acids in stored dairy-cattle slurry. *Agricultural Wastes* 13 (3): 159-178. http://dx.doi.org/https://doi.org/10.1016/0141-4607(85)90031-9.
Petersen, V., R. Markfoged, S. D. Hafner, and S. G. Sommer. 2014. A new slurry pH model accounting for effects of ammonia and carbon dioxide volatilization on solution speciation. *Nutrient Cycling in Agroecosystems* 100 (2): 189-204. http://dx.doi.org/10.1007/s10705-014-9637-6.
Philippot, L., S. Hallin, G. Börjesson, and E. Baggs. 2009. Biochemical cycling in the rhizosphere having an impact on global change. *Plant and Soil* 321 (1-2): 61-81.
Raich, J. W., and A. Tufekcioglu. 2000. Vegetation and soil respiration: Correlations and controls. *Biogeochemistry* 48 (1): 71-90. http://dx.doi.org/10.1023/a:1006112000616.
Rochette, P., D. A. Angers, M. H. Chantigny, N. Bertrand, and D. Côté. 2004. Carbon Dioxide and Nitrous Oxide Emissions following Fall and Spring Applications of Pig Slurry to an Agricultural Soil. *Soil Science Society of America Journal* 68 (4). http://dx.doi.org/10.2136/sssaj2004.1410.
Rodhe, L. K. K., J. Ascue, A. Willén, B. V. Persson, and Å. Nordberg. 2015. Greenhouse gas emissions from storage and field application of anaerobically digested and non-digested cattle slurry. *Agriculture, Ecosystems & Environment* 199 (Supplement C): 358-368. http://dx.doi.org/https://doi.org/10.1016/j.agee.2014.10.004.
Ryan, M. G. 1991. Effects of climate change on plant respiration. *Ecological Applications* 1 (2): 157-167.
Sandkvist, A., M. Hagelberg, and B. Mathisen. 1984. Effect of antibiotics and chemotherapeutics on biogas production from piggery waste. Paper read at Bioenergy 84. Proceedings of conference 15-21 June 1984, Göteborg, Sweden. Vol. III. Biomass conversion.
Sanz, J. L., N. Rodríguez, and R. Amils. 1996. The action of antibiotics on the anaerobic digestion process. *Applied Microbiology and Biotechnology* 46 (5): 587-592. http://dx.doi.org/10.1007/s002530050865.
Sawamoto, T., M. Nakamura, K. Nekomoto, S. Hoshiba, K. Minato, M. Nakayama, and T. Osada. 2016. The cumulative methane production from dairy cattle slurry can be explained by its volatile solid, temperature and length of storage. *Animal Science Journal* 87 (6): 827-834. http://dx.doi.org/10.1111/asj.12495.
Shah, G., G. Shah, J. Groot, O. Oenema, A. Raza, and E. Lantinga. 2016. Effect of storage conditions on losses and crop utilization of nitrogen from solid cattle manure. *The Journal of Agricultural Science* 154 (1): 58-71.
Shah, S., G. Grabow, and P. Westerman. 2006. Ammonia adsorption in five types of flexible tubing materials. *Applied Engineering in Agriculture* 22 (6): 919-923.
Smith, D. L., A. D. Harris, J. A. Johnson, E. K. Silbergeld, and J. G. Morris. 2002. Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria. *Proceedings of the National Academy of Sciences of the United States of America* 99 (9): 6434-6439. http://dx.doi.org/10.1073/pnas.082188899.
Smithson, P. A. 2002. IPCC, 2001: climate change 2001: the scientific basis. Contribution of Working Group 1 to the Third Assessment Report of the Intergovernmental Panel on Climate Change, edited by JT Houghton, Y. Ding, DJ Griggs, M. Noguer, PJ van der Linden, X. Dai, K. Maskell and CA Johnson (eds). Cambridge University Press, Cambridge, UK, and New York, USA, 2001. No. of pages: 881. Price£ 34.95, US $49.95, ISBN 0-521-01495-6 (paperback).£ 90.00, US $130.00, ISBN 0-521-80767-0 (hardback). *International Journal of Climatology* 22 (9): 1144-1144.
Sommer, S. G., E. Friis, A. Bach, and J. K. Schjørring. 1997. Ammonia volatilization from pig slurry applied with trail hoses or broadspread to winter wheat: effects of crop developmental stage, microclimate, and leaf ammonia absorption. *Journal of Environmental Quality* 26 (4): 1153-1160.
Sommer, S. G., and N. J. Hutchings. 2001. Ammonia emission from field applied manure and its reduction—invited paper. *European Journal of Agronomy* 15 (1): 1-15. http://dx.doi.org/https://doi.org/10.1016/S1161-0301(01)00112-5.
Sommer, S. G., and J. E. Olesen. 1991. EFFECTS OF DRY-MATTER CONTENT AND TEMPERATURE ON AMMONIA LOSS FROM SURFACE-APPLIED CATTLE SLURRY. *Journal of Environmental Quality* 20 (3): 679-683.
Sommer, S. G., and R. Sherlock. 1996. pH and buffer component dynamics in the surface layers of animal slurries. *The Journal of Agricultural Science* 127 (1): 109-116.
Speksnijder, D. C., D. J. Mevius, C. J. M. Bruschke, and J. A. Wagenaar. 2015. Reduction of Veterinary Antimicrobial Use in the Netherlands. The Dutch Success Model. *Zoonoses and Public Health* 62: 79-87. http://dx.doi.org/10.1111/zph.12167.
Steinfeld, H., and T. Wassenaar. 2007. The role of livestock production in carbon and nitrogen cycles. *Annu. Rev. Environ. Resour.* 32: 271-294.
Sutton, M. A., J. W. Erisman, F. Dentener, and D. Moller. 2008. Ammonia in the environment: From ancient times to the present. *Environmental Pollution* 156 (3): 583-604. http://dx.doi.org/10.1016/j.envpol.2008.03.013.
Sutton, M. A., J. K. Schjorring, G. P. Wyers, J. H. Duyzer, P. Ineson, and D. S. Powlson. 1995. Plant-Atmosphere Exchange of Ammonia [and Discussion]. *Philosophical Transactions: Physical Sciences and Engineering* 351 (1696): 261-278.
Thiele-Bruhn, S. 2003. Pharmaceutical antibiotic compounds in soils - a review. *Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde* 166 (2): 145-167. http://dx.doi.org/10.1002/jpln.200390023.
Thornton, B., P. Millard, E. I. Duff, and S. T. Buckland. 1993. The relative contribution of remobilization and root uptake in supplying nitrogen after defoliation for regrowth of laminae in four grass species. *New Phytologist* 124 (4): 689-694. http://dx.doi.org/10.1111/j.1469-8137.1993.tb03859.x.
Uchida, Y., T. J. Clough, F. M. Kelliher, J. E. Hunt, and R. R. Sherlock. 2011. Effects of bovine urine, plants and temperature on N2O and CO2 emissions from a sub-tropical soil. *Plant and Soil* 345 (1-2): 171-186. http://dx.doi.org/10.1007/s11104-011-0769-z.
Van Boeckel, T. P., C. Brower, M. Gilbert, B. T. Grenfell, S. A. Levin, T. P. Robinson, A. Teillant, and R. Laxminarayan. 2015. Global trends in antimicrobial use in food animals. *Proceedings of the National Academy of Sciences of the United States of America* 112 (18): 5649-5654. http://dx.doi.org/10.1073/pnas.1503141112.
Van der Krift, T., and F. Berendse. 2002. Root life spans of four grass species from habitats differing in nutrient availability. *Functional Ecology* 16 (2): 198-203.

Velthof, G. L., P. J. Kuikman, and O. Oenema. 2003. Nitrous oxide emission from animal manures applied to soil under controlled conditions. *Biology and Fertility of Soils* 37 (4): 221-230.
Zhao, L., Y. H. Dong, and H. Wang. 2010. Residues of veterinary antibiotics in manures from feedlot livestock in eight provinces of China. *Science of the Total Environment* 408 (5): 1069-1075.

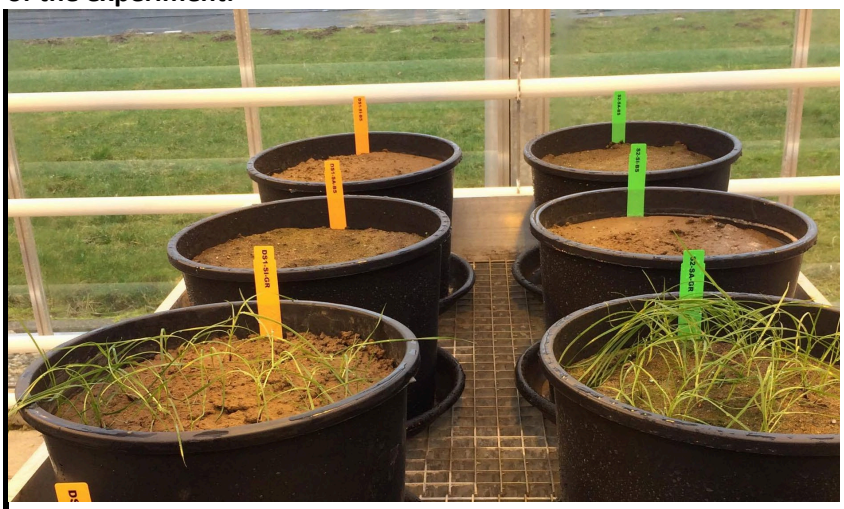
Appendices

Appendix 1: The greenhouse set up with respect to soil type, slurry treatment and pot type.


Soil Type
Sand Pot
Silt-loam Pot
Slurry treatment
200 mg/L Doxcycline
10 mg/L Doxcycline
0 mg/L Doxcycline

Appendix 2. Photos from the experiment in the greenhouse with reference to different developments and occurrences over the full course of the experiment.

Picture 1. The surface caking of the silt loam soil in between watering.



Picture 2. The slower emergence and initial development of the silt loam soil (left) compared to the sand soil (right).

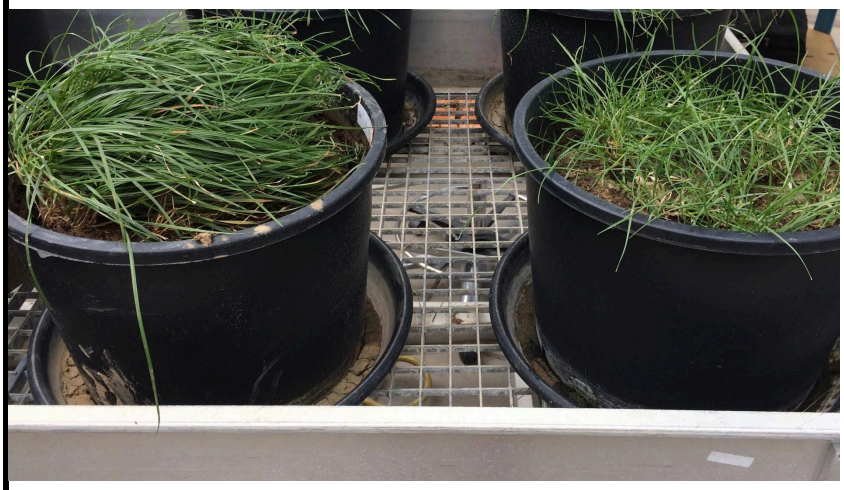
Picture 3. Weed Emergence in the sand soil before they were removed.

Picture 4. The relative visual color, development (flowering) and growth difference of the silt loam (left) and sand (right) pots just before the first cut.

Picture 5. The slurry application on the Bare soil Pots.

Picture 6. The slurry application on grass pots. Some of the slurry that was poured on the grass did not fall through with watering and was manually loosened and put beneath the stems after it had dried.

Picture 7. The Drying out and caking of the silt loam Bare soil Pots after man



Picture 8. The relative visual color, development and growth difference of the silt loam (left) and sand (right) Pots before the final Harvest. Note the lodging of the grass in the silt loam Pot.

Picture 9. The fine root growth on the surface into the slurry application of the silt loam Pots.

Picture 10. The washing of the roots after the final harvest.

Appendix 3: The relative sampling schedule of the gas emission measurements for the different pots.
The first number is the relative Pot number, thereafter the slurry treatment (O, 10, 200 mg/L Doxycycline or No slurry (NS)) followed by pot type (BS=Bares soil, GR=grass).