Nitrous Oxide-Novel Mitigation Methodologies

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Nitrous Oxide-Novel Mitigation Methodologies:
Objective 1 - Hippuric Acid effects on N_2O emissions.

to

Ministry of Agriculture and Forestry

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Prepared by

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IDENTIFICATION

Project Code	**CC MAF POL_2008-36**
Project Title	**Nitrous Oxide-Novel Mitigation Methodologies**
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Institutions	**Lincoln University & Agresearch**

Executive Summary

- Increasing the concentration of hippuric acid in the applied urine did NOT reduce the emissions of N_2O from the urine patches with emissions accounting for 1.28 to 1.65 % of the N applied. This was despite 3 previous studies demonstrating significant reductions.
- Hippuric acid treatments appeared to influence soil NO_2^--N concentrations (the gate way for N_2O production) but not definitive conclusions could be drawn.
- The application of a nitrification inhibitor, dicyandiamide, did suppress N_2O emissions with an N_2O emission factor equal to 0.6% of N applied. This was due to the prolonged occurrence of higher soil ammonium and lower soil nitrate levels.
- Microbial analyses identified changes in community composition in terms of nitrite oxidisers (nitrifiers; *nxr*A genes) particularly under the DCD treatment and the benzoic acid treatment. While nitrite reducers (denitrifiers; nirS gene) showed no differences over time or treatment.
- This work has highlighted gaps in our knowledge with regard to the movement, fate and longevity of hippuric acid and its break down products when applied in a pasture situation.
- Further work must be performed to examine the movement and fate of isotopically labelled hippuric acid over time. This will clarify if hippuric acid has any further potential as a novel N_2O mitigation methodology.

Goals & Rationale

Goal:

To identify novel mitigation strategies for reducing nitrous oxide (N_2O) emissions from ruminant urine patches AND to identify the microbial N_2O emission pathway affected by the mitigation strategy.

Rationale:

Information gap - What role does urine composition and its interaction with the soil physical and chemical environment play in the subsequent microbiological nitrogen (N) transformations that produce N_2O emissions?

Microorganisms are known to play a vital role in soil N cycling. Soil amendments designed to inhibit or alter N dynamics therefore are likely to function by changing the microbial composition and/or activity in affected soils. Such changes may be desired, for example mitigation of nitrous oxide emissions by inhibition of bacterial ammonia oxidation. Knowledge of the timing and duration of management practice effects on soil microbes is important for correct and meaningful interpretation of outcomes.

Reasons for the gap - There is a shortfall in our knowledge on what compounds are in ruminant urine and their relative concentrations, individual compounds effect(s) on soil microbiology and how these compounds react with soil constituents. Researchers are well aware that urinary-N is dominated by urea and that this is the most significant source of N_2O, which is produced via microbial pathways. There is sparse information on the compounds to be found in ruminant urine (e.g. hippuric acid) and their individual effects on N_2O emissions.

In the context of known research:

- Increasing the hippuric acid concentration in artificial urine has been shown to significantly decrease the average N_2O flux by 54% in a sandy pasture soil and it has been hypothesised that hippuric acid can inhibit denitrification (Groenigen et al. (2006)[1].
- When four varieties of artificial urine were applied to a sandy soil, increasing hippuric acid concentration resulted in a significant decline in the average N_2O flux Kool et al. (2006)[2]. It was hypothesized that the breakdown product, benzoic acid, either inhibited denitrification or decreased the N_2O/N_2 ratio.
- A study, recently completed in our laboratory at Lincoln University, using repacked soil cores and real urine collected from milking cows (Bertram et al. 2007)[3] found that cumulative N_2O emissions were reduced by 70% when the hippuric acid concentration was amended, from that of the collected urine, to the upper limit recorded in dairy cow urine at the Lincoln University dairy farm (Prof. Dewhurst, pers. comm.).
- The hippuric acid in ruminant urine is diet dependent and could potentially be manipulated.

[1] Groenigen J.W. van, Palermo V, Kool D.M, Kuikman P.J. (2006) Inhibition of denitrification and N_2O emission by urine-derived benzoic and hippuric acid. *Soil-Biology & Biochemistry* 38(8): 2499-2502

[2] Kool D.M, Hoffland E, Hummelink E.W.J, Groenigen J.W. van (2006). Increased hippuric acid content of urine can reduce soil N_2O fluxes. *Soil Biology & Biochemistry* 38(5): 1021-1027

[3] Bertram J.E., Clough T.J., Sherlock R.R., Condron L, O'Callaghan, M. *Submitted to Global Change Biology.*

2. Objectives

2.1 Aims:

(i) To compare the N_2O emissions (and the subsequent emission factors (EF)) of bovine urine with varying levels of hippuric acid applied to pasture.
(ii) To determine how hippuric acid 'ranks' with other known N_2O mitigation compounds.
(iii) To establish what microbial N_2O emitting pathway is affected by hippuric acid.

2.2 Outcomes:

(i) First field assessment of hippuric acid as an N_2O mitigation option from real urine.
(ii) First determination of hippuric acid's mode of N_2O flux inhibition at the microbiological level in correlation with measured gas fluxes.
(iii) A ranking of hippuric acid against a known urinary N_2O mitigation compound.
(iv) Potentially a new research pathway for N_2O mitigation.
(v) At least one manuscript to be submitted for international publication in a peer reviewed journal.

3. Approach

3.1 Field site and chamber design

In April 2008 a short term field trial was commenced at a field site situated at Lincoln University, on a perennial ryegrass (*Lolium perenne*)/white clover(*Trifolium repens*) pasture. Soil fertility test results for this site are shown in Appendix 1 and discussed below.

In order to monitor the N_2O gas fluxes stainless steel chambers were constructed and installed at the field site one week prior to treatment applications. Chambers were 38.5 cm in diameter giving a surface area of 1164 cm^2 (1.16 x 10^{-5} ha). The walls of the chambers protruded 9.5 cm into the soil surface. A headspace cover was used to form the chamber headspace. This headspace cover was also made of stainless steel and insulated with polystyrene foam to avoid temperature perturbations during gas flux measurements. A 'water trough' seal was used to prevent gas leakage during headspace cover use.

3.2 Treatments

Urine was collected from the Lincoln University dairy farm (43° 38.522S, 172° 26.450E Lat./Lon.) from cows grazing ryegrass/white clover pasture. The urine collected had nitrogen (N) and hippuric acid (HA) contents of 8.83 g N L^{-1} and 56.3 mMol L^{-1} respectively. The N content was raised to 10 g N L^{-1} by adding urea to the collected urine. This additional urea was either at natural abundance or enriched in ^{15}N, as discussed below, and was added to raise the N content of the urine to the upper bounds of that found during bovine urinary-N deposition.

Six treatments (replicated four times, giving a total of 24 plots) were applied on the 2nd of April 2008 and consisted of the following:

i) Control (nil urine) consisting of water only (Control),
ii) Urine with the hippuric acid concentration as collected 56 mMol L^{-1} (HA1),

iii) Urine with the hippuric acid concentration increased to 73 mMol L^{-1} (HA2),
iv) Urine with the hippuric acid concentration increased to 90 mMol L^{-1} (HA3),
v) Urine with the hippuric acid concentration as collected 56 mMol L^{-1} but with DCD added to the urine at 10 kg ha^{-1} (DCD)
vi) Urine with the hippuric acid concentration as collected 56 mMol L^{-1} but with additional benzoic acid added 33.7 mMol L^{-1} (BA)

The DCD (dicyandiamide) treatment constituted the known nitrification inhibitor (DCD) and provided a bench mark with which to assess any changes in N_2O emissions as a result of hippuric acid amendment. The addition of benzoic acid was performed because hippuric acid is believed to break down into glycine and benzoic acid, and it is the benzoic acid that is thought to be the antimicrobial agent. Treatments HA1 and HA3 had the additional urea added, ^{15}N enriched, so that the resulting urinary-N had ^{15}N enrichments of 1.757 and 1.778 atom % ^{15}N respectively. Thus the effect of hippuric acid addition, if any, on N_2O emissions resulting from the urea-N fraction of the urine could be determined.

One litre of the urine treatments were applied to each chamber thus the rate of urinary-N application was equivalent to 860 kg N ha^{-1}.

3.3 The molecular approach

Molecular analysis of soil microbes, e.g. increases or decreases in functional population size, gene expression, etc., assists in identification of soil microorganisms potentially responsible for observed soil N dynamics. In particular, these analyses can identify which, if any, subpopulations of nitrogen-cycling microbes respond to particular soil amendments and/or management regimes.

Soil NA extracts contain all the DNA and RNA present in soil, including that of plant, fungal, protozoan, bacterial and archaeal origin. In order to obtain information about microorganisms involved in soil nitrogen cycling, we used molecular techniques that targeted two specific subpopulations of nitrogen-cycling bacteria: nitrite oxidizers and nitrite reducers. These two groups were assayed by measuring the number and diversity of *nxrA* (nitrite oxidoreductase) and *nirS* (nitrite reductase) genes, respectively.

To determine community diversity of these bacteria, we employed denaturing gradient gel electrophoresis (DGGE) to distinguish DNA sequence diversity of these indicator genes in soil DNA samples. Real-time, or quantitative, PCR (qPCR) was utilised to enumerate gene copy numbers in soil samples, indicative of total population size. Changes in either community structure (DGGE) or community size (qPCR) were traced for both genes, and used to compare the effects of the different soil treatments on these populations over time. Once this information was obtained, it could be directly compared to the gas flux and soil nitrogen data to determine if any correlations exist between microbial community and soil N dynamics.

3.3.1 Nitrite oxidizers, nxrA gene

Nitrite oxidation comprises the 2nd half of soil microbial nitrification, with nitrite oxidizing bacteria utilising NO_2^- generated by ammonia oxidizers as a reducing equivalent, thereby generating the more oxidized NO_3^-. Bacterial capable of nitrite oxidation are phylogenetically diverse and have representative members in the alpha- and gamma-

proteobacteria. Little is known about the diversity or importance of nitrite oxidizers in agricultural soils, and nothing is known about their occurrence or importance in New Zealand agricultural soils. We therefore sought to investigate this particular subpopulation of nitrogen cycling bacteria, whose real contribution to soil nitrogen turnover and greenhouse gas emission in New Zealand pasture systems is unknown. Previous studies of German agricultural soil (Wertz et al, 2008; Poly et al, 2008) demonstrated that the predominant groups of nitrite oxidizers in the soils examined were formed by *Nitrobacter* and *Nitrospira* sp. *Nitrobacter* sp. are thought to be more tolerant to higher levels of NO_2^- than their *Nitrospira* sp. relatives, and may therefore be more relevant for examination in soils with high N-input (e.g. pasture systems).

3.3.2 Nitrite reducers (denitrifiers), nirS gene

Denitrification is distinct from nitrite respiration in that true denitrifiers are able to reduce nitrite all the way to dinitrogen gas. By-products of incomplete denitrification, however, include the greenhouse gases nitric oxide (NO) and nitrous oxide (N_2O). Denitrification is essentially anaerobic respiration in which NO_2^- is used as a terminal electron acceptor instead of oxygen (O_2). Denitrifiers are typically facultative anaerobes, using O_2 when possible but switching to denitrification when O_2 becomes scarce. Denitrification therefore occurs mainly in water-logged soils, or those soils with high water content (i.e. low O_2 diffusion). Precipitation is thus known to play a vital role in determining the rate of denitrification in soil. Bacterial nitrite reducers occur in two known flavours, those containing the *nirS* gene and those containing the *nirK* gene. In this study we chose to examine *nirS* denitrifiers as this gene is thought to have a greater phylogenetic range than *nirK*, and little is known about the occurrence or abundance of denitrifying bacteria in New Zealand pasture systems.

4. Measurements

4.1 Nitrous Oxide flux determinations and ^{15}N enrichments

Nitrous oxide determinations were made on 28 occasions over the 78 days of the study. These were achieved by filling the water trough, surrounding the chamber, with water and gently lowering the headspace chamber onto the base, so that it's base sat in the water trough and a gas-tight seal was achieved. Then a rubber bung was inserted into a 20 mm diameter hole in the chamber surface. The purpose of this hole was to avoid headspace gas pressure fluctuations as the chamber was lowered onto the base and thus avoid perturbing the potential N_2O fluxes. Gas samples were taken of ambient air and from the headspace chambers at 15 and 30 minute intervals. To take the gas samples a glass syringe, equipped with a 3-way stopcock and 0.5 mm needle, was used. The syringe was flushed with ambient air and then injected into the chamber headspace where upon the syringe was flushed twice and a 10 mL gas sample taken and injected into a pre-evacuated Exetainer® (6 mL volume).

Gas samples were analysed using a gas chromatograph (8610, SRI Instruments, CA.) interfaced to a liquid autosampler (Gilson 222XL, Middleton, WI.). The autosampler had been specially modified for gas analysis by substituting a purpose-built (PDZ-Europa, Crewe, UK) double concentric injection needle for the usual liquid level detector and needle. This enabled the entire gas sample to be flushed rapidly from its septum-sealed container (6 mL Exetainer®) into the GC.

The GC configuration was similar to that used by Mosier and Mack (1980) and included two 0.3 cm OD stainless steel columns packed with Haysep Q connected in series, oxygen-free dry nitrogen carrier gas (40 mL min^{-1}), and a ^{63}Ni electron capture detector at 320^{o}C. Gas samples were analysed within 1 to 2 days of sampling. Immediately prior to analysis the over-pressurised samples were all brought to ambient atmospheric pressure, using a double-ended hypodermic needle. One end of the needle was placed at a constant depth (0.5 cm) just below the surface of some water in a small beaker while the other end pierced the Exetainer® septum. A brief flow of bubbles resulted and when these ceased, the gas in the Exetainer® was at ambient air pressure. Dissipating the excess gas pressure through the water medium not only gave a visual indication of when the samples were at ambient air pressure, it also avoided any potential contamination of the sample with ambient air. Reference gases were prepared following the same over pressure-equilibration procedure as described above.

Gas samples were also taken in a similar manner, after the headspace chambers had been in position for 1 hour, for mass spectrometer analysis of the ^{15}N enrichment of the N_2O in the controls, HA1 and HA3 treatments.

4.2 Soil inorganic-N determinations

In addition to the headspace chambers a soil sampling plot was set up immediately adjacent next to each headspace chamber (50 x 50 cm) so that soil samples could be taken over time with out perturbing the soil within the chambers. These soil plots had identical treatments and management as the headspace chamber plots. Soil samples for inorganic-N determination were taken using a 2.5 cm diameter x 7.5 cm long soil corer. Three cores were taken on each sampling occasion.

4.3 Soil pH

Soil pH on the surface of the plots was determined following treatment application using a flat surface pH electrode.

4.4 Meteorological Data

Micrometoeroloigical data were obtained from a nearby site (3 km away). Soil and air temperatures, and rainfall data were gathered.

4.5 Soil bulk density

Soil bulk densities were determined by taking a 5.4 cm diameter by 10 cm long (229 cm^3) soil core from beside each chamber at the completion of the study immediately adjacent to the soil sampling plots in areas where no treading had occurred.

4.6 Dry matter yields

Dry matter yields were taken by hand harvesting the plots at a height of 5 cm, bagging the sample, and then drying at 70^{o}C for 48 hours.

4.7 Microbial analyses

4.7.1 Nucleic acid extractions from soil

Soil samples for microbial analysis were taken immediately after urine or water application (day 0) and on days 1, 2, 3, 4, 8, 13, 20, 27 and 48. Three soil cores (7.5 cm depth x 1.5 cm radius) were mixed in a plastic bag, and duplicate 1g soil samples were aseptically taken and immediately snap-frozen in liquid nitrogen, within 30 minutes after cores were taken. Samples were then stored at -80°C until nucleic acid (NA) extraction. In total, 4 replicates of 6 treatments at 10 sampling times (240 samples) were available for analysis. Nucleic acid extractions were performed on one of each duplicate sample using the method of Griffiths et al. (2000). Replicates were kept together as much as possible to avoid inter-replicate effects during extraction. Crude NA extracts were stored at -80°C and thawed as few times as possible to avoid freeze-thaw degradation of NA in each sample. 17 ul of each sample was removed for DNAse treatment, or to yield purified total RNA. These RNA samples are currently stored at -80°C pending further processing for DGGE and qPCR analysis.

4.7.2 DGGE

DNA primers F2842 F1 nxrAgc (with a 40 bp GC-clamp at the 5'-end) and F2843 R2 nxrA (Poly et al, 2008) were used to amplify *Nitrobacter*-like *nxrA* sequences from soil for DGGE analysis. All 240 soil samples yielded detectable amplification of a correct size fragment (362 bp). Approximately equal amounts of PCR product were analysed on 7% polyacrylamide (PA) gels with a denaturing gradient from 28-53% (Muyzer et al, 1993). DGGE was performed for 17 hours at 70 V and 60°C with a CBS DGGEK-4001-110 mutation detection system (CBS Scientific, Del Mar, CA)

For *nirS*, primers cd3af and R3cd[GC] (with a 33 bp GC-clamp at the 5'-end) (Throbäck et al, 2004) were used to amplify *nirS* sequences from soil NA samples. Approximately equal amounts of PCR product were analysed on 7% PA gels with a denaturing gradient of 40-60%, at 60°C and 80 V for 17 hours.

Gels were stained with silver nitrate (Merrill et al, 1981), preserved, dried, and scanned into digital images using a Bio-Rad G-800 calibrated densitometer and Quantity One software (both Bio-Rad, Hercules, California). Presence or absence of bands was assessed using Diversity Database (Bio-Rad), data from which were converted into text files for statistical analysis of relatedness using GenStat v.11 (VSN International, Hemel Hempstead, UK) for principle component analysis.

4.7.3 Real-Time PCR

Dilutions, 10^{-1}, of soil crude NA extracts were prepared in 20 µl volumes using DEPC-treated water (Ambion/Applied Biosystems, Austin, Texas) in UV-treated 0.2 ml PCR tubes. Samples were vortexed briefly to ensure mixing. 1ul of each dilution was used as template in 20 µl qPCR reactions employing the SYBR Green chemistry for detection. Standard curves were generated using a 10-fold dilution series of the pCR2.1-TOPO vector (Invitrogen Corporation, Carlsbad, California) containing a cloned *nxrA* gene fragment from a previous laboratory experiment with Wakanui silt loam soil. For *nirS*, a 10-fold dilution series of the pGEM T Easy vector (Promega Corporation, Madison, Wisconsin) containing a cloned *nirS* fragment from the same Wakanui silt loam was used to generate a standard curve for qPCR.

No template controls (NTC) and standard curves were performed in duplicate. 10^{-1} dilutions of DNA extracted from extra field trial soil samples was used as an internal control between replicate plates, with *nxrA*, *nirS* and archaeal *amoA* (Wuchter et al, 2006) primers. 10 random soil samples per replicate were amplified in duplicate, to test reproducibility of qPCR results for individual samples. Standard SYBR Green thermal programmes for the Bio-Rad iQ5 real-time thermocycler (Bio-Rad) were used for detection and melt curve analysis of products, with annealing temperatures of 57.5°C for *nxrA* and 57.1°C for *nirS*. 20 random samples were analyzed by agarose gel electrophoresis after qPCR to verify purity and specificity of reaction products. qPCR results were first $\log_{10}$-transformed to give a normal distribution, then analysed by two-way ANOVA using MiniTab15 (MiniTab Inc., State College, Pennsylvania), with $\log_{10}$ copy number as response factor and treatment and sampling day as variables.

4.8 Statistical anaylses

Statistics included one-way analysis of variance with urine treatment as a factor. Due to the skewed nature of the N_2O flux data statistical analyses of the N_2O fluxes were performed on log-transformed data (ln flux + 1). Principal component analysis was used to assess trends in microbial populations.

5. Results

5.1 Nitrous oxide fluxes, emission factors, and N_2O-^{15}N enrichments

5.1.1 N_2O fluxes

Nitrous oxide fluxes (μg m^{-2} h^{-1}) were lowest in the control treatment, ranging from 1 to 386 μg m^{-2} h^{-1}, and these were significantly lower ($P<0.01$) than fluxes in the other treatments on almost all occasions except for days 8, 10, 23, 29, 36, 45, and 48 when there was no statistical difference between the control N_2O fluxes and those from the DCD treatments (Figure 1a, 1b). These were periods of relatively low fluxes. Over the interval day 65 to 78 the control N_2O flux was not significantly different from any treatment. Fluxes in the DCD treatment ranged from 17 to 2967 μg m^{-2} h^{-1} and they were significantly lower ($P <0.01$) than the N_2O fluxes from the other applied urine treatments for the majority of the gas sampling times up until day 48 when the N_2O fluxes from the DCD treatment did not differ from the other urine affected treatments (Figure 1a, 1b). The N_2O fluxes from the benzoic acid treatment ranged from 55 to 9690 μg m^{-2} h^{-1} and these were generally higher than the DCD N_2O fluxes as noted above and they did not differ from the hippuric acid flux treatments on any occasion, except for day 2 when the benzoic acid N_2O fluxes (mean 238 μg m^{-2} h^{-1}) were lower ($P <0.01$) than in the HA1 treatment (mean 485 μg m^{-2} h^{-1}). In the hippuric acid treatments the N_2O fluxes ranged from 53 to 5890, 79 to 7987, and 92 to 6499, for the HA1, HA2 and HA3 treatments respectively. There was only one sampling date when fluxes from these three hippuric acid treatments differed from each other ($P <0.01$) and this was day 2 where there was a trend for the N_2O flux to decrease with increasing levels of hippuric acid addition. For the HA1, HA2 and HA3 treatments the N_2O fluxes on day 2 were 488, 364 and 290 μg m^{-2} h^{-1} respectively.

5.1.2 N_2O emission factors

As a percentage of the urine-N applied the N_2O-N fluxes over 78 days equated to 0.60(0.10), 1.65(0.26), 1.30(0.17), 1.50(0.28), and 1.28(0.09)% for the DCD, benzoic acid, HA1, HA2, and HA3 treatments respectively (s.e.m in brackets) as shown in Figure 2. This calculation is commonly termed the emission factor (EF). The EF of the DCD treatment was significantly ($P<0.05$) lower than that of the benzoic acid and HA2 treatments but did not differ significantly from the HA1 and HA3 treatments. There was no difference between treatments in terms of the EF when the DCD treatment was omitted from the analysis.

5.1.3 N_2O flux ^{15}N enrichments

The ^{15}N enrichment of the N_2O flux from the control, HA1 and HA3 treatments ranged from 0.324 to 0.484, 0.495 to 1.551, and 0.404 to 1.582 atom% ^{15}N respectively. As would be expected the ^{15}N enriched treatments had higher N_2O enrichments compared with the control. There were no significant differences between the enrichments of the N_2O from the HA1 and HA3 treatments on any sampling date. The N_2O ^{15}N enrichments from these treatments were lower than the original ^{15}N enrichment of the HA1 and HA3 treatments applied (1.757 and 1.778 atom % ^{15}N respectively) indicating that a fraction of the N_2O originated from unlabelled components of the urine or nitrogen in the pasture soil. The latter is more likely given the duration of the enrichment (Figure 3). There was, however, no effect on N_2O ^{15}N enrichment and thus N_2O source, due to increasing the hippuric acid concentration in the urine applied.

Figure 1. N_2O-N flux over time non-transformed with associated rainfall events (1a) and log transformed N_2O-N flux (1b) with error bars ± s.e.m.

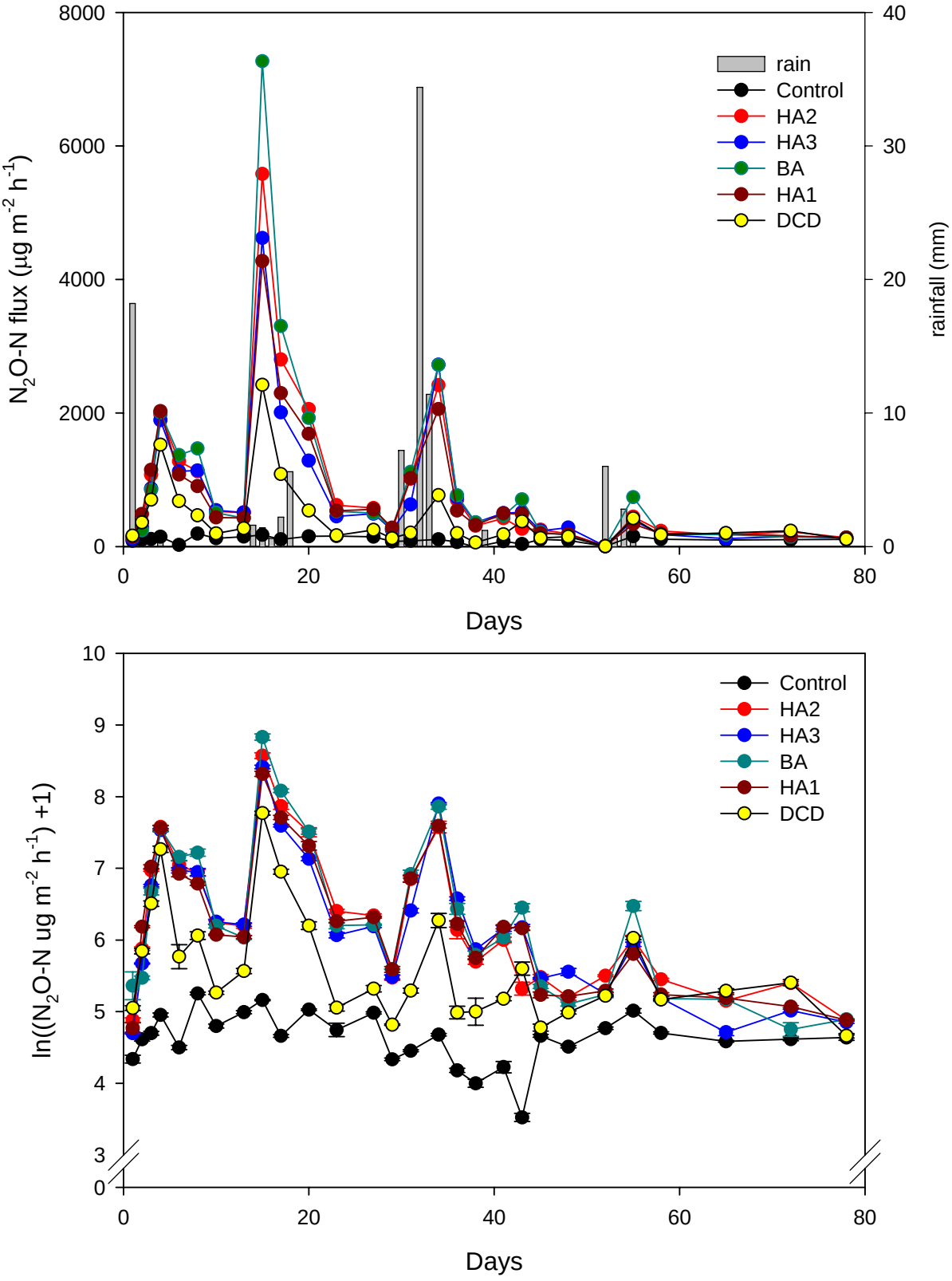


Figure 2. N_2O emission factors from urine treatments applied (Error bars are one s.e.m, $n = 4$).

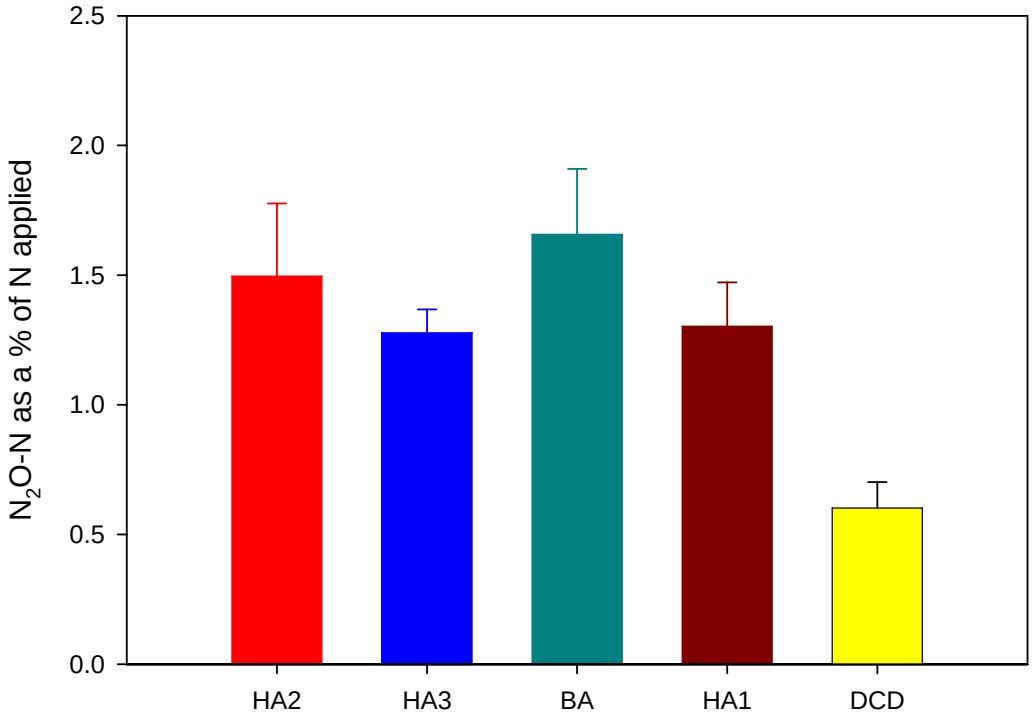
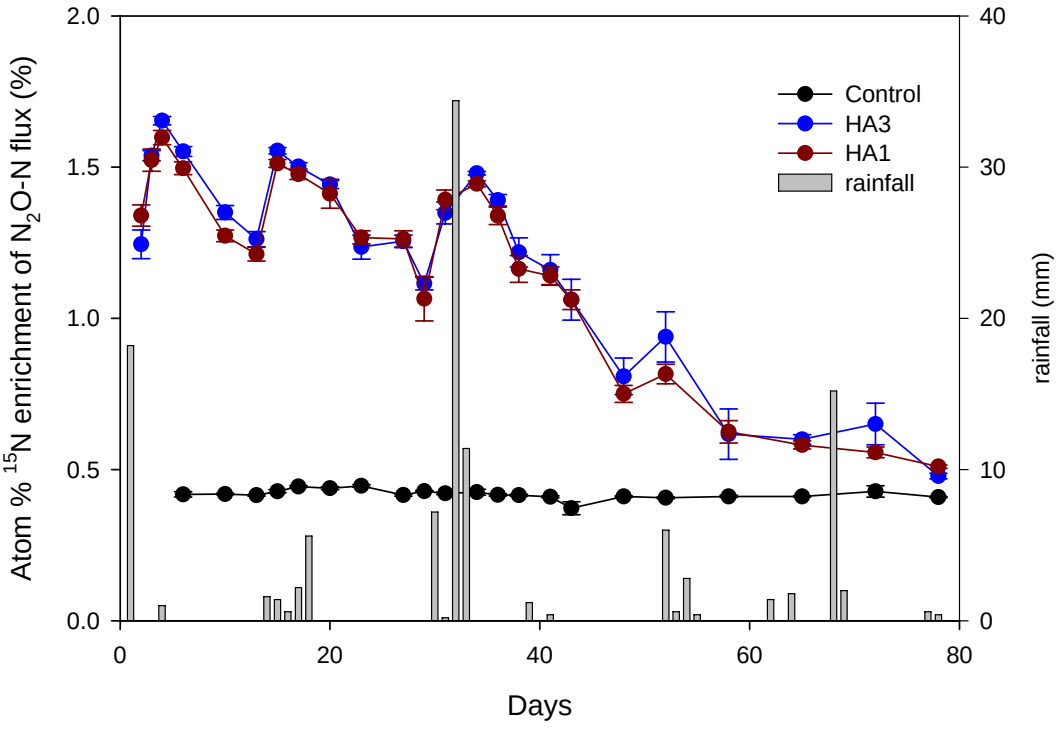


Figure 3. N_2O-N ^{15}N enrichment of the control, HA1 and HA3 ^{15}N enriched treatments (Error bars are one s.e.m, $n = 4$) and the rainfall over the experimental period.



5.2 Soil Inorganic-N fluxes

5.2.1 Soil ammonium concentrations

Soil ammonium-N (NH_4^+-N) concentrations were lower in the control compared to the urine treatments until day 27 (Figure 4a) and mean concentrations ranged from <1 to 31 μg g^{-1} dry soil. Soil NH_4^+-N concentrations increased almost immediately the urine treatments were applied with mean concentrations reaching a maximum of 357 μg g^{-1} dry soil on day 3. There were no statistically significant differences in soil NH_4^+-N concentrations due to urine treatment until day 20 where upon the DCD treatment had higher ($P < 0.01$) soil NH_4^+-N concentrations. This trend continued until the end of the experiment, with the exception of day 35. Meanwhile in the benzoic and hippuric acid treatments there were no statistically significant differences in the soil NH_4^+-N concentrations and by day 27 theses were not statistically different from the control treatment (Figure 4a).

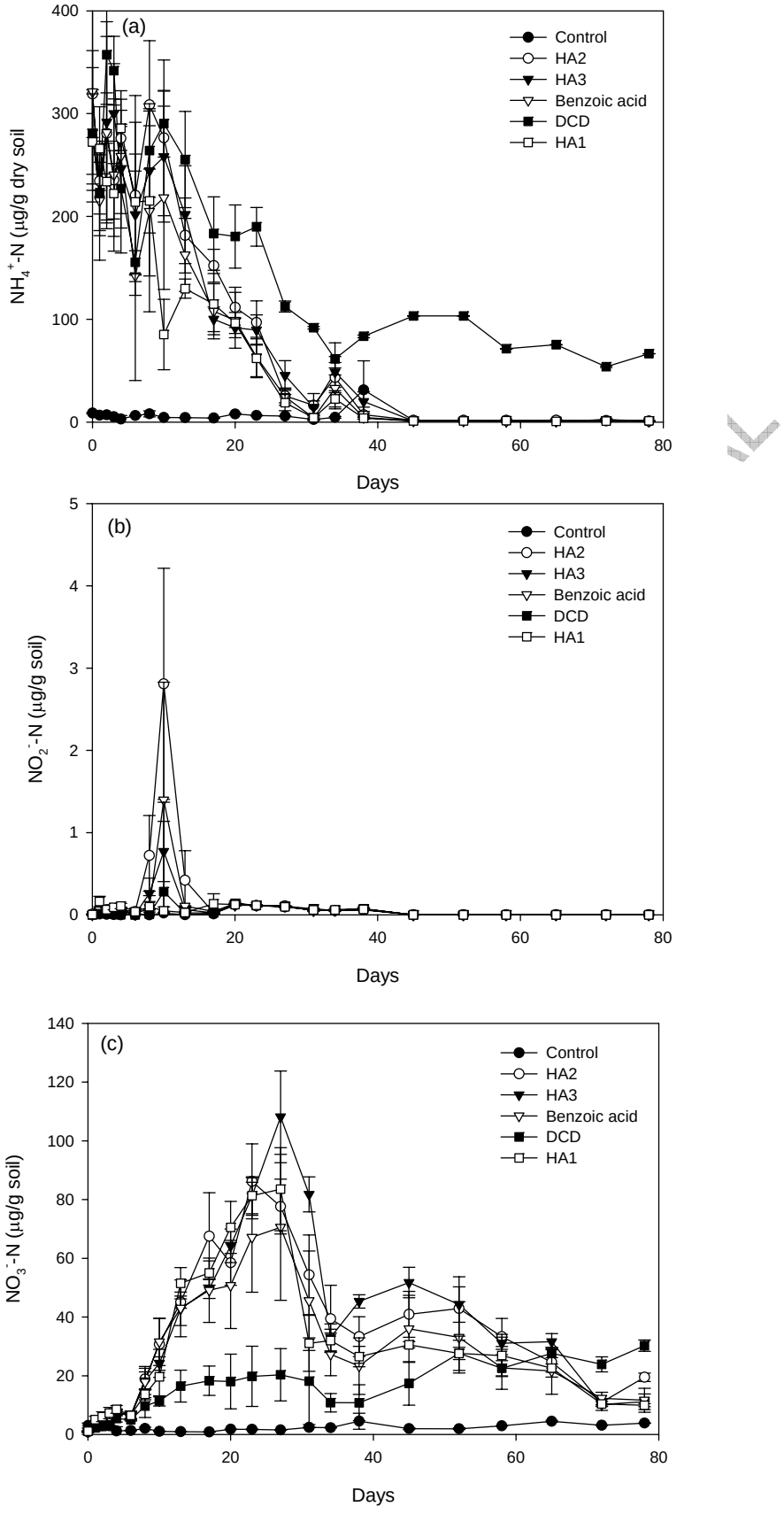
5.2.2 Soil nitrite concentrations

Concentrations of nitrite-N (NO_2^--N) remained low until day 8 where upon they increased, peaking on day 10 and then declining to levels found in the control by day 20. During this time there were no statistically significant differences between treatments (Figure 4b). Despite this on day 10 soil NO_2^--N concentrations differed by orders of magnitude with the HA2, HA3 and HA1 treatments having mean NO_2^--N concentrations of 2.81, 0.77 and 0.07 μg g-1 dry soil ($P = 0.159$).

5.2.3 Soil nitrate concentrations

In the control treatment the nitrate-N (NO_3^--N) concentrations were low (Figure 4c) throughout the measurement period. In all the urine treatments the soil NO_3^--N concentrations increased above that of the control from day 4 onwards and only became comparable to the levels in the control by day 78. The soil NO_3^--N concentrations in the DCD treatment increased the least and remained below 30 μg g^{-1} dry soil throughout the study at concentrations that were significantly lower ($P < 0.01$) than in the other urine treatments until at least day 31 whereupon the soil NO_3^--N concentrations in the other urine treatments had declined to be comparable with those in the DCD treatments. The soil NO_3^--N concentrations gradually declined further in the non-DCD urine treatments while in the DCD treatment the soil NO_3^--N concentrations slowly increased until by day 72 the soil NO_3^--N concentrations were higher ($P < 0.01$) in the DCD treatment (Figure 4c).

Figure 4. Soil inorganic-N concentrations over time.



5.3 Pasture dry matter yields and N uptake

5.3.1 Dry matter yields, percentage N in herbage and N uptake.

Pasture cuts were taken on days 14, 29, and 78. The first two cuts are reported on here as data are still being analysed for cut 3. The DM yields did not differ ($P > 0.05$) between treatments at cut 1 on day 29 with mean yields ranging from 21.4 in the control to between 30.3 to 40.9 g m^{-2} in the urine treatments. At this time the %N in the herbage did not differ statistically between treatments being 4.2% in the control and ranging from 5.0 to 6.0% in the urine treatments. Uptake of N at cut 1 was not different due to treatment ($P = 0.09$) with 0.88 g m^{-2} in the control and a range of 1.52 to 2.03 g m^{-2} in the urine treatments.

At cut 2 the DM yields were all higher ($P < 0.05$) in the urine treatments (14.4 to 18.8 g m^{-2}) than in the control (1.6 g m^{-2}) with no differences between the urine treatments. The same trend occurred with respect to %N at cut two ($P < 0.01$) with the control having 4.7% and the urine treatments ranging from 6.0 to 6.3% with no difference between the treatments. Uptake of N at cut 2 differed due to lower uptake in the control ($P < 0.01$) with 0.88 g m^{-2} in the control and a range of 0.88 to 1.15 g m^{-2} in the other urine treatments with no difference between these.

5.3.2 ^{15}N enrichment of dry matter.

In the control and those treatments where the urine treatments had been enriched with ^{15}N the atom% ^{15}N enrichment of the herbage at cut 1 was 0.3782, 1.1201, and 1.1624 atom % ^{15}N for the control, HA1 and HA3 treatments respectively. At cut 2 these values were 0.3712, 1.3839, and 1.4364 respectively with no statistical differences between the HA1 and HA3 values. The data indicate that at cut 2 more of the N in the herbage had come from the urine-N applied than at cut 1. But at both cuts there was native soil N contributing to the plant N uptake since neither treatment had ^{15}N enrichments equaling the ^{15}N enrichment of the applied urine-N (>1.7 atom % ^{15}N).

5.4 Soil surface pH

The surface pH in the control treatment remained relatively constant throughout the study averaging 6.9. In the urine treatments the soil pH increased reaching maximum mean values of 8.5 with no statistical difference between soil pH values in the urine treated soils through out the study. By day 41 the urine treatment soil pH values were below that of the control ($P<0.01$) but by day 78 there was no difference between the controls and the urine treated soils.

5.5 Climatic conditions

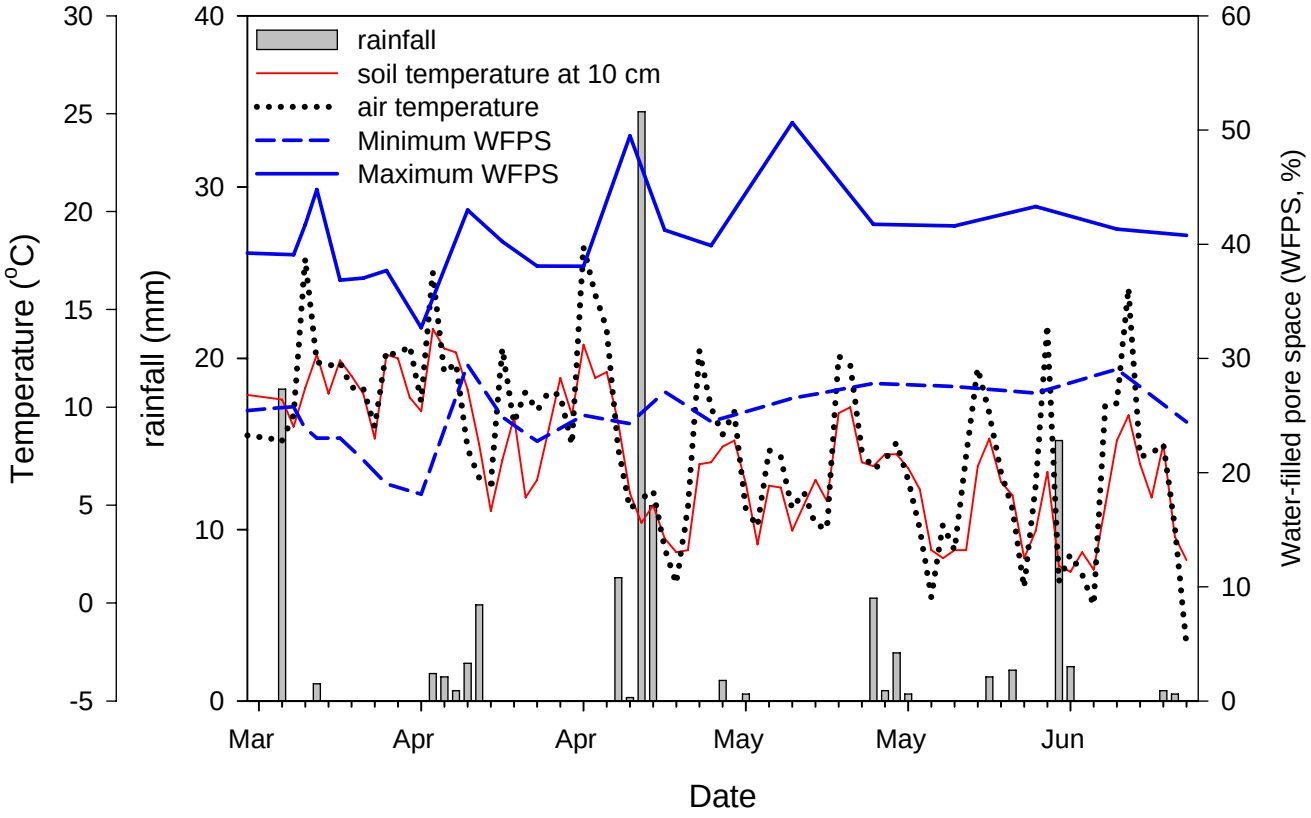
There was a significant rainfall event at the meteorological station approximately 8 hours after the experimental treatments were applied consisting of a thunderstorm and 18 mm of rainfall. This micrometeorological station is 3 km away from the field site. At another rainfall monitored field site 1 km away only 3 mm of rainfall fell. Thus it is not possible to know the precise rainfall at this time. Other rainfall periods were centered about days 16, 34, 40 and day 54 (Figure 6).

Hourly soil temperatures (10 cm depth) at the micrometeorological station decreased over the period of the study, and averaged 8.6°C with minimum and maximum values of 1.2°C and 20.9°C respectively. While air temperatures at the micrometeorological station (09:00 hours) averaged 8.3°C with minimum and maximum values of -2.1°C and 18.2°C respectively (Figure 5).

5.6 Soil moisture conditions

Soil bulk density, a determinant of water-filled pore space (WFPS) did not vary between replicates or treatments averaging 0.93 g cm^{-3} soil (s.e.m 0.01). Thus soil porosity averaged 0.65 cm^3 voids cm^{-3} soil (s.e.m <0.01). Values of water-filled pore space (WFPS) did not vary with treatments and over the entire 78 day period averaged 32.0% (s.e.m 0.2%) with a range of 18 to 51% WFPS (Figure 5).

Figure 5. Rainfall, soil and air temperatures, and water-filled pore space over the 78 day experimental period.



5.7 Microbial results

5.7.1 DGGE

DGGE anaysis of *nxrA* amplicons from the field trial soil samples demonstrated statistically significant treatment effects (Figure 6). For the BA treatment, samples taken at days 1-4 contained *nxrA* communities that were significantly different from the *nxrA* communities present on days 13, 20, 27, and 48. For treatment HA2, day 0 communities were significant different from communities on days 1, 3 and 13+. Day 1 communities were significantly different from those at day 2 and day 20, although the significance of the difference at day 2 is disputable (P=0.049). Day 2 communities demonstrated similarity to day 0 communities, and were significantly different from communities sampled at day 13 and later. Days 3, 4 and 8 communities were significantly different only from Day 20 communities, indicating that Day 20 may represent a temporary community shift that is observable only on that sampling day. While HA2 results were somewhat erratic, results for the HA3 treatment were much more solid, indicating a clear separation between early (before day 13) and later (after day 13) community structures. Interestingly, the same statistically significant separation was observed for the HA1 treatment, although the cut-off for separation was before day 8 in that case. Control communities did not differ significantly from each other or from DCD treated communities at any time point. The only time effect specifically observed was between day 20 communities, at which time HA3 and BA communities differed from control and DCD communities, but not from each other (Figure 7).

Suprisingly, *nirS* communities did not differ between treatments or over time at the 95% confidence level (Figures 8 and 9).

5.7.2 Real-time PCR

Due to inconsistencies with control samples (data not shown), one replicate from each qPCR sample set was excluded from statistical analyses. For *nxrA*, replicate 4 was excluded, while for *nirS* replicate 3 was excluded. Thus the results discussed here are from 3 replicates only.

Due to technical problems associated with absolute enumeration of gene copy numbers from soil samples (Smith et al, 2006), our analyses do not focus on absolute copy numbers. Because controls used to general standard curves for quantitation consisted of pure DNA, any quantitative figures we derive about soil samples would mostly likely be an underestimation of actual numbers. We chose rather to focus on relative difference in copy numbers between treatments. Such analyses provide information on how different soil amendments differentially influence functional bacterial populations. We therefore employed two-way ANOVA analysis of our quantitative data to determine which factors (time and treatment) had significant influence on detectable gene copy numbers.

The *nxrA* analysis of soil samples showed significant time and treatment effects. Copy numbers of *nxrA* per gram of soil (averaged over all treatments) did not differ for days 0 through 8 (Figure 10A). There was a slight increase in *nxrA* copy numbers on day 8 for all treatments, but this difference was not significant at the 95% confidence level. After day 8, however, average *nxrA* copy numbers began to increase, and had increased by about 7-fold by day 20. Already by day 48, average copy number for all treatments began to decrease, suggesting that the N pulse with urine application originally instigated the increase but was unable to maintain it due to N removal by immobilization or denitrification over time.

Because of the duration of this experiment, it is unknown whether numbers would continue to decrease or whether they would stabilize at that level. In any case, a 7-fold increase in gene copy number in response to a urination event is not dramatic, particularly when considering that each nitrite oxidizing bacterium present in soil samples may possess one to three different *nxrA* gene copies in their genome.

To examine whether inclusion of control results in analyses diluted any weak effects, we also performed two-way ANOVA on the *nxrA* qPCR data set without control results (Figure 10B). For days 0, 2, 3, 4 and 8, these results were not different from the complete analysis, with the exception that day 3 *nxrA* copy numbers were significantly lower (2-fold at most) than the numbers on day 8. This may indicate a temporary decrease in urine-exposed nitrite oxidizer populations three days post urine addition, and perhaps indicates a community shift to cope with the urine stress. DGGE results, however, did not confirm this possibility. For days 0, 8, 13, 20 and 27, ANOVA results showed a stronger increase in *nxrA* copy numbers from day 0 to day 20 (an increase of almost 10-fold).

When *nxrA* copy numbers for each treatment were averaged over days 0 through 8, all urine-treated soils possessed higher numbers of *nxrA* genes than the control treatment (Figure 11A). These results were only significant for the two elevated hippuric acid treatments (HA2 and HA3), which contained 2- to 3-fold higher numbers of *nxrA* genes compared to the control. When *nxrA* copy numbers for days 0, 8, 13, 20, 27 and 48 were averaged together for each treatment (Figure 11B), there was a valid difference between the control treatment and the BA, HA3 and HA1 treatments at the 95% confidence level. These differences, while significant, only amounted to a 4- to 6-fold increase in *nxrA* copy numbers relative to the control. The DCD and HA2 treatments had slightly higher numbers of *nxrA* gene copies relative to the control, but these differences were not significant.

Quantitative analysis of *nirS* gene copy numbers in soil samples somewhat corroborated the DGGE results in that no significant time or treatment effects were measured. For days 0 through 8, there appeared to be a slight increase (i.e. ~2-fold) in *nirS* copy numbers when averaged over all treatments (Figure 12A), but this effect was not significant. Nor were significant time effects observed for all treatments averaged over all days (Figure 13A), over days 0, 1, 2, 3, 4, and 8 (Figure 13B), over days 0, 8, 13, 20, 27 and 28 (Figure 13C), or when control qPCR data were excluded from analysis of urine-treated samples (Figure 12B). Interestingly, copy numbers of *nirS* were similarly higher at days 8, 13 and 20, with fewer gene copies detectable at earlier and later sampling points (Figure 12). This difference was at most, however, 2-fold and again not significant. When all time points (both early and late) were averaged for each treatment, there was found no significant variation in *nirS* copy number.

NOTE: In Figures 6 to 13 below, the treatment names described and used above (Control, BA, DCD, HA1, HA2, HA3) correspond to Control, BA, DCD, U, HA1, HA2 respectively.

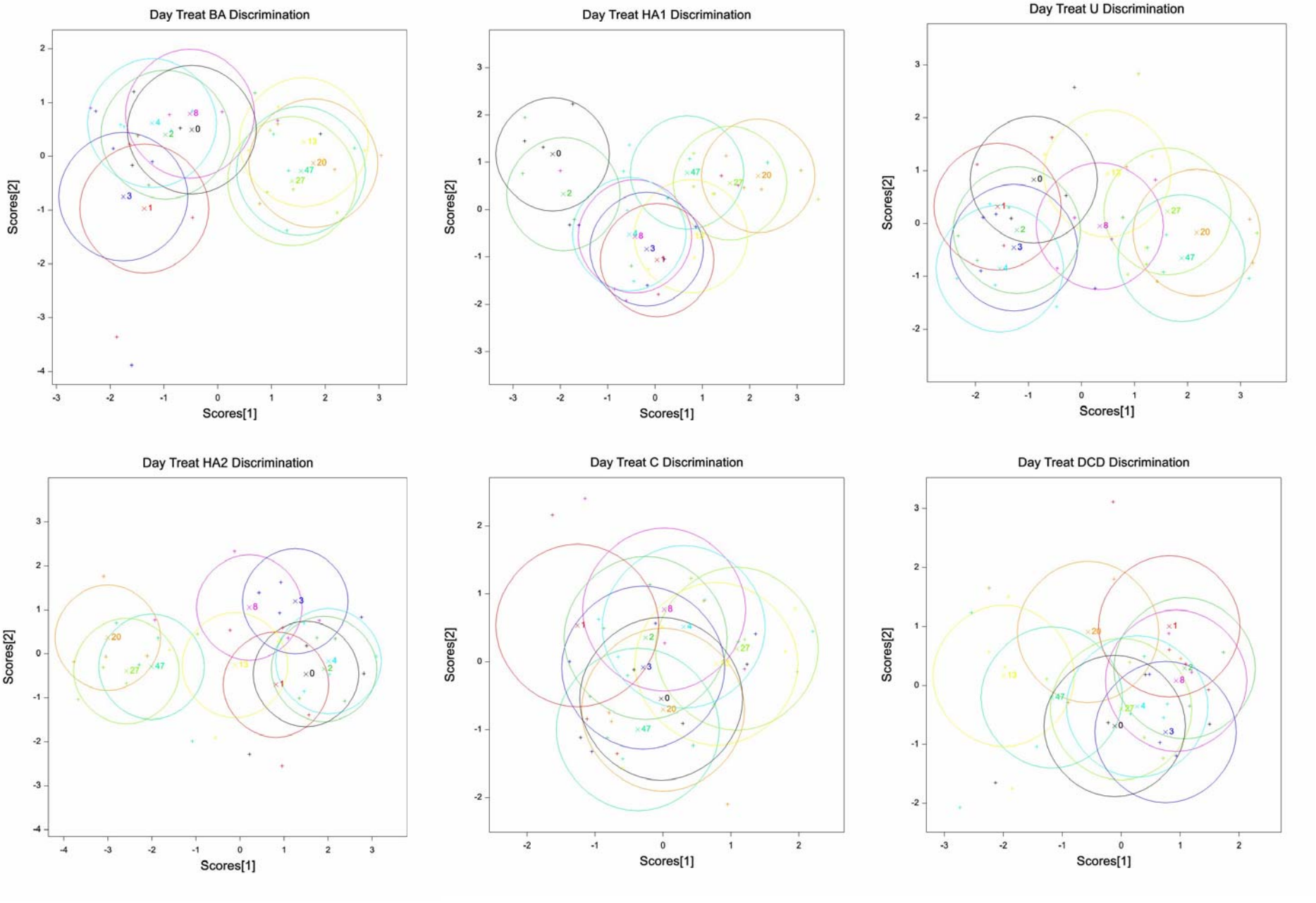


Figure 6. PCA results for *nxrA* DGGE analysis, grouped by treatment. Circles represent 95% confidence interval of the

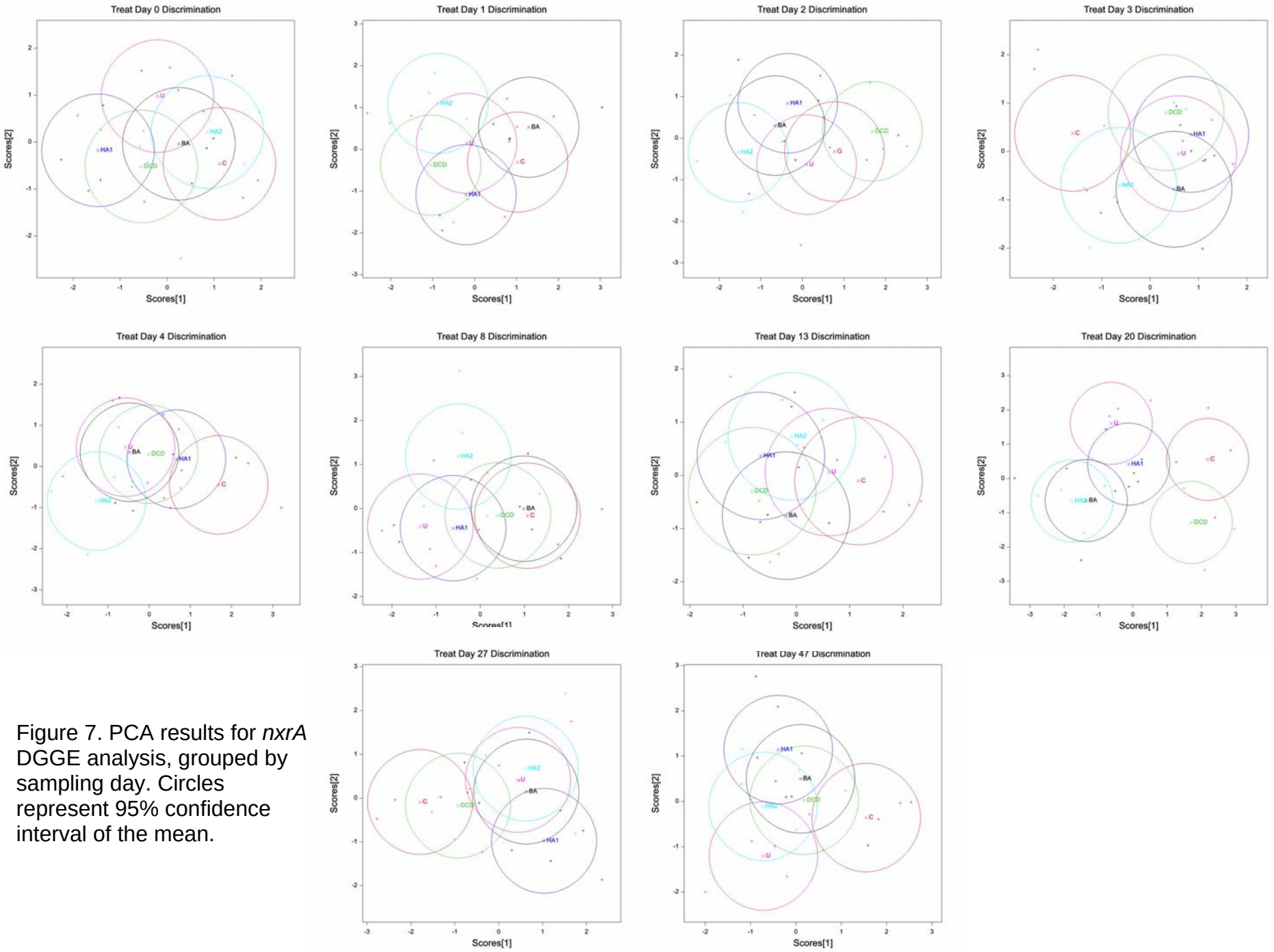


Figure 7. PCA results for *nxrA* DGGE analysis, grouped by sampling day. Circles represent 95% confidence interval of the mean.

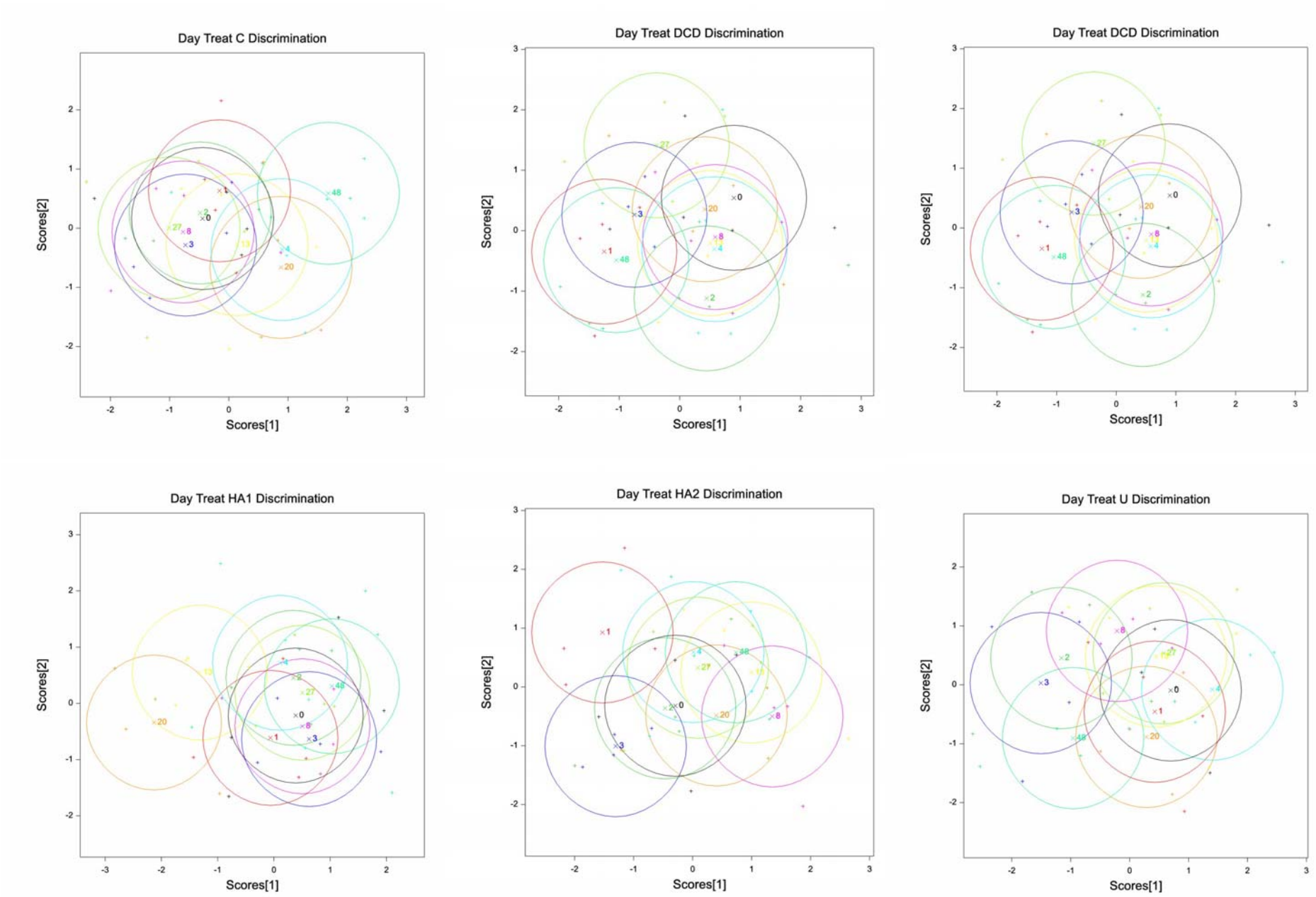


Figure 8. PCA results for *nirS* DGGE analysis, grouped by treatment. Circles represent 95% confidence interval of the mean.

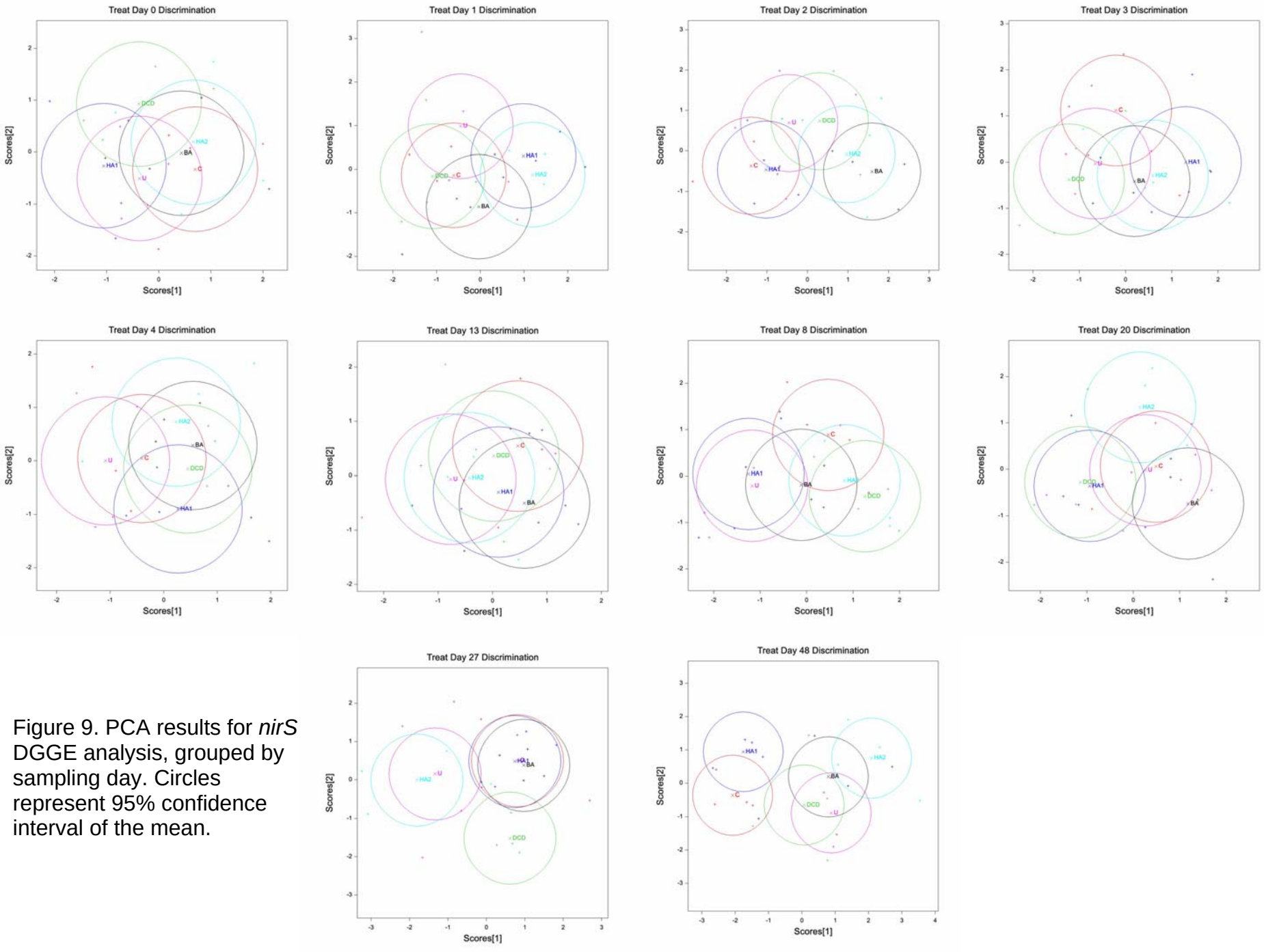
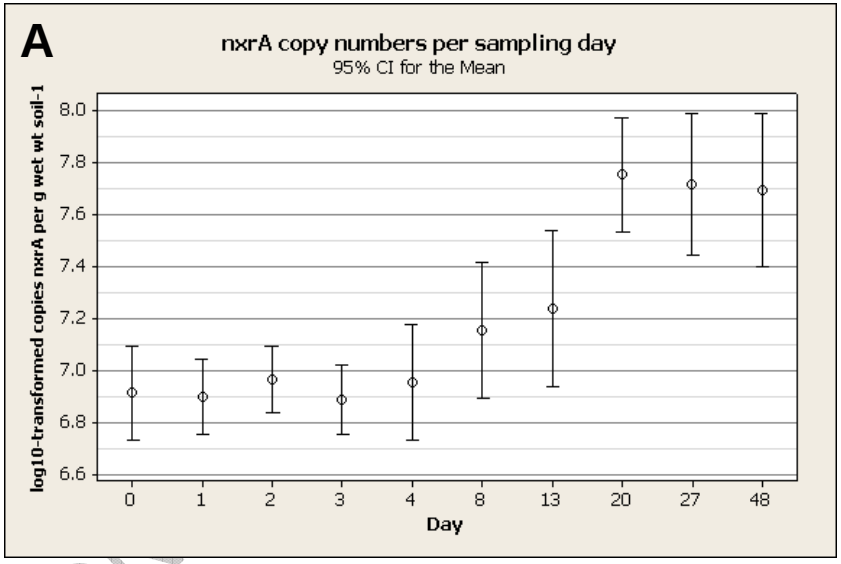
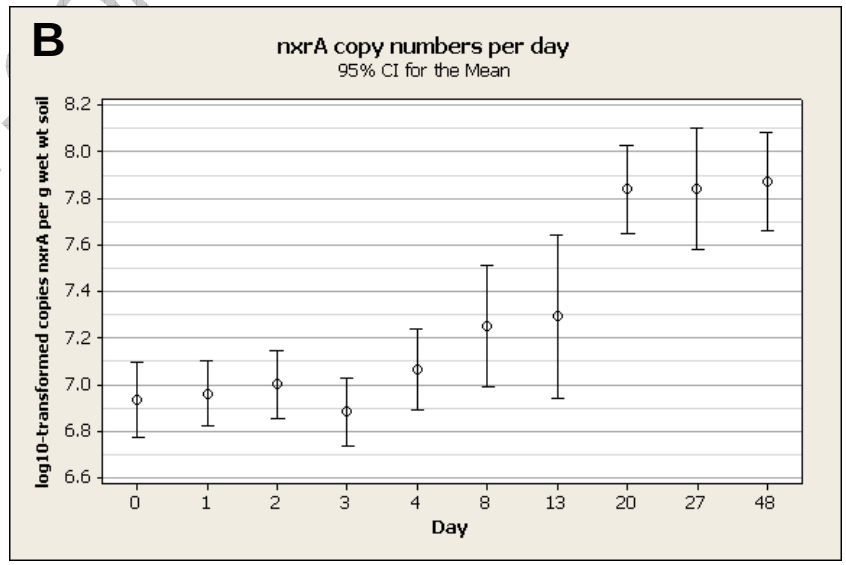
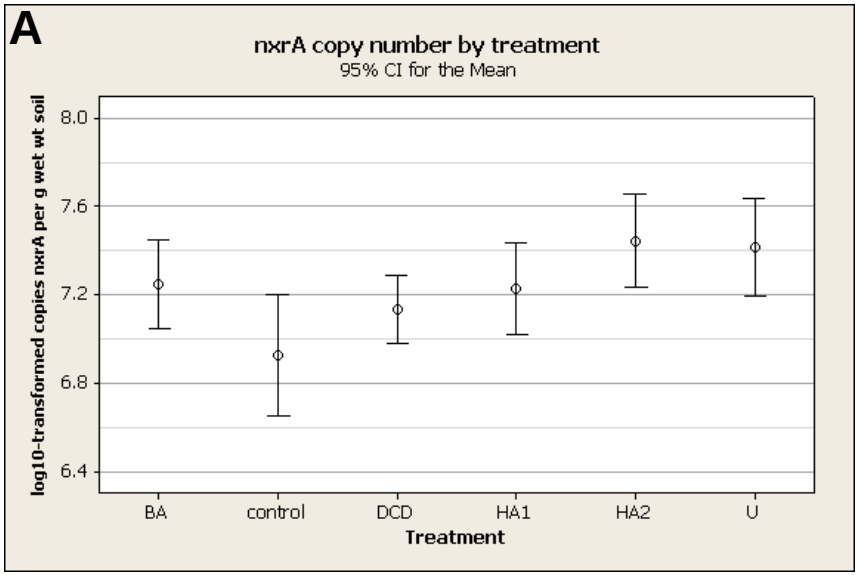


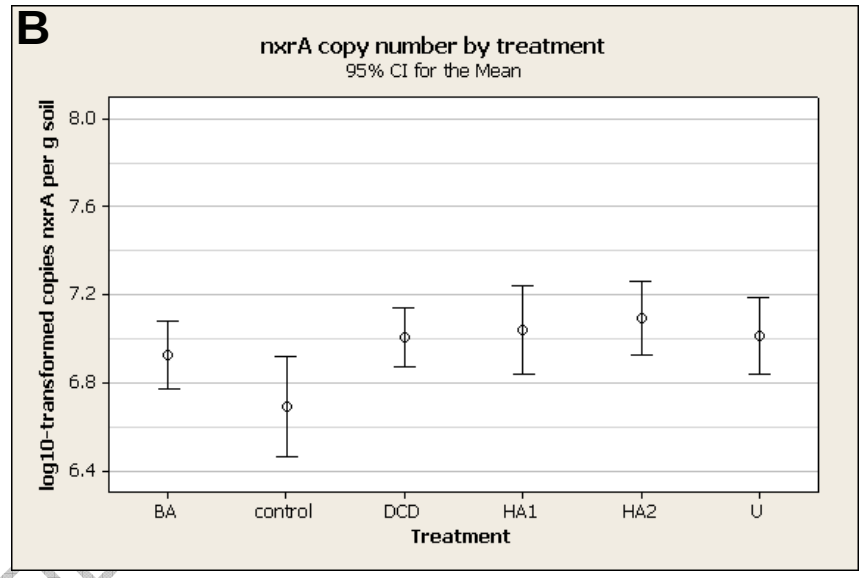
Figure 9. PCA results for *nirS* DGGE analysis, grouped by sampling day. Circles represent 95% confidence interval of the mean.

Figure 10. *nxrA* copy numbers in field trial soil samples from each sampling day averaged over all treatments with (A) or without (B) control data included. Error bars represent the 95% confidence interval for the mean.









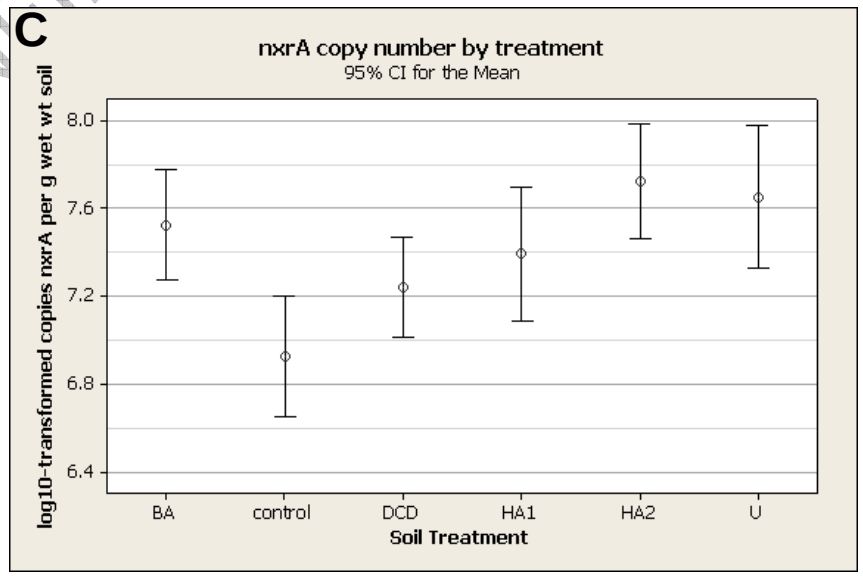


Figure 11. *nxrA* copy numbers in field trial soil samples from each treatment averaged over (A) all 10 sampling days, (B) days 0, 1, 2, 3, 4, and 8 or (B) days 0, 8, 13, 20, 27, and 28. Error bars represent the 95% confidence interval for the mean.

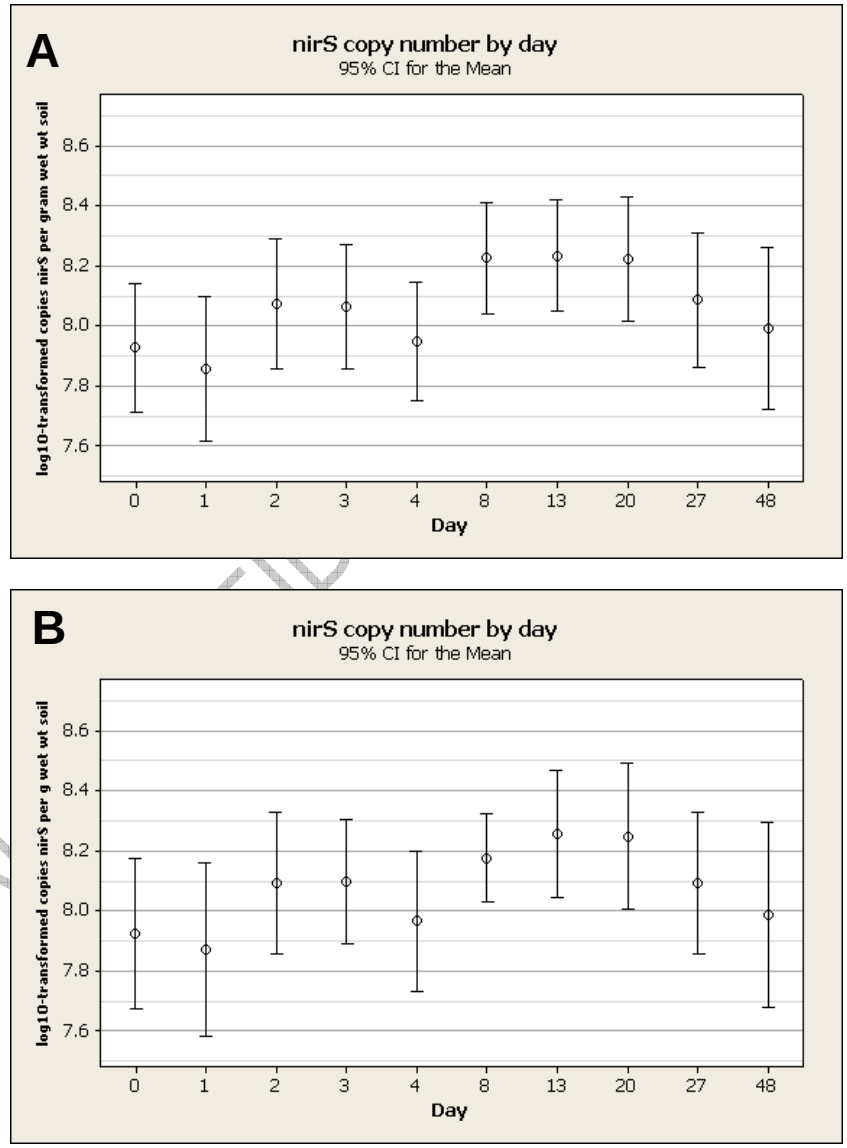
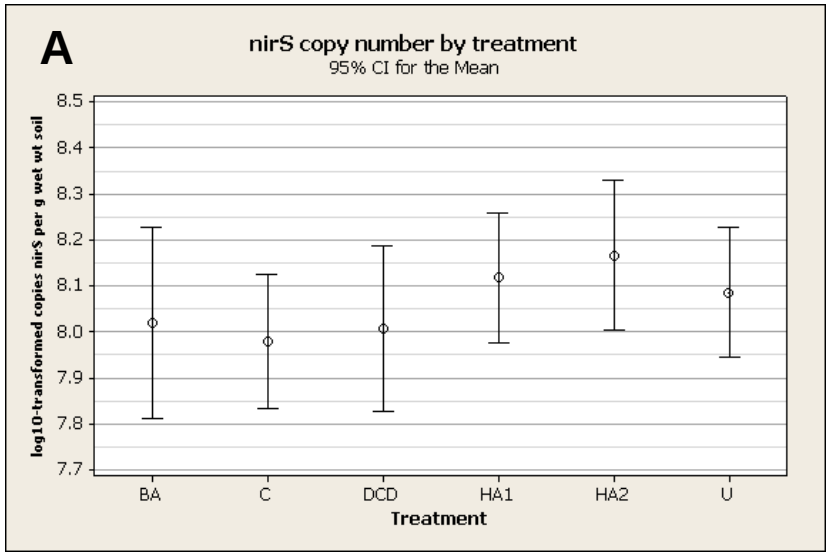
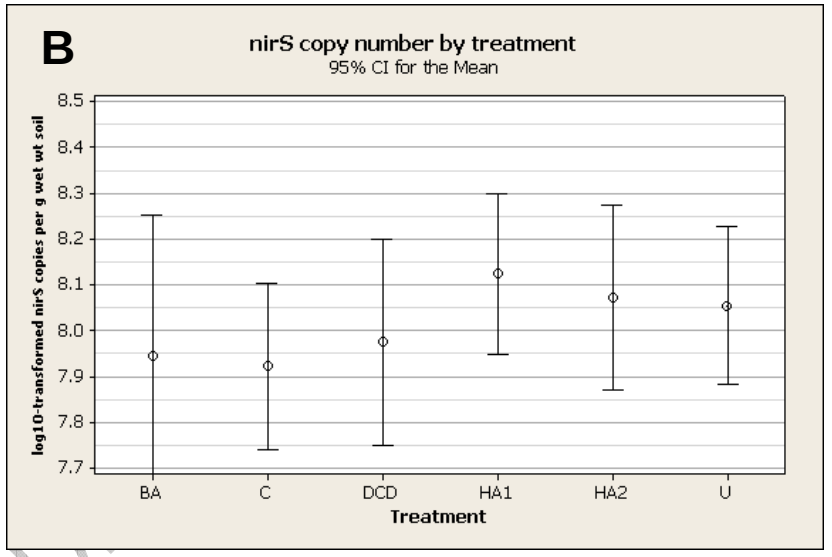


Figure 12. *nirS* copy numbers in field trial soil samples from each sampling day averaged over all treatments with (A) or without (B) control data included. Error bars represent the 95% confidence interval for the mean.





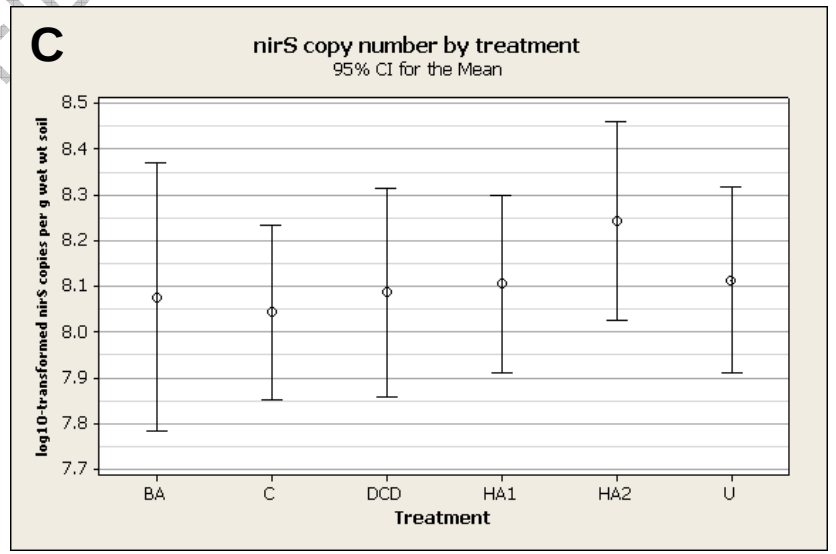


Figure 13. *nirS* copy numbers in field trial soil samples from each treatment averaged over (A) all 10 sampling days, (B) days 0, 1, 2, 3, 4, and 8 or (B) days 0, 8, 13, 20, 27, and 28. Error bars represent the 95% confidence interval for the mean.

6 Discussion

6.1 Nitrous oxide fluxes

Clearly the addition of hippuric acid and/or benzoic acid failed to inhibit N_2O fluxes from the applied urine patches with no difference between these treatments and the unamended urine (HA1). This was a totally unexpected result given the previous laboratory studies where significant reductions in N_2O emissions have been achieved following the addition of hippuric acid to either urea or synthetic urine solutions.

To try and explain this result we need to first compare the conditions of our field experiment with those of the previous studies[1, 2, 3]. In the study by Kool et al.[2] intact soil cores were used (% cm diameter, 5 cm height) from a poor sandy soil (85%) but they were taken from just below the sod, so presumably much of the microbial pool was absent e.g. root rhizosphere material. These cores were then maintained at 70 to 92% water-filled pore space (WFPS) at 16°C with soil pH ranging from 5.2 to 5.6. The study by van Groenigen et al.[1] used the same soil, sampled in the same way with cores kept at 90% WFPS. Bertram et al.[3] used sieved and repacked soil cores maintained at 70% WFPS.

In all these previous studies studies the soil cores were intact or repacked so the soil microbiology was not in a natural state for a pasture soil that would be receiving urine. The soils were highly disturbed due to the removal of the sod or sieving and repacking. These practices could have caused the removal or change in significant microbial communities that may have been able to utilize hippuric or benzoic acids as substrates e.g. fungi. The interaction between hippuric acid and soil microbes (*Pseudomonas* sp.) in the presence of plant roots has also been recently demonstrated. Thus laboratory studies without roots and/or microbial strains present in the surface soil explain the discrepancy between these previous published studies and out results.

All the previous studies[1,2,3] were performed at very high WFPS conditions thus denitrification was the predominant N_2O producing mechanism. Despite our study site reaching field capacity on several occasions due to rain events the WFPS never reached these levels of WFPS but maximum values of ca. 50%. However, this was sufficient for denitrification to occur as can be seen at day 31 when the soil nitrate concentrations decrease significantly as a result of rainfall at this time. Due to leaching and/or denitrification as evidenced by the N_2O flux. Thus denitrification, the pathway presumably inhibited by the hippuric acid derivative i.e. benzoic acid, was certainly not inhibited at day 31. This was supported by the DGGE *nir*S community data which showed no treatment differences over time.

This begs the question as to what is the fate or longevity of hippuric acid and its benzoic acid derivative in soils. At the completion of the experiment a selection of soil samples from a HA1, HA3 and the benzoic acid treatment, for days 0, 2, 4, 6, 8, 10, 13, and 23, that had been stored at 4°C, were analysed for their hippuric acid and benzoic acid content. No water-soluble hippuric acid or benzoic acid was present in any samples. Neither was any soil-bound hippuric acid found. However the soil-bound benzoic acid levels were

[1] Groenigen J.W. van, Palermo V, Kool D.M, Kuikman P.J. (2006) Inhibition of denitrification and N_2O emission by urine-derived benzoic and hippuric acid. *Soil-Biology & Biochemistry* 38(8): 2499-2502

[2] Kool D.M, Hoffland E, Hummelink E.W.J, Groenigen J.W. van (2006). Increased hippuric acid content of urine can reduce soil N_2O fluxes. *Soil Biology & Biochemistry* 38(5): 1021-1027

[3] Bertram J.E., Clough T.J., Sherlock R.R., Condron L, O'Callaghan, M. *Submitted to Global Change Biology.*

elevated according to treatments with higher levels around days 8 to 10. After 78 days storage little credence can be given to these results but the numbers do indicate that the hippuric acid and the benzoic acid derivative had been present in the soil at varying quantities. This is despite the possible heavy rain fall event that occurred within 24 hours of the trial being laid out.

Further support for the presence of hippuric acid and its benzoic acid derivative are found in the higher elevations of NO_2^--N under the HA2 and HA3 treatments. While this was not significant at the traditionally accepted 95% level of confidence (P =0.159) there was certainly a trend in the data. Similarly the DGGE results indicated soil microbial community changes in the HA2 and HA3 treatments over relatively prolonged periods.

This immediately raises other significant issues with respect to the potential use of hippuric acid as an inhibitor of N_2O fluxes. How mobile are hippuric acid and benzoic acid when applied to soil in urine and what if rainfall or irrigation follows? Does soil bound benzoic acid have any anti-microbial effect? How quickly does benzoic acid become soil-bound and what soil quality factors influence this? Before any more field trials of hippuric acid inhibition are performed we need to ascertain the longevity of the water soluble fraction of benzoic acid and its fate in the soil in a pasture situation.

The soil pH in our study was higher than in the previously reported studies from Europe but similar to the study of Bertram et al. thus there is no common ground in terms of soil pH with which to judge our present results.

6.2 Microbial results

This study is the first molecular analysis of *Nitrobacter sp.*-like nitrite oxidizers and *nirS* denitrifiers in New Zealand pasture systems. The purpose of these analyses was to follow changes in size and community structure of soil bacteria known to play roles in soil nitrogen cycling after treatment of test field plots with bovine urine or with water. Four of the urine treatments, BA, HA2, HA3 and DCD, contained known (DCD) or putative (elevated concentrations of benzoic acid and hippuric acid) nitrification/denitrification inhibitors. The first part of this study was to assess the function of the putative nitrification inhibitors in a pasture system, using a known nitrification inhibitor as a positive control. The results from this part of the study refute the utility of benzoic acid and hippuric acid elevation to lower nitrous oxide flux from pasture systems.

Molecular analyses of bacterial nitrite oxidizers and nitrite reducers in these soils were unable to demonstrate a correlation between either of these subpopulations and the observed nitrogen turnover dynamics. The peak in soil NO_2^- at day 10 for the BA, HA2 and HA3 treatments cannot be explained by the *nirS* copy number data, as these treatments yielded *nirS* copy numbers that were both slightly higher and slightly lower than the treatments lacking NO_2^- peaks (C, DCD and HA1), although still not significantly different. Neither does the *nxrA* copy number data seem to indicate nitrite oxidizer involvement in either formation of or utilization of the day 10 NO_2^- peak, which may have come from delayed ammonia oxidizer activity or from a burst of nitrate reduction and subsequent lag in nitrite reducer activity.

Our inability to demonstrate change in *nirS* denitrifier community composition or size may be due to several factors: 1) negative reaction of *nirS* denitrifiers to high N-input

urination events, 2) seasonally low *nirS* abundance or 3) unfavourable soil moisture (Wallenstein et al, 2006). The *nirS* gene is, however, only one of many genes known to be involved in soil microbial denitrification. In addition to *nirS* are *nirK* (copper cofactor nitrite reducase), *norB* (nitric oxide reductase) and *nosZ* (nitrous oxide reductase). These genes offer alternatives for molecular analysis of denitrifier populations in soil (Philippot and Hallin 2006).

Soil moisture plays an obviously important role in the N dynamics in this study, as the N_2O flux data correspond quite neatly to precipitation events, and are likely indicative of denitrification activity. On the contrary, low soil moisture on the eve of precipitation events (e.g. days 10, 30 and 48) may have restricted diffusion of inorganic nitrogen species to relevant bacterial populations. Precipitation and increase in soil moisture would then mobilize inorganic nitrogen, potentially causing a burst in microbial activity that may not be detectable by viewing total bacterial numbers or community structure. Chemoautotrophic bacteria, such as ammonia and nitrite oxidizers, are slow-growing bacteria due to the low energy yield using inorganic nitrogen as reducing equivalents. A single urination event, such as that applied in this field trial, may have had a priming effect on resident bacterial populations, but did not last long enough to product observable lasting changes in these populations. It would therefore be very interesting to determine how microbial community size and structure under urine patches changes after multiple urination events.

There are limitations to the methods used in this study that may have precluded the ability to identify microbial responses to urine and to putative nitrification inhibitors. First, PCR bias due to differential primer binding or other factors may yield results that are not representative of native soil populations, or stochastically introduce artificial variability between otherwise similar samples. Second, the qPCR data exhibited high variability in copy number between replicates. It is difficult to discern the source of this variability, as it could arise from inconsistent urine diffusion, variability in replicate plot temperature, moisture, plant biomass, etc., sampling variability, etc.

It should be noted that these DNA-based studies cannot definitely exclude treatment or time effects on these two subpopulations of bacteria, as gene activity/expression may be affected without altering existing community structure or population size. In addition, PCR detection of genes from environmental sample is unable to distinguish between genes from intact cells and genes from lysed cells that persist in the environment. In order to firmly conclude how these two subpopulations are affected by treatments, and to conclusively determine whether microbial activity is responsible for observed N dynamics, molecular analyses should be repeated on copy DNA (cDNA) prepared from total RNA from soil samples. Examination, for example, of the active *nxrA* and *nirS* communities at days 4, 8 and 13 (i.e. surrounding the day 10 NO_2^- peak) may provide conclusive evidence that attributes this peak to the activity of bacterial nitrite oxidizers or reducers.

Appendix 1 Soil fertility test result from field site.

Analysis		Level Found	Medium Range	Low	Medium	High
pH		6.2	5.8 - 6.3			
Olsen P	(mg/L)	12	20 - 30			
Potassium	(me/100g)	0.42	0.50 - 0.70			
Calcium	(me/100g)	6.9	6.0 - 12.0			
Magnesium	(me/100g)	0.98	1.00 - 3.00			
Sodium	(me/100g)	0.18	0.20 - 0.40			
CEC	(me/100g)	13	12 - 25			
Base Saturation	(%)	63	55 - 75			
Volume Weight	(g/mL)	1.02	0.60 - 1.00			
Sulphate-S	(mg/kg)	2	7 - 15			
Available N (15cm Depth)	(kg/ha)	70	150 - 250			
Base Saturation		K 3.2	Ca 52	Mg 7.3	Na 1.3	
MAF Units		K 9	Ca 9	Mg 23	Na 8	
Anaerobically Mineralisable N		46 ug/g				