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Strategic science of nitrate as a mitigation technology for grazing ruminants

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Executive summary

Supplementary nitrate is effective in reducing enteric methane emission from ruminants in a reliable and quantifiable manner. This project has addressed the process of nitrate metabolism in the rumen and of metabolism of nitrite and methaemoglobin in the animal. Nitrite accumulation in the rumen is best managed through controlled delivery of nitrate (by frequency of consumption or potentially by controlled rate of release within the rumen) and matching of nitrate and feed/substrate consumption to prevent nitrite accumulation in the rumen. Manipulating the rumen environment by a probiotic or by sulphur and molybdenum balance did not control methaemoglobin formation in sheep and therefore did not mitigate risk from nitrite poisoning. Between-animal variation in metabolism of nitrate (as judged by methaemoglobin levels) is apparent and may offer opportunity for selecting resistant or resilient animals and identifying a rumen microbiology that could be introduced to provide protection. Use of 'slow release' nitrate sources needs to be tested.

Nitrate feeding through lick-blocks for sheep and cattle as well as via liquid supplements and total mixed rations was conducted safely and lick-block demonstrations are currently operating via Action on The Ground projects. Recommendations for best management feeding practises for nitrate were developed in association with another project.

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1 Background

Nitrates occur naturally in a variety of ruminant feeds including natural forage and can provide a useful source of non-protein nitrogen to support microbial growth. Within the rumen a subset of microbes can metabolise nitrate to nitrite and through to ammonia. This metabolic cascade is both a threat and opportunity to the ruminant. It is an opportunity because the reactions competitively inhibit methane production, reducing the methane output of ruminants in a quantitative and reliable manner. It is a threat because there is a risk that the intermediate product, nitrite (NO_2), will be absorbed and cause toxicity in the ruminant host. The concentration of methaemoglobin (MetHB) in the blood is an indicator of the severity of nitrite toxicoses and so the risk to animal health. In order to capitalise on the methane inhibiting potential of nitrate, there is a need for the nitrite toxicity risk to be understood and strategies to minimise this risk within the animal developed.

"This program seeks to develop the science underpinning nitrate supplementation to assure this becomes a safe, sure and commercially attractive methane mitigation technology by June 2015, Intensive study of the modes of action of these processes in the rumen will be undertaken to optimise their efficacy and safety. Impact of nitrate supplements on animal productivity will be assessed. Large demonstrations of nitrate feeding will be supported elsewhere. The expected conclusion is that nitrate will be known as a safe, sure certified mitigation technology"

Contracted Project Outcomes:
"1. Dietary conditions for safe nitrate feeding containing (Nitrogen [N],Sulphur IS],Molybdenum [Mo] levels) and relayed to AotG nitrate demonstration sites.
2. Efficacy of nitrate mitigation in cattle known and available.
3. Growth and productivity data of nitrate supplemented livestock made available
4. Long-term impacts of methane suppression on animal growth promoted.
5. Nitrate feeding submitted for approval as a CFI methodology.
6. Nitrate used safely in major AotG demonstrations with over 1000 cattle"

2 Methodology

The methods applied are grouped with respect to achieving the six individual project outcomes as summarised in the section above:

2.1 Dietary conditions for safe nitrate feeding

Understanding the metabolism and biological impact of nitrate and its metabolites was the principal scientific task of the project and underpinned all investigations made. Concentration of methaemoglobinin the blood was used as a measure of the risk of 'safe nitrate feeding' in all animals' studies. The methods involved in managing this risk included:

2.1.1 Review of current understanding of the processes and biology of nitrate and nitrite reduction in the rumen.

A comprehensive literature review was conducted by Prof R.A. Leng (Leng et al., 2013) and published on the University of New England's web site.

2.1.2 Dynamics of nitrate metabolism assessed by infusion of nitrogen isotopes into the rumen and blood

Two investigations were conducted to quantify the interconversion and fluxes of rumen N species.

Initially a previously unpublished study of nitrate kinetics in 8 Merino sheep was assessed. The second study was extended to include infusions into blood to quantify the blood-gut transfer of NO_2 and NO_3. A detailed methodology of Experiment 1 is shown below followed by details of a second infusion study addressing intraruminal nitrate and nitrite kinetics.

Experiment 1.

Eight Merino-cross wethers (3 years old; 38 (SD 2) kg) with established rumen cannulas were housed indoors in individual metabolism cages in two adjacent rooms A and B (4 sheep per room). The cages were placed in pairs at random about 1 m apart in one of 3 sites in each room. The close proximity of the cages enabled a single overhead feeder with two moving conveyor belts to deliver equal portions of each sheep's daily ration each hour. Two batches of feed based on chaffed oaten hay (9.7 MJ ME, 60 g CP, 2.9 g NO_3^-/kg DM) were prepared by adding iso-nitrogenous amounts of N as urea or KNO_3 (May and Baker Ltd, Dagenham, England). The first diet (1.2% urea) was made by sprinkling a urea solution onto the hay while it was tumbled in a feed mixer (Diet 1). After thorough mixing, the feed was removed and dried overnight on a concrete floor in a warm ventilated room. A second batch of feed with 4% KNO_3 (2.5% NO_3^-, Diet 2) was made by sprinkling a KNO_3 solution onto oaten chaff in the same mixer and drying it in the same way. The sheep were offered 1 kg/d of air-dry feed from the overhead feeders.

Four sheep were acclimated to the urea diet (Diet 1) over a period of 18-20 d. The other 4 sheep were acclimated to the 2.5% NO_3^- diet by mixing increasing amounts of Diet 2 with Diet 1 so that by Day 7, the sheep were offered only the diet with 2.5% NO_3^-. After Day 7, sheep were maintained on these diets until the end of the study. On Days 18-20, the sheep were moved into respiration chambers to enable their methane (CH_4) output to be determined and, one day later, ^{15}N tracers to be administered. The results for diet digestibility characteristics and CH_4 output have been reported separately[19].

Intraruminal injections of $K^{15}NO_3$ and $^{15}NH_4Cl$

Intraruminal injections of $^{15}NH_4Cl$ (99 mol ^{15}N/mol N; approx. 2.0 mmol in 120 mL) were given to the 4 sheep in Room A, and intraruminal injections of $K^{15}NO_3$ (99 mol ^{15}N/mol N; approx. 2.5 mmol in 120 mL) were given to the 4 sheep in Room B (see Table D1).

Table D1. The timing of events during the week when ^{15}N tracers were administered intraruminally. Filled cells denote 2 d when rumen samples were collected from sheep after a single dose of $^{15}NH_4Cl$ was administered intraruminally; unfilled cells denote 2 d when rumen samples were collected from sheep given $K^{15}NO_3$ intraruminally.

Sheep and diet	Tues	Wed	Thurs	Friday
Room A. Sheep 3 and 4. Urea supplemented	$^{15}NH_4Cl$		$K^{15}NO_3^-$	
Room B. Sheep 7 and 8. Urea supplemented.	$K^{15}NO_3^-$		$^{15}NH_4Cl$	
Room A. Sheep 1 and 2. Supplemented with 2.5% NO_3^-	$^{15}NH_4Cl$		$K^{15}NO_3^-$	
Room B. Sheep 5 and 6. Supplemented with 2.5% NO_3^-	$K^{15}NO_3^-$		$^{15}NH_4Cl$	

The ^{15}N-labelled tracers were dissolved in deionised water and delivered using a 140 mL syringe to which was attached a stiff plastic tube (2 mm i.d.). The solution was injected continuously over about 0.6 min while the tube was moved through the rumen contents to disperse the ^{15}N-labelled solutions. Samples of ruminal fluid were taken from each sheep via samplers consisting of a wire cage wrapped in nylon gauze and placed in the dorsal sac, before the dose was administered, and afterwards at 20, 40, 60, 90, 120, 180, 330, 425, 760 and 1270 min. The samples (20 mL) were placed in labelled 50 mL centrifuge tubes in crushed ice and delivered to the laboratory within 20 min. In the laboratory, they were immediately placed in a high speed centrifuge (Beckman, Model J2-21M) and centrifuged at 15,000 ***g*** for 15 min. The supernatant solution was then removed to storage containers containing 0.15 mL 18 M-H_2SO_4 before being stored at -18°C to await further analysis. The upper bacterial layers from the residues in the centrifuge tubes were transferred to new tubes and mixed with 40 mL water to wash the bacteria. The tubes were then centrifuged at 15,000 ***g*** for 15 min as before, and the

new supernatant solution was discarded. The top bacterial layers in the residues were then transferred to a 10 mL bottle, frozen and freeze-dried. The dried bacterial samples were stored at room temperature to await total N and ^{15}N analysis.

Samples of whole rumen contents were also taken (at 200, 360 and 1250 min after tracers were administered) with a core sampler consisting of a Perspex tube (20 mm i.d.) that was inserted quickly into the rumen contents so that a core of mixed rumen contents was forced up the tube. These contents were trapped by pulling on a wire, passing through the tube, with a rubber stopper on the far end. The stoppered tube was then removed and its contents were added into a 25 mL plastic container containing liquid N_2 so that the contents were quickly frozen (within 1.5 min) before storage at -18°C.

The above procedures were repeated 2 d later, so that all 8 sheep received intraruminal doses of both $K^{15}NO_3$ and $^{15}NH_4Cl$about 2 d apart, in a cross-over pattern.

Experiment 2

Animals and diets

All protocols for the care of the animals used in this experiment were approved by the University of New England Animal Ethics Committee (AEC14-027). Four rumen cannulated crossbred ewes (~1year of age, average live weight of 39 kg ± 5 kg) were housed in metabolic crates and fed 1.0 kg air-dried commercial mix of chaffed lucerne hay and cereal straw (basal diet) during two experiments.

The first experiment lasted 18 days divided in three periods (P1 to P3). Intravenous ^{15}N-nitrite, intraruminal ^{15}N-nitrate and ^{15}N-ammonia were infused into the sheep when they were offered the basal diet (before introduction of a nitrate supplement) (P1), during the first week of introduction to nitrate supplementation (P2) and again during the second week after introduction to nitrate supplementation (P3). Ewes were fed hourly using automatic feeders, in P1 the feed was supplemented with 1.06 % urea. Urea supplementation was replaced with 2.0 % nitrate in P2 and P3.

A second experiment was conducted to study the effect of feeding frequency on nitrite toxicity. Three ewes from the group of sheep used in P1 to P3 with similar body weights (average of 37.3 ± 3.2 kg) were selected and randomly allocated to one of three treatments (hourly feeding, twice a day feeding: 500 g at 8:00 and 20:00, or once a day feeding: 1kg at 8:00) in a randomized 3x3 Latin square design. Allocation to treatments was done every two days at 8:00, the first day consisted of an adaptation period, and measurements were taken on the second day.

Feed was prepared by sprinkling diluted urea or calcium nitrate ($5Ca(NO_3)_2.NH_4NO_3.10H_2O$, Bolifor CNF, Yara, Oslo, Norway) onto the chaff while the chaff was tossed in a rotary mixer. One mix of feed was offered in experiment 1, another batch was mixed for experiment 2.

Isotope infusions

Catheters were inserted into both jugular veins of each sheep the day before each experimental period (day 1, 7, 14) one to be used for isotope administration, and one for collecting blood samples. Catheters consisted of a polyethylene tube (60cm of length, OD 1.50 mm x ID 1.00 mm, Sterihealth) inserted into the jugular vein by a double bever introducer needle (14G, Surflo I.V. catheter, Terumo, Japan). Catheters were flushed with 2 mL heparinised saline (25 iu/mL) after each sampling and with a stronger solution overnight (100 iu/mL). Catheters were replaced between periods 1, 2 and 3 as routine sanitary practice.

On days 2, 8 and 15, an isotonic solution of $NaNO_2$, enriched with 8.4% ^{15}N-$NaNO_2$ (0.019mg/min ^{15}N-$NaNO_2$ and 0.21mg/min N-$NaNO_2$, 0.35mL/min) was continuously intravenously infused over 9h (09:00-18:00). Ewes were intraruminally infused with a solution of ^{15}N-KNO_3 (0.038 mg/min, 1mL/min) for 9 h on days 3, 9 and 16. On days 4, 10 and 17, ewes were intraruminally infused with a solution of ^{15}N-NH_4Cl (0.038mg ^{15}N-KNO_3/min, 1mL/min) over 11 h. A single intraruminal injection of Co-EDTA (4.8g of Co (II) acetate.$4H_20$ dissolved in 100mL, (Udén *et al.* 1980)) was made into each ewe on days 5, 10 and 17 with the dose being directed to multiple sites within the rumen to facilitate rapid mixing.

Infusion lines (diameter?) were connected to a pump (Masterflex® L/S, model 7510-05, Cole Parmer) and attached to the animal via the jugular catheter or to a rumen probe with two inlets, allowing a simultaneous infusion dripping onto the gaseous phase of the rumen and rumen fluid sampling from the ventral sac through a ventrally directed probe covered with a 40µm nylon filter.

2.1.3 Conducting batch, continuous culture and animal investigations of the effect of molybdenum and sulphur on the ruminal metabolism of nitrate and the risk of methaemoglobinaemia.

An in-vitro assay and a sheep productivity study were conducted to assess the effects of S and m on nitrate reduction, animal performance and MetHB concentration. The findings of the in-vitro study were reported in Li et al., (2013a) but the full methods used have not been published so are reported below:

One level of nitrate, two levels of sulphur and two levels of molybdenum were investigated in an *in vitro* fermentation system. The design was a 2 × 2 factorial arrangement with 2 replications within each treatment. The expected average concentrations of N, S and Mo in the buffer solutions were calculated based on the estimated dietary concentration required by cattle (Freer *et al.* 2007). The $NaNO_3$, $NaHSO_4$ and $Na_2MoO_4 \cdot 2H_2O$ were dissolved in the buffer solution and then supplied directly to the fermentation vessels. The level of $NaNO_3$ was 4 g NO_3-N/kg DM (24.3 g sodium nitrate/kg DM). The levels of $NaHSO_4$ were 0 and 1.8 g S/kg DM (6.75 g sodium hydrogen sulphate ($NaHSO_4$)/kg DM) and the levels of added molybdenum were 0 and 2.18 mg Mo/kg DM (0.005 g sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$,)/kg DM); The ratio of N:S contributed by the feed and incubation medium was 7:1. All vessels were iso-nitrogenous. The treatments are listed in Table D2.

Table D2. Ingredients in the substrate (g/30g DM). If the supplementary compound (nitrate-N, sulphate or molybdate) is added to the incubation medium, the '+' symbol is used; the symbol '–' represents the absence of the supplementary compound.

Treatment No.	Treatment ID	Italian ryegrass (g DM/d)	$NaNO_3$ (g/d)	$NaHSO_4$ (g/d)	$Na_2MoO_4 \cdot 2H_2O$ (g/d)
T1	+ N – S – Mo	30	0.72849	-	-
T2	+ N + S – Mo	30	0.72849	0.20250	-
T3	+ N – S + Mo	30	0.72849	-	0.00016
T4	+ N + S + Mo	30	0.72849	0.20250	0.00016

The study was carried out using the rumen simulation technique RUSITEC (Sanshin Industry Co. Ltd., Japan). The general incubation procedure was as described by (Kajikawa *et al.* 2003). The whole unit consisted of 8 fermentation vessels with an effective volume of 600 ml each. Each fermentation vessel was loaded with 2 polyester bags (10 x 20 cm with a pore size of 50 ± 15 µm). On day 1, one bag was filled with 100 g of solid rumen contents to inoculate particle-associated microorganisms into the system and the other bag with the daily diet, 15 g of ground mixture of Italian ryegrass and red clover hay. The nutrient composition of the representative Italian ryegrass and red clover hay samples is given in Table 2.

Rumen content was obtained from two fistulated Jersey steers (approximately 17 months, average LW 280 kg) approximately 60 minutes prior to the commencement of inoculations and maintained at 39 ^{0}C. The steers graze on a pasture of phalaris, rye grass and white clover throughout the period during which the experiment was conducted. During the winter season, the steers were being supplemented with Italian ryegrass and red clover hay (× 2 per week) offered to the steers to ensure they have enough dry matter intakes to maintain an active protozoal population.

A growth and metabolism study testing for nitrate and sulphur interactions was conducted in sheep. The methods for this are fully reported by Li et al., (2013b).

2.1.4 Assessing intake of nitrate blocks relative to urea blocks by sheep

In this study the possibility of restricting intake of nitrate supplement by including in a lick block was assessed. A full description has been published (Li et al., 2012 AAAP), so only a summary is provided here. The experiment involved 3 components:

- Quantifying the daily intake of lick blocks containing either urea or nitrate (as calcium nitrate) using individually housed sheep
- Providing nitrate or urea blocks in a cross-over grazing study in which the average daily block intake of sheep was determined by monitoring block weight.
- A subgroup of sheep were provided the same quantity of nitrate (in ground up block) as was consume din the paddock and the daily methane production (DMP) was determined on nitrate and urea fed sheep.

2.1.5 Assessing the role of a probiotic (Propionibacterium sp.) on nitrite accumulation in rumen fluid and the efficacy of this probiotic in reducing nitrate toxicity in sheep

Since many propionibacteria have highly active nitrite reductase enzyme systems, it was considered highly likely that introducing them into the rumen as a probiotic would prevent the accumulation of sodium nitrite. An initial in-vitro study was conducted (unpublished) and then a sheep feeding study oif a pure culture of *Propionibacterium acidipropionicum* was undertaken which has been published (de Raphelis et al., 2014). A brief overview of the methods of these two studies is provided below:

In-vitro study:
A 4x2 factorial experiment was conducted to investigate the effects of N source (none, urea, NO_2, NO_3) and of the inoculum *Propionibactierium acidipropionici* on fermentation and nitrogen metabolism in rumen liquor. Incubations were carried out testing 64 samples per run; each run was repeated three times. The 64 samples consisted of eight treatments of which there were eight replicates/run. Each replicate was examined at different time points (0, 1.5, 3, 5, 7, 9, 11, 21h after starting the incubations) to have an overview of the evolution of metabolite concentrations and gas production over time.

The treatments (Table D3) aimed into investigating the effects of different nitrogen sources: H_2O (negative control), Urea (positive control), NO_3 and NO_2 combined with a probiotic PA or H_2O as control. Urea, NO_3, and NO_2 were introduced in iso-nitrogenous proportions, high amounts of NO_2 being toxic for ruminal bacteria, the treatments assigned to NO_2 consisted in 20% NO_2 and 80% Urea.

Table D3. Experimental treatments used in *in-vitro* incubation and their abbreviations. The treatments were replicated 8 times per run, with 3 x 21h runs being conducted. The three N supplements were all iso-nitrogenous, however due to the toxicity of NO_2 to microbes, N supplement in the NO_2 treatment consisted of NO_2+ urea. The probiotic inoculum (*Propionibacterium acidiproopionici;* PA) was present or absent.

	H_2O	**Urea**	**NO_3**	**NO_2**
Control	ConN+ConP	Urea+ConP	NO_3+ConP	NO_2+ConP
PA	ConN+PA	Urea+PA	NO_3+PA	NO_2+PA

The substrate in each syringe consisted in ground oaten chaff (0.200g; 0.5mm sieve size). Pooled rumen fluid from five rumen cannulated wethers was added with an artificial buffer into each syringe.

Animals and feeding
Five rumen cannulated wethers were grazing without nitrogen supplementation prior to the experiment and were not adapted to NO_3 or NO_2 feeding. At least 7 days prior the first rumen fluid samplings these sheep were group-housed and fed oaten chaff *ad libitum*.

Rumen fluid collection

Fresh rumen fluid (60mL) was collected from each sheep before morning feeding at 9:00 am, approximately 30 minutes prior to the commencement of incubations and maintained at 39°C. Before starting the incubations, the presence and activity of protozoa was verified in each rumen fluid sample with a quick observation under a microscope. At the start of the incubations, rumen fluid from the five sheep was pooled using equal amounts from each sheep (44mL per sheep).

In vitro Incubation

Preparation of vessels

One vessel (1000mL) was used to prepare the incubation medium. The vessel was fitted with two tubes (internal diameter: 4.4mm), one to allow withdrawal of incubation medium and another one) for constant bubbling of CO_2 (BOC anaerobic grade) through the incubation medium. The vessel was placed in a 39°C water bath and a modified buffer (418 mL) was added (Soliva and Hess, 2007; Figure 1). After flushing the vessel for approximately 10 minutes with CO_2, a reducing solution (containing cysteine and NaOH) (22mL) was added. Approximately 15 minutes before starting incubations rumen fluid (220mL) freshly collected pooled rumen fluid was blended with the anaerobic buffer in the heated storage bottle.

Figure D1: Flushing of the rumen fluid and buffer mixing bottle prior to transferring buffered rumen fluid into individual incubation syringes (top of picture).

Preparation of syringes

Plastic syringes (n=64: Terumo 60mL; Figure D1) were fitted with a three-way luer-lock tap (BD-Connecta). Ground oaten chaff (200 ± 10 mg chaff/syringe) was directly weighed into each syringe. Syringes were pre-warmed in a 39^{o}C shaking water bath until inoculation. Treatments among the same time point were randomly allocated to the syringes.

PA (EQ-42 stock culture, 1150 x10^9 CFU/g, Lallemand specialities, USA) was introduced in each syringe respecting the dosage of 10^9 CFU per mL incubation medium. 0.3478 g PA was weighed and dissolved in 20.0 mL water, 0.5 mL of the solution was injected into the appropriate syringes as defined in the experimental design; control groups were injected with 0.5 mL of milliQ H_2O.

According to the treatments, 0.5 mL diluted NO_3, NO_2, urea or milliQ H_2O were added into the syringes, the dosage was calculated to supply 0.5 % N in substrate DM (Table D4).

Table D4. Quantities of sodium nitrate ($NaNO_3$), sodium nitrite ($NaNO_2$), and urea added to each incubation syringe. (One N source per syringe)

N source	Mass per syringe
$NaNO_3$	6.071 mg
$NaNO_2$	0.9857 mg NaNO2 +1.714 mg Urea
Urea	2.142 mg

Once the vessel and the syringes were ready to use, the buffered incubation medium (10 mL) was drawn up into the syringe. After shaking the syringe, its initial gas volume was read on a scale and the syringe was returned into the shaking water bath for incubation.

Time measurements for each syringe started when the incubation solution was drawn up. After the introduction of the substrates and incubation medium syringes with incubation duration of 0h were shaken and immediately emptied for incubation medium analyses as described in "Post incubation measurements".

Incubation
Syringes were introduced into the water bath for 1.5, 3, 5, 7 9, 11 or 21 hours of incubation in a program summarised in Figure 3. Incubations lasted a maximum of 21 hours; syringes were kept in 39 °C shaking water baths.

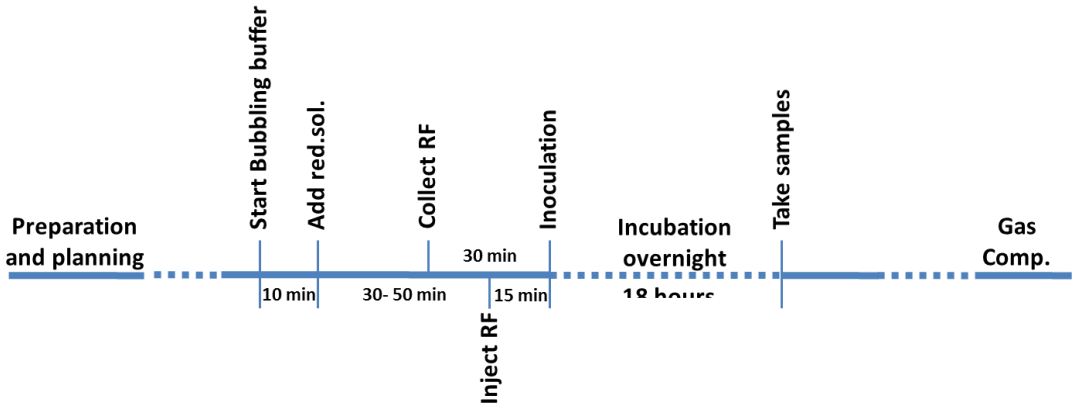


Figure D2. Summary of the in-vitro incubation procedure

Post-incubation measurements

Once removed from the shaking water bath, the final gas volume was read. The liquid part from the syringe was ejected into a 25 mL plastic vial, and a new 3 way tap was carefully fitted to the syringe avoiding losses of gas. The pH of the liquid was subsequently measured with a pH-meter (Ecoscan pH 6, Eutech instruments). Two subsamples (4.0 and 6.0 mL) were taken and mixed respectively with a few drops hydrochloric acid solution (to obtain a pH lower than 3), or a formalin solution (3.0 mL). Acidic samples were stored at -20°C for further NH_3 analyses. Samples preserved with formalin were kept at room temperature upon NO_2 and NO_3 analyses.

Once emptied from its liquid fraction, the syringe was connected to the tap of a 1 mL syringe with a luer tip fitted to a 3 way tap. 0.5 mL gas was transferred into the 1mL syringe and then injected with a needle into a 12 mL vacuumed air tight tube (Labco Exetainer®). The 1 mL syringe was flushed with headspace gas prior to the transfer. The vacuumed tubes were filled until slight positive pressure with approximately 11.5 mL purified gaseous N_2 (BOC, 99.999% N_2).

Analysis of gas composition (methane, nitrous oxide, hydrogen)
Concentrations of N_2O were measured by gas chromatography (Varian 450-GC) with an ECD detector. CH_4 and H_2 were also measured by a gas chromatography (Varian CP4900), fitted with thermal conductivity detectors. Three channels consisting of a Molsieve 5Å (MS5A) column, with

argon as carrier, a Molsieve 5Å column with helium as carrier and a CP-PoraPLOT U (PPU) column with helium as carrier were used to determine H_2 and CH_4 concentrations.

Ammonia determination
Frozen acidified incubation medium samples were thawed. A subsample of each sample was introduced into 1.5 mL micro-centrifuge tubes (Eppendorf®) and centrifuged for 10min at 13,000xg. Supernatant (0.5 mL) was introduced into a 12 mL tube containing 9.5 mL milliQ H_2O. Standards were prepared with known NH_3 concentrations. NH_3 was determined by a continuous flow analyser (Skalar San++). The detection procedure was based on the modified Bethelot reaction; NH_3 was buffered and chlorinated with monochloramine which reacts with 5-aminosalicylate. A green colour is formed after oxidation and measured at 660nm.

Nitrate and nitrite determinations
Both NO_2 and NO_3 were detected by continuous flow analyser (Skalar San++, Breda The Netherlands).

Statistical analyses
A three-way factorial ANOVA (probiotic by N-source by time) was conducted using GenStat (2013), incorporating all two- and three-way interaction terms. Repeated-measures analysis was not required, as separate replicate (syringes) were used for each time. As these data were positively skewed with heterogeneous variances, the in-transformation was adopted, and the underlying statistical assumptions were valid on this basis.

2.1.6 Managing feeding frequency of sheep to minimise nitrite accumulation and MetHB accumulation.

An observation throughout our studies was that MetHB concentration was always low (<5%) in sheep and cattle in which the animals were offered feed repeatedly during the day or had continuous access to feed. Consequently an investigation was made of the effect of feeding frequency on the concentration of blood MetHB and nitrate products.

A short targeted experiment was conducted to study the effect of feeding frequency on nitrite toxicity. Three ewes similar body weights (average of 37.3 ± 3.2 kg) were selected and randomly allocated to one of three treatments (hourly feeding, twice a day feeding: 500 g at 8:00 and 20:00, or once a day feeding: 1kg at 8:00) in a randomized 3x3 Latin square design. Allocation to treatments was done every two days at 8:00, the first day consisted of an adaptation period, and measurements were taken on the second day.

Feed was prepared by sprinkling diluted urea or calcium nitrate (5Ca(NO3)2.NH4NO3.10H2O, Bolifor CNF, Yara, Oslo, Norway) onto the chaff while the chaff was tossed in a rotary mixer. Both blood and rumen fluid were sampled, but only one 10 mL sample of rumen fluid was kept and preserved with 2 mL 38% (v/v) formaldehyde. Samples were taken at 7:45, 9:00, 10:00, 11:00, 12:00, 13:00, 15:00 and 17:00. Feed refusals were weighed and returned to each ewe at each sampling time.

2.1.7 Managing form of substrate to maximise nitrite reductase activity and minimise NO_2 absorption.

Seeing the role of carbohydrate supply on MetHB concentration stimulated an investigation into whether the form of carbohydrate could differentially affect nitrite accumulation and so MetHB. The fastest nitrite reductase system in non-rumen anaerobic ecosystems is the “Nir” system but it is not known if this operates in the rumen. The Nir system is responsive to the concentration of NADH in the cell, so incubations nourished by isoenergetic supplies of glucose or of glycerol (which differ in their NADH yield/MJ of substrate), were established.

Experimental design
A 4x2 factorial design was used for the *in vitro* incubations. Glucose (Glu), glycerol (Gly) or a negative control (Con) were supplemented into incubation media with NO3 (NO_3) or urea (U) as background N

source (Glu-NO_3, Glu-U, Gly- NO_3, Gly-U, Con- NO_3, Con-U). Each treatment was replicated 9 times; each of these replicates was analysed after a different incubation time (0, 1.5, 3, 5, 7, 9, 12, 15, 24h). All incubations were conducted in triplicates simultaneously by 3 operators.

Animals and feeding

Six rumen cannulated crossbred ewes were fed chaffed oaten hay indoors for several months prior the experiment. Ewes were randomly allocated to NO3 or urea treatments (n=3) and gradually acclimated to dietary treatments over 15 days. Chaffed oaten hay was fed as basal diet mixed with diluted NO3 (2% NO_3/DM) or urea (1.1% urea/DM). Diets were isonitrogenous and offered once daily *ad libitum*.

In vitro incubations

In vitro incubations were carried out with fresh rumen fluid (100mL/sheep) obtained from 6 sheep before morning feeding (7:30 AM) and maintained at 39°C until the inoculation. Rumen fluid from sheep on NO_3 and urea diets were introduced in two different vessels and diluted in a buffer medium (2:1) as described by Soliva and Hess (2007). Briefly, the buffer medium contained (per litre): a main element (250mL; Na_2HPO4 1.42g, KH_2PO_4 1.55g, $MgSO_4$ $7H_2O$ 0.31g), a buffer solution (250mL; $NaHCO_3$ 8.75g, $(NH_4)HCO_3$ 1.0g), a trace element (0.13mL; $CaCl_2$ $6H_2O$ 0.56mg, $MnCl_2$ 0.32mg, $CoCl_2$ $6H_2O$ 0.06mg, FeC_3 $6H_2O$ 0.43mg) and a resazurin solution (1.29mL; Resazurin 0.032mg). A freshly prepared reducing solution (76.5mL; Cysteine HCl 2.39g, 0.4M NaOH 14.35mL) was also added to the vessels. The buffer medium was continuously flushed with CO_2 and rumen fluid was added after 30min, once the medium was reduced (colour changing from blue to pink).

Plastic syringes (162 x 60mL, Luerlock, Terumo) containing ground oaten hay (500 □m sieve size; 200 ± 10 mg DM hay/syringe) were fitted with three-way luer-lock taps (BD-Connecta, BD). Syringes were pre-warmed in a 39^{o}C shaking water bath until inoculation. Diluted NO3 or urea (0.5%N per DM) and glucose (15% per DM) or glycerol (18% per DM) were introduced isoenergetic proportions into the incubation syringes shortly before the addition of buffered rumen fluid.

2.1.8 Testing of possible formation of imidazoles (toxins) in molasses blocks containing nitrate.

Molasses based lick-blocks are a chemically heterogeneous matrix and extraction and chromatography of components is difficult. In the case of imidazoles such as 4 methyl imidazole as could be expected to form when ammonia (present in the calcium nitrate) is heated with molasses, the challenge extreme with major challenges in ionic strength during both solid phase extraction and liquid chromatography. None the less, the analytical method was developed over 2 years and then blocks manufactured by Olsson's with containing 5% (L1N) or 27% (L4N) calcium nitrate or 1.67% (L1U) or 9% (L4U) urea. The extracts were made at a rate of 500 mg of block in 250 ml of water (2 g/L), purified by solid phase extraction and then analysed by HPLC.

Regarding the extraction of Imidazoles, the SPE method of Klejdus was tested using aqueous solutions of 4MI (1 ppm). After preconditioning the SCX cartridge with methanol and water, the sample (treated with 0.1M HCL (20□□L / 3 ml of solution), to achieve a pH of 7.0 for the sample) was loaded onto the cartridge, which was then washed with methanol and eluted with 5M HCl / methanol (1:3). LC-MS of the eluent gave a peak with very poor shape and a much lower retention time (RT) that that found for 4MI previously but the mass spectrum of the peak confirmed that it was definitely 4MI. It seemed likely that the high acidity of the fraction was interfering with the ion exchange retention mechanism of the mixed-mode HPLC column, resulting in a greatly altered retention time.

Mass spectrometric conditions were developed by infusion of a 1 ppm solution of 4MI into the ESI interface of a Varian 1200L LC-MS system, operating in positive ion mode. While other authors had found that selective reaction monitoring of the 83-56 fragmentation in positive ion mode gave good sensitivity for 4MI, we found that little fragmentation of the molecular ion occurred until high collision energies were used, resulting in considerable amounts of other fragmentation productions as well. Selective ion monitoring of the $M+H^+$ peak (m/z 83) gave adequate sensitivity and much more reproducible results and this method was chosen for the analysis.

Solvent mixtures ranging from 50% to 90% methanol (incorporating 0.1% formic acid) were tested for the HPLC method using the Restek Ultra PFP Propyl column. All gave acceptable results, with the retention time increasing slightly as the percentage of methanol increased, with 70% methanol giving a retention time of ca 2.6 minutes (about 4 column volumes) and 4MI being easily detectable at concentrations of 100 ppb.

2.2 Efficacy of nitrate mitigation in cattle known and available

The efficacy of nitrate in mitigating enteric methane emission from cattle was quantified by 3 studies:

- Assessing methane emission from feedlot cattle on a urea or iso-nitrogenous nitrate containing diet when the N source was included in the liquid supplement. Emissions were measured by the Greenfeed emission monitoring unit (GEM). A full report on this study is published (Velazco et al., 2014) so methods are not reported here.
- Assessing the methane mitigation of roughage fed cattle with supplementary nitrate (or urea) included in the liquid supplement (diluted molasses). Emissions were measured by respiration chambers.

Crossbred Angus cattle (15 months old, X= 231.4 kg) randomly stratified by weight, were placed in group pens (n=6; 4 animals/pen) then randomly allocated to either urea (U) or calcium nitrate (NO3) supplementation treatments. They received an *ad libitum* basal diet of oaten chaff (DM 90.4%; NDF 56%; CP 7.8%; ME 9.9MJ/kgDM), plus a liquid, molasses based supplement with isonitrogenous concentrations (29gN/kg) of either (NO3) or (U). Pen intakes of the basal diet were recorded, and the daily pen intake of supplement was determined gravimetrically. Blood MetHb concentrations were monitored weekly from jugular venepuncture using a blood gas-analyzer. During the last two weeks of the trial, 2h CH_4 production was determined once weekly by open-circuit calorimetry for each group (at 1000 h), using equipment whose construction and operation has been described in detail by Waghorn (2012).

- Assessing the methane emission from rangeland cattle supplemented with lick-blocks containing 40% calcium nitrate. Emissions are being measured by GEM units.

In association with an AoTG project, emissions from nitrate-fed cattle are being measured at Augathella in the central west of Qld. Four hundred cattle are accessing a single water point in a 3000 acre paddock. The cattle have been trained to go through an auto-drafter that weighs animals as they walk over the platform then drafts them into one of 4 treatment groups. The four treatment groups are:

  - Urea-based multi-nutrient block
  - Nitrate based multi-nutrient block
  - Urea containing single nutrient blocks (highN, S, or P blocks)
  - Nitrate containing single nutrient blocks (highN, S, or P blocks)

2.3 Growth and productivity data of nitrate supplemented livestock made available

In all of the above mentioned studies, where animals were involved, growth was monitored and wherever possible feed intake also monitored. For sheep studies wool growth was always monitored. These studies included:

- Study 1. Sheep: Nitrate and Sulphur interactions (Liet al., 2013bRAAN)
- Study 2. Sheep: Nitrate with/without propionibacterium probiotic. (Methods section 2.1.5 above)
- Study 3. Sheep: Long term efficacy of dietary nitrate and other dietary mitigation agents (oil, bentonite, cysteamine) in affecting body and wool growth of lambs. (Methods in section 2.4)
- Study 4. Sheep: Synergy of nitrate and defaunation in affecting lamb growth, productivity and emissions (Methods follow)

- Study 5. Cattle: Growth on feedlot diet with 1.88% dietary nitrate in association with AoTG project (Hegarty et al., 2013)
- Study 6. Cattle: Growth on roughage provided with nitrate- or urea-containing molasses supplements (Methods provided above in Section 2.2)

A summary of the methods for studies listed above that are not previously published are provide below. In addition, because reductions in feed intake are a common but undesirable commercial consequence of nitrate feeding, a review of nitrate's effects on voluntary intake was conducted by a post-graduate student.

Study 4. Synergy of nitrate and defaunation in affecting lamb growth, productivity and emissions

Animals and diets

All protocols for care and treatments of the animals were approved by the University of New England Animal Ethics Committee (AEC 14-083).

Twenty cross-bred merino lambs with an average body weight of 38 ± 1.9 kg were used in this study. Lambs purchased from Chiwick were moved to Kirby research station, The University of New England and adapted to lucerne cereal mix chaff for 4 days. Lambs were weighed and randomly selected and assigned into defaunated and faunated groups by stratified randomisation. Defaunated lambs were then changed to a dietary coconut oil distillate (COD) with initial inclusion of 3% to 5% of COD over 7 d to eliminate protozoa while faunated lambs remained to lucerne cereal mix. On d 46, lambs from defaunated and faunated groups were randomly selected and assigned into 4 subgroups (n = 5) to form a 2×2 factorial design of experiment (faunated stage and nitrate supplement). Lambs had been adapted to nitrate (Calcium nitrate, $(5Ca(NO_3)_2.NH_4NO_3.10H_2O$, Bolifor CNF, Yara, Oslo, Norway) for 2 weeks with initial addition of nitrate from 1 to 2% (3.12 % Bolifor). After the period of nitrate adaptation, lambs were given nitrate for 6 weeks before the comparative data were collected. Lambs had *ad libitum* access to the diets (Table D5) until methane production measurement period. On d 100 all lambs were on restricted intake (fixed intake 80%) 5d before they came in their respiration chambers for methane measurement and continuously received fixed intake in the period of faeces collection for analysis of digesta kinetics from d 112 to 120. On d 120 all lambs were scanned their body composition and rumen volumes. Water was made available at all time.

Table D5. Analysed chemical compositions of the diets (% as fed)

Component	**Diet (D1) Oaten chaff**	**Diet (D2) 2% nitrate**
Dry matter	90.2	89.6
Dry matter digestibility	71	70
Digestible organic matter in DM	67	66
Inorganic ash	6.4	7.3
Organic matter	93.6	92.7
Neutral detergent fibre	49	48
Acid detergent fibre	26	25
Crude protein	4.1	7.1
Metabolisable energy (MJ/kg)	10.6	10.4
Nitrate nitrogen (mg/kg)		4100

Defaunation of lambs

After 7 d feeding COD, all feed was withdrawn for a day and lams were orally dosed with sodium 1-(2-sulfonatooxyethoxy) dodecane (Empicol ESB/70AV, Allright and Wilson Australia Ltd, Melbourne) administered at 100 mL/d 10% v/v solution to remove protozoa. Lambs were fasted on day 0 and were dosed on three consecutive days and feed was withheld during this period. Animals required 7 d to recover their pre-drenching voluntary intake. The three day dosing with Empicol was repeated commencing 7 d after the first dosing. A further 7 d after the second drenching program, rumen fluid samples were collected for protozoa enumeration.

Rumen fluid sampling, ammonia, volatile fatty acid concentrations, and protozoal enumeration
Samples of rumen fluid (20 mL) were collected using oesophageal intubation from each animal before feeding for protozoal enumeration after second drenching program. Samples were immediately checked under a microscope to confirm that the animals were protozoa-free or otherwise. Rumen pH was measured immediately using a portable pH meter (Orion 230 Aplus, Thermo scientific, Beverly, MA, USA). A 15 mL subsample was placed in wide-neck McCartney bottle acidified with 0.25 mL of 18 M sulphuric acid and stored at -20^{0}C for VFA and ammonia analyses. A 4 mL subsample was placed in wide-neck McCartney bottle containing 16 mL of formaldehyde-saline (4% formalin v/v) for protozoa enumeration. Protozoal staining technique was an adaption of the procedure described by Dehority (1984). Protozoa were counted using a Hawksley Cristalite B.S. 748 counting chamber (Sussex, UK). The protozoa were differentiated into large (>100 µm) and small (<100 µm) holotrich and entodiniomorph grouping. The VFA analysis was determined by gas chromatograph using a Varian CP 3800 Micro Gas Chromatograph instrument (SMARTGAS, Varian CP 3800) and ammonia analysis was determined based on modified Berthelot reaction using a continuous flow Skalar San^{plus} Analyser (Skalar Analytical B.V.4800 DE Breda, The Netherlands).

Methane chambers and production measurement
The animals were placed in the chambers for a period of 22hrs where their methane emissions were recorded. Air was drawn through each chamber at a rate of approximately 120 l/min using a household vacuum cleaner (Sony Corp. Japan) connected to 37mm i.d. flexible hosing from outside the chamber room. Air within the chambers was continuously mixed by an oscillating fan mounted in the roof of each chamber. Total airflow through each chamber was measured using an AL800 airflow meter (American Meter Co.) with samples of outgoing air analysed for CH_4 concentrations over 20sec every 6mins.

Concentrations of methane emitted by each animal were determined using an Innova 1312 photo acoustic gas analyser (Air Tech Instruments, Denmark), calibrated with a two point calibration (0 and 100ppm CH4). Data was recorded on a personal computer using proprietary software. Temperature and humidity was measured via temperature and humidity probes placed inside the chambers and attached to Easysense advanced data loggers (Data Harvest, UK). Methane production was calculated as the product of the average net increase in methane concentration (outflow – inflow) and the total airflow through the chamber. As a second measure of methane production, average methane concentrations were analysed from a Tedlar bag containing sub-samples of air from within each chamber, drawn into it by a peristaltic pump continuously over the 22hr period. Recovery of methane released from a cylinder of pure methane (BOC Ltd – 99% purity) was between 98 and 100%.

Preparation of digesta kinetic markers, and faeces and urine collection
A solid marker, chromium (Cr)-mordanted fibre was prepared in accordance with the method described by Uden et al. (1980). Oaten chaff was sieved through a 1mm screen and the chaff particles smaller than 1mm discarded. The remained chaff was dried in a fan-force oven at 65^{0}C for 24h, washed, boiled and treated with sodium lauryl sulphate (15g per 100g chaff dry weight), acetone, sodium dichromate (30-33% of chaff dry weight), ascorbic acid (50% of chaff dry weight) and dried in the oven at 65^{0}C for 24h to leave Cr-mordanted fibre (NDF). The Cr-mordanted feed was then ground through a 5mm screen and stored in a dry place. A liquid marker, Cobalt (Co)-EDTA was purchased from the commercial company (AVA Chemicals (P) Ltd. Mumbai. India).

Faeces collection
For a period of six continuous days post administration of the digesta kinetics markers, a total faecal collection was conducted to determine mean digesta retention times of both liquid and solid phase digesta for each animal. Plastic bags were attached to the bottom of the metabolism crates in order to collect any faeces passed by each animal. The first bag was replaced with a new one 8hrs after administration of the markers, with subsequent changes occurring at set intervals over the following six days – every 2hrs for the first 24hrs, every 4hrs for the next 48hrs, every 8hrs for the next 24hrs and every 12hrs for the next 48hrs. A total of 28 samples were collected from each. Twice a day, samples were weighed, thoroughly mixed and sub sampled. Each sample bag was recorded with the associated animal's ID, time and date of collection.

Urine collection
Total urine output was collected over six days from each animal. The urine was collected into 10lt plastic buckets placed underneath the metabolism crates. Each bucket contained approximately 100ml of 10% (v/v) H_2SO_4 to prevent bacterial degradation of purine derivatives (PD). Once a day, each animal's urine was decanted into a pre-tared bucket and weighed. The collected urine was then diluted to 3lts with water

and thoroughly mixed to prevent precipitation of PD. Subsamples were taken and the pH adjusted to <3 by the addition of 18M H_2SO_4 before being frozen at -20°C until required for analysis.

Estimation of microbial protein outflow from urine
Allantoin excreted in sheep urine represents about 60-80% of total purine derivatives (PD) present, it was assumed that the concentrations in the urine samples accounted for 70% of total PD excretion and adjusted accordingly. Purine absorption was calculated by means of the Newton-Raphson iteration process with microbial N supply being 0.727 x the absorbed purines (IAEA 1997). The procedure to estimate allantoin concentration was of (IAEA 1997) using a UV-1201 spectrophotometer (Shimadzul, Japan) set at 522nm.

Methaemoglobin (MetHb) concentration in blood
Blood was sampled 3 h after feeding on d 46, 53, 61 and 105. A sample of 8 mL was taken from the jugular vein, using lithium heparinised vacutainers (BD Franklin Lakes, NJ, USA). MetHb concentration in blood was determined using blood analyser.

Live weight gain and wool growth
All lambs were weighed after 4 days of their arrival to determine their initial body weight. After defaunation period, the comparative study began on d 0 all lambs were weighed and their body weights were recorded every two week during the 6 weeks *ad libitum* feeding of nitrate-supplemented diet.
Clean wool growth rate, yield, fibre diameter were determined on the mid-side of the sheep between d 46 to d 96 by clipping a patch approximately 10×10 Cm (Oster Golden A5 clippers, blade size 30 model Cryogen X, USA). After the wool from the patch was clipped, four sides and one diagonal were measured and the area of the patch was calculated using Heron's formula. Wool samples were sent to New England Fibre Testing PTY Limited to determine yield, micron, curve and comfort.

Statistical analyses
Data were statistically analysed using SAS 9.3 (SAS Inst., Cary, NC). Data from rumen fermentation characteristics, digesta kinetics, wool growth, microbial protein N and methane production and yield were subjected to analysis of variance in PROC GLM, factors being protozoa, NO_3 and protozoa × NO_3. For analysis of live-weight and live-weight gain the model used the initial weight as a covariate. Means were analysed using the least squares means (LSMEANS) procedure. Differences among means were tested using the PDIFF option.

2.4 Long-term impacts of methane suppression on animal growth

The effects on body- and wool-growth by sheep from supplementing a chaff diet with one of 4 potential methane reducing technologies was investigated. Thirty Suffolk x Merino SAMM hogget ewes, approximately 12 months old and weighing 41 ± 2 kg, were housed in individual pens and adapted to a diet of lucerne and wheaten-chaff for 2 weeks before being fed *ad libitum* throughout the 70 d experimental period, with any refusals being collected, weighed and recorded to determine feed intake. The animals were assigned to one of five groups by stratified randomisation according to live-weight. Diets were:

- Chaff + 2% urea Control –
- Chaff + 2% nitrate (as calcium nitrate)
- Chaff + 2% sodium bentonite
- Chaff + 5% oil (as canola oil)
- Chaff + 80mk/kg LW cysteamine, provide as an encapsulated Chinese product.

Growth and feed intake was measured over 70d and wool growth measured after further adjustment to diet. Methane production was measured and blood and rumen samples taken for methaemoglobin.

2.5 Nitrate feeding submitted for approval as a CFI methodology

FtRG projects were specifically excluded from developing/submitting CFI methodologies, however, the intent of this objective was that the project provide underpinning data and understanding that could be used in developing and supporting a CFI methodology. This project has contributed to the development of best management practices for nitrate feeding to underpin the current nitrate CFI methodology in association with Andrew Sedger and with providing advice on levels of ammonium-

and calcium-nitrate that should be fed to cattle. This advice was developed using the following methods.

2.5.1 Development of Best Management Practices

A simple set of 'dot points' was sought to adequately describe for producers the purpose, risks and management strategies to minimise risk and provide confidence in nitrate feeding for methane mitigation. An initial set of 8 points was developed by UNE then circulated to other nitrate researchers around the world (Canada, The Netherlands, Europe and Australia). These edits were compiled, revised through the same team and submitted to MLA. This list was then worked on by others within the NLMP (especially Andrew Sedger and John Black and a revised advisory document of Best Management Practices".

As a further step, GrazFeed simulations of pasture intake and performance of northern cattle (British x brahman cross) cattle were conducted to give guideline figures of weights of nitrate supplements required/animal/day. In addition these allowed changes in daily emission and likely emission intensity (methane/kg live weight gain) to be anticipated.

The list of key practices and this practical data on nitrate inclusion levels were then combined in a modified set of Best Management Practices, with Andrew Sedger taking primary responsible for creating this industry-friendly copy as part of another project. These best management practices are included below and the description of the GrazFeed calculations giving rise to the recommendations on nitrate inclusion are provided subsequently.

2.5.2 Guidelines on nitrate inclusion levels

The GrazFeed animal nutrition model was used to estimate feed intake and growth rate of crossbred steers (Bos indicus x Bos taurus cross; 300, 400, 500 kg LW) when consuming pasture of 45, 55, 65 or 75% DM digestibility. The protein content of pasture with each digestibility was estimated by establishing a regression equation between DMD and pasture crude protein using approximately 92 pasture cuts representing 17 tropical and subtropical species (Milford 1960). Grazfeed estimated the DMI, ME intake, protein intake and methane output for each feeding scenario. These cattle and pastures were chosen as being most relevant to the rangeland cattle production systems in which the nitrate feeding methodology will be applied.

Based on the estimated DMI, the quantity of nitrate required per day to provide 7g nitrate/kg DMI was estimated and expressed in terms of daily intake of ammonium nitrate (77.5% NO3) or calcium nitrate (as Bolifor; 63.6% NO3 as fed).

GrazFeed simulations were then re-run for each LW and DMD, with the CP content of the diet raised in keeping with the quantity of nitrate nitrogen added.

For GrazFeed simulations, assumed ages for 300, 400 and 500 kg cattle were 15, 24 and 36 months respectively. Degradability of protein was considered to be 67% in all situations. A supplementation goal of achieving a LWG of 1.5 kg/d was specified so that cattle would eat as much DM as feasible. Pasture on offer was set at 0kg/ha to prevent Grazfeed implementing selectivity routines that make interpretation difficult, so DMD reported is DMD of the feed consumed by the cattle (rather than that on offer). Effects of weather were ignored during the simulation.

2.6 Nitrate used safely in major AotG demonstrations with over 1000 cattle

In association with an AoTG project, this project has supported development and delivery of molasses-based lick blocks containing up to 40% calcium nitrate in 3 major rangeland studies; being:

- "Arabella" (Charleville Qld). Comparative intake of NO3 lick blocks 50 + 220 head (n)
- "Burleigh" (Richmond, Qld). Principle trial with 40% calcium nitrate blocks, n=650
- "Dungowan" (Augathella Qld). Trial with ACC, n = 400. Methane to be measured by GEM

3 Results

Results are presented against each activity undertaken to deliver each of the required outcomes:

3.1 Dietary conditions for safe nitrate feeding

Three toxicity risks were perceived to be associated with the inclusion of nitrate in animal feeds, being: (1) absorption of nitrite from the rumen and formation of MetHB which reduces oxygen carrying capacity of the red blood cells and can cause death by anoxia; (2) formation of 4-methylimidazole compounds in molasses based lick blocks,(3) formation of nitrosamines within the animal and potential human poisoning due to nitrosamines. Studies to address imidazoles and methaemoglobin were undertaken in this project. Study of nitrosamines in the meat of nitrate-fed cattle was undertaken in an AoTG project and is not reported here (but was negative for nitrosamines).

3.1.1 Review of current understanding of the processes and biology of nitrate and nitrite reduction in the rumen.

This review assessed literature from all fields of nitrate research and application and its findings included the following points;

- Nitrate reduction by micro-organisms in natural and contrived environmental situations that have economic consequences (e.g. oil fields, sediments, sewage works, biodigestors etc) has led to an enormous literature on the microbiology, biochemistry and genetics of these organisms which are not only widespread but are extremely diverse in their metabolic strategies.

- Researches of microbial physiology and biochemistry have described several microbial enzymes that effect the reduction of nitrate to ammonia in the rumen and other anaerobic ecosystems.

- These enzymes are characterised as nitrate and nitrite reductases and often are present in the same organism.

- Dissimilatory nitrate reductase functions as an electron acceptor re-oxidising reduced coenzymes and playing the same role as would be played by methanogenesis in some ecosystems.

- Assimilatory nitrate reductase produces ammonia intracellular at rates commensurate with cellular growth.

- Recently, an assimilatory nitrate reductase that is also coupled with oxidation of sulphide to sulphur or sulphate has been identified in bacteria from a number of anaerobic ecosystems. The most studied organism occurs in oil-contaminated water where the oil organics are being fermented. These organisms are known as nitrate reducing, sulphide oxidising bacteria (NR-SOB). They have been shown to produce ammonia and sulphate or poly- sulphur under nitrate-limited growth media conditions and when nitrate is in excess and sulphide is limiting they produce nitrite. Organisms with these capabilities appear to be present in the rumen. For example W. succinogenese has the capacity to oxidise hydrogen sulphide and reduce nitrate and nitrite. The organism uses the conversion of nitrite to ammonia to generate ATP for growth.

- Sulphur reducing bacteria (SRB) and nitrate reducing bacteria (NRB) are diverse and are found together in many anaerobic ecosystems including the rumen. There are major interactions between nitrate and sulphate metabolism in the consortiums of micro-organisms that are present in these diverse anaerobic ecosystems. Of major importance is that many of the SRB appear to have dual roles, i.e. they reduce inorganic and organic sulphur and also most of the species also actively reduce nitrite to ammonia. However, in a high nitrate medium, these organisms or their sulphur and nitrite reducing abilities appear to be suppressed. In the oil-field studies, the nitrate suppression of sulphur reduction in the sulphur reducing organisms decreases hydrogen sulphide production and stimulates the NR-SOB to produce nitrite from nitrate. This inhibition is strengthened in substrate limited media (low amounts of fermentable carbohydrate providing limited electron donors). It is hypothesised that a similar interaction may explain the accumulation of nitrite when nitrate is suddenly introduced into the rumen.

- It is concluded that when animals are acclimated slowly to dietary nitrate, nitrite accumulation in the rumen is avoided and toxicities are not experienced.

- Use of nitrate as a supplement for to grazing animals will require the development of supplementary feeding strategies that either permits the animal frequent access to nitrate, or a slow-release nitrate formulation is provided in the supplements that are ingested less frequently, (Slow release nitrate preparations are already in use particularly for production of turf). The same problems beset managers wanting to use urea as nitrogen supplement and there are a number of strategies already developed that may be brought to bear on this area for research.

- There are numerous basal feed resources throughout the world used for ruminant production that are deficient in crude protein and require supplementation with a source of fermentable nitrogen to balance the diets for production. These include traditional grain based feeding systems as adopted by the US and Australian beef feedlot industry. Urea has been the source of additional rumen ammonia. However, with careful slow adjustment (a requirement which also applies to urea feeding), there seems to be no impediment to replacing urea with nitrate and possibly with better results (more efficient microbial growth and less energy losses as methane).In addition in the grazing livestock industries nitrate has major potential to replace urea in block licks, loose mixes and liquid mixes which have proved so successful in promoting growth in animals fed crop residues, stubbles and dry pastures throughout the world.

- Numerous possibilities exist for replacing urea with nitrate in unconventional high energy, low protein forage crops that are being used for ruminant production in various parts of the world, for example sugar cane and molasses or cassava-based diets.

- Recent technical developments using straw treated with alkali to improve its digestibility may promote production systems for ruminants, where replacing urea with nitrate is entirely feasible. With these new treatment technologies, production levels approach those obtained on grain based feedlot diets. Some 35 million tonnes of straw is produced annually in Australia and with the adoption of the new technologies, the straw presently wasted, often by burning, could support 5- 10 million cattle. The present use of straw treated or otherwise as a ruminant feed is enhanced by supplementing the diets with urea. Inhibition of methane by replacing urea with nitrate in these diets is an attractive proposition that could assist such industries to develop, particularly if credit is provided for reduction in greenhouse gas emissions. Sugar cane and cassava thrive on the coastal fringe of Queensland and could potentially be the feed of choice for fattening cattle for the overseas meat trade, or in drought when there is the need to de-stock pastures and provide other means of supporting, in particular, the breeding stock.

- The application of nitrate to ruminants under ideal pasture grazing conditions is not likely to be practical for a number of reasons including the potential for toxicities, the probable lack of intake of high nitrate supplements and the increased load of nitrogen in animal excreta which may increase NOx release into the atmosphere. However, research leading to the successful application of nitrate in low protein diets could provide the incentive for the development of pasture species designed to improve pasture biomass low in protein. This would require a paradigm shift in pasture plant breeding and management

3.1.2 Dynamics of nitrate metabolism assessed by infusion of nitrogen isotopes into the rumen and blood

The following results were derived from the 15N nitrogen kinetics studies.

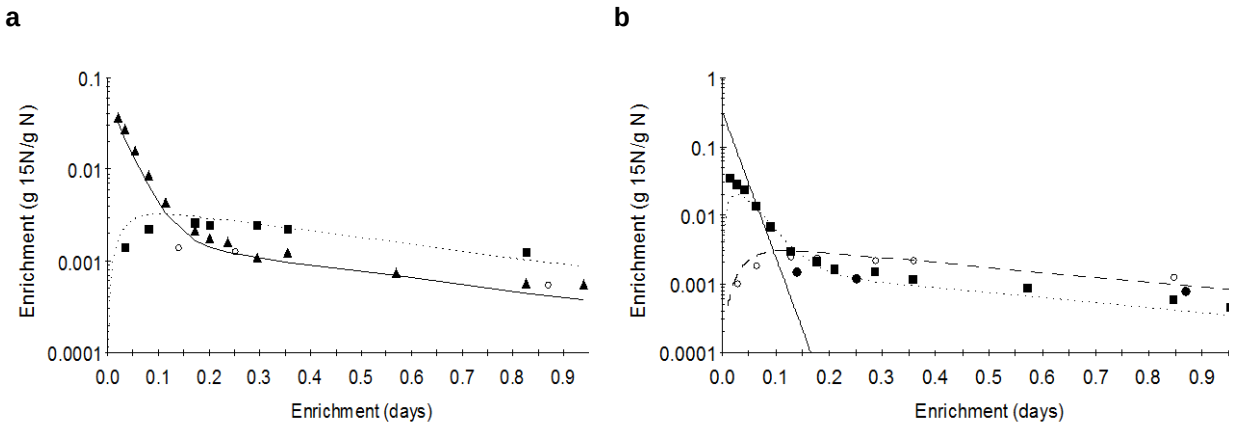
Results of experiment 1 (conducted before NLMP but processed in NLMP)

One–compartment N model (whole rumen contents)
Estimates of the mass of the total rumen N compartment (Fig.1) and outflow of N from this compartment did not differ between diets nor between the forms in which ^{15}N was administered intraruminally, i.e. $K^{15}NO_3$ or $^{15}NH_4Cl$, and results for all individual tracer experiments were averaged. Accordingly, the mass of the total N compartment in the rumen was estimated to be 21g (SEM 0.99, n=8), and the N outflow to be 20 g N/d (SEM 1.52, n=8).

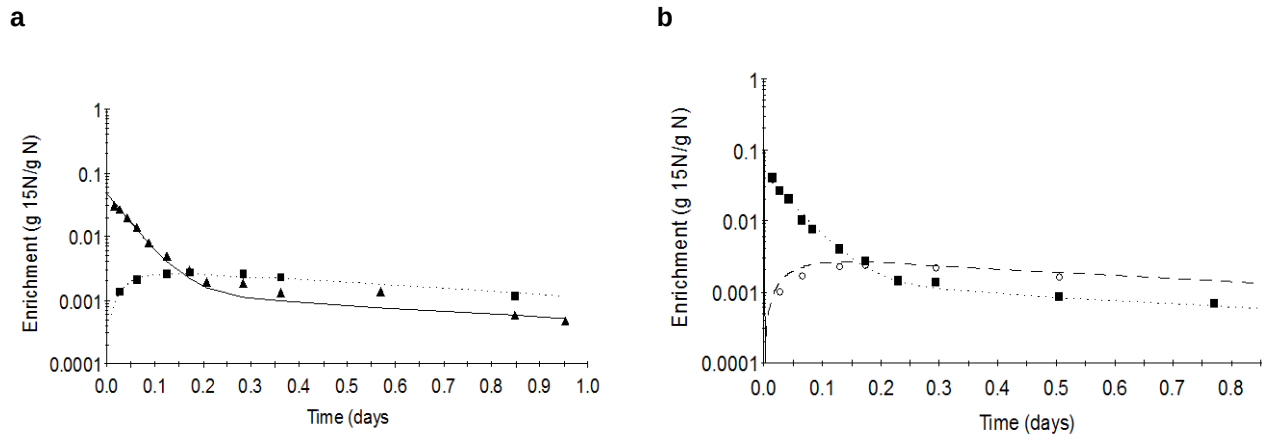
Three-compartment N model (nitrate, ammonia and bacteria)

Visual appraisal of the curves generated by solving and optimizing the compartment sizes and flow rates indicated that the enrichment v. time data for rumen NH_3-N and bacterial N were well fitted by the 3-compartment model (Fig 1). Using criteria described by other workers[27; 26], the parameters were well determined statistically (mean CV <0.1 except for L(0,1) and F(3,1) as discussed above); parameter correlations were within the range of -0.8 to 0.8 (except for R(0,2) and U(3,0) which showed higher correlations for model output after fitting data for some individuals) and residual sums of squares between compartments were relatively low. There was, however, some evidence of bias in the fits to the bacterial enrichments in the 10 h after tracers were administered. The estimates of mean flows of N (designated R(i,j) in WinSAAM) from the individual optimized model solutions had relatively low SEM.

Representative examples of fits to the enrichment v. time results for individual sheep are given in Figs D3 and D4. For all sheep, simulated turnover of the NO_3^- compartment was extremely rapid and the enrichment of NH_3-N after $^{15}NO_3^-$ injection, which was zero at the moment of tracer injection, had risen to its maximum value by the time the first or second sample of rumen NH_3 was taken (Fig. D3b). The fitted enrichment curves for NH_3-N and bacterial N after administration of either $^{15}NH_3$ or $^{15}NO_3^-$ were remarkably similar, indicating that conversion of NO_3^- to NH_3 occurred rapidly on all diets.



FigureD3. Output from a 3-compartment model (shown as lines over-plotted on experimental data represented by symbols) for enrichment of NH_3-N (▲) and bacterial N (■) after intraruminal injection of $^{15}NH_4$-Cl into the rumen (a), and of $^{15}NO_3$-N (no data, simulated solid line), NH_3-N (▲) and bacterial N (■)after intraruminal injection of the same amount of ^{15}N in the form of $K^{15}NO_3$ (b) for Sheep 2 ingesting a diet containing 1.2% urea. All 4 sets of enrichment data were fitted simultaneously using the one model.



FigureD4. Output from a 3-compartment model (shown as lines over-plotted on experimental tracer data represented by symbols) for enrichment of NH_3-N (▲) and bacterial N (■) after intraruminal injection of $^{15}NH_4$-Cl into the rumen (a), and of $^{15}NO_3$-N (no data, simulated solid line), NH_3-N (▲) and bacterial N (■)after intraruminal injection of the same amount of ^{15}N in the form of $K^{15}NO_3$ (b) for Sheep 10 ingesting a diet containing 2.5% NO_3^-. All 4 sets of enrichment data were fitted simultaneously using the one model.

The mean compartment sizes (bolded values) and N flow rates determined by fitting the ^{15}N tracer results to the steady-state model for each sheep are given in Figs D5 and D6, respectively.

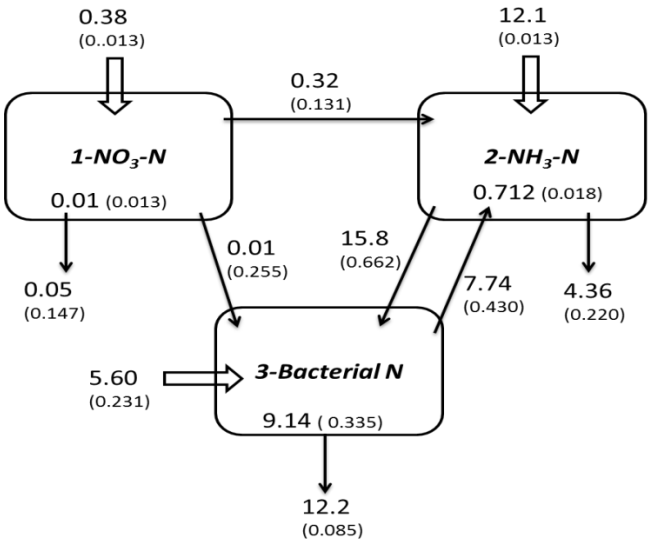


Fig. D5. A steady state model showing compartment sizes (M(i), g N) and flow rates (R(i,j), g N/d) in the rumen of sheep (mean ± SE). The same model was fitted to experimental tracer data from compartments 2 and 3 for each of the sheep given a diet of chaffed oaten hay supplemented with 5.52 g urea-N/d (see example of fit for one sheep in Fig. D3).

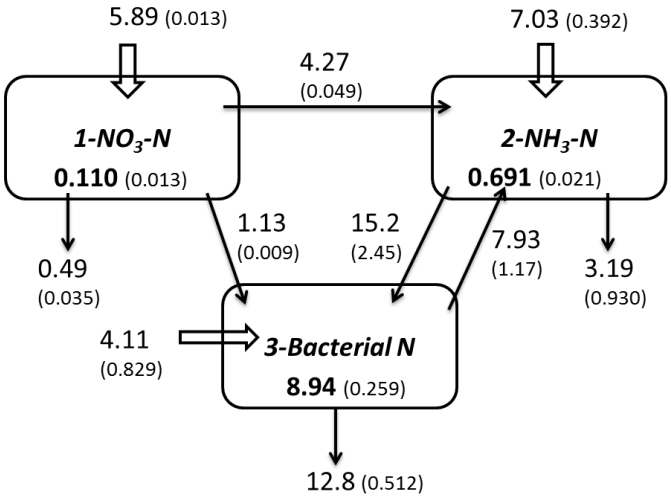


Fig. D6. A steady state model showing compartment sizes (M(i), g N) and flow rates (R(I,j), g N/d) in the rumen of sheep (mean ± SE). The same model was fitted to experimental tracer data from compartments 2 and 3 for each of the sheep given a diet of chaffed oaten hay and a supplement of KNO_3 supplying5.52 g N/d (see example of fit for one sheep in Fig. 3D).

The model-optimised rumen NH_3-N compartment mass estimate (0.69-0.71 g N) was similar to the mean value determined from other results for these sheep[19] and the bacterial compartment size (8.4-9.5 g N) was about 30% higher than the values used to initialize the models.

In NO_3^- -supplemented sheep, 72% of NO_3^- entering the rumen from the feed was apparently reduced rapidly to NH_3 via R(2,1); 19% was directly assimilated by bacteria (R(3,1) and 9% (R(0,1) was either absorbed across the rumen wall, stored in compartments that were not sampled (e.g. protozoa or fungi) or passed out of the rumen in digesta (Fig. D6). Bacteria obtained the majority of their N for polymer synthesis from NH_3 and the remaining 20-26% from NAN via U(3) (probably mainly as peptides and amino acids) and via R(3,1) (NO_3^-). The irreversible loss of bacterial N from the sampled compartment (net synthesis) was similar for sheep on both diets (12.2-12.8 g N/d) but the total synthesis of bacterial crude protein (CP) was about 26% higher than the net synthesis because there was extensive recycling of NH_3-N between the bacterial N and NH_3-N compartments. The transfer of N via R(0,2) would represent NH_3 absorbed via the rumen wall and also NH_3 in digesta flowing out of the rumen (about 1.1 g N/d).

Results of experiment 2.

All $_{15}N$ analyses in ammonia and in rumen microbes have been completed but it has not yet been possible to determine the enrichment of rumen nitrate and nitrite at this time, therefore the flux models have not been solved. We have investigated a large number of masss spectrometer methods for analysis for these compounds without success.

3.1.3 Conducting batch, continuous culture and animal investigations of the effect of molybdenum and sulphur on the ruminal metabolism of nitrate and the risk of methaemoglobinaemia.

In-vitro study

NO_3-N infused to the fermentation vessel on average was 103 ± 7.8 mg/d, the percentage of NO_3-N added daily remaining in the vessel effluent was less than 0.5% and percentage remaining as NO_2-N was even lower, with a mean value of 0.02%, confirming that the majority of NO_3-N supplied to the incubation medium was reduced by the microbiota in the vessels.The addition of SO_4^{2-} to the NO_3^- containing diet produced 75% less CH_4 when compared with the SO_4^{2-} absent NO_3-containing diet (Table D5. P<0.05).

Table D5. Effects of different concentrations of Mo and S on *in vitro* rumen fermentation and nitrogen parameters (mean over 4 days). The '+' or ' –' symbol represents the present or absence of the supplementary compound.

	+ NO_3 – S – Mo	+ NO_3 – S – Mo	+ NO_3 – S – Mo	+ NO_3 – S – Mo	**s.e.d.**	P value		
						Mo	S	Mo × S
CH_4 production (mmol/d)	0.64	0.19	0.80	0.16	0.056	0.313	<.001	0.176
Nitrate (ug N/d)	115.4	175.3	119.9	422.6	34.79	0.022	0.006	0.025
Nitrite (ug N/d)	10.1	39.7	13.8	47.3	2.91	0.126	<.001	0.537

The addition of SO_4^{2-} and MoO_4^{2-} did not stimulate NO_3^- or NO_2^- reduction, which is contrary to our hypothesis. However, the negligible amounts of NO_3 and NO_2-N present in the effluent suggests that NO_3^- was almost completely reduced to ammonia and or utilized in microbial synthesis. Therefore, the level of NO_3 tested in this study (4 g NO_3-N/kg DM) could be safely applied to an in-vivo study.

Sheep study

Many of the lambs developed diarrhoea by Day 14 (a mean rumen pH of 5.8 was observed for lambs experiencing diarrhoea). Thiamine (Vitamin B1) deficiency was diagnosed in one lamb (T2) in the final week, and it was removed from the trial.

Dry matter intake, live weight gain and clean wool growth

Dry matter intake, LWG and feed conversion ratio (FCR) did not differ (P> 0.05) between treatments (Table D6). The average clean wool growth (CWG) was increased (P < 0.001) by approximately 30% when 1% urea was replaced by 1.88% NO_3 in the diet (T3 *vs.* T1). The rate of CWG was improved (P < 0.001) when the content of elemental S in the NO_3-containing diet was increased from 0 (T2) to 0.18% (T3); however, this additional elemental S supplementation did not further increase CWG in animals fed a NO_3-containing diet (T4 *vs.* T3). Sheep offered diets containing added NO_3 instead of urea also tended (P = 0.07) to have higher skin surface temperatures.

Table D6. Average dry matter intake (DMI), daily liveweight gain (LWG), feed conversion ratio (FCR), clean wool growth (CWG) and skin surface temperature of wether lambs fed diets with and without nitrate and/or elemental sulphur over a 38-day measurement period (Days 27–65). Data were based on 11 lambs per treatment

	Treatment					
	T1	T2	T3	T4		
	1% urea + 0.18% S	1.88% NO_3 + Nil S[A]	1.88% NO_3 + 0.18% S	1.88% NO_3 + 0.4% S	s.e.d.	*P* value
Initial LW (kg)	22.4	22.5	21.9	22.2	0.54	0.72
DMI (g/d)	761	687	813	741	103.9	0.682
LWG (g/d)	114	91	153	122	30.7	0.264
FCR (g DMI/g LWG)	7.58	7.62	5.49	8.53	1.680	0.28
CWG (µg/cm^2/d)	486[a]	530[a]	668[b]	738[b]	52.0	<0.001
Skin surface temperature (ºC)[B]	29.4	30.0	29.8	30.2	0.29	0.07

[A]: One lamb diagnosed with thiamine deficiency was removed.
[B]: skin surface temperature was measured on Days 71, 72 and 73 after sheep were shorn.

Nitrogen and sulphur utilisation

Throughout the seven-day total collection, DMI and LWG were not affected by diet (P >0.05). N intake, microbial N outflow and N retention did not differ between treatments (P >0.05; Table D7). Whereas the addition of 0.4% elemental S to a 1.88% NO_3 diet increased S intake and retention (P < 0.001*)*, addition

of elemental S to the diet did not affect N retention of sheep receiving NO_3-containing diets ($P > 0.05$). Lambs offered the T2 diet ingested less S ($P< 0.001$) and retained less ingested S ($P < 0.001$) than the other treatments. The N and S content of wool were not affected by any of the four treatments.

Table D7. Main effects of treatment (means ± s.e.d., $n = 5$) on DM digestibility (DMD), N and S utilisation of sheep fed *ad-libitum* diets with and without nitrate and/or elemental sulphur over a seven-day period of total collection

	T1	T2	T3	T4		
	1% urea + 0.18% S	1.88% nitrate + Nil S	1.88% nitrate + 0.18% S[A]	1.88% nitrate + 0.4% S	s.e.d.	*P* value
DM intake (g/d)	981	956	891	960	69.60	0.61
WG (g/d)	175	135	160	122	29.8	0.32
DMD (%)	69[ab]	73[c]	67[a]	71[bc]	1.30	0.01
Microbial N outflow (g/d)	10.2	9.76	9.41	10.1	1.818	0.97
N intake (g/d)	21.43	21.34	20.48	21.99	1.57	0.81
N retention (g/d)	6.41	8.02	6.81	8.52	1.30	0.36
$N_{retention}/N_{intake}$ (%)	29.2	37.9	32.8	40.3	5.13	0.17
S intake (g/d)	3.51[a]	1.86[b]	3.19[a]	4.95[c]	0.40	<.001
S retention (g/d)	1.35[a]	0.67[b]	1.23[a]	2.29[c]	0.16	<.001
$S_{retention}/S_{intake}$ (%)	38.2[a]	36.6[a]	38.4[a]	47.1[b]	2.95	0.01
Wool N (%)	15.3	15.3	15.3	15.3	0.10	0.87
Wool S (%)	3.72	3.55	3.61	3.51	0.130	0.46
Wool N:S ratio	4.13	4.31	4.24	4.37	0.162	0.50

A: One sheep was removed from the analysis due to severe diarrhoea.

Enteric methane production

There was a substantial increase in daily DMI as the experiment progressed; however, there was no effect of treatment on DMI over Days 27–65 (Table D6), during the digestibility study (Table D7) or during the measurement of methane emission in the final week of the study (Table D8). Methane production (g/d) and methane yield (g/kg DM intake) were reduced ($P < 0.05$) by approximately 24% when urea was replaced by NO_3 in the diet (T1 *vs.* T3). The addition of 0.4% elemental S to the 1.88% NO_3 diet also reduced methane production ($P = 0.021$) and methane yield ($P = 0.028$).

Table D8. Least square means for dry matter intake (DMI), methane production and methane yield of wether lambs ($n = 6$) fed diets with and without supplementary nitrate and/or elemental sulphur

	T1	T2	T3	T4		
Variable	1% urea + 0.18% S	1.88% NO_3 + Nil S	1.88% NO_3 + 0.18% S	1.88% NO_3 + 0.4% S	Average s.e.d.	*P* value
DMI (g/d)[A]	789	771	878	904	74.8	0.331
Methane production (g/d)	14.3[a]	10.0[b]	10.7[b]	7.41[c]	1.875	0.021
Methane yield (g/kg DMI)	17.2[a]	13.2[b]	13.1[b]	8.22[c]	1.872	0.028

[A]*DMI on the day that methane production*

Methaemoglobin concentration in blood

There was no treatment difference in the blood MetHb concentration of lambs ($P > 0.05$), which averaged 0.3% of total haemoglobin across all animals. The blood MetHb concentrations in lambs receiving 1.88% NO_3 did not change over the entire MetHb monitoring period, averaging approximately 0.38%. None of the sheep had blood MetHb concentrations greater than 2.5% during the MetHb monitoring period.

3.1.4 Assessing intake of nitrate blocks relative to urea blocks by sheep

Experiment 1.
A fixed allocation of basal diet was offered, so there was no effect of N source (urea *vs.* nitrate) or N inclusion level on intake of the basal diet, with all animals consuming more than 98% of feed offered (nitrate group 1187 *vs.* urea group 1129 ± 71.2 g/d, P > 0.05).

Block intake showed a N-source × N-level interaction (P = 0.001), such that while intake of urea containing blocks increased as N content increased (25 to 76 ± 12.3 g/d), intake of nitrate containing blocks decreased (81 to 21 ± 12.3 g/d) as N content rose (Figure D7).

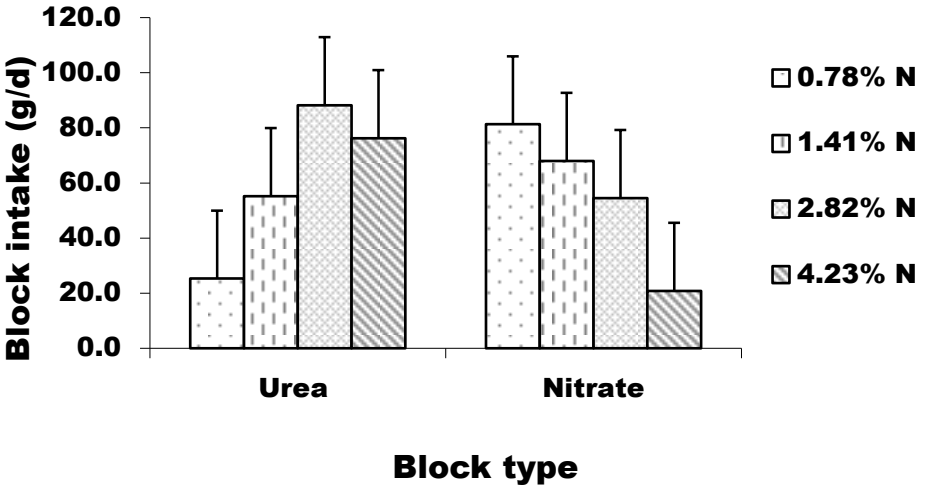


Figure D7. Daily lick-block intake of sheep sequentially offered blocks containing 0.78%, 1.41%, 2.82% or 4.23% N respectively for periods of 8d, with N added as either urea (n = 4) or nitrate (n = 4).

No significant differences (P > 0.05) in blood MetHb concentrations were observed between nitrate supplemented sheep (0.97 ± 0.285%) and urea supplemented sheep (0.46 ± 0.285%). The levels of MetHb in blood of nitrate supplemented sheep, however, were very low and posed no threat to animal health.

Experiment 2.
When sheep were fed their average voluntary daily intake of the nitrate blocks containing 2.82% N as supplement (55 g/d prepared as a slurry and mixed in the basal feed), methane yield (g CH_4/kg DM) was significantly reduced (P < 0.05;Fig D8) relative to that of sheep fed an equal quantity of supplementary urea N (17.3 *vs.* 22.0 ± 1.85 g/kg DMI).

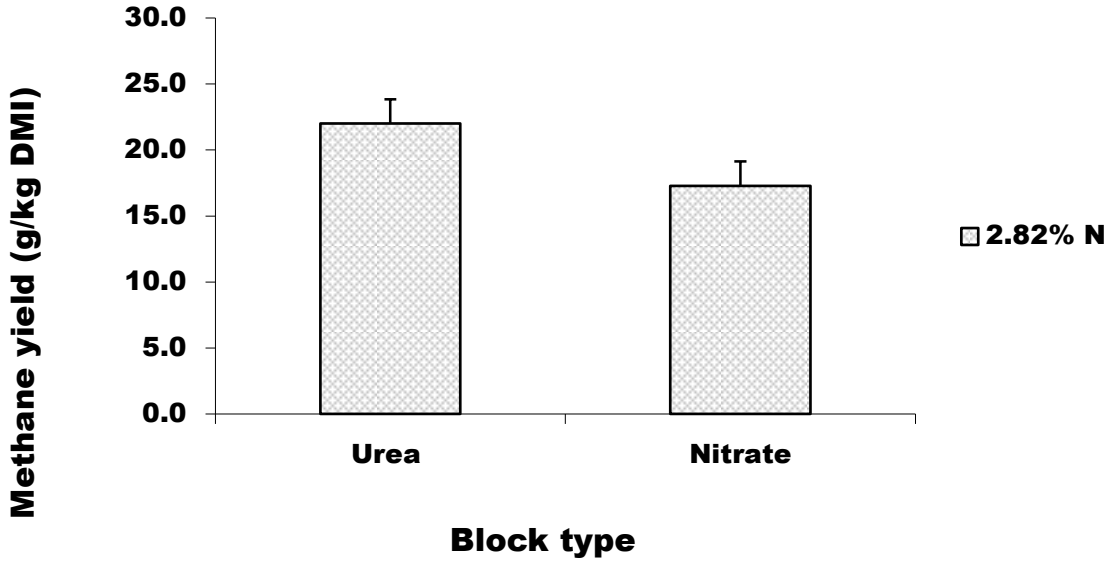


Figure D8. Methane yield (g/kg DM intake) of sheep supplemented with 55 g of blocks containing either 2.82% nitrate-N or 2.82% urea-N.

Experiment 3.
This split paddock trial was characterised by sheep growing faster in one paddock than the other. In the first 2 weeks, urea supplemented sheep grew faster than nitrate supplemented sheep (5.22 *vs.* 4.46 ± 0.389 kg, $P < 0.05$), but following the rotation between paddocks, the reverse was true (2.42 *vs.* 1.52 ± 0.389 kg, $P < 0.05$), indicating that the diet selected was different in quality between the two paddocks.

Average daily intake of lick-block by sheep was significantly less for nitrate containing blocks than for urea containing blocks (19.7 vs. 34 ± 6.69 g/d; $P < 0.05$; Figure D9). This intake was lower than intake of the block when it was continuously available to penned sheep in experiment 1

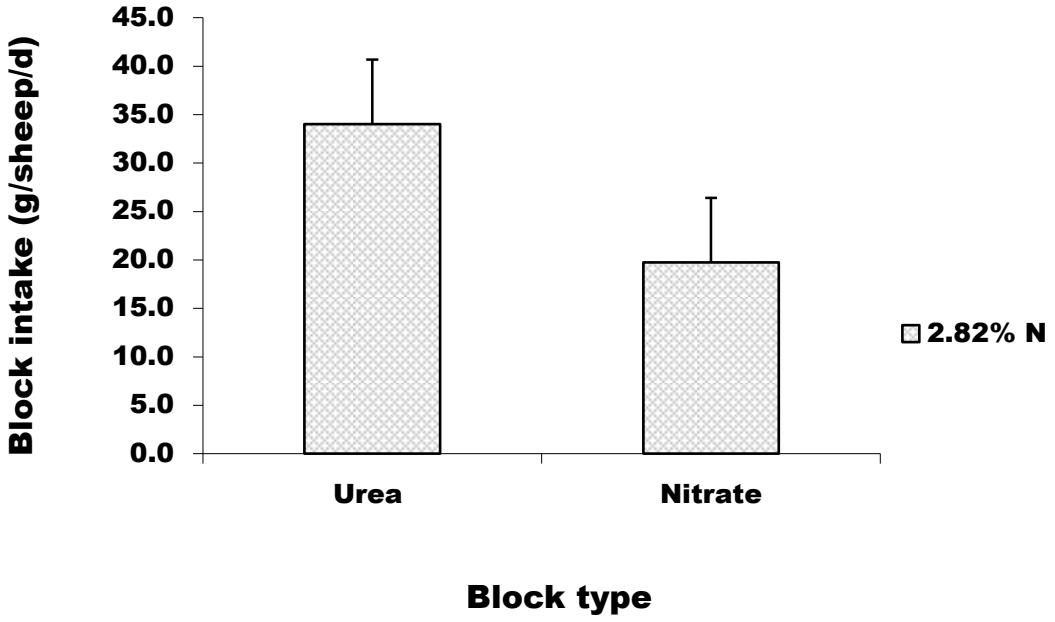


Figure D9. Average daily intake of lick-block per sheep within flocks of 25 grazing sheep provided with either 1 nitrate block or 1 urea block containing 2.82% N as the additive. Data are averaged over 2 ×14 d periods.

As in experiment 1, nitrate supplemented sheep tended to exhibit a higher average blood MetHb concentration than did urea supplemented sheep (P < 0.1; Fig D10), but significant between-day variation in MetHb concentration also occurred (Figure 4). Also in keeping with previous observations (Sokolowski *et al.* 1969), wool growth of nitrate-supplemented sheep was faster than that of urea-supplemented sheep (9.6 *vs.* 8.8 ± 0.344 mm per month P < 0.05).

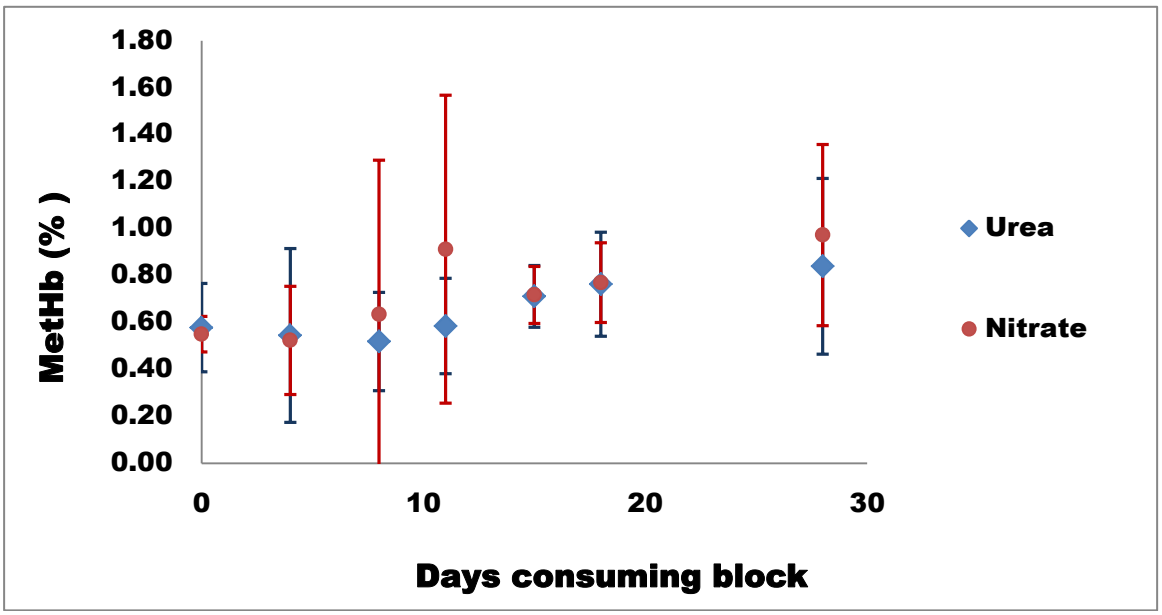


Figure D10. Blood methaemoglobin concentration (MetHb%: percentage of haemoglobin present as methaemoglobin) in grazing sheep offered iso-nitrogenous urea or nitrate-containing lick-block supplements over a period of 28 d. Error bars indicate standard deviation. Day 0 samples were collected the day before lick-blocks were introduced.

3.1.5 Assessing the role of a probiotic (Propionibacterium sp.) on nitrite accumulation in rumen fluid and the efficacy of this probiotic in reducing nitrate toxicity in sheep

An in-vitro study was conducted followed by an in-vivo sheep study, with results reported below. For the in-vitro study, all measurements were affected by the incubation duration (P<0.001), by the N-source (P<0.001) and by the interaction between N-source and incubation duration (P<0.001; Table D9).

Table D9: Results of fermentation and gas parameters averaged across time during a 21h in-vitro incubation (TGas: total gas, N: N-source, N*PA: Interaction between PA and N-source, ConN: negative control N-source, ConP: negative control probiotic)

	ConN-ConP	ConN-PA	NO_2-ConP	NO_2-PA	NO_3-ConP	NO_3-PA	Urea-ConP	Urea-PA	sem	P-value		
										PA*N	PA	N
pH	6.59	6.55	6.66	6.59	6.67	6.62	6.62	6.60	0.007	**<0.01**	**<0.001**	<0.001
TGas(mL)	9.16	10.32	6.46	7.68	7.06	8.19	9.21	9.74	0.003	0.24	**<0.001**	<0.001
NH_3 (mM)	6.27	6.00	11.36	10.81	9.32	9.25	11.24	11.52	0.022	0.31	0.223	<0.001
NO_2 (mM)	0.05	0.05	0.10	0.11	0.19	0.20	0.05	0.05	0.059	0.98	0.30	<0.001
NO_3 (mM)	0.16	0.17	0.20	0.21	2.19	2.72	0.15	0.15	0.05	<0.10	**<0.05**	<0.001
CH_4 (%)	10.98	9.41	1.45	1.33	4.07	3.11	10.09	9.03	0.14	**<0.001**	0.12	<0.001
CH_4 (mL)	1.01	0.97	0.09	0.10	0.28	0.25	0.93	0.89	0.14	0.91	0.78	<0.001
H_2 (%)	0.20	0.12	0.49	0.42	0.30	0.29	0.12	0.16	0.27	0.49	0.60	<0.001
H_2 (mL)	0.02	0.01	0.03	0.03	0.02	0.02	0.01	0.02	0.27	0.53	**<0.01**	<0.01
N_2O (mL)	0.0004	0.0004	0.0010	0.0019	0.0014	0.0017	0.0006	0.0005	0.13	**<0.05**	0.07	<0.001

Nitrate metabolism (Nitrate, nitrite, ammonia, nitrous oxide)

Nitrogen was added to incubations to achieve concentrations of 7.1 mM NO3 or 1.4mMNO2. The measured initial concentrations were, 8.1mM NO3 and 1.2mM NO2 respectively in the incubations so close to those theoretically present.

The data showed a slow conversion of NO3 to NO2, with the NO2 concentration in the nitrate incubation building to a slow peak of the NO2 pool (of 1.1mM NO2, or approximately 12% of added nitrate N) after 9h incubation. In contrast, nitrite added to anincubation disappeared rapidly (Figure D11). There appears to be an asynchrony of several hours, between the decline in nitrate and the accumulation of nitrite, suggesting the nitrate reductase system becomes saturated or otherwise inactivated over time (for example as pH drops below 6.6). Alternately, nitrate-N may be metabolised to unknown intermediates en-route to conversion to nitrite. NO2 clearance was proportionally faster compared to NO3, with 92% of NO2 having disappeared from the incubation medium after 3h while it took 11h of incubation before 93% of NO3 had disappeared from the incubation medium. It must be remembered that the total load of nitrite to be cleared was much less than was the total nitrate load (0.99mg NaNO2; 6.1 mg NaNO3).

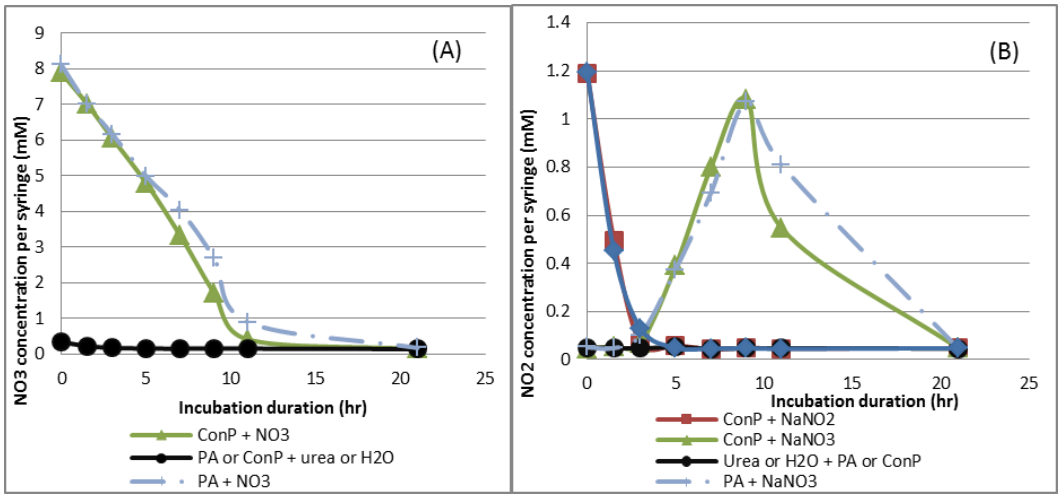


Figure D11. Nitrate (A) and nitrite (B) concentrations (mM) in incubation syringes supplemented with nitrate or nitrite and with or without Propionibacterium acidipropionici (PA) over a 21 h incubation duration.

The presence of PA did not affect overall NO2 concentration, but significantly increased NO3 concentration in the incubation medium ($P<0.05$), suggesting inhibition of nitrate reductase by PA.

Across all time points, NH3 was significantly lower for syringes incubated with NO3 ($P<0.001$) and ammonia concentration did not surge in the first few hours after nitrate addition as it did with nitrite and with urea (Figure D12). This is further evidence that the rate of nitrate metabolism to nitrate in 'fresh' rumen fluid (in the first few hours of incubation) is much slower than the rate of nitrite to ammonia concentration in this initial period. Failure to provide any N source (control) caused a progressive decline in ammonia concentration over the first 11h with little change thereafter, despite gas production and pH continuing to change in near linear manner out to 21h.

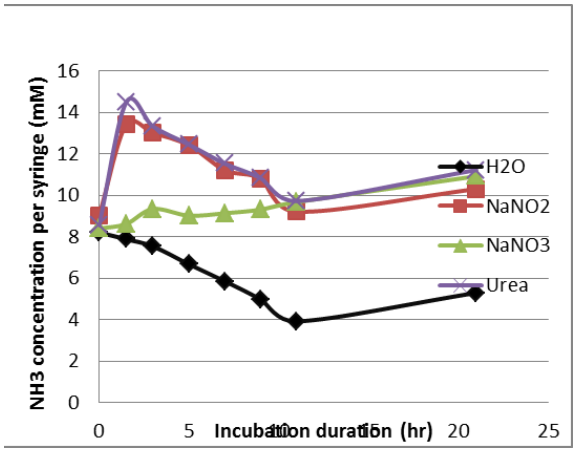


Figure D12. Effect of N-source (water, urea, nitrate, and nitrite) on ammonia concentration (mM) during a 21h in-vitro incubation.

Nitrous oxide (N2O) is little reported from rumen studies but was identified as present in these studies. Its production was significantly affected by N-source (P<0.001), being readily produced when NO3 was added to the medium but not when NO2 was added (Figure D13). The addition of PA however increased N2O production from nitrate after the first few hours of incubation and also induced its production from NO2, suggesting the PA organism was pushing some NO3 into nitrous oxide. It is not clear if nitrous oxide was been further metabolised or only voided to the gas phase. The total quantities of N2O accumulating were very small (typically <0.01 ml/incubation).

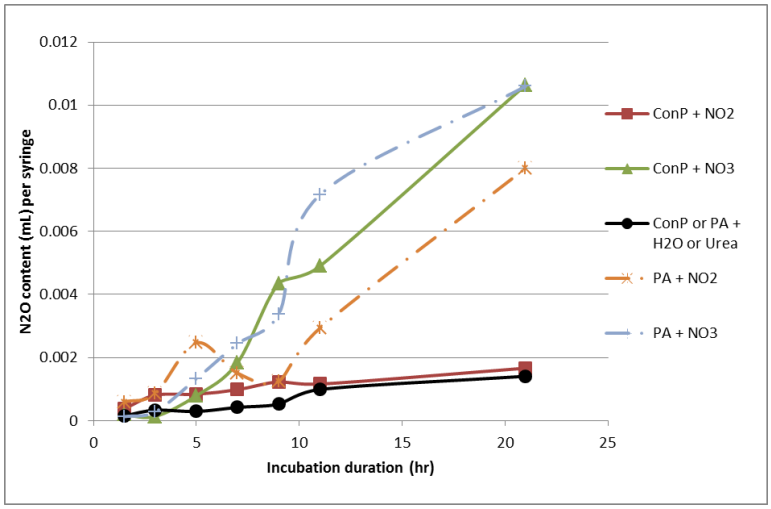


Figure D13. Effect of PA and N-source (nitrate, nitrite, water or urea) on nitrous oxide production (mL) versus incubation duration (hr)

Fermentation characteristics: pH and total gas production

As summarised in Table D9, pH of the incubation medium was lower in syringes incubated with PA (P<0.001) and was affected by the N-source (H2O< Urea< NO2< NO3) (P<0.001). The near linear decline in pH was married with a near linear rise in total gas production (Figure 8), indicating viable fermentations were retained over the entire period on all treatments. Total gas production increased when PA was in the incubation medium (P<0.001). Syringes treated with H2O (negative control for N-source) had a higher total gas production than syringes with urea, which produced more gas than NO3 and NO2 containing incubations (P<0.001).

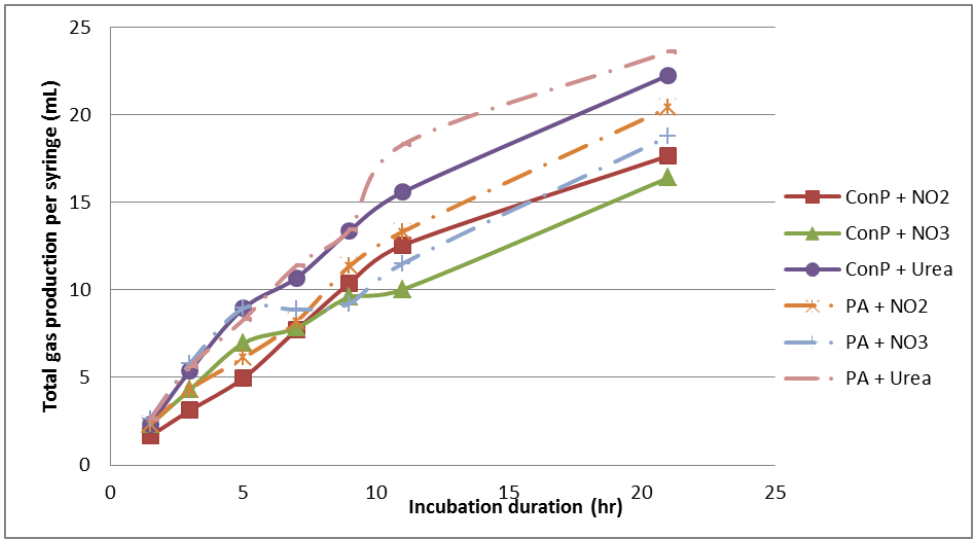


Figure D14. Total gas production versus incubation duration (hr) for syringes incubated with nitrate, nitrite or urea with or without PA

Methane and hydrogen production

Only N-source had a significant effect on volume of CH4 produced (P<0.001); both NO2 and NO3 decreased CH4 production (expressed in mL per syringe, averaged over time) by 70 and 89% respectively. Regarding CH4 as percentage of total gas production, the interaction between PA and N-source was significant (P<0.001). Figure D15 illustrates how PA reduced CH4 production in syringes incubated with NO2. A different pattern of CH4 production for NO3 or NO2 incubations is also illustrated. In NO3 incubations CH4 increased until 5h incubation, reaching 5.04% of total gas production, and decreased until 21h (2.75 and 4.22 % for PA and control respectively). In incubations with NO2, CH4 production increased in a more linear pattern with a stabilization for syringes incubated with PA after 11h.

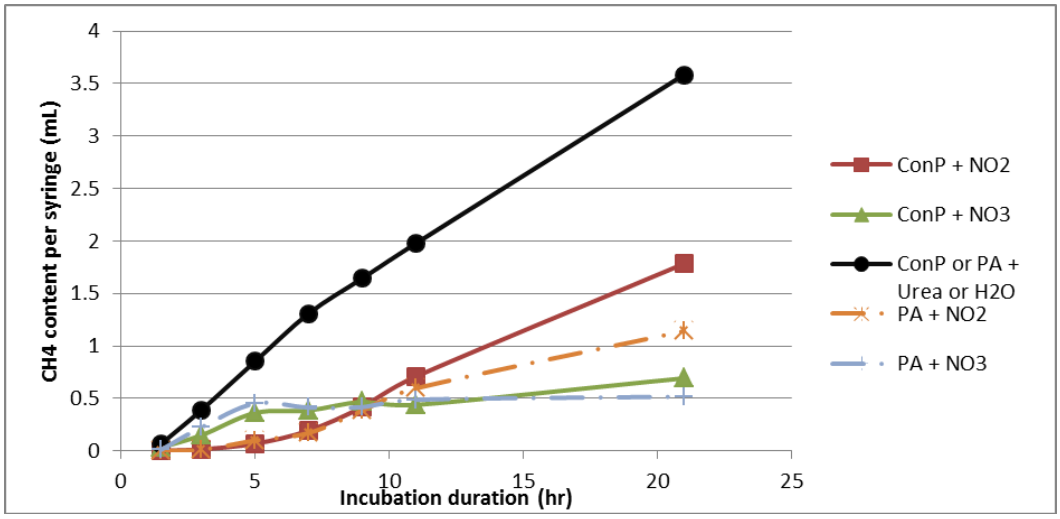


Figure D15. Effect of N-source and PA on methane production (mL) versus incubation duration (hr). Note as ControlP. ControlPA with urea or water all had near identical curves, these data have been collated in a single curve.

H2 production expressed per mL or percentage of total gas was significantly affected by N-source (P<0.001), H2 production being the highest for NO2 followed by NO3 and negligible for Urea and H2O. Expressed per mL, H2 was significantly increased by PA (P<0.01). Figure D16 illustrates H2 production as percentage of total gas. The increase in H2 production is starting after a longer incubation duration for NO3 (21h) than NO2 (7h).

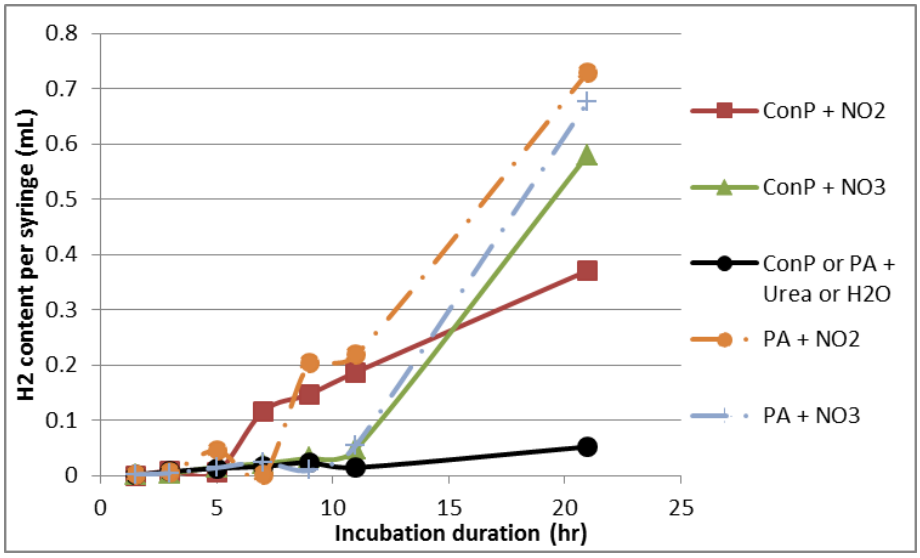


Figure D16. Effect of N-source and PA on hydrogen production (mL per syringe) versus incubation duration (hr) (Urea or H2O: negative or positive control for N-source with or without PA). Note as ControlP. ControlPA with urea or water all had near identical curves; these data have been collated in a single curve

3.1.6 Managing feeding frequency of sheep to minimise nitrite accumulation and MetHB accumulation.

The principal discovery from this work was the high level of between-animal variation in rumen and blood parameters following consumption of nitrate. This makes us particularly cautious in drawing generalizations regarding 'best management practices' because what happens in one sheep does not happen in another and this identifies the need for great conservativeness in drafting recommendations.

The Latin Square analysis is yet to be analysed but the figures (Fig D17 = average data; D18-D20 = individual animal data) below show the diversity in response to consumption of a nitrate-containing diet fed daily, twice daily, or at hourly intervals.

The principle results arising from his study were that:

- Blood NO3/NO2 more stable than in rumen
- Blood NO3 often higher than blood NO2
- Blood MHB takes several hours to decline post-feeding
- Rumen NO2 rapidly appears as blood NO3
- Between-animal variance in intake pattern & profiles is large

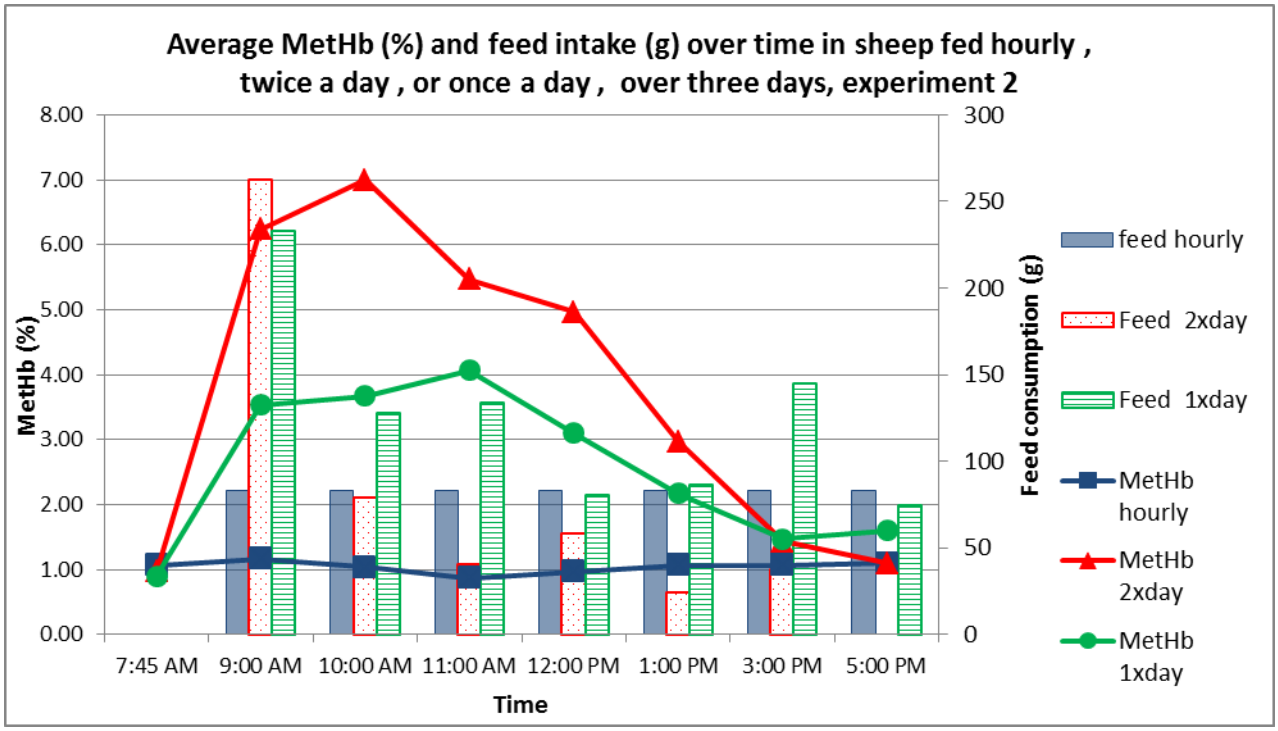


Figure D17. Blood methaemoblobin percentage and feed consumption averaged for 3 sheep when they were fed a nitrate containing feed hourly, twice daily or once daily.

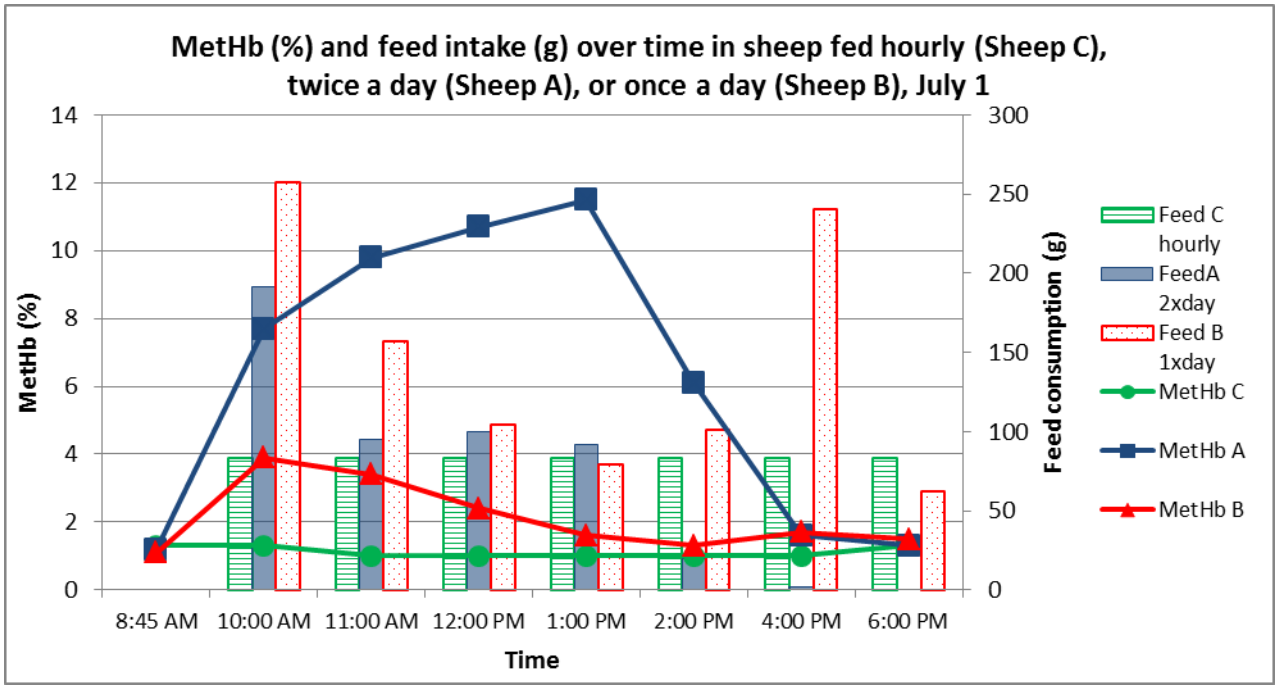


Figure D18. Blood methaemoblobin percentage and feed consumption for 3 individual sheep when they were fed a nitrate containing feed hourly, twice daily or once daily. If metabolism of all sheep was similar, Figures D18-D20 should all look identical.

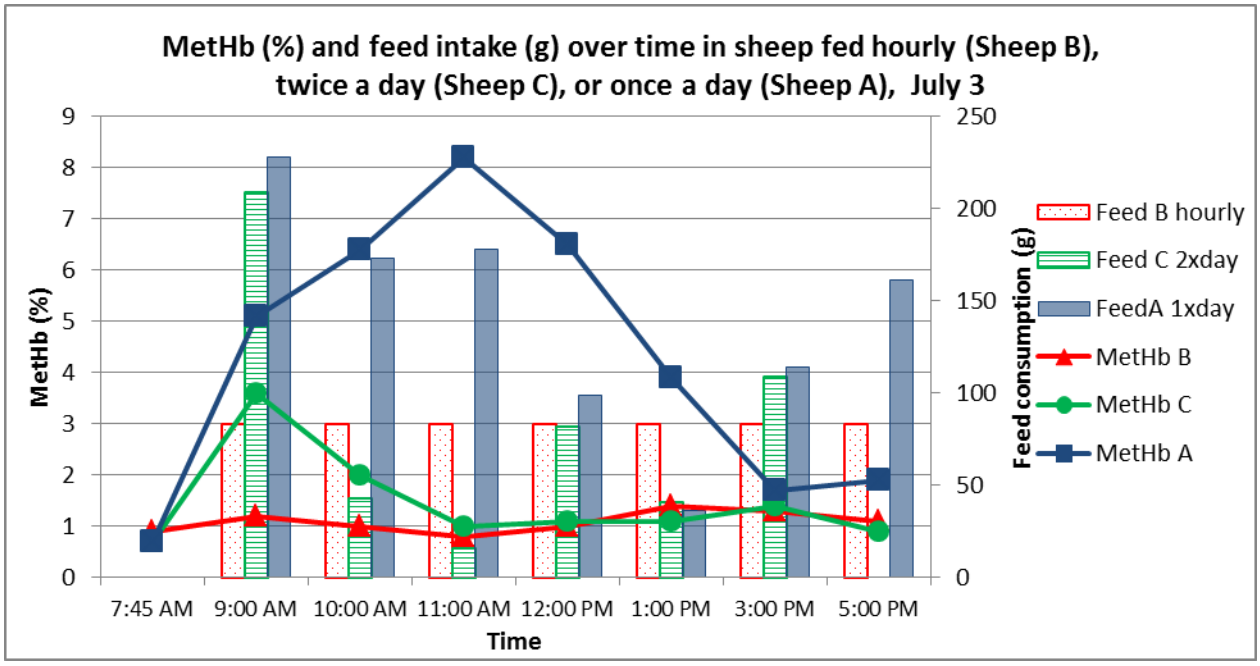


Figure D19. Blood methaemoblobin percentage and feed consumption for 3 individual sheep when they were fed a nitrate containing feed hourly, twice daily or once daily. If metabolism of all sheep was similar, Figures D18-D20 should all look identical. Figure D19 has a different sheep on each feeding regime than in Figure D18 or D20

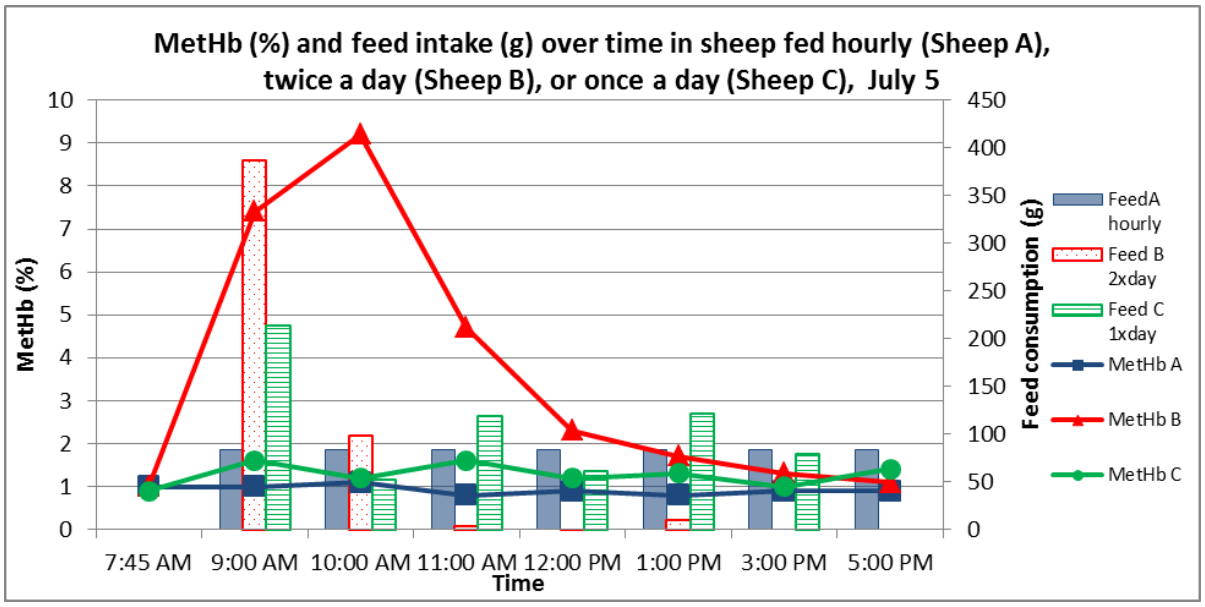


Figure D20. Blood methaemoblobin percentage and feed consumption for 3 individual sheep when they were fed a nitrate containing feed hourly, twice daily or once daily. If metabolism of all sheep was similar, Figures D18-D20 should all look identical.. Figure D20 has a different sheep on each feeding regime than in Figure D18 or D19.

3.1.7 Managing form of substrate to maximise nitrite reductase activity and minimise NO2 absorption.

In syringes incubated with NO3, NO3 disappeared from the incubation media within 3h for Gly treatments or 5h in media containing Glu or Con. Glycerol seemed to increase the kinetics of NO3 reduction; the

NO2 peak was higher and appeared earlier when Gly was added to NO3 (0.80 mg NO2-N at 3h of incubation). For Con and Glu treatments, NO2 concentration reached 0.67 and 0.56 mg NO2-N after 5h of incubation (Figure D21). There was only a minor difference between both treatments; Glu causing a slightly lower NO2 peak. Nitrite disappeared from the incubation media within 12h of incubation. In all the three treatments, area under NO2 curves was almost twice as large as the area under the NO3 curve, meaning that NO2 disappearance was much slower than the disappearance of NO3.

Some NO3 (0.3mg NO3-N) was also present in incubation media incubated with U. No NO3 was detected in rumen fluid taken from the sheep fed either NO3 or U. Consequently the chaffed oaten hay probably contained initially some NO3 (confirm with the feed analysis).

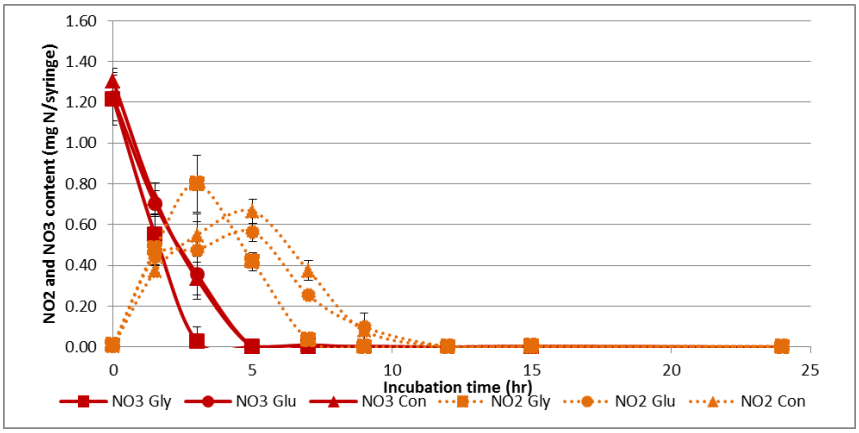


Figure D21. Nitrate and nitrite concentrations (mg N) in incubation syringes supplemented with an energy source (glucose, glycerol or a negative control) and with nitrate over 24h of *in vitro* incubation

Within the first 5h of incubation treatments containing U had higher NH3 concentrations (Figure D22). Among NO3 treatments, Con seemed to have higher NH3 concentrations than Gly and Glu.Nitrate supplementation produced less gas than U, but only in the Con treatments (Figure D23).

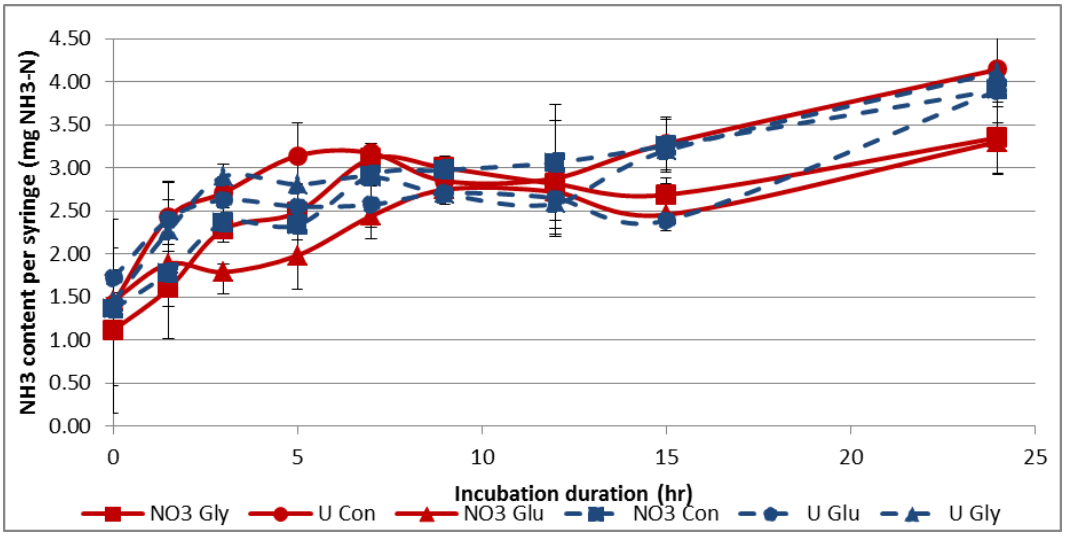


Figure D22. Ammonia content (mg N) in incubation syringes supplemented with an energy source (glucose, glycerol or a negative control) and with a nitrogen source (nitrate or urea) over 24h of *in vitro* incubation

During the first 10h of incubation, Glu increased total gas production more rapidly than Gly with both N-sources. Nevertheless, from 10h to 24h of incubation, total gas production did not appear to differ due to energy source. Furthermore, in Con treatments an inhibitory effect of NO3 on gas production was observed, which was less pronounced in Glu and Gly treatments.

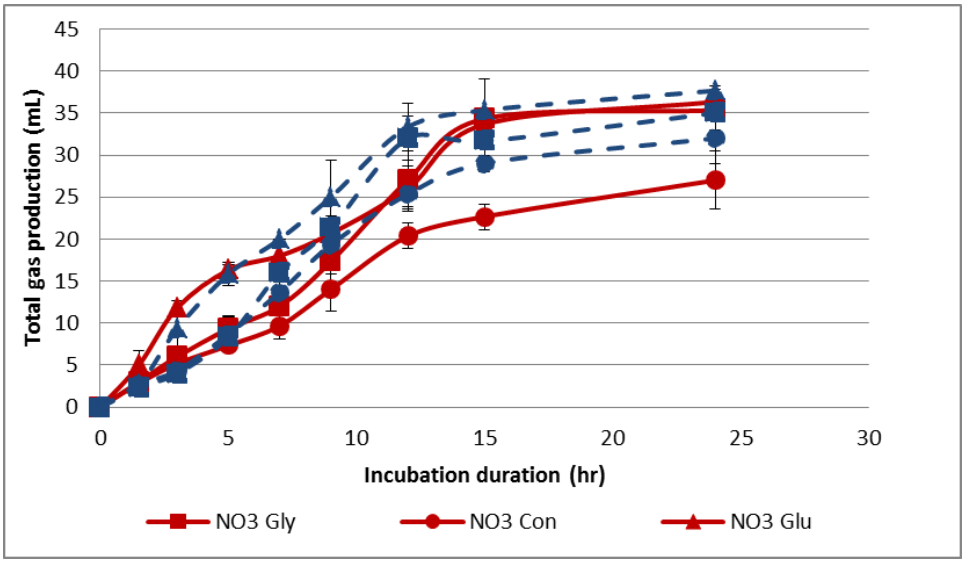


Figure D23. Total gas production (mL) in incubation syringes supplemented with an energy source (glucose, glycerol, or negative control) and with a nitrogen source (nitrate or urea) over 24h of *in vitro* incubation

Methane:

Nitrate lowered methane emission with both added energy sources. Beyond 12h of incubation, Gly supplementation resulted in higher methane emissions than Glu. Methane mitigation by NO3 was greatest in the NO3-Con treatment (Figure D24). In theory 1mg NO3-N (7.14 mM NO3) should lower methane by 7.14mM or 1.8mL (ideal gas law for 39°C, at atmospheric pressure). However, for NO3-Con methane mitigation was 46% higher than the theoretical methane mitigation potential of the added NO3.

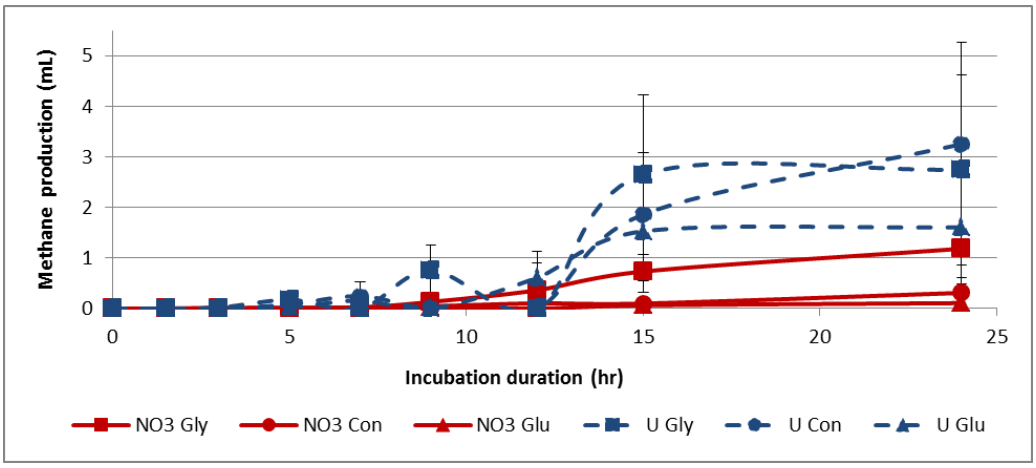


Figure D24. Methane accumulation (mL) in incubation syringes supplemented with an energy source (glucose, glycerol or a negative control) and with a nitrogen source (nitrate or urea) over 24h of *in vitro* incubation

Hydrogen

In incubations treated with U, hydrogen production reached a peaked at around 12h and diminished afterwards (Figure D25). Hydrogen appeared in the gaseous phase in particular towards the end of the incubation period for NO3 treatments. In incubation media containing NO3, hydrogen accumulation started after 9h of incubation, when the entire NO3 and the majority of NO2 had disappeared from the incubation medium.

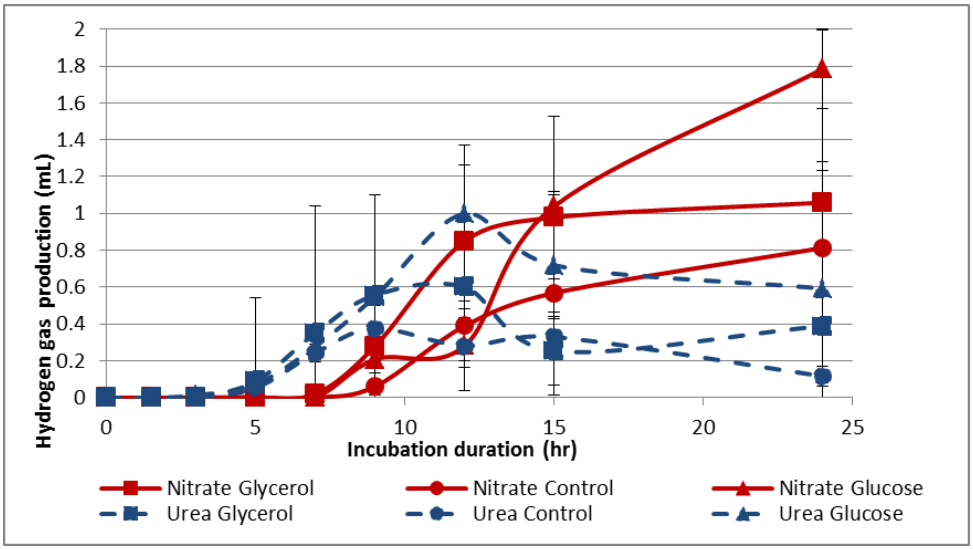


Figure D25. Hydrogen accumulation (mL) in incubation syringes supplemented with an energy source (glucose, glycerol or a negative control) and with a nitrogen sources (nitrate or urea) over 24h of *in vitro* incubation

Nitrous oxide production

Contrary to previous *in vitro* and in vivo findings where nitrous oxide production was stimulated by NO3 supplementation (Kaspar and Tiedje 1981; de Raphélis-Soissan et al. 2014), the current study showed that nitrous oxide production was inhibited by NO3 supplementation (Figure D26). The main difference with the previous *in vitro* study is that sheep were adapted to dietary NO3 supplementation in the current study and not in the previous study (MSc thesis).

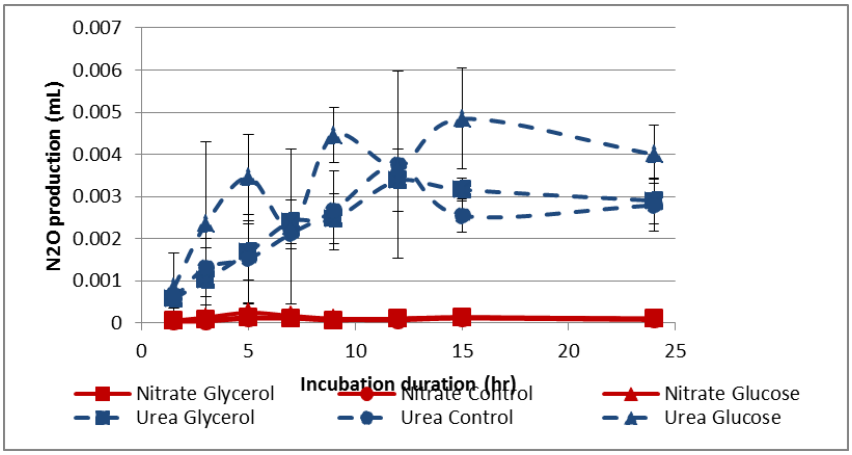


Figure D26. Nitrous oxide accumulation (mL) in incubation syringes supplemented with an energy source (glucose, glycerol or a negative control) and with a nitrogen sources (nitrate or urea) over 24h of *in vitro* incubation

VFA

Total VFA production followed a similar pattern across treatments with slightly lower total VFA production in NO3 than U treatments (Figure D27). Acetate proportions were generally higher for NO3 than for U. Among the same N-source, incubations without Glu or Gly had considerably larger acetate proportions than glycerol and glucose treatments. Propionate proportions were higher for U than NO3 treatments. Among those treatments, glycerol resulted in the highest propionate proportion, followed by glucose and the negative control. The acetate to propionate ratio was higher for NO3 than U and also higher in Con vs Gly or Glu treatments. NO3-Con in particular had a high acetate to propionate ratio.

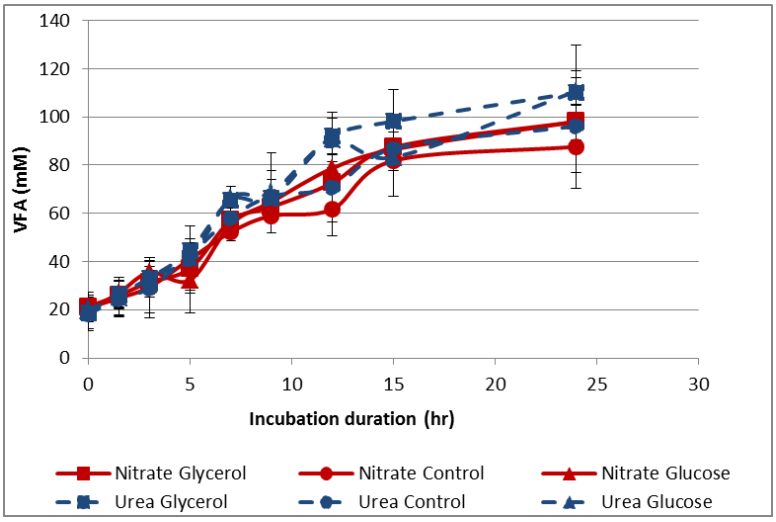


Figure D27. Concentration of total VFAs (m moles/L) in incubation syringes supplemented with an energy source (glucose, glycerol, or negative control) and with a nitrogen source (nitrate or urea) over 24h of *in vitro* incubation

Hydrogen balance

Hydrogen balance was calculated by adding hydrogen equivalents (mmol) produced in the incubation medium via acetate and butyrate production (Figure D28). Hydrogen sinks incorporating hydrogen during propionate, methane, NO3 reduction, bacterial growth and gaseous hydrogen production were subtracted. Hydrogen supply via NADH production during glycolysis from glucose or glycerol was also added, resulting in a relatively stable hydrogen balance across glucose and glycerol treatments.

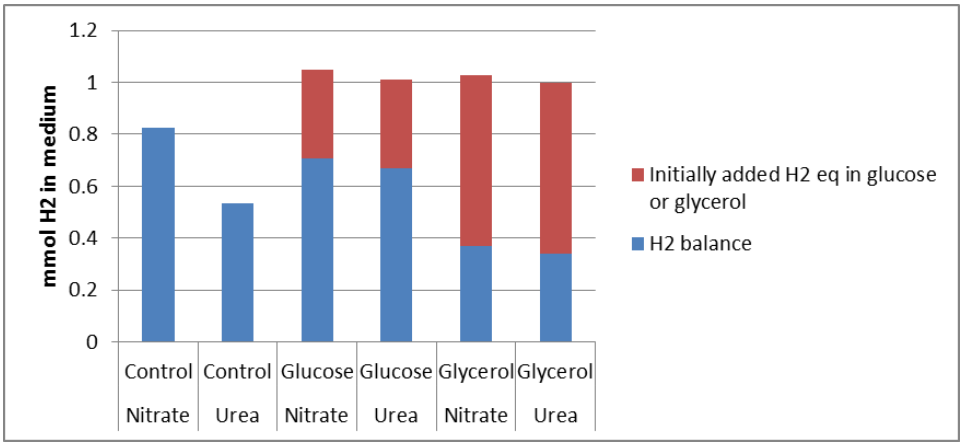


Figure D28. Net hydrogen content (mmol H2) in the incubation medium after 24h *in vitro* incubation

N-balance

Nitrogen balance was calculated by adding measured sources of nitrogen (mg N) contained in NH3, NO2 and NO3. Nitrogen present as bacterial cells as estimated by calculating ATP yields from VFA (Bergen 1977) and assuming a N content of 8% in bacterial cells. Nitrogen in U was only added to the initial time point, since the degradation rate of U is very rapid. Due to the unknown degradation rate of feed N, the N content in feed was also only added to the initial time point. No major differences were observed across treatments beside a lower N-balance at the end of the incubation in Con groups (Figure D29).

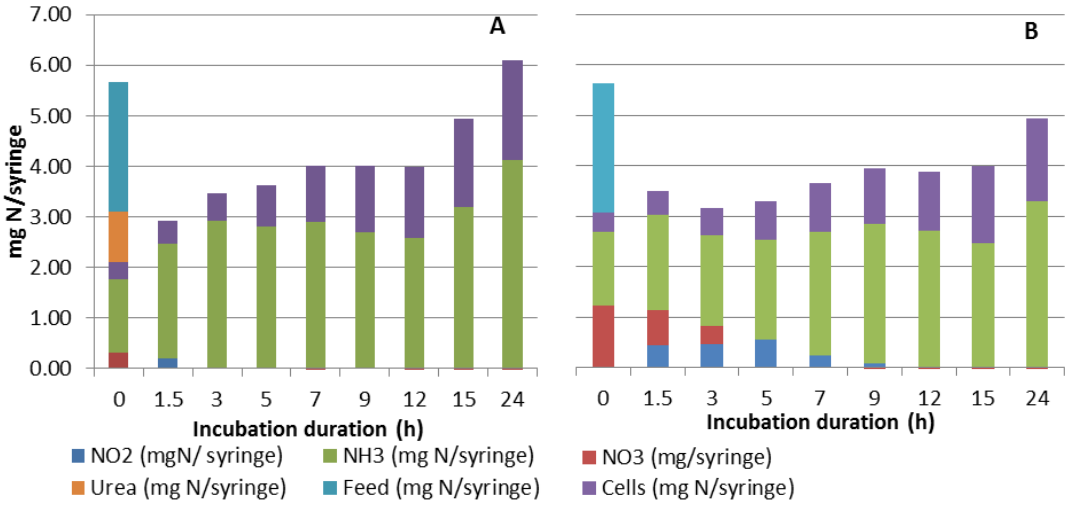


Figure D29. Nitrogen balance after 24 h incubation in incubation media containing no added energy source and urea (A) or nitrate (B)

3.1.8 Testing of possible formation of imidazoles (toxins) in molasses blocks containing nitrate.

Extensive time and effort was invested in trying to apply existing extraction and analysis techniques to 4-methylimidazole from molasses-based lick blocks. While miniscule changes in pH, temperature and ionic strength caused significant changes in the extraction from this very complex medium, analysis was conducted of test blocks made in the laboratory (with varied nitrate:sugar ratios and temperature of manufacture)and commercially. Methylimidazole was able to be recovered when artificially added to blocks (Fig D30) but the natural level in blocks was below the detectable limit in all cases, indicating that formation of 4-methylimidazole is not a threat to commercial nitrate-block production.

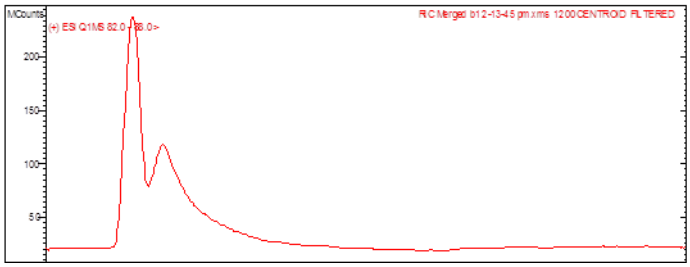


Figure D30. LC-MS of a fraction derived from SPE of an extract from a molasses feed block containing 100 ppm 4MI

3.2 Efficacy of nitrate mitigation in cattle known and available

While most of the metabolic and rumen fermentation research used sheep, three cattle studies mitigation of methane through consumption of dietary nitrate were conducted. These were:

- Measurement of emission rate *in vitro* from cattle fed nitrate or urea treated chaff
- Measurement of emission from cattle fed nitrate or urea in a molasses liquid supplement
- Measurement emission from cattle fed nitrate or urea in a feedlot ration

3.2.1 Emission rate in vitro from cattle fed nitrate or urea treated chaff

Rates of methane production were significantly lower by digesta from nitrate-fed cattle than from urea-fed cattle, with the nitrate effect greater in defaunated relative to faunated cattle. Part of both the nitrate and defaunation effects was attributable to reduced total gas production in nitrate-fed or defaunated rumen fluid, indicating a lower rate of microbial fermentation in incubations from these sources (Table D10).

Table D10. The pH, ammonia concentration, volatile fatty acid (VFA) concentration and molar proportions and methane production as influenced by the presence or absence of protozoa (F, fauna) or nitrate (NO_3) addition for incubations of rumen fluid *in-vitro*

Item	Treatment				P-Values		
	Defaunated		Refaunated				
	-NO_3	+NO_3	-NO_3	+NO_3	F	NO_3	F × NO_3
pH	6.19^{b}	6.49^{a}	6.0^{c}	6.32^{b}	<0.01	<0.001	0.98
Ammonia (µg/mL)	10.12^{c}	18.57^{b}	16.7^{b}	21.17^{a}	0.01	0.001	0.18
Total VFA (mM)	102.96^{ab}	83.15^{b}	137.6^{a}	98.01^{b}	0.08	0.04	0.14
VFA molar proportion (%)							
Acetate (%)	69.33	70.30	67.8	68.74	0.45	0.63	0.10
Propionate (%)	20.34	22.48	19.6	21.54	0.47	0.11	0.94
Butyrate (%)	9.57^{ab}	6.78^{b}	11.0^{a}	8.49^{b}	0.20	0.04	0.89
Acetate / propionate	3.42	3.15	3.5	3.26	0.76	0.39	0.92
Total gas (mL/0.2g DM)	31.00^{a}	20.33^{c}	29.9^{a}	23.56^{b}	0.21	<.001	0.01
CH_4 (mL)	3.72^{b}	0.60^{d}	4.4^{a}	2.55^{c}	<.001	<.001	<.001

^{a,b,c,d} *Means in the same row with different superscripts differ (P < 0.05)*
SEM = Standard error of the mean
F = Faunation status

3.2.2 Emission from cattle fed nitrate or urea in a molasses liquid supplement

In most sheep and cattle studies undertaken by us or otherwise published, nitrate inclusion reduces the feed intake of the ruminant. Consequently it was no surprise that the intake of a molasses based liquid supplement was reduced (0.45 v 1.28 kg/head/d) when in was modified to contain nitrate rather than urea (isonitrogenous 29g N/kg supplement). In contrast, consumption of the basal diet tended (P=0.06) to be higher in nitrate fed cattle (Figure D11).

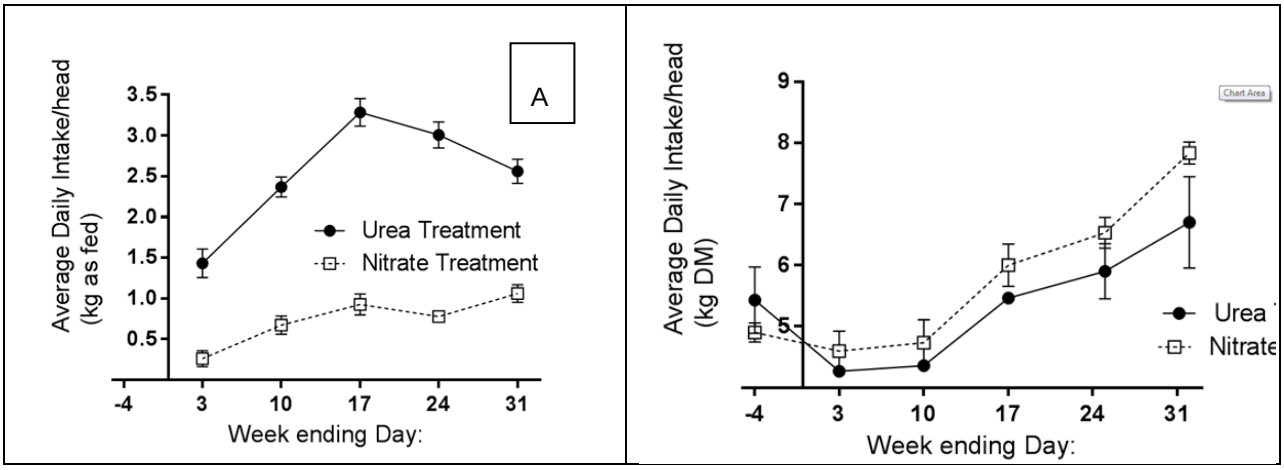


Figure D31. Intake of (a) molasses based liquid supplement and (b) of a basal diet of blended Lucerne/oaten chaff by angus x brahman cross steers

3.2.3 Emission from cattle fed nitrate or urea in a feedlot ration

In association with a large (380 head) feeding trial undertaken by an AoTG team, GEM units were used to monitor the emission from nitrate supplemented (1.88% NO3) and urea enriched (isonitrogenous) feedlot diets (Velazco et al., 2014).

While nitrate tended to reduce methane emissions (P<0.1), the study also showed nitrate inclusion led to a significant reduction in meal size in the feedlot environment and an increase in the number of meals consumed.(Table D10b). This changing feeding behaviour may have confounded estimation of methane yield (MY; g CH_4/kgDMI) as MY was significantly higher as measured by a GEM which has not been previously recorded.

Table D10b. Least square means for average dry matter intake , number and weight of feeding events, delay between feeding event and methane measurement, measured daily methane production , predicted methane production, methane yield live weight , live weight gain , feed conversion ratio and methaemoglobin concentration in blood in feedlot cattle given diets containing urea or nitrate.

	Urea	Nitrate	*P*-value
Dry matter intake (kg/d)	12.9	10.8	0.088
Number of meals per day (meals/d)	7.39	14.69	0.007
Meal weight (kg/meal)	1.82	0.77	<0.001
Delay between feeding event and GEM (h)	5.15	3.44	0.006
Measured daily methane production (gCH_4/d) [B]	205	170	0.071
Predicted methane production (gCH_4/d)[BC]	239	209	-
Methane yield (g CH_4/kg DMI)	15.8	16.9	0.064
Live weight (kg)	430	419	0.388
Live weight gain (kg/d)	2.00	1.61	0.188
Feed conversion ratio (kg feed/kg LWG)	6.47	6.69	0.824
Methane intensity (g CH_4/kg LWG)			
Final methaemoglobin(%)	0.83	1.31	0.22

3.3 Growth and productivity data of nitrate supplemented livestock made available

There are too many experiments to report individually but findings from all relevant experiments conducted in2012-2015 by the team are compiled in Table D11.

Table D11. Percentage change in production and emission traits of nitrate supplemented animals in UNE studies 2012-2015 relative to urea supplemented cohorts in the same experiment. (Positive indicates parameter was higher in nitrate supplemented animals)

Animal	Reference	DMI	Wool	LWG	DMP	MY
Sheep	Li et al.,2012 APS	-6		-19	-41	-35
Sheep	Li et al., 2012 AAAP			17		-21
Sheep	Li et al., 20123APS	7	37	34	-25	-24
Sheep	Barnett (unpub)	-2	-6	-1		-24
Sheep	de Raphelis et al2014	-1	22	-19	-19	-16
Sheep	de Raphelis et al2014	-1	6	-28	-19	-14
Cattle	Velazco et al 2014	-6		-7	-17	7
Cattle	Goopy (unpub)	-9				
AVERAGE		**-3**	**15**	**-3**	**-24**	**-18**

From Table D11 it is apparent that the average response to nitrate inclusion in the diet was a reduction in feed intake (3%) and liveweight gain (3%), an increase in wool growth (15%) and a substantial reduction in the daily methane production and methane yield of supplemented animals.

The effects of nitrate on feed intake were further reviewed for sheep, goats, beef cattle and dairy cattle. The effect is important but variable as evidenced by the data reviewed on beef cattle shown in table D12

Table D12. Effects of dietary nitrate on feed intake in beef cattle (from review conducted by Gamaliel Simanungkalit for this project)

Treatment		BW (kg)	Feed Intake					P value	Source
			Control		Nitrate (kg/d)	Alteration			
Substance	Time (d)		Diets	DMI (kg/d)		Kg/d	%		
1% $NaNO_3$	160	350	Concentrate	11.38	10.39	**- 0.99**	8.70	**0.01**	Weichental *et al.* (1963)
1% KNO_3	183	229	CF+ 1%U	7.58	6.85	**- 0.73**	9.60	**0.01**	Lichtenwalner *et al.* (1973)
2.84% CAN	96	245	C+1.16U	7.70	9.00	**+ 1.30**	16.88	ns	Ascensao (2010)
2.2% NO_3^-	46	283	CF+1.25%U	7.10	6.60	**- 0.50**	7.00	ns	Husholf *et al.* (2012)
4% $CaNO_3$	90	170	TRS+1.5%U	5.04	5.42	**+ 0.38**	7.54	**0.001**	Hiep *et al.* (2012)
1% NO_3 (EN)	84	339	CF	9.6	8.8	**- 0.8**	8.3	**0.02**	Pereira *et al.* (2013)
2% NO_3 (EN)	84	339	CF	9.6	8.7	**- 0.9**	9.3	**0.02**	Pereira *et al.* (2013)
3% NO_3 (EN)	84	339	CF	9.6	8.4	**- 1.2**	12.5	**0.02**	Pereira *et al.* (2013)

3.4 Long-term impacts of methane suppression on animal growth

Thirty Suffolk x Merino SAMM hogget ewes, approximately 12 months old and weighing 41 ± 2 kg, were housed in individual pens and adapted to a diet of lucerne and wheaten chaff for 2 weeks before being fed *ad libitum* throughout the 70 d experimental period.Animals were assigned to one of five groups by stratified randomisation according to liveweight. Group 1(n=6) were fed chaff plus 2% urea to make the diet iso-nitrogenous (control), group 2 (n=6) received chaff plus 2% nitrate

(5Ca(NO3)2.NH4NO3.10H2O, Bolifor CNF, Yara, Oslo, Norway), group 3 (n=6) received chaff plus 3% sodium bentonite (Riverina (Australia) Pty Ltd, Brisbane, Australia), group 4 (n=6) received chaff plus 5% canola oil, and group 5 (n=6) were fed chaff plus coated CSH (30%; Shanghai Bangcheng Biological Sciences and Technology, Shanghai, China) at a rate equivalent to 80 mg / kg BW.

From Table D13 it is apparent that nitrate did not significantly affect feed intake of liveweight gain by the lambs over the 70 period, although methane mitigation was apparent late in the 70d study when emission were measured in respiration chambers. This data is now being examined to test for evidence of changing impacts of nitrate on intake and growth as time on feed increases. On another occasion where we have observed no effect of nitrate on feed intake, we observed no effect on wool growth and no difference in LWG.

Table D13. Productivity and emission attributes of crossbred lambs measured over 70d. lambs were individually penned and fed throughout.

	Urea	Nitrate	Bentonite	Canola Oil	CSH	P-value
Body weight gain (g/d)	170 ± 12a	168 ± 8a	174 ± 11a	221 ± 4b	232 ± 9b	<0.001
DM intake (g/d)	1609 ± 19a	1580 ± 13a	1530 ± 21ab	1613 ± 26a	1587 ± 14a	0.04
Clean wool growth (µg/cm2/day)	1322 ± 62	1243 ± 79	1260 ± 76	1258 ± 66	1242 ± 23	NS
FCR	9.6 ± 0.6a	9.5 ± 0.5a	9.0 ± 0.6ab	7.3 ± 0.2bc	6.9 ± 0.3c	<0.001
GFR fat depth (mm)	15.6 ± 2.3a	12.8 ± 1.4a	13.6 ± 1.2a	24.3 ± 1.1b	18.1 ± 1.3a	<0.001
Methane yield (g CH_4 / kg DM)	17.5 ± 0.7a	13.3 ± 0.3b	17.4 ± 0.4a	16.4 ± 0.6a	17.1 ± 0.6a	<0.001
Emissions intensity	167 ± 6a	130 ± 8b#c	156 ± 10ab	117 ± 7c	115 ± 6c	<0.001

3.5 Nitrate feeding submitted for approval as a CFI methodology

We were explicitly prohibited from developing a CFI methodology in FtRG, but our data has been used in supporting development of the approved methodology with input into its content (RH). We have also contributed to best-management practices with respect to supplementing with nitrate as reported in the final milestone submitted with this report. This was the core material required in the final progress report and is provided below.

3.5.1 Best management practices for feeding nitrates to cattle

The following text is taken directly from the Best Management Guidelines developed by Andrew Sedger.

Many cattle producers, particularly in northern Australia, feed non-protein nitrogen to cattle in the form of urea during the dry season to improve pasture consumption and animal productivity. It has been shown that substituting nitrate for urea can have the added benefit of also reducing methane emissions. For example, feeding nitrate at 10 grams per kilo of dry matter intake can reduce methane by 10 per cent. Producers wanting to improve the overall environmental impact of their production system may be interested is applying this technique. However there is a downside to feeding nitrates.

If too much is consumed too quickly it is toxic to cattle as it reduces the oxygen-carrying capacity of the blood. In extreme cases, nitrate poisoning can lead to the death of the animal. The risk of poisoning is greater in cattle fed low digestibility diets.

These nine guidelines have been developed to help producers decide how to feed nitrates to their herd safely.

1. Assess the level of nitrate in the current pasture, feed and water supplies. If any are likely to be already high in nitrate, do not provide supplementary nitrate.

The level of nitrate that animals may be exposed to can be highly variable. Key risk factors include: plant species including their stage of maturity and growing conditions (e.g. the risk is greater during drought), the use of nitrogen fertilizer, and water source (e.g. if water drains from highly fertile soils, or has been contaminated with fertilizer or decaying organic matter it may be high in nitrates). Hays made from cereal crops, especially those grown under drought conditions and cut while 'sappy' can develop toxic levels of nitrate when they heat up. Oaten hay is particularly risky.

For a more detailed list of nitrate risk factors, including plant species associated with nitrate poisoning, see NSW DPI's Primefact 415 (Nitrate and nitrate poisoning in livestock)[1].

2. Introduce nitrate into the diet of your herd gradually.

For the first two weeks, the maximum amount of a nitrate compound (calcium nitrate and ammonium nitrate) that should be fed is shown below for cattle of different weights and fed diets with different dry matter digestibility (DMD) and crude protein (CP) content (Table D14).

Table D14.

Live Weight below	Calcium nitrate g Forage DMD and *(CP)* content					Ammonium nitrate g Forage DMD and *(CP)* content			
	45% (*6.4%*)	55% (*7.6%*)	65% (*8.8%*)	75% (*10%*)		45% (*6.4%*)	55% (*7.6%*)	65% (*8.8%*)	75% (*10%*)
300 kg	23	28	31	35		19	23	26	29
400 kg	25	32	36	39		21	27	30	32
500 kg	27	34	39	41		22	28	32	34

For example, a herd with an average live weight of 350 kg on a diet with 50% DMD and 7% CP should be fed no more than 25g calcium nitrate and 21g ammonium nitrate in compound per day during the two-week adjustment period.

3. After the adjustment period, never feed cattle more than the maximum safe level of nitrate.

The maximum safe limit is 50 grams of nitrate per animal per day or 7 grams per kilo of dry matter intake per day for a 450 kg animal. The maximum amount of a nitrate compound (calcium nitrate and

ammonium nitrate) that should be fed is shown below for cattle of different weights and fed diets with different dry matter digestibility (DMD) and crude protein (CP) content.

Table D15

LW below	Calcium nitrate g					Ammonium nitrate g			
	Forage DMD and *(CP)* content					Forage DMD and *(CP)* content			
	45% (*6.4%*)	55% (*7.6%*)	65% (*8.8%*)	75% (*10%*)		45% (*6.4%*)	55% (*7.6%*)	65% (*8.8%*)	75% (*10%*)
300 kg	**45**	**56**	**63**	**70**		**37**	**46**	**51**	**57**
400 kg	**51**	**65**	**73**	**79**		**42**	**53**	**60**	**64**
500 kg	**53**	**68**	**77**	**82**		**44**	**56**	**63**	**67**

For example, a herd with an average live weight of 450 kg on a diet with 70% DMD and 9% CP should be fed no more than 77g calcium nitrate and 63g ammonium nitrate in compound per day (Table D15).

4. **Keep a close eye out for signs of nitrate poisoning during and after the adjustment period as some individual animals may have a naturally lower ability to adjust effectively to nitrate supplementation.**

5. **Exercise caution when feeding nitrate to cattle that are on restricted intake, that have recently been fasted, or lack adequate levels of digestible dry matter.** In these circumstances nitrate is consumed faster and therefore the risk of nitrate poisoning is greater.

6. **When feeding nitrates to grazing cattle, they should be given continuous access to the nitrate source (usually in the form of a lick-block). Avoid pulse-feeding nitrates to cattle.**

7. **In a feedlot system where cattle are on total mixed rations, the nitrate should be dissolved in the liquid supplement, prior to mixing the ration, thus ensuing even distribution.**

8. **Ensure that cattle being fed nitrates also have adequate sulphur intake, in order to support rumen health. The recommended level is 2 grams of sulphur per 100 g of nitrate.**

9. **Stop feeding nitrates to cattle for 24 hours before any stressful or physically demanding activity, such as mustering.**

Warning signs of nitrate toxicity

Nitrates have a direct caustic action on the lining of the gut. The first signs that cattle may have been fed too much nitrates are diarrhoea, salivation and abdominal pain.

The next stage of nitrate poisoning involves an accumulation of nitrite – a by-product of nitrate - in the blood, where it reduces the blood's ability to carry oxygen.

Signs that nitrate poisoning has occurred include difficulty in breathing, with gasping, rapid breaths the most obvious symptom. Affected animals are weak and tremble and will stagger. Severely affected animals will go down, convulse and die.

What should I do?

Contact your nearest vet as soon as possible to confirm nitrate poisoning and to treat affected animals.

If this is impractical, immediately remove the stock suspected of nitrate poisoning from any source of nitrate, and handle as little and as quietly as possible.

If possible, feed the affected animals hay that has not been fertilised with a nitrogen source, or some other form of low-nitrate herbage in association with no more than 20% (by weight) cereal grain. This will help rumen microbes to deplete accumulated nitrite.

Affected animals can be treated with an injection of methylene blue. It is important to note, however, that the Australian Pesticides and Veterinary Medicines Authority (APVMA) no longer approve the use of methylene blue in food-producing animals.

3.5.2 Guidelines on Nitrate inclusion levels

On forage <55% DMD, cattle lost weight while light weight cattle grew at approximately 1kg/d on 75%DMD rations (Table D16). In most cases, protein supply limited animal growth via restricting feed intake (Table D17). These numbers indicate that the Grazfeed model is realistic, though perhaps conservative, in the animal growth and metabolism it is modeling

Table D16. Liveweight change (kg/d) of brahman x british crossbred steers of various weights consuming rations of various Dry matter digestibility (DMD%). Crude protein of the pasture is shown in parenthesis.

LW below	Forage DMD%			
	45 (*6.4*)	55 (*7.6*)	65 (*8.8*)	75 (*10*)
300 kg	-0.77	0.00	0.38	0.92
400 kg	-0.78	-0.07	0.32	0.79
500 kg	-0.89	-0.22	0.24	0.65

While for 300kg cattle, intake was probably limited by energy intake, forage intake by all heavier cattle (Table D17), was probably limited by the intake of rumen degradable protein, as this was below 10gCP/MJ ME which rumen microbes typically require for growth and function.

Table D17. Voluntary feed intake (kg DM/d) of unsupplemented cattle consuming forages of differing DMD%

LW below	Forage DMD%			
	45 (*6.4*)	55 (*7.6*)	65 (*8.8*)	75 (*10*)
300 kg	4.11	5.07	5.7	6.34
400 kg	4.62	5.87	6.63	7.14
500 kg	4.82	6.17	7.03	7.44

These forage intakes (kg DM/d) were then used to estimate the quantity of nitrate animals in each scenario would consume per day in supplements (being 7 g nitrate/kg DMI) reported in Table D18.

Table D18. Daily intake (g/d) of calcium nitrate (Bolifor CNF) and of ammonium nitrate required to provide 7g nitrate/kg DM intake to cattle of known weight with estimated pasture intakes

	Calcium nitrate					Ammonium nitrate			
	Forage DMD and *[CP]* content					Forage DMD and *[CP]* content			
LW below	45 (*6.4*)	55 (*7.6*)	65 (*8.8*)	75 (*10*)		45 (*6.4*)	55 (*7.6*)	65 (*8.8*)	75 (*10*)
300 kg	**45**	**56**	**63**	**70**		**37**	**46**	**51**	**57**
400 kg	**51**	**65**	**73**	**79**		**42**	**53**	**60**	**64**
500 kg	**53**	**68**	**77**	**82**		**44**	**56**	**63**	**67**

It is worth noting that this level of calcium nitrate consumption through lick blocks is feasible. While AoTG studies have observed nitrate blocks are far less readily consumed than are comparable urea blocks, it is feasible to make calcium nitrate blocks with 40% calcium nitrate (by weight) and daily intake of 100g block/d (=40g/d calcium nitrate)may be achievable. This level of nitrate supplementation would be associated with a 10-15% increase in the total nitrogen intake of cattle (Table D19). Since feed intake and therefore animal growth were largely constrained by feed intake the effect of this N supplement on animal performance was then crudely estimated by repeating the GrazFeed simulations in which the CP content of the diet was inflated by the appropriate percentage (Table D19).

Table D19. Percentage increase in daily crude protein intake by cattle associated with consumption of a nitrate supplement providing 7g nitrate/ kg DM intake.*

	Forage DMD%			
LW below	45 (*6.4*)	55 (*7.6*)	65 (*8.8*)	75 (*10*)
300 kg	**15.5**	**13.0**	**11.2**	**9.9**
400 kg	**15.4**	**13.0**	**11.2**	**9.9**
500 kg	**15.5**	**13.0**	**11.2**	**9.9**

*(*remember if the nitrate is supplied as NH_4NO_3 that the ammonium will also contribute CP which is NOT included in this table)*

Re-running Grazfeed with this higher dietary N intake typically increased LWG by 40g/d across 55-75% DMD forages where intake had been constrained by CP intake, so this represents an anticipated productivity gain associated with nitrate use in contrast to no N supplementation.

Further, the inclusion of nitrate at a rate of 7g/ kg DMI would lead to the following change in daily methane production. It was assumed that 1 mole of methane should be saved per mole of nitrate consumed (= 259g methane spared/kg nitrate), but an efficiency of 80% in this process is allowed, so a methane saving of 207 g methane/kg nitrate was applied (Table D20).

Table D20. Effects of feeding nitrate at 7g/kg DMI on methane emissions due to change in DMI or due to direct mitigation of methanogenesis by nitrate

LW	DMD (%)			
	45	55	65	75
	Change in methane (g/d) due to change in DM intake			
300 kg	0	15	13	8
400 kg	0	12	10	11
500 kg	0	14	9	13
	Change in methane due to nitrate (g/d)			
300 kg	-6.0	-7.4	-8.3	-9.2
400 kg	-6.7	-8.5	-9.6	-10.4
500 kg	-7.0	-8.9	-10.2	-10.8
	Net change in methane as % of original DMP			
300 kg	-7.2	6.6	3.4	-0.8
400 kg	-7.2	2.6	0.2	0.4
500 kg	-7.2	3.6	-0.7	1.2

It is apparent that when the extra methane arising from addition feed consumed as a result of N supplementation is allowed for, the mitigation effect of nitrate is largely lost. However, calculation of the methane emission intensity (methane/kg LWG) for those situations in which cattle would be gaining rather than losing weight (being 65% and 75% DMD feeds), showed a consistent 14% reduction in emission intensity.

In summary it is apparent that the maximum limit of nitrate supplementation supported by the CFI methodology when applied in rangelands:

- Could feasibly be delivered in lick-blocks
- Will increase the daily nitrogen intake by 10-15%
- Will increase feed intake and LWG by a small margin (~40 g LWG/d) during protein limited growth
- In so doing the net effect on daily methane emission is largely lost, but a reduction in emission intensity of 14% during times of feed intake is apparent, so a desirable change in the emission cost of growth from individual animals is also apparent and this value will still be lower than if urea had been used to provide non-protein nitrogen to the rumen.
- Further to improved feed intake and growth, it may be expected that (relative to unsupplemented animals) that increased intake by breeding females due to NPN supplementation would also reduce the methane cost per calf sold due to to increase in cow condition score, conception and calf rearing.
- Practical cautions remain with nitrate lick blocks. For instance, while AACo have their own process for block production, standard molasses based blocks are somewhat hygroscopic and dissolve/decay during the northern wet season. Together with cattle having a clear preference for urea-based blocks over nitrate blocks, this means a workable block mechanism for roll out of the methodology is not available to general producers at this time.

3.6 Nitrate used safely in major AotG demonstrations with over 1000 cattle

While a huge amount of AoTG effort has gone into this, there are few results to date, largely as a result on need to continually evolve the hardware (walk over weighers, autodraft and automated block intake measurement). An initial study near Charleville identified block intake decrease with calcium nitrate content (as found for sheep by Li et al., 2012 AAAP in FtRG). Currently nitrate and urea blocks are being provide to 400 cattle in an ACC grow-out study neat Augathella. A larger replicated study (650 breeders) in the southern Gulf region (nth of Richmond Qld.) was run in 2014 and will recommence in late May 2015 when cattle are handled and can bescanned for pregnancy (Figure D34ab).

In the feedlot study (n=383), in which cattle were fed nitrate or urea in isonitrogenous feedlot finisher diets, there was a significant reduction in feed intake and in live weight gain (LWG) of cattle, but no indication of any difference in feed efficiency or feed conversion ratio

Table D21. Dry matter intake, feed:gain ratio and feed efficiency of cattle with a dietary inclusion of supplementary nitrogen at a Low or High inclusion level provided as urea (U) or nitrate (N). Data are presented for 3 periods within the feedlot finishing study. (Individual data for AF pens + pen means (bunks), total N = 383). DM1-3 are successive portions within the 70 d feeding period.

	DMI (kg DM/d)	*FG:F (g/g)*	*DMI1 (kg)*	*DMI2 (kg)*	*DMI3 (kg)*
Level - Low	*10.95*	*0.154*	*10.42*	*11.69*	*11.14*
Level - High	*10.38*	*0.153*	*9.75*	*11.03*	*10.72*
Difference	*0.572*	*0.001*	*0.668*	*0.663*	*0.420*
P	*0.000*	*0.736*	*0.005*	*0.001*	*0.062*
Urea	*11.02*	*0.154*	*10.36*	*11.73*	*11.15*
Nitrate(NO3)	*10.32*	*0.154*	*9.81*	*10.98*	*10.71*
Difference	*0.699*	*0.0002*	*0.552*	*0.753*	*0.442*
P	*0.000*	*0.950*	*0.020*	*0.000*	*0.036*
Low_U	*11.18^{a}*	*0.156*	*10.59^{a}*	*11.83^{a}*	*11.12^{a}*
Low_NO3	*10.73^{b}*	*0.152*	*10.24^{a}*	*11.55^{a}*	*11.16^{a}*
High_U	*10.86^{ab}*	*0.151*	*10.13^{a}*	*11.64^{a}*	*11.18^{a}*
High_NO3	*9.91^{c}*	*0.155*	*9.37^{b}*	*10.42^{b}*	*10.26^{b}*
Ave SED	*0.209*	*0.004.475*	*0.334*	*0.288*	*0.307*
Diff LU-HNO3	*1.271*	*0.0013*	*1.220*	*1.416*	*0.862*
P(LU ≠ HNO3)^{2}	*0.000*	*0.772*	*0.000*	*0.000*	*0.005*
P (interaction)	*0.087*	*0.172*	*0.371*	*0.021*	*0.023*

There was also no difference in consumption pattern across the whole herd due to nitrate (Table D22).

In keeping with the slower ADG of NO_3 fed steers, the effect of N inclusion on final carcass attributes over 100 d later showed NO_3-fed steers provided carcasses that were lighter (326.0 v 334.9 kg; $P < 0.001$) so of lower total carcass value ($1235 v $1270; $P < 0.001$). After allowance for carcass weight, there were no differences in measures of carcass fatness but the H-NO_3 carcasses were of lower value than those of other treatments at the same carcass weight. Average meat colour of all treatments was mid-range but meat of NO_3- fed cattle was slightly darker (P=0.001) than that of U-fed cattle, at least at the low N inclusion level. Fat colour was also slightly affected by N level and source, such that fat colour of NO_3-fed steers was darker than that of U-fed cattle at the low inclusion level. At high inclusion levels, neither the color of meat nor fat was affected by NO_3. After adjustment for carcass weight, there was no difference in carcass value, eye muscle area, fat depth or marbling parameters due to N source.

No evidence of NO_3 being present in any meat (raw or cooked) was apparent, with all samples being less than the detection limit of the analysis. While only 8 analyses were done, these samples were comprised of subsamples from 100 cattle per treatment. Nitrite was detected in raw meat samples from both H-U and H-NO_3 fed cattle but was not detectable in cooked meat. None of the 8 samples assayed contained any nitrosamines at or above the detection limit.

Table D22. Number of meals, duration of each mean and total time spent feeding by cattle (n=139) with a dietary inclusion of supplementary nitrogen at a Low or High inclusion level provided as urea (U) or nitrate (N). Data are presented for 3 periods within the feedlot finishing study.

		NPN source			Level of NPN			
	Days	Urea	Nitrate	*P*-value	Low Level	High Level	*P*-value	*P*- value interaction
Time in feeder (min/day)	21 - 51	98.0	98.9	0.84	96.9	100.0	0.46	0.87
	52 - 82	78.3	84.2	0.28	80.9	81.6	0.88	0.99
	83 - 112	65.4	73.5	0.09	68.2	70.4	0.65	0.78
Number of feeder visits per day	21 - 51	11.7	12.7	0.52	12.3	12.1	0.88	0.39
	52 - 82	14.6	15.6	0.66	14.9	15.3	0.88	0.88
	83 - 112	13.1	15.4	0.24	13.9	14.6	0.74	0.85
Average time per visit (min)	21 - 51	10.7	10.5	0.86	10.7	10.5	0.90	0.42
	52 - 82	7.5	7.4	0.97	7.8	7.1	0.63	0.60
	83 - 112	6.5	5.9	0.55	6.4	6.0	0.71	0.75
DMI per minute (g)	21 - 51	113	106	0.32	115	104	0.11	0.87
	52 - 82	152	138	0.13	149	142	0.38	0.74
	83 - 112	162	145	0.10	156	151	0.65	0.99

The pilot trail at Charleville showed a lower intake of nitrate containing blocks, whether the block was primarily an NPN supplying block or a block rich in S or P but also carrying nitrate within it.

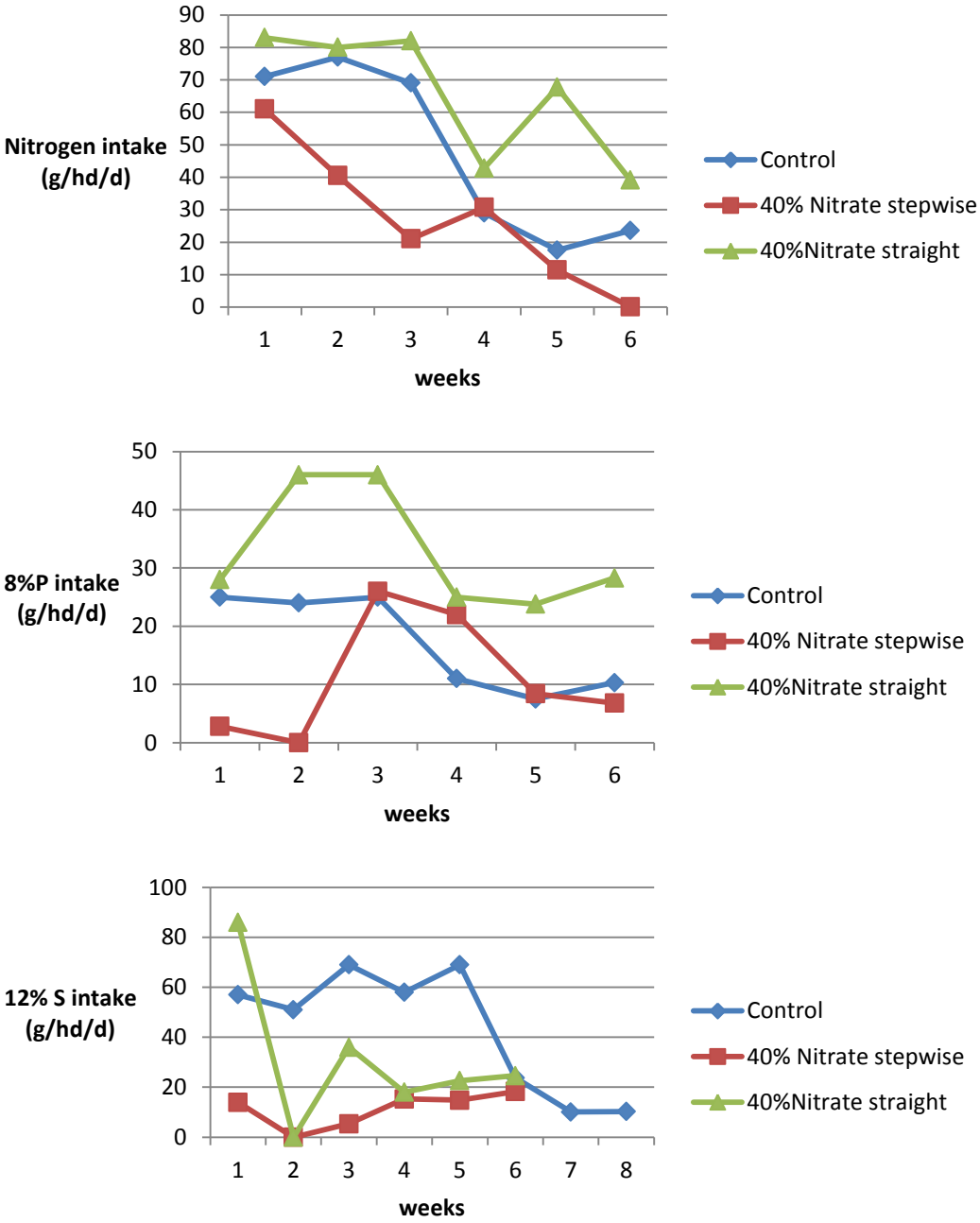


Figure D32. Intakes of Phosphorus, Nitrogen and Sulfur containing blocks over 5 week period up to the methaemoglobin bleeding sampling. Cattle on the nitrate stepwise treatment were acclimated to 40% nitrate level from 20% nitrate in steps of 10% every fortnight.

Most importantly, neither mortalities nor toxicity symptoms were observed at any stage throughout the trial. Cattle that were acclimated to high nitrate concentrations in the lick block (obviously diluted when other factors of diet are taken into consideration). Increasing step-wise had slightly lower supplement intake than cattle that were introduced to the final concentration immediately (as accepted by industry as standard practice). This supports the notion that cattle provided the final concentration immediately will self-regulate consumption due to an initial off-putting experience if a high intake is experienced, thus these animals will not eat excessive amounts. However, cattle that were introduced step-wise showed no-intakes that would indicate risk of toxicity.

The follow-on trial at Charleville was reduced from 600 to 220 heifers due to drought but showed no adverse effect of nitrate supplementation (relative to urea) on animal performance, with all treatments recording similar LW loss over the Autumn/Winter period (Table D23). There was no difference in intake

of urea v nitrate in the single multi-nutrient blocks (122 g/head/d each) or total block when N,S,P blocks were all on offer (175 g/d NO3 blocks v 213 g/d urea-blocks).

Table D23. Average animal performance by Supplement treatment of heifers offered one of 4 lick block options; being an industry standard urea block, a 30% nitrate, or a choice of N,S,P blocks offered in the same lick yard.

Average Liveweight/Treatment (Kg)				
Week Commencing	**1. Urea Single**	**2.Nitrate Single**	**3.Nitrate Free/Ind**	**4.Urea Free/Ind**
22-Apr-13	330	325	322	328
29-Apr-13	330	323	324	333
6-May-13	329	322	324	328
13-May-13	328	323	318	326
20-May-13	326	316	317	324
27-May-13	320	312	309	314
3-Jun-13	314	307	308	313
10-Jun-13	313	308	306	315
17-Jun-13	321	302	308	315
24-Jun-13	305	295	295	313
Total Δ LW (kg)	-25	-29	-27	-15

At these intakes, the quantity of methane mitigated/d was very small, being typically 1-2g CH_4/d (Table D24).

Table D24. Estimated methane reductions from nitrate supplementation

Treatment				
	2.Nitrate Single		3.Nitrate Free/Ind	
Week Commencing	CH4 saved g/hd/d	%CH4 mitigation	CH4 saved g/hd/d	%CH4 mitigation
22-Apr-13	1.8	1.5	0.3	0.2
29-Apr-13	4.2	3.4	0.4	0.4
6-May-13	4.6	3.8	0.6	0.5
13-May-13	2.9	2.4	1.4	1.1
20-May-13	2.8	2.3	1.4	1.1
27-May-13	1.3	1.1	2.8	2.3
3-Jun-13	0.2	0.1	1.7	1.4
10-Jun-13	1.2	1.0	1.7	1.4
17-Jun-13	0.3	0.3	0.4	0.4
24-Jun-13	0	0	0.8	0.7

**No Methane (CH_4) reductions were expected for urea supplemented animals and thus no estimations were made*

** All calculations were based on the assumptions that animal weight was 330kg, DMI was 6.6kg Block intake was as observed in the averages from the observed intakes, a CH_4 reduction efficiency of 90% and CH_4 emission without intervention being 122g/hd/day. Assumptions: Methane (CH_4) mitigation has been calculated as 1.1x estimated methane emissions as estimated by the calculations (Minson & McDonald 1987; SCA 1990; Blaxter & Clapperton 1965; Brouwer 1965) set out in Australian methodology for estimation of greenhouse gas emissions and Sinks (1998). Animal parameters used in this calculation were for a 330kg Heifer with an ADG of 0.5kg/hd/d. Feed digestibility was set to 60% and the proportion of gross energy intake emitted as methane was assumed to be 55.27MJ/kg CH_4 (Brouwer 1965). Obviously during this time of the season at which the trial was executed and the type of climatic conditions, feed digestibility is like to be approximately half that used in the calculations, at best. However a higher value was used to better illustrate the still only modest methane mitigation.*

Data from Burleigh is only just being received but it is apparent from observations of blocks over the recent wet season that current molasses based blocks do not have the structural integrity to retain their hardness over the wet season (Fig D33). While this may be seen as a risk to safety if animals could eat a large quantity of nitrate block, it is also apparent that cattle did not choose to eat these blocks over the wet season.

Figure D33. Molasses based lick block in use at Burleigh over a Nth Queensland wet season. Block ash lost its strength.

Figure D34a. Intake of lick blocks by cattle being monitored at "Burleigh" Qld in one of 4 supplement pens to which animals are directed when they come to water.

Figure D34b. Steers accessing nitrate blocks and having methane emission measured at Augathella Qld. using ACC cattle

4 Discussion

The industry implications of key findings or realisations are explored below. This is quite different than the discussion which would be provided in a scientific paper. Discussion is not grouped according to the 6 outcomes as used in methods and materials and in discussions sections, but according to three larger groupings

- Implications of new understandings relating to risks with nitrate feeding
- Implications of nitrate on productivity of ruminants
- Implications with applicability in industry

4.1 Implications of new understandings relating to risks with nitrate feeding

Three risks were perceived with respect to animal or human health from nitrate inclusion in ruminant diets. These were the risk of compromised animal productivity health from reduced feed intake and from nitrite toxicoses following excess absorption of ruminal nitrite (NO_2); risk of imidazole formation in molasses-based lick blocks containing nitrate and leading to neural dysfunction; and thirdly formation of nitrosamines in animal tissues that could be toxic to consumers of meat. This project (and additional work requested by Cargill) was able to show that neither the risk from nitrosamines nor imidazoles were significant in the studies undertaken.

So the most significant risk, the understanding and management of which was the principal focus of the project, was the adverse effects of nitrate on animal health and animal production. For all the years in which nitrate has been of interest as a factor causing animal deaths, astoundingly little is known about its metabolism in the rumen and by the animal.

Questions such as

- From what part(s) of the digestive tract are nitrate and nitrite absorbed and by what mechanisms?
- Does the Nir mechanism for dissimilatory nitrite reduction so important in other ecosystems even exist in the rumen?
- What is the basis of between animal variation in animal vulnerability to nitrite poisoning? This was not addressed during these studies

We did not pursue these because we commenced by ‘looking for a practical solution’ and only found these fundamental issues were not understood later in the life of the project. So what did we discover and what are the implications around nitrite toxicity?

Firstly, nitrite’s adverse impacts are likely to result from both changing the blood’s oxygen carrying capacity (based on MetHB formation) and on vasodilation through conversion of NO_2 to nitric oxide (NO) in the host. MetHB formation following intraruminal dosing has been shown in FtRG (Callahan) to substantially reduce the ability of cattle to cope with exercise such as when being mustered. We have not in our experiments or recent review of literature, discovered any way of either preventing MetHB formation once nitrite enters the blood, nor accelerating the regeneration of haemoglobin once MetHB has been formed. So in the interests of practical nitrate feeding, most attention went to avoid nitrite being formed in, or being absorbed from, the rumen.

Data clearly identified that risk of nitrate in the feed was not only affected by the nitrate content, but by the feeding behaviour of the animal. The safest feeding scenario (not necessarily the most productive) was when animals consumed multiple small nitrate meals/d (in difference to one or 2 short feeding bouts) AND when nitrate intake was coupled with ingestion of fermentable carbohydrate. This leads to the following implications:

- Provision of nitrate rather than urea in feedlots is safe and feasible, with acclimation to dietary nitrate coinciding with acclimation to higher cereal grain contents. Two caveats on this warrant mention. (1) Reduction in feed intake associated with feeding (as observed in AoTG) would not be acceptable to the feedlot operator & intake reduction must be overcome for commercial use. In all our studies of prolonged nitrate feeding, MetHB rises over weeks and months of nitrate feeding and safety cannot be presumed just because animals are adapted to nitrate (Figure D10).

- Provision of nitrate in drinking water (eg by reticulation off bores or dams) would be extremely high risk in extensive environments where cattle may only drink once per day or once per 2 days.
- Provision of nitrate in lick blocks may be a safe route to administration as intake is necessarily slow, but as nitrate/nitrite is cleared from the rumen within 3 h, there may be a serious asynchrony between supplementary N intake and carbohydrate intake. This currently occurs with urea lick blocks but the risk of NO_2 accumulation in the rumen when animals are not also consuming carbohydrates is novel for nitrate blocks and needs to be tested.

Numerous ways were investigated to prevent nitrite entering the blood and these focussed primarily on accelerating the reduction of nitrite to ammonia. A probiotic approach by introducing an exogenous organism with a high nitrite reductase level (*Propionibacterium acidipropionici*) proved ineffective for ruminants consuming a roughage diet, though may be effective in starch fed animals (French study), as these organisms are primarily starch fermenters. There was no evidence that we could accelerate the conversion of nitrite to ammonia in the rumen and make nitrate feeding safer by manipulating dietary Mo or S levels, though provision of sulphates generated further methane mitigation and wool growth. What proved very powerful in managing MetHB (so we believe nitrite concentrations) was the rate of intake of nitrate and of fermentable substrate. It is very likely that this reflects the maximum kinetic reaction rate of the nitrite reductase enzymes (with the maximum clearance rate [V_{max}] exceeded during large meals allowing NO_2 to accumulate). To overcome this, the rumen requires either more enzyme (as we observed occurs with acclimation or enzymes of higher capability. Our efforts to modify the type of reductase operating by modifying substrate type (glycerol v glucose) did not support the hypothesis thatsubstrate type can be managed to modify nitrite reductase enzyme activity. It is worth noting that increasing the reaction rate of nitrite reductase is only of merit IF it is increased more than the rate of nitrate reductase. It is the differential between these two reaction rates that will affect nitrite accumulation in the rumen and MetHB formation.

While there are no apparent advantages from MetHB formation, the formation of nitric oxide (NO) and the vasodilation in the host resulting from NO derived from nitrite in blood, is likely to offer advantage and is discussed in the following section.

Regarding making nitrate safe for use, we have not found microbial, nutritional or management tools to make dietary nitrate inclusion universally safe. We have found ingestion of nitrate in multiple small meals a fundamental approach to safety which has application in feedlot production. Any nitrate delivery system that delivers large intakes in a small amount of time, especially in the absence of fermentable carbohydrate should be avoided.

4.2 Implications of nitrate on productivity of ruminants

Urea is a conventional form of non-protein nitrogen (NPN) supplement for ruminants; nitrates can also serve this function with the differences that (a) their N content is generally lower (approx. 17-25%N v 46%N) so more must be fed and (b) they bring the risk of nitrite toxicity in difference to ammonia toxicity from urea.

On a diet deficient in nitrogen (as most dry season pastures in Northern Australia are), we showed calcium nitrate is an effective NPN supplement, increasing digestibility and DM intake. So its efficacy as an NPN supplement in protein deficient areas (such as where the current CFI methodology may be applied) should not be forgotten or overshadowed by comparison to urea

It is also apparent that in most cases when the diet is adequate in southern Australia (most of southern Australia), nitrates will lower the total feed intake. While this can be seen simplistically as an 'inbuilt safety' with nitrate feeding as it must reduce the risk of over consuming nitrate it will, however, reduce liveweight gain and therefore adversely affect productivity.

The data on nitrate effects on intake shows some inconsistency (both in our data and that reviewed). Since this is a pivotal response affecting profitability of nitrate, a clearer understanding of the mechanisms by which nitrite affects intake is needed and should be pursued.

One likely mechanism is the formation of NO from absorbed NO_2, as NO is a powerful vasodilator and extremely likely to lead to lowered blood pressure in the animal. Lowered blood pressure has many physiological consequences, but one of them is inappetance. Another consequence of NO influencing

productivity is increased blood flow to the skin. This is probably the reason that skin surface temperature and wool growth were found to be increased in nitrate-fed sheep.

Dietary nitrate did not affect indicators of body and carcass composition in cattle (FtRGAOTGR1-182) and we await final data on body composition of nitrate and urea fed lambs measured by CT scanning.

In summary, nitrates offer enhanced productivity with reduced emission for ruminants experiencing a protein deficient diet but not when replacing urea. However in protein-adequate feeding environments, there is a risk of feed intake being reduced and this will reduce growth rate of these animals and returns from carbon markets for nitrate-derived methane mitigation will not financially compensate for this in the near future.

4.3 Implications for applicability in industry

One of the unique attributes of nitrate as a methane mitigation technology is that the mitigation achieved is consistently delivered and is directly proportional to the quantity of nitrate absorbed (being approximately 10% emission reduction per 1% nitrate inclusion in feed). This surety does not exist for other mitigation strategies such as oils or saponins or tannins, where results are variable within and between sources. For this reason it is worth persisting with development of a nitrate as a mitigation strategy. There is concern about nitrite toxicity but 40 years ago there was comparable concern about the risk associated with dispensing urea as an NPN supplement. The array of investigations undertaken in this project has shown quite clearly that while achievement of mitigation from nitrate is a certainty and can be relied on, the adverse effects of nitrate (risk of nitrite toxicoses and reduced feed intake) do not always occur, meaning there is opportunity to manage nitrate for mitigation without inducing adverse consequences.

The most obvious opportunity is in feedlots where risk of nitrite toxicity would be minimised by normal management practices (slow adaptation to diet, uniform mixing of feeds, feed always available). However, the little information available indicates reduced feed intake occurs in such a feeding system and this is unacceptable in such an enterprise. A clearer understanding of the mechanism of nitrate's impact on feed intake and how to manage this is pivotal to nitrate's application in feedlot systems.

For extensive livestock, delivery of nitrate via lick blocks is possible, and while the CFI methodology uses only a low nitrate level, we know this is able to be included in a multi-nutrient salt or molasses block, and our evidence is no harmful methyl-imidazoles are generated. Intake of blocks is reduced through nitrate inclusion and realistic intakes of 120g/head/d, of a block that is 30% Bolifor CNF (@64%NO3) will not exceed the CFI threshold. Delivery via liquid supplement is also feasible but again intake reduction due to nitrate is apparent. Development of a CFI methodology around liquid molasses supplement delivery of nitrate would increase the proportion of Australia in which nitrate could then be used, applying in the cropping and coastal regions.

With our current limited understanding of ruminal nitrate/nitrite metabolism and lack of anything but feed management tools to regulate nitrite toxicity risks, it would be hazardous to expand nitrae use into industry applications with less control over nitrate consumption. In particular, supplementation via loose licks (salt based blends of high nutrient mineral granules and occasionally protein meals) or via water medication would place the animals at unnecessary risk.

In terms of increasing productivity (rather than reducing emission or emission intensity), the finding that nitrate changed the skin temperature and wool growth of sheep is indicative of a novel mechanism by which nitrate may improve this production. We have not comprehensively addressed whether nitrate feeding should increase the capture of feed energy in body reserves as a result of reducing methane production. On principle we would not expect this to be the case. Unlike rumen modifiers, nitrate simply robs the rumen of hydrogen and high energy electrons to produce ammonia. In so doing it draws through acetogenic hydrogen-producing reactions, while other rumen modifiers cause hydrogen accumulation so suppress acetate production and stimulate propionate production.

4.4 Summary of implications for Australian agriculture

Dietary nitrate offers a sure and predictable means of mitigating enteric emissions from ruminants. The risks associated with its inclusion in feeds and supplements closely parallel those of urea that now forms the principle non-protein nitrogen supplement in Australia. This work has shown nitrate can serve effectively as an non-protein nitrogen supplement promoting growth on low protein feeds and that feed management offers the most immediate means of controlling methaemoglobinaemia. Specifically a controlled release of nitrite into the rumen will prevent toxic nitrite accumulation and this is ideally managed through the total mixed rations of feedlots or controlled supplement delivery via lick blocks or liquid molasses supplements. There are a number of potential feed additives that can augment feed management in improving the safety of nitrate feeding.

5 Future research needs

The safe commercial implementation of nitrate feeding requires research at both the on-farm and the fundamental science level.

At the farm level there is still need to develop commercially practical blocks which will have the weather resistance for prolonged use in the tropical environment. This is likely to involve use of ammonium nitrate rather than calcium nitrate on account of the higher N content in ammonium nitrate and the hygroscopic nature of calcium nitrate blocks, but also factors like block hardnesss need refinement. There is also opportunity to broaden application of nitrate use through inclusion of liquid supplements but with only one experiment with this mechanism conducted, a greater number of studies are required to provide confidence in the delivery mechanism. Lack of “on-farm” data also poses significant risk to implementation of the current nitrate block CFI methodology. More experience is needed with the practicality of delivery and efficacy of mitigation to adequately advise graziers who may be wishing to implement this methodology.

In the fundamental science, we need to understand:

- The mechanisms by which nitrate affects feed intake and how these can be moderated to eliminate the risk of intake suppression. This currently prevents nitrate use in feedlots and will be a consideration in all southern grazing systems.
- The extent and cause of between animal variation in MetHB formation following nitrate ingestion. Is this due to differences within the rumen or in animal metabolism, and if ruminal, is a microflora leading to low MetHB identifiable as a potential probiotic?
- The prospects for reducing nitrite toxicity risk by slowing the release of nitrate into the rumen (eg. by fat- or polymer coated prills) rather than striving to accelerate nitrite removal. In effect this would deliver a continuous stream of nitrate to the rumen microflora, not pulses of nitrate associated with the time of ingestion.
- How and where nitrate and nitrite in the gut are absorbed so strategies to inhibit nitrite absorption can be made.

6 Publications

- Li L, Malisang, S, Hegarty RS, Nolan JV, Leng RA (2012). Delivery of dietary nitrate to sheep through lick-blocks for enteric methane abatement. Proceedings of the 15th AAAP Animal Science Congress 26-30 November 2012, Thammas at University, Rangsit Campus, Thailand
- Leng, R.A. (2013). Reducing Enteric Methane Production in Ruminants with Dietary Nitrate; anaerobic nitrate metabolism in bacteria with special reference to the roles of sulphur and molybdenum. https://www.une.edu.au/about-une/academic-schools/school-of-environmental-and-rural-science/research/animal-science/ruminant-nutrition-group
- Li L,. SilveiraC. I.,. NolanJ. V,. GodwinI. R, Leng R. A. and R. S. Hegarty (2013). Effect of added dietary nitrate and elemental sulfur on wool growth and methane emission of Merino lambs. Animal Production Science 53, 1195-1201.
- Velazco, JI, Bremner G, Li L, Luijben K, Hegarty RS, Perdok H (2013) Short-term emission measurements in beef feedlot cattle to demonstrate enteric methane mitigation from dietary nitrate. Advances in Animal Biosciences 4,579.
- Godwin, IR, Oelbrandt, N, Velazco de los Reyes, J, and Hegarty RJ (2012) Adaptation of cattle to dietary nitrate may involve adaptation to methaemoglobinaemia. Proceedings of the Nutrition Society of Australia, 36, 11.
- Li, L., Ines Silveira Rojas and Hegarty, R.S. (2014) The interactions between nitrate, sulphur and molybdenum in reducing nitrite accumulation and methane production from ruminants: an in-vitro incubation study with Italian ryegrass as substrate. Proceeding of the 30th Biennial Conference of the Australian Society of Animal Production (30). P. 60.
- Nguyen, S. Li, L., Hegarty R.S. (2014), Effect of rumen protozoa on fermentation and methane production. Animal production in Australia. Proceeding of the 30th Biennial Conference of the Australian Society of Animal Production (30). P. 340.
- Nguyen, S. Li, L., Hegarty, R.S. (2014) Effect of defaunation and nitrate on fermentation and methane production in vi-tro. Animal production in Australia. Proceeding of the 30th Biennial Conference of the Australian Society of Animal Production (30). P. 362.
- Velazco JI, Cottle DJ, Hegarty RS (2014). Feeding behaviour needs to be considered in validating methane mitigation strategies for feedlot cattle using short-term measurements of methane emissions. Full paper submitted to ISRP-ISNH-ASAP conference, Canberra, Australia Animal Production Science, 54(10), 1737-1740.
- de Raphélis-Soissan V,LiL., GodwinI.R., BarnettM.C., Perdok H. and R.S. Hegarty (2014). Use of Nitrate and Propionibacterium acidipropionici to Reduce Methane Emissions and Increase Wool Growth of Merino Sheep. Animal Production Science, 54(10), 1860-1866.
- Godwin I, Li, L., Luijben K., Oelbrandt, N., Velazco, J., Miller, J., Hegarty, R.S. (2015) The effects of chronic nitrate supplementation on erythrocytic methaemoglobin reduction in cattle. Animal Production Science http://dx.doi.org/10.1071/AN13366.

Other manuscripts that will be submitted prior to the financial close of this project include:

Nolan JV, Hegarty R, Li L, and RC Boston. Modelling the kinetics of reduction of supplementary nitrate to ammonia and incorporation of nitrate and ammonia into bacteria in the rumen of sheep given roughage diets (Under revision for J. Agric. Sci (Camb))

Barnett M.C., Forster N.A., Ray G.A. , L.Li,. Guppy C.N and R.S. Hegarty. Using portable X-ray fluorescence (pXRF) to determine faecal concentrations of non-absorbable digesta kinetic and digestibility markers in sheep and cattle (90% Prepared for Journal of Animal Science).

Nguyen S. H., L. Li and R. S. Hegarty (2014). “Effects of rumen protozoa and nitrate on fermentation and methane production”. (Prepared for British Journal of Nutrition; 90% drafted)

Presentations regarding nitrate have been given to:

- The Primary industry Centre for Science Education 2013, 2014
- Japanese TV
- Wagyu Breeders tour group
- Science development schools,
- NLMP Update to Government and Industry 2013
- NSW DPI and UNE leadership tours,
- NSW DPI Rural Climate Solutions conference.
- A webinar was held on nitrate feeding and general nutritional management and is available at http://futurebeef.com.au/topics/nutrition/#nitrateWebinar (28/11/2p13) “A new northern PDS trial: Nitrate supplements, methane and remote technologies.
- Breeder Management Field day. Herd dynamics, remote technologies, nutrition. Held at Burleigh demonstration site, Southern Gulf Region, N Qld. 58 attendees, 47 producers.
- NSW Beef Producer Forum, Armidale, 80-producers.
- Agribusiness Today Beef Forum, Orange, 120 participants
- A range of high school and university groups through UNE open days and classes