Final report:

NOVEL METHANE ASSESSMENT IN RUMINANTS

(Project Code CC MAF POL_2008-37)

to

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PROJECT DETAILS

PROJECT CODE	CC MAF POL_2008-37
PROJECT TITLE	**Alternative methods of rumen methane assessment**
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EXECUTIVE SUMMARY

The research presented here is a pilot study to investigate the suitability of two novel, independent methods of direct methane assessment in cattle rumens.

The first 'arm' is the investigation of use of a portable methane sensor used in environmental research adapted for use as an indwelling rumen probe via the cannula in rumen fistulated cattle. The second method is the use of microbial metabolic 'proxies' of methane production in rumen contents, which has been previously used in such digesta for other end products of microbial metabolism, but not methane.

This preliminary study concludes that the METS methane sensor technology used is capable of measuring small changes in methane concentration in rumen liquid phase *in vivo* over time intervals as low as 30 minutes. This represents a solid advance in rumen methane research capacity, opening opportunities in detailed rumen methanogenesis studies.

There was also an apparent positive relationship between recorded methane concentration in the liquid phase and the rumen pH/ redox environment ($r^2 = 0.91$). If this relationship was demonstrated by future research to be consistent across diet treatments, it represents a potential 'proxy' for methanogenesis that is far more easily obtained. The other 'proxies' for short interval methanogenesis investigated – real time polymerase chain reactions (RT-PCR) enumeration of methanogen populations and mRNA quantification – gave mixed results. PCR enumeration suggested the methanogen populations were relatively slow to shift, which in turn suggests that it is their metabolic activity rather than population size that determines methane production in short interval measurement periods. However, due to technical difficulties with the development of the method, assessment of this activity by mRNA quantification was not achieved in the time frame of this project.

The results of this project suggest there are several possible methods suitable for direct assessment of rumen methanogenesis across short interval periods.

Project Goals

To assess the suitability for use of two novel methods of direct assessment of rumen methanogenesis in cattle.

Introduction

Methane emissions from ruminants are very significant in New Zealand's GHG production. However, the research underpinning our current understanding of methane production in ruminants is sparse, and the methods used to measure emissions have large errors and require long (days) experimental measurement periods. This lack of 'real time' measurement capacity limits the development of specific mitigation tools in response to diurnal or grazing patterns of methane production.

There is a need for a different approach that is able to assess changes in methane production over short (ie. hours) periods. Recent developments in two separate fields – rumen microbiology and methane sensor technology - have presented opportunities to do this. This project is a pilot study to investigate the suitability for use of two methods that have not been used for methane production measurement in rumens to date. The first is a portable sensor that has recently been developed for under water methane measurement. The second is the use of metabolic 'proxies' of methane production in the rumen to indirectly estimate the immediate production of methane at a given time. These methods have been used to estimate the production of other rumen/ hindgut bacterial end products, but to date has not been used for methane assessment.

Materials and Methods

The two methods have preliminary assessments that were conducted separately.

<u>Methane sensor *in vivo*</u>

The portable methane sensor (METS #11, D-Opto Technologies, Lausanne, Switzerland) was designed for applications in aqueous environments, for detection of methane concentrations, usually at relatively low levels (<150umol/L). The sensor was obtained from the manufacturer after initial estimates of rumen methane concentrations in rumen fluid were provided to enable calibration of the apparatus for this novel application (upper limit 350umol/L).

Experiment 1
Four holstein friesian cattle (liveweight 350 kg) with rumen cannula fitted had SF6 boluses added seven days before being pen fed rye grass silage (ME – 10.5 MJ/ kg DM) for another seven days *ad libitum*. The cattle were then fitted with breath assessment harnesses and then placed in individual metabolism crates (day zero of the experiment) to be fed *ad libitum* grass silage for two days in split meals at 8.30am and

4.30pm, with the residual feed recorded each day. On day one indwelling pH, temperature and redox probes were placed in the rumens of two cattle, and measurements datalogged every 15 seconds. On day two at 6.30am the methane sensor was placed in the rumen of one animal that was fitted with the pH, temperature and redox probes, and a simultaneous ventral sac rumen sample was obtained for subsequent analysis. A similar sample was also obtained from each of the remaining three animals. The methane sensor was datalogged every second to a computer until 12pm (two hours prior to and four hours after feeding), when it was removed. Concomitant eructated/exhaled SF_6 for the corresponding six hour blocks were obtained from all four animals using the breath assessment harnesses.

The methane sensor was replaced in the same animal at 2pm and a simultaneous ventral sac rumen sample was obtained. The probe measurements were datalogged every second until 8pm when it was removed. Eructated/ exhaled gas assessments were obtained from all animals for this period.

Rumen samples were also taken from the two animals that did not have an indwelling probe at 6.30am, 12.30pm, 2pm and 8pm for subsequent analysis.

At 8pm all probes were removed from all cattle and they were removed from the metabolism crates.

Experiment 2
After reviewing the results of the initial experiment, a second experiment was undertaken with several significant changes to the original design.

To reduce the methane concentration recorded in the liquid phase of the rumen, barley straw was fed instead of grass silage, and at less than half of maintenance energy requirement. The cattle were individually fed the straw for 3 days before entry to the metabolism crates at 5pm on day 3, then fasted until feeding at 9.00am the next day. At 8.30am the pH, redox and SF_6 collection equipment was fitted to the four cattle, and the methane sensor to a single animal, and datalogged measurements were made as in Experiment 1.

Rumen samples were obtained from the three cattle without the methane sensor at 8.30am and every hour until 12.30pm. Rumen samples were obtained from the single animal with the methane sensor fitted at 8.30am and 12.30pm only. At 12.30pm the cattle had all measurement and collection equipment removed and were released to pasture.

Methane and SF_6 concentrations in the vacuum containers were analysed by Mass Spectrophotometry at Lincoln University.

Rumen microbiological metabolic 'proxies'

From the rumen samples described above a sub-group of samples was selected for further analysis on the basis of the rumen function parameters measured. These, along with the rumen metabolism measurements obtained simultaneously were used to

assess the suitability for use of microbial methods adapted from similar work in independent fields at the Queensland Department of Primary Industry (QDPI).

Three independent methods were used, with all three used in comparison with methane sensor records of methane flux across the pre and post prandial period, as follows. SF6 marker assessments of methane eructated/ exhaled obtained from the trials were collected for use as alternative independent assessment of methanogenesis, and were used for comparison on the proviso that the generally large standard errors associated with use of this method limit the accuracy of the technique in short interval measurements. Rumen samples were treated for extraction of nucleic acids prior to the application of these methods using the standard QDPI protocols for rumen microflora.

The first method used was the flux in pH, temperature and redox values across this period compared (analysis of variance) with the recorded methane concentrations in the rumen fluid. The second was quantification of methanogen (*Methanobrevibacter ruminantium* and *Methanobrevibacter smithii)* populations by real time Polymer Chain Reaction method (16S RNA and mcrA genes) pre and post feeding (2 hour intervals), on the basis of existing research suggesting a post prandial increase in methanogens in ruminants. This was done using QDPI methodologies, equipment, and standards for experiment 1 only. The third method was examination of the methanogen metabolic activity via mRNA (methane production) quantification. All methods used except the rumen function measurements were according to the protocols developed by the QDPI, and all three were run in collaboration with the QDPI staff both at Lincoln University and at the QDPI laboratory in Brisbane, Australia.

These methods were used in combination to assess if in combination they could provide the capacity to sensitively measure methane flux in short (< two hours) periods.

Results and Discussion

Methane sensor *in vivo*

General suitability of methane sensor for use *in vivo* in cattle
The METS sensor was effectively adapted to physical use in the rumen, and there were no logistical or technical issues that impeded its use in the pen fed cattle. The power supply used was designed by this project for use in free grazing cattle, and these trials have shown this is readily achievable. The size, weight, durability and datalogging capacity of this particular sensor render it suitable for these applications. On the assumption that the apparatus retains the accuracy specified by the manufacturer in this application, the results obtained in this project suggest that on this basis it is a valuable advancement in methane studies as it effectively logs the measured methane concentration in the liquid phase under these conditions in a reliable, consistent manner.

While there is work that remains in determining the accuracy of these methane solute measurements, this project has demonstrated that there is no impediment to the use of this sensor in any other area of its application in *in vivo* methane assessment in cattle.

Experiment 1
The cattle consumed 5 kg DM silage daily in the crates. The methane concentrations recorded in the liquid phase of the ventral sac rumen contents when rye grass silage was fed *ad libitum* very quickly (<30 minutes) reached sensor saturation (calibrated to <350umol.L in this application) (Table 1). The recorded concentrations did not significantly decline between meal events in the period examined in this experiment.

This lack of flux in the recorded methane concentrations in the rumen prevented the use of these recordings in comparisons with either the microbial assessments or the physico-chemical assessments obtained in experiment 1.

However, subsequent sensor assessment after experiment 1 in low or neglible methane aqueous media demonstrated the sensor remained sensitive to methane flux from zero to saturation, although extended periods (*c*. 8 hrs) in rumen digesta appeared to reduce the function of the replaceable filter on the sensor head and reduce sensitivity until washed. The uniformly high readings suggests the methane concentration in the liquid phase is consistently higher than 350 umol/L, at least in the ventral sac of the rumen, which is a surprising finding as it is greater than would be expected on standardised abomasal flow rates (>150 L/d) for cattle of this liveweight on this diet, given the accepted methane emissions (20-30g/ kg DM fed) and generally very low methane solubility at physiologic temperatures (< 1.0 mole %). There is limited literature on methane concentrations in the liquid phase of rumen contents, and none at all in NZ pasture based systems, but given that the highest fluid flow rates out of the rumen are through the ventral sac floor region where this sensor was positioned, it suggests that a considerable amount of methane is not being released from the liquid phase in the rumen. This is contrary to the conventional view of the source of methane emissions that suggests almost all of the methane produced is released as gas within the rumen space.

The diet and intake levels used were not considered high methane production factors, but the finding that methane levels in the liquid phase were consistently above 350 umol/L demonstrates the need for a sensor calibration range well above that. Discussions with the sensor manufacturer have indicated that there is no technical problems with achieving this, requiring only a factory calibration procedure to do so.

Experiment 2
The use of straw at low energy intakes was designed to reduce overall rumen metabolism and digesta flow rates, and therefore the methane concentration in the fluid phase of the digesta, although recognising high fibre diets are associated with higher methane yields per kilogram intake.

Table 2 shows the methane concentrations recorded, demonstrating that by use of this diet we were able to observe a methane concentration flux due to a feeding event that remained within the calibration range of the sensor. These results demonstrate that the sensor is able to measure relatively small (<50 umol/L) changes in methane concentrations in rumen digesta over short (<30 minutes) periods. Although there is

considerable validation work still required to conclusively demonstrate the specific accuracy and sensitivity of this sensor application, we conclude these results suggest that this method opens a new opportunity in detailed, short interval rumen methane assessment.

Conclusions on methane sensor suitability for *in vivo* use in cattle rumens

We are persuaded by these results that this method can deliver suitable information on cattle rumen methane concentrations in the liquid phase *in vivo* in a manner that enables the detection of small changes (<50 umol/L) across short intervals (*c*. 30 minutes). Provision of such information would be a significant step forward in rumen methane research capacity.

Methane and SF6 concentrations in eructated and exhaled gas

The use of SF6 as a marker to estimate rumen methane production is the primary research tool for assessment of methane emissions, but the convention is to use a mean value from four days of measurement due to the large standard errors involved. In this project, the time frame under investigation was in six hour blocks, and the hourly methane yields estimated by this method in the period immediately prior to and shortly after feeding in both experiment 1 and 2 were higher - above 10g methane / kg DM fed in the six hour measurement period – than the conventionally accepted pasture based estimates (*c*. 25g methane / kg DM fed in 24 hours) would suggest is likely, with a standard deviation of 24.5% and a standard error of 12.3%. There is no means of using these short interval measurement values in robust comparison with the methods trialled in this project when the errors involved are this high, and the attempts to do so were abandoned.

Rumen microbiological metabolic 'proxies'

Rumen pH, redox and temperature

Tables 1 and 2 present the recorded rumen pH, redox and temperature values of experiments 1 and 2. Both Table 1 and 2 display a post prandial reduction in pH and redox, and Table 2 shows increases in temperature and methane concentrations. Previous Lincoln University dairy rumen research has demonstrated that rumen temperature is strongly affected by drinking events for up to 30 minutes, and this is seen in Table 1. Due to the saturation of methane values in Table 1, the recorded values displayed in Table 2 only were used in an analysis of variance model to estimate the strength of association of recorded methane concentration in rumen fluid with the pH, redox and temperature of that fluid. Redox and pH alone were the most highly correlated with methane concentration (R^2 = 0.91). The broad trend of the relationship – increasing methane concentration with decreasing pH and redox values – is consistent with the accepted understanding of rumen metabolic dynamics.

Given the small sample size used in this project, this highly correlated result suggests there is merit in using these rumen function parameters in estimating rumen methane *production,* as expressed in the fluid concentration**.** There are current, robust *in vivo* methods of very accurate pH, redox, pressure and temperature recording of rumen contents, and a large amount of data across the years, seasons, individual animals and

diet treatments has been obtained in recent Lincoln University research in the field. This opens the possibility of using the methane sensor in constructing a physico-chemical model for estimating rumen methane production at given points under closely defined circumstances, and that would be of considerable benefit in modelling critical points for mitigation in specific feeding systems – for example, twice a day milked dairy herds on 24 hour fresh break pasture systems.
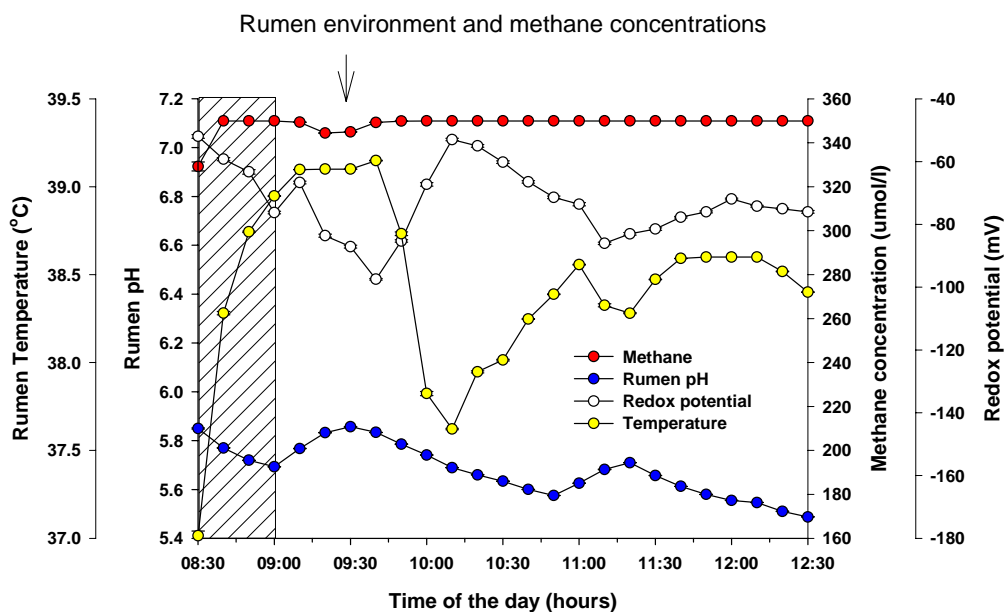
Table 1 The rumen methane concentration (liquid phase) (micro moles/ litre – umol/l), pH, redox (millivolts – mV) and temperature (Celcius – ^{0}C) recorded in the period 8.30am – 12pm in Experiment 1.
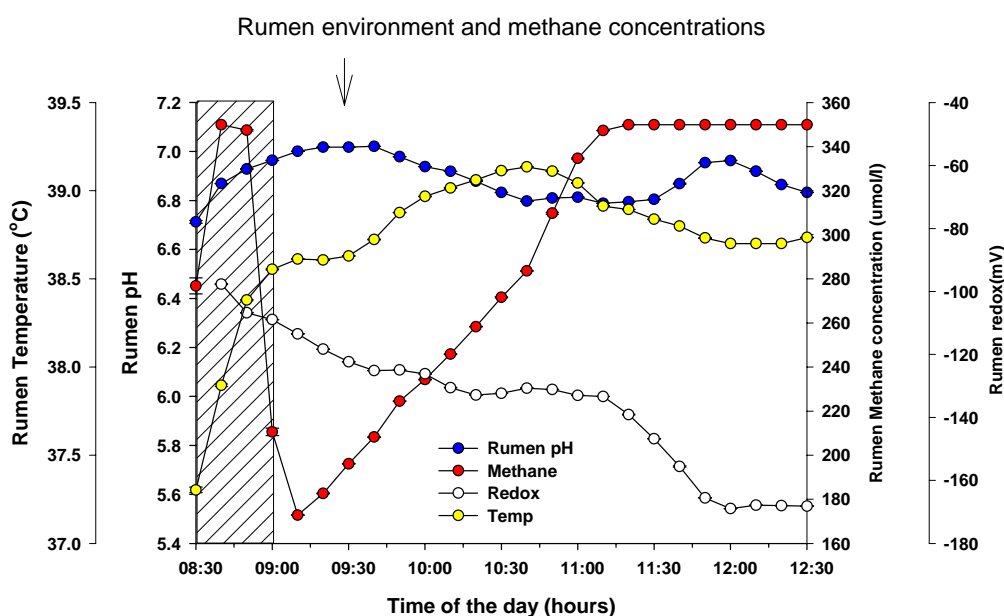

Table 2 The rumen methane concentration (liquid phase) (micro moles/ litre – umol/l), pH, redox (millivolts – mV) and temperature (Celcius – ^{0}C) recorded in the period 8.30am – 12pm in Experiment 2.

RT-PCR enumeration and mRNA quantification.

RT-PCR

The enumerations of the four different pre and post prandial intervals selected for the PCR assessments of the four animals used in experiment 1 are presented in Table 3. The methanogen populations are broadly stable across the intervals measured, and there was broad agreement between the two approaches used in regard to the direction of population change. Given the intervals varied between 4 hrs before and after feeding, it would not appear that enumeration of population changes is an effective 'proxy' of rumen methane production over short (< 6 hour) periods.

Table 3 The *Methanobrevibacter ruminantium* and *Methanobrevibacter smithii* populations as assessed by 16S RNA and mcrA frequency, respectively, using real time polymerase chain reaction. The units are expressed as log values.

Animal	Interval	Count (10^) (16S RNA)	Count (10^) (mcrA)
1	2h before	3.45E+08	1.38E+10
	2h after	1.58E+07	2.79E+09
2	2h before	2.22E+08	5.68E+09
	at feeding	2.92E+08	8.18E+09
3	at feeding	3.37E+08	4.02E+10
	2h after	3.39E+08	2.38E+10
4	4h before	2.15E+08	2.24E+10
	4h after	9.50E+07	2.21E+10

mRNA quantification

The quantification of mRNA associated with methane production would enable a very specific and potentially sensitive method of assessment of methane production at a given point in time. There is a body of opinion amongst rumen researchers that rumen microflora populations are extremely stable within a given feeding system, and that the principal means by which bacterial production of compounds is increased is not by population change, but by increased metabolic activity. This cannot be observed by conventional genomic assessments such as RT-PCR of 16S RNA, but would be accessible via mRNA quantification. This area of research is in its infancy, but QDPI is one group working in the area with end products of fermentation other than methane.

During this project the preliminary work in development of the method was undertaken, but there were a number of technical impediments that delayed progress, and the results obtained by the date of this report were unsatisfactory and incomplete. However, QDPI work is ongoing in the area, and very recent results (June 08) in allied species have demonstrated that some of the most fundamental impediments have been identified and overcome, which suggests there is value in continuing development in this area.

Conclusions

The need for accurate and specific assessment of rumen methanogenesis over short intervals will inevitably drive further research in this area. This preliminary project has demonstrated that the use of portable methane sensors in vivo is logistically possible, and while appropriately robust validation is still required, the small amount of data to date suggests the method is capable of delivering information on small changes in methane concentrations over periods as short as 30 minutes. This would be a major advance in detailed rumen methanogenesis studies of a type required for future mitigation research.

In addition to this, the results of this project suggest there is value in the use of alternate approaches to rumen methanogenesis involving the assessment of microbial production of inorganic and organic solutes present in the rumen and influence parameters such as pH and redox. As these are readily available with current and highly reliable technologies, this opens a means of modelling rumen methanogenesis independent of methanogen biology. However, future development of mRNA quantification methods may provide a corollary means of direct methane production assessment for use in concert with such an approach.

Project Summary

- The aim of this project was to investigate the suitability of two novel methods of short interval rumen methanogenesis assessment.
- Any such methods are highly desirable because at present the available methods for methane production assessment do not enable accurate or short interval measurements, and these are needed for the examination of mitigation strategies targeted at specific diurnal or temporal patterns of methane production.

- The first component of the investigation was the use of a portable methane sensor in vivo in cattle rumens. Although independent validation of the accuracy of the methane measurements proved difficult in this project, the results in this work demonstrate the probe can be effectively used in cattle rumens, and suggest the sensor is a potentially valuable method of obtaining information on small changes in methane levels over very short intervals.
- As the first project goal was to determine the suitability of this sensor for this purpose, these results are a major outcome of this project.

- The second component of the investigation was the use of several metabolic 'proxies' for rumen methanogenesis, adapted from allied fields of microbial research.
- The results obtained in this component of the project suggest there is significant potential in the use of assessments of the physico-chemical status of the rumen environment in estimating methanogenesis at specific points in time, but limited use for RT-PCR of methanogen populations for short interval assessment. Due to technical difficulties encountered, the use of mRNA for estimation of methanogenesis was not able to be satisfactorily assessed in the timeframe of this project.