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Review of current *in vivo* measurement techniques for quantifying enteric methane emission from ruminants

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Review of current *in vivo* measurement techniques for quantifying enteric methane emission from ruminants

K.J. Hammond, et al.

- Methods used to measure *in vivo* enteric CH_4 emission are reviewed
- Methods are chambers/enclosures, SF_6, short-term gas concentrations in exhaled air
- No ‘one size fits all’ method for measuring CH_4 emission by individual animals
- All methods require attention to detail, rigour and routine data quality assessment

Abstract

Ruminant husbandry is a major source of anthropogenic greenhouse gases (GHG). Filling knowledge gaps and providing expert recommendation are important for defining future research priorities, improving methodologies and establishing science-based GHG mitigation solutions to government and non-governmental organisations, advisory/extension networks, and the ruminant livestock sector. The objectives of this review is to summarize published literature to provide a detailed assessment of the methodologies currently in use for measuring enteric methane (CH_4) emission from individual animals under specific conditions, and give recommendations regarding their application. The methods described include respiration chambers and enclosures, sulphur hexafluoride tracer (SF_6) technique, and techniques based on short-term measurements of gas concentrations in samples of exhaled air. This includes automated head chambers (*e.g.* the GreenFeed system), the use of carbon dioxide (CO_2) as a marker, and (handheld) laser CH_4 detection. Each of the techniques are compared and assessed on their capability and limitations, followed by methodology recommendations. It is concluded that there is no 'one size fits all' method for measuring CH_4 emission by individual animals. Ultimately, the decision as to which method to use should be based on the experimental objectives and resources available. However, the need for high throughput methodology *e.g.* for screening large numbers of animals for genomic studies, does not justify the use of methods that are inaccurate. All CH_4 measurement techniques are subject to experimental variation and random errors. Many sources of variation must be considered when measuring CH_4 concentration in exhaled air samples without a quantitative or at least regular collection rate, or use of a marker to indicate (or adjust) for the proportion of exhaled CH_4 sampled. Consideration of the number and timing of measurements relative to diurnal patterns of CH_4 emission and respiratory exchange are important, as well as consideration of feeding patterns and associated patterns of rumen

fermentation rate and other aspects of animal behaviour. Regardless of the method chosen, appropriate calibrations and recovery tests are required for both method establishment and routine operation. Successful and correct use of methods requires careful attention to detail, rigour, and routine self-assessment of the quality of the data they provide.

Keywords: enteric methane, rumen fermentation, *in vivo* methodology, emission

Abbreviations: CH_4, methane; CO_2, carbon dioxide; DMI, dry matter intake; GHG, greenhouse gases; H_2, hydrogen; LMD, laser methane detector; MER, methane emission rate; N_2O, nitrous oxide; NH_3, ammonia; O_2, oxygen; PAC, portable accumulation chamber; RQ, respiratory quotient; SF_6, sulphur hexafluoride tracer; STP, standard temperature pressure.

Introduction

Ruminant husbandry is a major source of anthropogenic greenhouse gases (GHG) with the main contributors of methane (CH_4), nitrous oxide (N_2O) and carbon dioxide (CO_2). Factors determining enteric CH_4 emission from ruminants and potential mitigation strategies have been the subject of considerable research efforts for many years (Hristov et al., 2013). The need for high throughput measurements of enteric CH_4 emission has led to the development of a variety of approaches for measuring this emission *in vivo*.

Research methods used to study enteric CH_4 emission and mitigation practices have common elements, but differ in terms of the apparatus and approaches used. This highlights the need for method standardization dependant on purpose, and validation of newer techniques. Accurate and/or precise measurements of CH_4 emission from individual animals are required for establishing national inventories, assessment of mitigation strategies, development of quantification protocols and genetic selection. There are diverse technologies

being used worldwide for quantifying enteric CH_4 emission, all of which differ in their application, cost, accuracy and precision, but all direct methods rely on measuring the concentration of CH_4 in air. We aim to extend previous reviews (*e.g.* Storm et al., 2012; Hegarty, 2013; Pickering et al., 2015), and consider methods currently used to directly measure enteric CH_4 emission from ruminants in terms of their underlying principles of use, capabilities and limitations. We do not intend this to be a technical review or 'user manual', nor to go into extensive technical and operational detail of the approaches, but to be an appraisal with appropriate references provided for obtaining further information. Methods considered here include respiration chambers and enclosures, sulphur hexafluoride (SF_6) as a tracer, and techniques based on short-term measurements of gas concentrations in exhaled air samples. The focus is on individual animal CH_4 measurements, thus we are not considering the use of micrometeorology and housing flux approaches for groups of animals (*e.g.* McGinn, 2013). Additionally, we are not delving into proxy techniques based on indicators of CH_4 emission (*e.g.* van Lingen et al., 2014). Historically, measurements of individual animal CH_4 emission have been obtained to determine CH_4 energy loss as a component of energy balance and for the estimation of heat production based on respiratory exchange (Reynolds, 2000). More recent research has focused on dietary and other factors that determine CH_4 emission and potential mitigation options, including genetic selection of animals with lower CH_4 emission based on ranking of individuals. In this regard the objectives of the research undertaken are an important consideration for the techniques used, as greater precision and/or accuracy maybe required for specific purposes and will determine the number of measurements required.

Our objective is to use published literature to summarize, identify and report on techniques that are currently used to directly measure *in vivo* enteric CH_4 emission from animals, and give consideration to their use for specific purposes or objectives.

1. Background on methodologies for determining enteric methane emission

It is important to define terminology and the underlying biology of enteric CH_4 emission before considering the methodology used. Methane arises from microbial activities in the lumen of the gastrointestinal tract. The CH_4 produced can be released from the animal via three routes (Ricci et al., 2014): 1) CH_4 from the rumen and lower gut is absorbed into the blood and exhaled from the lungs via expiration, 2) CH_4 emitted directly from the rumen by eructation, and 3) CH_4 emitted from the hindgut in the flatus. Collectively, expiration and eructation have been encompassed in this review by the term 'exhaled' gas, as the majority of eructated gas from the rumen is inhaled into the lungs before being exhaled (Hoerneckie et al., 1965; Berends et al., 2014). Measurements of CH_4 produced in the rumen and hindgut have been obtained using radiolabelled CH_4 dilution. It was found that virtually all CH_4 produced in the hindgut of sheep fed lucerne chaff at maintenance intake was absorbed and removed from blood by expiration whilst the majority of CH_4 produced in the rumen was eructated as opposed to being absorbed into blood (Figure 1; Murray et al., 1976). In this regard, only 2% of total CH_4 emission occurred via flatus for sheep (Murray et al., 1976). According to Murray et al. (1976), approximately 87% of the CH_4 exhaled from the mouth and nose of the animal arises from the forestomach via eructation and absorption into blood. Approximately 13% of CH_4 is produced in the hindgut, where 89% of that (11% of total CH_4 produced) is absorbed into the bloodstream and eliminated via expiration (Ricci et al., 2014). Older work using tracheotomized cattle found that the proportion of CH_4 emission attributable to CH_4 absorbed into blood and expired was much greater before feeding, compared to after feeding (50-70% *vs*. 20-23%; Hoernicke et al., 1965). More recent estimates from lactating dairy cattle suggested that as much as 12% of estimated daily CH_4 emission is absorbed into the portal vein and expired (Reynolds et al., 2013). This has

implications for estimates of CH_4 emission that may be based solely on eructation events (*e.g.* Garnsworthy et al. 2012), but suggests that measurements based on breath and eructation do account for most of the CH_4 produced in the rumen and hindgut.

Figure 1 near here.

When referring to CH_4 *emission rate (or flux)*, the typical unit is mass or volume (g or L) per unit of time (min, h, or d). In most publications, ruminant CH_4 emission rate is expressed and compared in units of g/d or L/d. Methane *concentration* is the quantity of CH_4 relative to other gases corrected for temperature, pressure and humidity. Methane concentration is typically measured using infrared analysis or gas chromatography and is expressed on a volume percentage basis (ppm). Methane *yield* describes CH_4 emission expressed relative to diet or dietary component intake (*e.g.* g CH_4/kg dry matter intake [DMI], which is used for IPCC Tier 2 inventory estimates (IPCC, 2006). Similarly, CH_4 *intensity* is a term used to express CH_4 emission relative to level of production, such as energy corrected milk yield (g CH_4/kg ECM).

This review is focussed on CH_4 measurement techniques related to emission rate and concentration only. Associated techniques that can be used to estimate CH_4 emission based on feed intake (*e.g.* g CH_4/kg DMI) or production (*e.g.* g CH_4/kg ECM), whilst of considerable value, are not discussed. For the purposes of the present review, CH_4 yield refers to g CH_4/kg DMI on a daily basis. Although expression of CH_4 yield on a daily basis is commonly used, it should be noted that there is large diurnal variation in CH_4 emission, as discussed later, depending on feed intake pattern and dietary characteristics. This can have implications for deciding which technique to use and interpretation of results.

1.1 Respiration chambers and enclosures

Over the last decade concerns over the contribution of ruminants to the global CH_4 emission inventory has stimulated a substantial increase in research on CH_4 emission from ruminants, resulting in the construction of numerous and varied types of respiration chambers. This is not a new area of research or a new technique. Respiration chambers have been used as indirect calorimeters for the measurement of respiratory exchange and CH_4 energy losses of ruminants for more than 120 years (*e.g.* Armsby, 1903; Kellner, 1913), with numerous publications describing how to construct, calibrate and use them (*e.g.* Flatt, 1969; Blaxter, 1971; McLean and Tobin, 1988). Since 1958, the European Association of Animal Production has held a series of Symposium on Energy Metabolism of Farm Animals (now combined with the Protein Metabolism Symposium) that has provided a forum for knowledge exchange on energy metabolism research, including the construction and operation of respiration chambers (for a partial list of the published proceedings see Reynolds, 2000). As mentioned previously, in recent years much of the research focus using respiration chambers has shifted to measuring and reporting enteric CH_4 emission. A more recent publication is the 'Technical Manual on Respiration Chamber Designs' (Global Research Alliance, 2012), which provides examples of respiration chambers used by various research groups around the world for measuring CH_4 emission.

Whole animal open-circuit respiration chambers are currently the most commonly used with varying degrees of complexity. Designs range from poly-tunnels and shower curtains placed over stalls (Powell et al., 2007; Aguerre et al., 2011), to more sophisticated and expensive dedicated calorimeters that represent longer term investments (Global Research Alliance, 2012). The principle of whole animal respiration chamber systems is that inflowing air is circulated through the chamber and around the animal to mix incoming air and emitted CH_4 within the volume of the chamber, while sampling incoming and exhaust air

for gas (*i.e.* CH_4) analysis. Methane emission is determined by multiplying the airflow through the system by the concentration difference between inflowing and outflowing air. Common to all open-circuit respiration chambers is the need to correct measurements of concentration and flow to standard temperature and pressure (STP) conditions and account for humidity. These corrections are crucial due to their effects on gas volume. The CH_4 contained in the chamber at the beginning and end of measurements must also be accounted for.

Measurement of CH_4 emission using respiration chambers is performed usually over periods of 1-7 sequential days (e.g. van Zijderveld et al., 2010; Herd et al., 2014; Schwarm et al., 2015), dependent on the purpose and resources. Measurements for an extended period (*e.g.* a few days) require some form of environmental control to maintain temperatures (usually within the thermal neutral zone) and to control humidity, which is especially important for high yielding lactating dairy cattle emitting heat via vaporization of water through respiration. The rate of accumulation of water vapour will depend on a number of factors including ambient air humidity and flow rate through the chambers. Flow rate should ideally be varied based on the rates of CH_4 and CO_2 emission by the animal, as the differential in concentrations between incoming and exhaust air must be measureable. In saying this, the CO_2 concentration in the chamber must be kept below a level (1%) that would affect metabolism of the animal (McLean and Tobin, 1988). Respiration chambers should be sufficiently air-tight to minimize air loss from the system or ingress of air that is not of the same composition as outside ambient air. Respiration chambers are typically operated under negative pressure, but positive pressure systems are also in use (van Gastelen et al., 2015).

Two critical sources of variation for measurement of CH_4 emission through respiration chambers are airflow rate through the chamber and the dynamics of air mixing in the chambers, which determines response time. In a recent ring test calibration of respiration

chambers in the UK (Gardiner et al., 2015), three potential sources of experimental error were evaluated by testing the recovery of a calibrated reference source of ultra-high purity CH_4 standard. These sources of error were analyzer error, ducting efficiency (from chambers to analyzers, including measurements of airflow), and chamber mixing. Of these, ducting (and airflow measurement) was the largest source of variation in CH_4 standard recovery within- and between-respiration chambers and facilities (1.3, 15.3 and 3.4% variation for analyzers, ducting/flow and chamber mixing, respectively). Measured recovery of a known amount of CH_4 (and/or CO_2) should be a standard procedure for testing and calibrating any respiration chamber used for measuring CH_4 and/or CO_2 emission (McLean and Tobin, 1988). If the absolute accuracy of CH_4 release is known and can be shown to be constant over time, the recovery can be used as a correction factor to calibrate measurements for individual respiration chambers and compare measurements across research centres (Gardiner et al., 2015). However, the use of such corrections (so-called 'fiddle factors') for low or high recovery rates is not a substitute for good practice. If recovery rates are significantly different from 100% (McLean and Tobin, 1987) or appear to vary, the source and the variation of the measurement error should be identified and the error should be corrected. The results of Gardiner et al. (2015) demonstrate that respiration chambers are not a 'gold standard' in terms of accuracy and precision unless they are routinely calibrated and are shown to achieve gas recovery rates of approximately 100% both before and after each experimental deployment.

Ventilated hood chambers or head boxes also can be used to quantify CH_4 emission (*e.g.* Odongo et al., 2008; Suzuki et al., 2008; Place et al., 2011). The technique involves the use of a box or hood that surrounds the animal's head and air drawn through the hood is analyzed for incoming and exhaust air CH_4 concentration. Airflow is measured and used to calculate CH_4 emission. Head chambers are typically large enough to allow the animal to move its head in an unrestricted manner and obtain feed and water. Like respiration

chambers, they can be used to obtain continuous measurements over successive 24 h periods. Troy et al. (2013) performed simultaneous measurements of CH_4 emission from beef cattle (*n* = 55) using respiration chambers and feed-mounted hoods located within respiration chambers. It was found that increases in hood CH_4 concentration over respiration chamber background were positively correlated with daily CH_4 outputs (r^2 = 0.47, *P* < 0.001), but there was considerable variability. Animals need to become accustomed to the hood apparatus, and require extensive training, which limits its use for screening large numbers of animals. Face-masks for 'spot-sampling' of respiratory exchange and CH_4 emission have also been used in cattle, sheep and goats trained to remain in sternal recumbence for 30 min measurements repeated every 2 h over the course of 24 h periods (Figure 2;Washburn and Brody, 1937).

Figure 2 near here.

Portable accumulation chambers (PAC) are essentially an airtight box without airflow. Methane emission is measured from individual animals over 1 or 2 h, such that CH_4, CO_2 and other gases accumulate while oxygen (O_2) depletes (Goopy et al., 2011; Hegarty, 2013). The PAC acts to trap all exhaled gases during the collection period and takes a single CH_4 measurement at the end. Methane emission is estimated as the concentration of CH_4 (corrected for background) multiplied by net chamber volume, adjusted for STP, divided by time of measurement (Goopy et al., 2011). The time period of use should be limited to avoid negative effects of increased chamber CO_2 concentration, as discussed previously, and thus the PAC techniques provides a single spot sample of accumulated gases emitted by an animal. Moderate repeatability (correlation of 0.33-0.43) of measurements of CH_4 emission by individual sheep (*n* = 207) using PAC was reported in studies at different sites (Goopy et

al., 2015). The time period of measurements relative to feeding and any postprandial changes in CH_4 emission is a potential source of variation in these measurements and thus should be accounted for when the technique is used.

1.2 Sulphur hexafluoride tracer technique

The SF_6 technique was developed by Zimmerman (1993) and the first reported use for estimating ruminant CH_4 emission was by Johnson et al. (1994). The technique is suitable for penned as well as free ranging and grazing animals, and relies on the placement of a permeation tube with a known SF_6 gas release rate into the reticulorumen of the animal. Samples of exhaled air are continuously collected using tubing with in-line flow restrictors (to regulate sampling rate) that are placed near the nose and mouth of the animal and are connected to a pre-evacuated collection vessel (canister). Samples are recommended to be taken over 24 h intervals, over a minimum period of five sequential days, with background air samples collected alongside animals at the same time. Daily CH_4 emission is calculated using the ratio of CH_4:SF_6 in the canister, with each gas corrected for background concentration, in conjunction with the pre-determined SF_6 permeation rate of the tubes, according to Williams et al. (2011; Equation 1) where the M subscript indicates a measured sample, and the BG subscript indicates a background concentration:

$$R\mathrm{CH_4}\ (\mathrm{g/d}) = R\mathrm{SF_6} \times (([\mathrm{CH_4}]_M - [\mathrm{CH_4}]_{BG})/([\mathrm{SF_6}]_M - [\mathrm{SF_6}]_{BG})) \times (MW_{CH4}/MW_{SF6}) \times 1000 \quad \text{(Eq. 1)}$$

Where: $R\mathrm{CH_4}$ is the calculated emission rate of ruminal CH_4 (g/d); $R\mathrm{SF_6}$ is the measured release rate of SF_6 from the permeation tube (mg/d); MW_{CH4} is the molecular mass of CH_4 (16), and MW_{SF6} is the molecular mass of SF_6 (146). The concentrations of $[CH_4]$ are expressed in ppm and concentration of $[SF_6]$ in ppt. The factor of 1000 is a unit converter so that $R\mathrm{CH_4}$ is expressed in units of g/d.

The accuracy and precision of the SF_6 technique for estimating ruminant CH_4 emission has been evaluated in a number of comparison studies with respiration chambers (*e.g.* Boadi et al., 2002; Grainger et al., 2007; Pinares-Patiño et al., 2008, 2011; Muñoz et al., 2012; Deighton et al., 2014b). Usually, the SF_6 technique provides a mean CH_4 emission that can differ (some 5-10% lower or higher) from that obtained for the same animals in respiration chambers (Table 1). Although mean CH_4 emission may not differ, within- and between-animal variation has been considerably larger using the SF_6 technique for sheep (Pinares-Patiño et al., 2011) and dairy cattle (Grainger et al., 2007), relative to the respiration chamber technique. Such variability needs to be taken into account to establish the number of animals and number of measurements within-animal required. Recently, an international panel of experts published guidelines which offer a comprehensive, citable, and peer-reviewed reference for the theory and practice of the SF_6 technique (Berndt et al., 2014). Underlying factors affecting the accuracy and precision of the SF_6 technique are continuing to gain better understanding, resulting in recommendations for modifications to the original procedures. For example, the release rate of SF_6 from permeation tubes declines over time after the permeation tube has been filled with SF_6. It has been shown that the discrepancy in CH_4 emission measured using respiration chambers, compared with the SF_6 technique, increased with the duration that permeation tubes were resident in the rumen (Deighton et al., 2013). Thus, variation in the rate of SF_6 release from permeation tubes has potential to introduce an error of gas measurements. This is especially evident when experiments are conducted more than 30 days after calibration of permeation tubes (Lassey et al., 2001; Moate et al., 2015), unless the permeation tubes can be recovered after the experiment and release rate re-calibrated (Pinares-Patiño et al., 2011). Most investigators have used zero order kinetics (a constant rate) to predict SF_6 release rate from permeation tubes, but it has since been demonstrated by Moate et al. (2015) that Michaelis-Menten kinetics are more

appropriate and can improve the accuracy of CH_4 emission estimates for periods up to 800 d (compared to the typical period of 60-90 d). Berends et al. (2014) showed that estimated CH_4 emission is sensitive to parameters affected by the difference in CH_4:SF_6 ratio in exhaled and eructed air, and to proportions of exhaled and eructed air samples, including distance from sampling point to mouth and nostrils. Deighton et al. (2014a) reported that temperature, but not submersion or orientation, of the permeation tube influences the rate of SF_6 gas release, whereby the detection of differences in CH_4 emission due to diets or between animals may be compromised by nutrient status or other effects on body temperature. However, changes in rumen temperature are typically small and therefore unlikely to have a substantial effect, with a 1°C change in rumen temperature affecting a 2.2% difference in permeation rate (Deighton et al., 2014a).

The SF_6 technique is a time-averaged technique reliant upon the constant release of SF_6 from the reticulorumen, and so collection of gas samples for periods shorter than 24 h are not ideal when using the SF_6 technique to estimate daily rates of CH_4 emission (Berndt et al., 2014). Otherwise, the concentration of gases sampled within a daily (and multi-day) measurement period can potentially bias the sample collection, particularly when feeding patterns and meal frequency vary and affect peak CH_4 concentration within the rumen (as discussed later for short-term techniques). The SF_6 technique has commonly employed the use of capillary tubes to allow the collection of exhaled air samples from the animal at a constant rate. However, Deighton et al. (2014b) has shown a systematic bias of up to 16% in calculated CH_4 emission when using capillary tubes as flow restrictors. These authors have proposed a 'modified' SF_6 technique which incorporates the use of orifice plate flow controllers to lower technique error.

Although the SF_6 technique allows the measurement of CH_4 emission from many individual animals whilst in their natural environment, Pinares-Patiño et al. (2008) reported

that halter and collection canisters placed on young sheep can interfere with grazing behaviour. This has not been a reported problem with young cattle and it is likely such a problem was not due to the SF_6 method per-se, but rather the use of a method that has not been optimized to animal size. The relative size of the gas sampling equipment (e.g. size, weight, attachment of equipment placed around an animal's neck) in relation to the size of the animal can be detrimental and impact on grazing behaviour. The SF_6 methodology requires that rumen SF_6 boluses be administered and frequent animal handling is needed, all of which are labour intensive and may interfere with animal behaviour. Although SF_6 itself is a potent GHG with a global warming potential of 23,900 and an atmospheric lifetime of 3,200 years (US EPA, 2014), the amount of SF_6 used with the SF_6 methodology is very small. Assuming that cattle permeation tubes each contain 2.5 g of SF_6 and sheep tubes contained 1.0 g SF_6, Williams et al. (2011) estimated that since the establishment of the SF_6 technique, world-wide ruminant research has used a total of less than 12 kg SF_6, which is minor compared to its industrial use.

The SF_6 technique has been used to estimate CH_4 emission using samples of rumen gas, rather than breath samples (Bayat et al., 2015), however Beauchemin et al. (2012) recommended against the use of the SF_6 technique in rumen cannulated animals. Beauchemin et al. (2012) concluded that the rumen cannulae would need to be tight fitting to minimize gas leakage, and more animals would be required to overcome additional variability. It is unlikely that the same proportion of SF_6 released into the rumen enters the portal vein as occurs for CH_4, thus absorbed CH_4 may not be properly traced because the proportion of total SF_6 released via cannulae may differ from the proportion for total CH_4 production. Moate et al. (2013) found that compared to non-cannulated cattle, the composition of gases in the rumen head space gas of cannulated cattle was altered and CH_4 yield was 10% lower, thus some

consideration should be given to the effects of rumen cannulation on CH_4 emission measures using any technique.

One major requirement for any tracer gas is that concentrations in the environment should be very low, relative to the concentration of the tracer in collected samples, with background gas concentrations accounted for (Berndt et al., 2015). Measurements of background CH_4 and SF_6 gases are important requirements of the SF_6 methodology and its precision for estimates of CH_4 emission (Williams et al., 2011; Lassey, 2013; Berndt et al., 2015). High background SF_6 concentrations create uncertainties, with loss of precision and lower accuracy, and a high background gas concentration will contribute substantially to a high variance associated with SF_6 determinations (Lassey, 2013). In well ventilated settings such as grazing, background sampling of CH_4 and SF_6 concentrations is relatively straightforward (Lassey, 2013). However, when experiments are conducted in enclosed barns or near industrial factories/sites, there tends to be higher concentrations of CH_4 and SF_6 in background air samples (Berndt et al., 2015). Williams et al. (2011) demonstrated that even when a building housing animals is deemed to be well ventilated, background concentrations of CH_4 and SF_6 can be substantially elevated compared to outdoor concentrations of these gases. Williams et al. (2011) reported errors in calculated CH_4 emission to range from -31 to +4 g CH_4/cow/d, despite the animal house being highly ventilated. Oh et al. (2015) has also reported larger variability in CH_4 yield using the SF_6 technique in a tie-stall dairy barn whereby the difference with the season of measurement (*i.e.* winter *vs.* summer months) was not consistent over time. It is recommended that distributed sentinel canisters are used for monitoring the accumulation of gases within animal house, as well as continuous ventilation of the building during the entire gas collection period. It was also mentioned by Berndt et al. (2015) that some laboratories discard results from breath samples when the background

concentration of SF_6 is >10% of the SF_6 concentration in the breath sample, and it was recommended that background concentrations of SF_6 should not exceed 10 ppt.

1.3 Short-term measurements

Respiration chambers and the SF_6 technique are typically used to obtain measurements of CH_4 emission over 24 hour periods. However, some research questions require accurate determination of CH_4 emission of large numbers of animals, and short-term measurement techniques are attempting to meet this objective with the spot measurement of exhaled CH_4 at certain time points (*e.g.* at milking or during feeding). Such techniques are usually automated, non-invasive and non-intrusive, allowing a high throughput of animals (and therefore CH_4 measurements). As mentioned previously, the use of repeated short-term measurements using face masks has been used in earlier studies (Washburn and Brody, 1937) to obtain estimates of daily rates of respiratory exchange and CH_4 emission. In this case, the success of the approach was attributable to the number and timing of measurements relative to diurnal patterns of CH_4 emission and respiratory exchange, with measurements made over 30 min at 2 h intervals.

1.3.1 Short-term measurements using automated head chambers (GreenFeed)

The GreenFeed system (C-Lock Inc., Rapid City, South Dakota, USA) is a static short-term measurement device that measures CH_4 (and other gases including CO_2) emission from individual cattle by integrating measurements of airflow, gas concentration, and detection of head position during each animal's visit to the unit (Zimmerman and Zimmerman, 2012; Huhtanen et al., 2015). The system (Figure 3) measures gas emission using a combination of an extractor fan and sensors which induce a measured airflow past the animal's head, allowing emitted air to be collected and sampled. The animal is enticed to

voluntarily visit the unit using a feed supplement that is delivered within a hood. Animals can visit the unit at any time, but in practice a 'visit' only results in a feed reward and measurement of CH_4 emission after a specified time has elapsed between visits (determined by the investigator). Methane emission measurements using a GreenFeed unit are typically over short (3-7 min) periods, several times within a day, over several days/weeks/months, and dependant on each animal's voluntary visitation to the GreenFeed unit. Software provided for use with the unit allows the investigator to control the timing of feed availability and to distribute CH_4 measurements across various times of the day. The system can be used in a variety of environments, including grazing conditions. A detailed and visualized explanation of the technique is provided in Hristov et al. (2015a).

Figure 3 near here.

Short-term CH_4 emission measured by a GreenFeed unit are determined by using an extractor fan to draw air over the animals head and past the nose and mouth into an exhaust pipe. The collected air is mixed, filtered and airflow rate measured using a hot-film anemometer. The concentration of CH_4 (and CO_2 and O_2) in the sample is measured using non-dispersive infrared analysis. Daily CH_4 emission (F_c; L/min) is calculated using volumetric airflow rate (Q_{air}) adjusted to STP and corrected for capture rate, as detailed by Huhtanen et al. (2015; Equation 2):

$$F_{c(i)} = [C_{p(i)} \times (Conc_{(i)} - BConc_{(i)}) \times F_{air(i)}]/10^6 \quad \text{(Eq. 2)}$$

Where, C_p is the fractional capture rate of air at any time (i); Conc is the CH_4 concentration of captured gas (ppm); BConc is the background concentration of CH_4 (ppm); and F_{air} is the volumetric airflow rate (L/min) measured on a dry-gas basis.

The C-Lock Inc. supplied software aggregates the data and calculates CH_4 emission during the measurement period (*i.e.* during the time the animal visited the GreenFeed unit, received a food reward, and maintained an appropriate head position within the sampling hood for enough time to measure sufficient numbers of eructation). Data are available through a web-based data management system provided by C-Lock Inc. The concept of the GreenFeed system is that numerous short-term CH_4 emission samples from an individual animal, taken several times within a day, over several days/weeks/months, can be aggregated to estimate an animal's average daily CH_4 emission.

Because the animal can move about freely, head position relative to airflow is important for successful CH_4 measurements. Distance of the animals head from the sampling port is determined using an infrared sensor which, combined with C-Lock Inc. programming recommendations, ensures only data with adequate head position and uninterrupted measurements are retained for statistical analysis. When used outdoors, wind can reduce the fraction of CH_4 captured and therefore units can be purchased containing wind anemometers which enable the use of a correction factor for wind effects on gas recovery. It is also important to ensure that no other animals are near the sampling hood when another animal is visiting.

The GreenFeed system has been reported by Hammond et al. (2015) to provide an overall estimate of CH_4 emission by growing dairy cattle that was not different from measurements made in respiration chambers (n = 8) but lower than the values obtained using the SF_6 technique (n = 12; Table 1). Dorich et al. (2015) found average CH_4 emission from lactating dairy cows (n =16) measured using the GreenFeed and SF_6 technique were similar, but the SF_6 technique showed higher variability in the relationship between CH_4 (g/d) and DMI (r^2 = 0.17, P < 0.02), compared with GreenFeed (r^2 = 0.42, P < 0.01). The authors attributed this higher variability for SF_6 measurements to the high concentration of

background gases combined with poor barn ventilation. Oh et al. (2015) arrived at similar conclusions in an experiment with lactating dairy cows (n = 48) housed in a tie-stall barn with tunnel ventilation whereby GreenFeed visitation was scheduled at set times by the investigators. These authors reported mean CH_4 yield and SD (g/kg DMI) and CV (%) for GreenFeed of 12.8, 3.63 and 27.2 (16.9, 3.40 and 20.1 for control; 12.4, 2.54 and 20.4 for inhibitor), respectively. Respective values for SF_6 were 14.7, 5.60 and 35.3 (19.1, 7.48 and 39.1 for control; 13.5, 4.59 and 34.0 for inhibitor). Hammond et al. (2015) reported significant treatment and individual animal differences in CH_4 emission that were detected using both respiration chamber and SF_6 techniques, but were not measured using a GreenFeed unit. This was attributed to a limited number of measurements obtained with the GreenFeed unit and the timing of the measurements relative to daily patterns of CH_4 emission, highlighting the importance of obtaining sufficient numbers of observations using the GreenFeed system.

The GreenFeed system requires provision of supplemental feed or an alternative 'enticement' (*e.g.* water) for the animal to use the unit. Amounts fed depend on the enticement used and the intended duration of visit. Supplemental feed may be a concern in both pastoral grazing systems and animal nutrition studies where there is the possibility of an excessive contribution of enticement feed to the diet, even if restrictions are imposed (Waghorn et al., 2013; Dorich et al., 2015; Hammond et al., 2015).

On pasture or in a free-stall facility, successful use of the GreenFeed system is reliant on animal visitation to the unit, and, for some, the number and timing of CH_4 measurements obtained relative to diurnal patterns of CH_4 emission may not be truly representative of daily fluctuations in CH_4 emission (Renand et al., 2013; Hammond et al., 2015). In a trial with young beef bulls (n = 18) fed pellet diets, Renand et al. (2013) reported that CH_4 emission estimated with GreenFeed was significantly higher during the day (263 g/d from 08:00 to

16:00 h) than at night (216 g/d from 00:00 to 08:00 h). The authors concluded that the visit hours had a significant effect on estimated CH_4 emission, particularly for animals whose number of visits were low and irregular during the day. Methane emission may be biased by sampling times; generally being highest after feeding and during rumination bouts (Storm et al., 2012; Hegarty, 2013; Dorich et al., 2015; Hristov et al., 2015b). As explained in Branco et al. (2015) and Hristov et al. (2015a, b), best results with GreenFeed are obtained when the number and timing of animal visits are controlled by the investigator, which is easily achievable in a tie-stall barn situation. These authors suggested collecting eight gas samples in 3 d staggered over time to cover an entire 24-h feeding cycle: 09:00, 15:00 and 21:00 h (sampling d 1), 03:00, 12:00, and 17:00 h (sampling d 2), and 00:00 and 05:00 h (sampling d 3). Recommended GreenFeed calibration and background gas collection procedures have to be strictly adhered to (Hristov et al., 2015a). As the GreenFeed system is reliant on animal visitation to the unit, animal behaviour, particularly in a grazing environment may change, however, Miller et al. (2015) found that mean daily speed, time spent travelling, livestock preference index for the main grazing area, stationary livestock residency index and travelling livestock residency index were not altered by the presence of a GreenFeed unit in the paddock.

Similar to other CH_4 measurement techniques, animals need to be trained, and not all animals' become a frequent GreenFeed user (Waghorn et al., 2013). Unlike respiration chambers, the system is not recommended for use with rumen cannulated animals (Garnett 2014) due to the potential loss of CH_4 via the cannulae and the effects of fistulation and CH_4 leakage on eructation peak profiles. A recent addition by C-Lock Inc. (called the "fistula attachment") successfully captures gases lost through the rumen fistula and directs gases to the main airflow. The devise is designed for cattle and the animals have to be restricted while the fistula attachment is in use (A. N. Hristov, personal communication).

Table 1 near here

1.3.2 Carbon dioxide as a tracer to estimate daily methane emission

The CH_4:CO_2 ratio method uses estimated CO_2 emission and measures CH_4 and CO_2 concentrations to predict CH_4 emission by individual animals. This method has no requirement for respiration chambers or use of tracers as with the SF_6 technique (Madsen et al., 2010; Lassen et al., 2012). The majority (e.g. 70 to 90%; Hoernicke et al., 1964) of CO_2 is produced through intermediary metabolism of the animal, but enteric fermentation contributes substantially as well. Carbon dioxide emission can be predicted based on estimates of energy metabolism, heat production and respiratory quotient (RQ), or carbon balance (as is often done for body tissue energy balance). Estimating the amount of CO_2 produced by the animal and exhaled in the breath allows quantitative CH_4 emission to be estimated from simultaneous measurements of CH_4 and CO_2 concentrations in exhaled air samples; much the same as estimating CH_4 emission using a known emission rate of SF_6 and the analyzed CH_4:SF_6 ratio in samples of exhaled air. Methane emission from the animal is estimated based on background corrected CH_4:CO_2 concentration ratio and predicted CO_2 emission, according to Madsen et al. (2010) (Equation 3):

$$CH_4\ (g/d) = CO_2 \times ([CH_4]_M - [CH_4]_{BG}/([CO_2]_M - [CO_2]_{BG}) \quad \text{(Eq. 3)}$$

Where, CO_2 is estimated CO_2 produced by the animal (g/d); $[CH_4]_M$ and $[CO_2]_M$ are measured CH_4 and CO_2 concentrations in breath samples, respectively; and $[CH_4]_{BG}$ and $[CO_2]_{BG}$ are background concentrations.

Using lactating dairy cows (n = 157), predicted CH_4 emission from CH_4:CO_2 ratio using estimated CO_2 emission has been compared using measurements of CH_4 and CO_2 in respiration chambers by Hellwing et al. (2013). These authors reported a positive relationship

(r^2 = 0.55) between predicted and observed CO_2 production (Table 1). However, the CH_4:CO_2 ratio significantly underestimated (by 17%) CH_4 emission as measured by the chamber method, and Hellwing et al. (2013) concluded that prediction of within-day variation in CO_2 emission from animal characteristics needs to be improved to obtain better individual animal CH_4 emission estimates.

The accuracy of the estimate of CH_4 emission using the CH_4:CO_2 ratio method will depend on several factors including the source of gases in the air sampled (*i.e.* exhaled air, flatus, and fermentation of manure or bedding, *etc.*) and diurnal variation in the ratio of CH_4:CO_2 due to differences in animal activity and fermentation rate associated with meal size and feeding frequency (Madsen et al., 2010). Therefore, as with the GreenFeed system, sampling protocols should include sufficient numbers and sampling times to account for diurnal and postprandial variation in CH_4 and CO_2 emissions, and thereby predict daily CO_2 emission. Also noteworthy is that the CH_4:CO_2 ratio is influenced by both CH_4 and CO_2; thus at a given production level, cows that are more efficient emit less CO_2 than predicted and so the CH_4:CO_2 ratio increases, compared to less efficient cows at a similar feed intake. Consequently, CH_4 emissions will be overestimated for the more efficient cow, partly due to true increases in the CH_4:CO_2 ratio and partly because of the overestimation of CO_2 production with improved feed efficiency (Huhtanen et al. 2015). Furthermore, changes in digestive and metabolic activity at a fixed level of feed intake can affect CO_2 emission, as well as variation in rumen fermentation, and thereby change the CH_4:CO_2 ratio, the estimated CO_2 emission and thus predicted CH_4 emission (Huhtanen et al., 2015).

1.3.3 Eructated methane concentration in exhaled air samples to estimate methane emission

First reported by Garnsworthy et al. (2012), the measurement of CH_4 concentration in air eructed by cattle during milking (often called the ‘sniffer’ technique) provides an estimate

of total daily emission by individual animals on-farm. As detailed by Garnsworthy et al. (2012), a sampling inlet is placed in the feed manger of an automatic milking system and gas concentrations in manger air are continuously sampled, analyzed and logged at 1-sec intervals. A custom designed program identifies and quantifies CH_4 concentration peaks (eructation) together with peak frequency (eructation rate). An index of CH_4 emission rate (MER) is calculated during each milking for each animal as the frequency of eructation per min multiplied by the area under the curve (integral) of each eructation peak. The length of each eructation peak is defined as the start of a rapid rise in CH_4 until the start of the next rise or return to baseline (Bell et al., 2014). The MER is converted to concentration of CH_4 emitted by the animal using an estimated dilution of eructated air determined at the end of each sampling period using calibration gas release and calculated as the mean ratio of CH_4 concentration in released and sampled gases. Methane concentration is determined using the following equation according to Bell et al. (2014; Equation 4):

$$CH_4 \text{ (mg/L)} = (\text{average integral of } CH_4 \text{ per eructation} \times \text{frequency of eructation}) \times \text{dilution factor} \quad \text{(Eq. 4)}$$

Measurements of CH_4 concentration in manger air obtained during eructation whilst cows were being milked have been used to estimate daily CH_4 emission from a calibration equation relating on-farm and chamber measurements (Garnsworthy et al., 2012). A positive relationship ($r^2 = 0.79$, $P < 0.001$) between MER during milking on-farm and total daily CH_4 emission measured in respiration chambers for the same animals ($n = 12$) was reported (Table 1). In contrast, Huhtanen et al. (2015) compared measurements of eructated CH_4 concentration with CH_4 emission measured using the GreenFeed system (two experiments with $n = 32$ and $n = 59$ lactating dairy cows) and found between-cow coefficient of variation (CV) was smaller for GreenFeed compared with eructated CH_4 concentration, and there was no relationship between the measurements of the two methods ($r^2 = 0.09$; Table 1).

Measurements of CH_4 concentration in manger air can be obtained repeatedly from a large number of individual animals in their normal environment. However, the method excludes CH_4 in exhaled air between eructations and any flatulence (Garnsworthy et al., 2012).

A concern with techniques that measure gas concentrations in exhaled air samples (such as GreenFeed and sniffer techniques) is that CH_4 and CO_2 concentration are highly influenced by the distance of the animal's head from the point of sampling, which is not a factor with total air sampling (Hegarty, 2013). Even small changes in head position create large differences in measured gas concentrations (Huhtanen et al., 2015), with variation in CH_4 concentration associated with differences in head movement and position, as well as variable air-mixing conditions created by feed manger geometry, rather than emission per se (Huhtanen et al., 2015). A high repeatability of CH_4 concentration in exhaled air samples could simply reflect the repeatability of head position and behaviour during each milking event. This could have implications if selecting for low CH_4 emitting animals, which may actually be targeting animals that are more restless during milking or habitually position their head further from the sampling tube. Another concern is differences in individual animal behaviour with respect to milking frequency (and thus moment of sampling of air), related to factors including milk yield, parity, number of cows per milking unit and social dominance (Lyons et al., 2014) as well as differences in feed intake and pattern (and thus moment of sampling of air relative to amount and moment of feed ingestion). This may result in targeting animals that emit relatively low amounts of CH_4 at the moment of measuring, but average daily CH_4 emission may not be lower compared with other animals. Another concern is the extent to which there is normal animal to animal variation in the dilution rates for gases in exhaled air relative to the amount produced.

1.3.4 Handheld laser methane detector

Another approach to monitor exhaled air CH_4 concentration is the use of handheld laser CH_4 detectors (LMD) to measure CH_4 concentration in the air between the animal's nose or mouth and the LMD (Chagunda, 2013; Ricci et al., 2014). Measurements of CH_4 concentration are taken manually by a portable apparatus approximately 1-3 m from the animal and are based on infrared-absorption spectroscopy for CH_4. The sequence of data acquisition consists of short periods of 2-4 continuous min. The resulting data consist of a series of peaks which represent the animal's respiratory cycle. Only peaks reflecting the increase in CH_4 concentration due to exhalation or eructation are used in the analysis (Ricci et al., 2014). The measured concentrations are adjusted for distance and background concentrations by the LMD.

Both Chagunda et al. (2013) and Ricci et al. (2014) have reported positive, but rather weak relationships between CH_4 concentrations derived using LMD compared with those obtained in respiration chambers (n = 2, r^2 = 0.22, P < 0.001 by Chagunda et al., 2013; n = 67, r^2 = 0.28, P < 0.001 by Ricci et al., 2014; Table 1). Although the LMD can easily deliver mean values of CH_4 concentration, it was illustrated that the collected data needs to be segregated into respired and eructated CH_4. This is to improve comparisons of the LMD data with measurements made using respiration chambers, and increase the sensitivity of the technique to detect differences in CH_4 emission between individual animals.

The LMD approach is similar to automated measurements of CH_4 concentration in exhaled air samples during milking (Garnsworthy et al., 2012) or feeding (Troy et al., 2013), except that the measurements are made on the air 'plume' at the animal's nostrils, and thus not affected by head position. The technique also enables more frequent measurements to be obtained whilst animals are in their 'normal' environment (as long as they remain still for a sufficient period of time), as opposed to being restricted to periods of milking or feeding.

However, the current system is 'hand held' which is labour intensive and introduces variation. Another concern is the effect that ambient conditions (wind speed, temperature, humidity, atmospheric pressure) have on the accuracy and precision of the measurements, with wind speed being a particular concern for grazing studies and outdoor measurements (Chagunda, 2013). This is a limitation that must be considered along with those discussed above for measurements of CH_4 concentration in air samples taken from mangers during milking or feeding.

2. Assessment of methodologies and their applicability for accurate methane emission measurements

Accurate and repeatable measurements of CH_4 emission from large numbers of animals are needed for investigating possible mitigation options, screening animals for breeding programmes, assessment of alternative management strategies, and decreasing uncertainties associated with national GHG inventories (Pickering et al., 2015). The list of criteria for appropriate and acceptable CH_4 measurement techniques encompasses both non-invasive and non-intrusive technologies that enable measurements for animals in their 'normal' environment, which can be applied under conditions relevant to commercial production, and are rapid, cost effective and ideally automated. Across all techniques, error in estimating CH_4 emission needs to be minimized. An understanding of physics associated with airflow, air mixing, background gas concentrations and ambient conditions is important, as well as an appreciation of animal behaviour (including head movement in some situations) is required, and an understanding of the applicability of data to the environment under evaluation.

Although CH_4 emission can be accurately measured from animals using respiration chambers, their limitations have been well documented in the literature, particularly with

regards to animal throughput (numbers of measurements over time), animal behaviour changes, constraints by their 'artificial' environment, and the cost of their construction and operation (Reynolds, 2000; Hegarty, 2013). Respiration chambers allow for measurements beyond that of CH_4 emission, such as the ability to derived energy and nitrogen balance and to exactly quantify the fate of feed and of digested energy in animals *etc.* Respiration chambers are typically designed for measurements of one animal at a time, normally over the course of successive 24 h periods until 3-7 daily measurements are obtained to account for between-day variation. Depending on respiration chamber and subject size, respiration chambers can also be used for measurements from pairs or groups of animals, to give an average rate per animal, with the pair or group as the experimental unit. However, the rate of throughput and cost limit their use for larger scale experiments. Although animals used for respiration chamber experiments should have an appropriate disposition and be acclimatized to the chambers and equipment before measurements begin, the lack of activity within the chambers inevitably lowers energy expenditure compared with loose housing or grazing environments. Even when animals are thoroughly acclimated to the chamber environment and housed as pairs or with animals visible in adjacent chambers, DMI and therefore CH_4 emission may decrease during chamber housing, depending on the level of production, diet composition and feeding level (Reynolds, 2000). Whilst the relative cost of respiration chamber construction and equipment is certainly lower than in the past (*e.g.* Flatt et al., 1958), lower cost alternatives have been used (*e.g.* Powell et al., 2008; Global Research Alliance, 2012; Dittmann et al., 2014), but with limited success in some cases. Similarly, portable measurement technology and dome structures over feedlots have been used to obtain measurements of CH_4 emission from groups of cattle or sheep in feedlot, housed or grazing environments (Cooprider et al., 2011; Storm et al., 2012). Measurements also can be made for entire buildings housing animals if incoming and outgoing air can be isolated, sampled

correctly, and the flow rate determined or estimated using tracer-gas methods (Storm et al., 2012). These temporary chamber approaches are likely prone to large measurement variability.

Methane concentration in exhaled air is variable and low when derived from absorbed CH_4 in blood, whereas CH_4 concentration is high when the exhaled air includes eructated gas from the rumen (Figure 1). The rate of CH_4 emission also varies considerably throughout the day in relation to feeding pattern and rate of fermentation (Figures 4 and 5). Thus, the pattern of CH_4 emission from the animal is an important consideration when deciding on the type of CH_4 measurement technique to be used and when interpreting data. In this regard, the relative amounts of CH_4 emitted via eructation *vs*. expiration may have implications for the use of breath analysis techniques that only consider CH_4 concentrations during eructation events (Garnsworthy et al., 2012).

All CH_4 measurement techniques are subject to experimental variation and minimizing variation is important. Considering the potential sources of variation when measuring CH_4 concentration in exhaled air samples, there appears little justification for this type of measurement without a quantitative and high collection rate, or use of a marker to indicate (or adjust) for the proportion of exhaled CH_4 actually collected. Accurate collection of air emitted from the animal (or use of a tracer) is important, as is sufficient mixing of the air to obtain a representative sample. Monitoring head position is crucial if total air emitted is not collected for measurement of gas concentration, especially for comparative and meaningful short-term measurements of CH_4. It is also important to measure both absorbed and eructated CH_4 emitted through expiration (both of which can vary amongst animals, diets and time of day; Ricci et al., 2014) otherwise emission may be underestimated if only eructation emission is measured. In this regard, there remains a risk of bias if data are rejected on the basis of number of eructations (*e.g.* using the GreenFeed or sniffer methods). Given

diurnal variation in feed intake and CH_4 emission between- and within-individuals, measurements should cover a full 24 h period and spot samples should be avoided as these are not randomly distributed in time and are therefore prone to bias due to factors such as social dominance of individual animals and consistent differences in sampling time after a meal.

Feeding behaviour and diurnal pattern of methane emission

The rate of CH_4 emission from the animal is not constant throughout the day, with diurnal patterns affected by the diet, feed allowance and feeding pattern. Crompton et al. (2011) showed that the daily pattern of CH_4 emission from lactating dairy cows varied when the same total mixed ration was fed once, twice or four times daily (Figure 4). Diurnal CH_4 emission patterns are best accounted for using techniques that sample continuously over 24 hour periods, but are also very important when using short-term sampling techniques as this can dictate the timing and number of samples required to accurately estimate daily CH_4 emission. In particular, differences in individual animal eating behaviour (*e.g.* many small meals *vs.* a few large meals each day) can cause substantial measurement error when using short-term sampling techniques. The timing of sampling is critical as there is potential to bias the estimate of overall average CH_4 concentration and therefore emission (*i.e.* measurements taken before feeding *vs.* the postprandial period). Substantial between-hour and between-day variation (Jonker et al., 2014) highlights the challenge of sampling gases that are truly representative of daily CH_4 emission. The required number and timing of these short-term measurements will depend on a number of factors, including animal type, the diet or dietary supplement fed, level of feed intake, and the pattern of feeding or supplement dosing. As an extreme example, pulse dose addition of a CH_4 inhibitor into rumen cannulated lactating dairy cows produced transient decreases in CH_4 emission, measured using respiration

chambers, which would be more difficult to measure using short-term measurements (Reynolds et al., 2014; Guyader et al., 2015). In contrast, when the same inhibitor was mixed into the diet such that the inhibitor was delivered to the rumen with feed consumed, the inhibitory effect on CH_4 emission, measured at specific time points using a GreenFeed unit, was evident throughout the day (Figure 5; Hristov et al., 2015b). As discussed above, diurnal variation in CH_4 emission will vary depending on meal pattern and amount of feed consumed (Figure 4). Therefore for dietary regimes where the rate of CH_4 emission is more constant over the course of each day, fewer short-term measurements will be needed, and *vice versa*.

Figures 4 and 5 near here.

Numbers of animals and duration of sampling required

Depending on the experimental objectives (*i.e.* ranking of animals for CH_4 emission or determining CH_4 emission differences between experimental treatments), it is important to consider the numbers of animals and duration of sampling required for each technique. This includes the extent to which animal subjects are representative of the animal population for which estimates of CH_4 emission are being made. Deighton et al. (2014b) identified and corrected a number of errors within the SF_6 technique and compared this 'modified' version with measurements of enteric CH_4 emission in the same cows using respiration chambers. These authors found the between-animal CV for CH_4 yield was similar for cows using either the modified SF_6 technique (6.5%) or respiration chambers (7.5%), which was also much lower than previously published CV between-cows of 11 to 21.5% (Deighton et al., 2014b). It was concluded that because the modified SF_6 technique was able to reduce the between-animal CV, it allowed the statistical power of the experiments to be increased, employing 1/3

of the animal numbers required to detect a 10% treatment effect in CH_4 emission, compared with the unmodified SF_6 technique.

Compared with respiration chamber and SF_6 techniques, it will take more time and more animals to undertake a treatment comparison on CH_4 emission using GreenFeed with voluntary visitation if some animals avoid the GreenFeed unit or do not visit it each day in a free-stall barn or pasture environments (Hammond et al., 2015). Cottle et al. (2015) assessed the duration and number of GreenFeed measurements required for estimating daily CH_4 emission from cattle with a pre-specified accuracy and confidence level. This analysis was based on a feedlot situation using 24 beef steers over 64 d with an average of two GreenFeed visits/d. These authors found that it was not possible to define CH_4 emission of a treatment group within 5% of the 64-d mean with 95% confidence using 10 animals, and it would require more than three months of data collection using 20 animals. Arbre et al. (2016) evaluated the repeatability (R) of CH_4 measurements in two different trials using cattle. The SF_6 technique was used for 20 d in six non-lactating dairy cows fed a hay-based diet and the GreenFeed system was used for 91 d in seven lactating dairy cows fed a maize silage-based diet. To achieve an R value of 0.70 for CH_4 (g/kg DMI), 3-d periods were necessary for SF_6 and 17-d periods for GreenFeed. The total number of animals required to detect a significant difference in CH_4 emission of 20% between two treatments (e.g. diet) was similar ($12 < n < 14$ per group) for both SF_6 and GreenFeed techniques.

It is important to note that these calculations were based on the actual number of cattle that visited GreenFeed and that this level of replication changes with contrasting environments, including intensive *vs.* extensive grazing, cultivars and crops *etc*. Not all animals become frequent GreenFeed users, especially at grazing and in feedlots. This is a particular concern when ranking individual animals for CH_4 emission, as only those animals that visit the GreenFeed can be ranked; so the number, proportion and representation of

animals is important in studies where GreenFeed is used. This should be considered when estimating the number of animals required based on statistical power calculations. As discussed in previous sections, in tie-stall barns gas sampling events can be controlled by the investigator, thereby eliminating the problem of unrepresentative sampling due to infrequent or lack of visitations.

3. Methodology recommendations

There are a number of methods being used to determine CH_4 emission from ruminants, all of which have strengths, weaknesses, advantages and disadvantages for specific purposes, depending on their conditions of use. No one method is appropriate for all conditions and objectives. Respiration chambers are often referred to as the 'gold standard' for measurement of CH_4 emission by individual animals and remain arguably the most accurate. However, the chamber technique does require significant technical skill to operate in order to generate accurate CH_4 emission measurements, and like any other method this is determined by conditions of use. Variation across a selection of new and existing respiration chambers in the UK demonstrated considerable variation in accuracy (Gardiner et al., 2015). In this respect, the types of chamber and techniques used are an important consideration. Respiration chambers can be costly, labour intensive, impose restrictions on animal behaviour, and have limited 'throughput'. However, when used with rigour they can be highly accurate and precise, capture total CH_4 emission, including losses from rumen fistulas. Respiration chambers have the added advantage of measurement of gas production or consumption of other gases (*e.g.* O_2, CO_2, hydrogen [H_2], ammonia [NH_3]), and the ability undertake other measurements such as energy metabolism. Chambers are well suited for testing the relatively small effects of diet composition or supplements on CH_4 emission on a small number of animals, and are an important resource for fundamental studies of the

biology of CH_4 emission and mitigation. Respiration chambers utilizing repeated measurements over the course of daily diurnal patterns of CH_4 emission have a clear advantage in being able to characterize CH_4 emission patterns. Measurement of diurnal variation allows insight into underlying mechanisms of enteric CH_4 formation, including relationships with H_2 production (*e.g.* van Zijderveld et al., 2011). However, respiration chambers are less practical for strategic 'applied' research that relies on evaluation in greater numbers of animals under commercial production environments. Their potential negative effects on feed intake and milk production, in the case of lactating cows, can be minimized with good technique (*e.g*. Hellwing et al., 2012), but must also be considered.

The SF_6 method can be used with lesser effect on animal behaviour under typical animal management conditions and has a higher throughput in terms of animal measurements obtained relative to time and cost. With recent recommendations for improving the technique, the SF_6 method can be used with a high level of precision, but it is labour intensive and dependent on implementation and technical skill to minimize experimental error. In addition, the accurate prediction of SF_6 permeation tube release rate is essential for long term experiments. It is also important that investigators recognise the role of background gas concentrations and their sampling, particularly with regards to building ventilation when using the SF_6 technique indoors and the potential to mislead estimates of CH_4 emission.

The short-term methods described in our review, in particular the 'sniffer' type methods of breath analysis, can be applied to very large numbers of animals under 'normal' management conditions, and may be appropriate for screening animals on farm. However, there are concerns over their accuracy, repeatability, and precision of the data obtained. These concerns extend to their sensitivity to detect treatment differences in CH_4 emission, even when used with large numbers of animals. Because the sniffer methods do not measure CH_4 emission, they can only provide estimates based on previously obtained relationships between

CH_4 concentrations and CH_4 emission measured using other techniques (*e.g.* respiration chambers). In both Garnsworthy et al. (2012) and Huhtanen et al. (2015), the intercepts of regressions predicting CH_4 emission from CH_4 concentration were highly positive, with a high predicted emission at zero CH_4 concentrations (e.g. intercept >250 g/d and >360 g/d for Garnsworthy et al. 2012 and Huhtanen et al. 2015, respectively). Of the 'short-term' measurement methods reviewed, GreenFeed has the advantage that expired CH_4 emission is measured. The technique, however, requires sufficient numbers of measurements over time to obtain accurate estimates of daily emission, and relies on animals voluntarily visiting the unit, which is important to consider for animal ranking purposes. On pasture and in free-stall barns, a larger experimental group of animals will be required if some animals do not visit GreenFeed and it is recommended that investigators carefully assess required sampling protocols against objectives and experimental conditions. The use of GreenFeed requires a feed supplement or other 'enticement', which may introduce between-day variation in supplement consumption and thus interact with or confound treatments being assessed. Therefore, the amount of feed supplement required to obtain sufficient numbers of measurements per day, and the day-to-day variation in the amount of supplement consumed (in free-stall and pasture conditions), has to be evaluated in analysis of nutrition experiments. In tie-stall barns, GreenFeed visitations and amount of 'bait' feed provided and consumed can be controlled much more by the investigator, thus eliminating the potential problem of unrepresentative sampling to a large extent. Under these conditions the method is more appropriate for comparing effects of diet and other treatments.

4. Conclusions

There is a need to standardize operating procedures and develop guidelines for conducting and assessing data from *in vivo* studies designed to measure enteric CH_4 emission

by ruminants, and evaluate nutritional strategies, or strategies that include the simultaneous evaluation of nutritional aspects. There is no 'one size fits all' method for measuring CH_4 emission by individual animals. Ultimately, the decision as to which method to use should be based on the experimental objectives and the resources available, but the need for high throughput methodology, *e.g.* for screening large numbers of animals for genomic studies, does not in itself justify the use of methods that are inaccurate, imprecise, or biased. Similarly, although the most sophisticated respiration chambers can in principal achieve the highest degree of accuracy (100% recovery of CH_4 emitted by the animal), they are only a 'gold standard' when used with sufficient rigour and technical expertise. Regardless of the method chosen, appropriate calibrations and recovery tests (*e.g.* McLean and Tobin, 1988; Gardener et al., 2015) are required for both method development and routine operation. Respiration chambers and short-term measurements of CH_4 emission via breath have been used to obtain measurements of ruminant CH_4 emission for more than 120 and 75 years, respectively, but successful and correct use of methods requires careful attention to detail, rigour, and unbiased and routine self-assessment of the quality of the data generated.

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Table 1. Published technique comparisons for the measurement of methane (CH_4) emission (g/d) from cattle.

Technique		CH_4 measured simultaneously	Diet	Animal	No. animals	Technique X *vs.* Y CH_4 emission g/d*		Significance (*P*-value): X *vs.* Y	Regression coefficient (r^2)	Slope	Ref
X	Y					X	Y				
RC	GF	No	Maize:Grass silage	Dairy heifers	4	215	198	0.54	0.06	0.23	1
RC	GF	No	Haylage	Dairy heifers	4	209	208	0.74	0.01	0.09	1
RC	GF	No	Lucerne chaff	Beef cows	5	216	209	>0.05	N/A	N/A	2
RC	GF	No	Lucerne chaff	Beef steers	10	198	215	>0.05	0.85	N/A	2
RC	GF	No	Lucerne silage	Dairy heifers	6	134	150	0.45	-0.36	N/A	3
RC	SF_6	No	Lucerne silage	Dairy heifers	6	134	128	0.80	0.13	N/A	3
RC	SF_6	No	Barley/lucerne cubes	Beef heifers	6	93.0	98.0	0.24	N/A	N/A	4
RC	SF_6	Yes	Ryegrass pasture	Lactating dairy cows	16	322	331	N/A	N/A	N/A	5
RC	SF_6	Yes	Grass silage/conc.	Lactating dairy cows	20	422	469	<0.01	0.69	0.64	6
RC	SF_6	No	Ryegrass pasture/conc.	Dairy cows	8	455	431	0.14	N/A	N/A	7
RC	LMD	Yes	TMR	Dairy cows	2	356 ppm	396 ppm	<0.01	0.22	N/A	8
GF	SF_6	Yes	Grazing forages	Dairy heifers	12	164	186	<0.01	0.40	0.41	1
GF	SF_6	Yes	TMR	Lactating dairy cows	16	468	467	N/A	N/A	N/A	9
GF	SF_6	Yes	TMR	Lactating dairy cows	48	12.8 g/kg DMI[a]	14.7 g/kg DMI[a]	<0.01 to 0.38[a]	N/A	N/A	10
GF	SF_6	Yes	Lucerne silage	Dairy heifers	6	150	128	<0.05	N/A	N/A	3
Technique comparisons using regression equations to predict CH_4 emission											
RC	CH_4:CO_2	Yes	30 different diets	Lactating dairy cows	157	412	345[b]	<0.01	0.55	0.58	11
RC	Sniffer	No	PMR	Lactating dairy cows	12	395	2.2 mg/L[c]	<0.01	0.79	0.57	12
RC	LMD	No	High/low conc. diets	Steers	67	175	53.4 µL/L[d]	<0.01	0.39	N/A	13
GF	Sniffer	Yes	Forage	Lactating dairy cows	32	453	1405 ppm[e]	0.11	0.09	0.07	14
GF	Sniffer	Yes	TMR	Lactating dairy cows	59	447	758 ppm[f]	0.02	0.09	0.10	14

* units unless stated

RC, respiration chambers; GF, GreenFeed; SF_6, sulphur hexafluoride tracer; LMD, laser methane detector; CH_4:CO_2, methane to carbon dioxide ratio; TMR, total mixed ration; PMR, partial mixed ration; DMI, dry matter intake; N/A, not available; Ref, reference

1 Hammond et al. 2015
2 Velazco 2015
3 Garnett 2015
4 Boadi et al. 2002
5 Grainger et al. 2007
6 Munoz et al. 2012

7 Deighton et al. (2014b). Used a 'modified' SF_6 technique that incorporated orifice plate flow restrictors with an initial canister pressure of 0.03 atm.
8 Chagunda et al. 2013
9 Dorich et al. 2015
10 Oh et al 2015[a]: Methane expressed as g/kg DMI. Experiment was conducted over two phases, with *P*-values (indicating significance of the difference between GF and SF_6 methods) ranging from <0.001 to 0.38 across the two phases.
11 Hellwing et al 2013[b]: The regression of predicted CH_4 (PCH_4, determined using estimated CO_2 production) on measured CH_4 in RC (MCH_4) gave the equation $PCH_4 = 147 + 0.58 \times MCH_4$, where MCH_4 was 412 g/d, and PCH_4 was calculated to be 345 g/d using the regression equation.
12 Garnsworthy et al. 2012. [c]The orthogonal-regression relationship between sniffer CH_4 (mg/L) measured during milking (MERm) and total daily CH_4 (g/d) measured subsequently in RC (MEc) gave the equation: MEc (g/d) = 252 + 57.2 × MERm; where overall means of MEc were 395 g/d and MERm were 2.2 mg/L.
13 Ricci et al. 2014: Methane measured from the same steers, averaged over high and low conc diets, was 175 g/d using RC, and 54.4 µL/L using LMD. The overall mean LMD-CH_4 of the raw data was correlated with RC-CH_4. Through a model fitting process, the regression between RC-CH_4 and LMD-CH_4 resulted in the equation CH_4 (g/d) = -514.9 + 9.81 × Eruc Time + 31.63 × MaxResp, where ErucTime is the mean eructation time recorded during eructation events (min); and MaxResp is the maximum CH_4 concentration during respiration events (µL/L).
14 Huhtanen et al. 2015. [e]For experiment 1, the regression between GF CH_4 (g/d) and sniffer CH_4 (ppm) resulted in the equation CH_4 (g/d) = 360 + 0.07 × sniffer CH_4, where sniffer CH_4 was measured as 1,405 ppm, and GF CH_4 emission (g/d) was 453 g/d. [f] For experiment 2, the regression between GF CH_4 (g/d) and sniffer CH_4 (ppm) resulted in the equation CH_4 (g/d) = 365 + 0.102 × sniffer CH_4, where sniffer CH_4 was measured as 758 ppm, and GF CH_4 emission (g/d) was calculated to be 447 g/d using the regression equation.

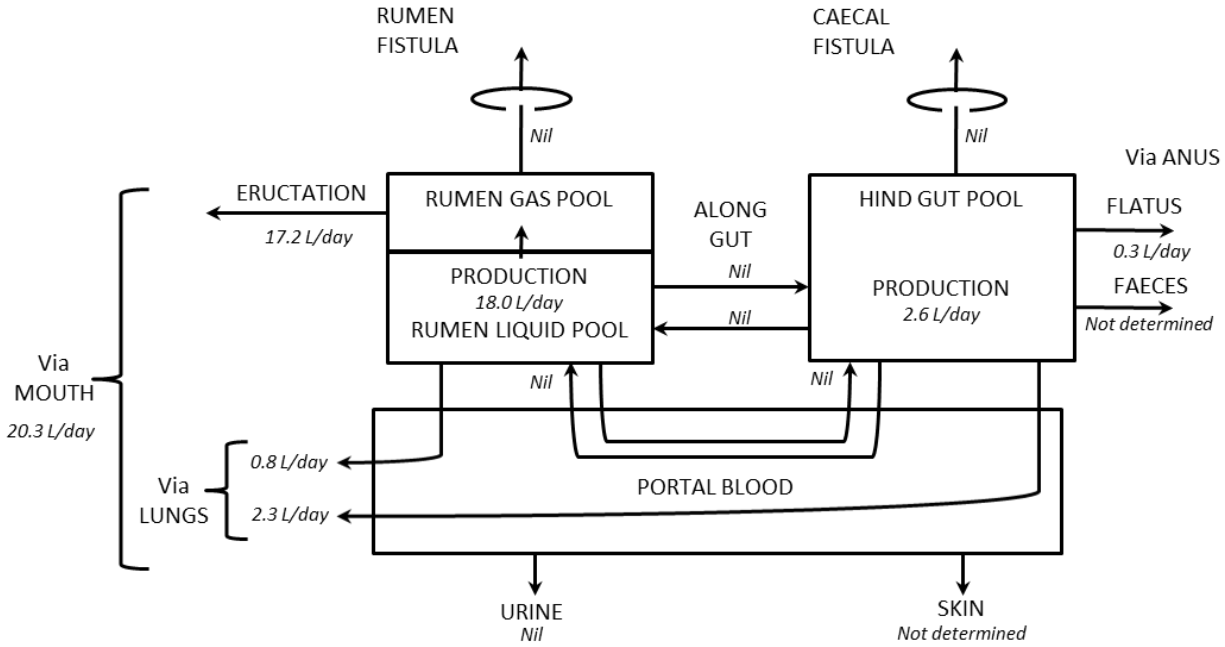

Figure 1. A model of the production and movement of methane in sheep on a lucerne chaff diet. Diagram sourced from Murray et al. (1975).

Figure 2. Face masks for respiration and methane emission measurements in the 1930's. Sourced from Washburn and Brody (1937).

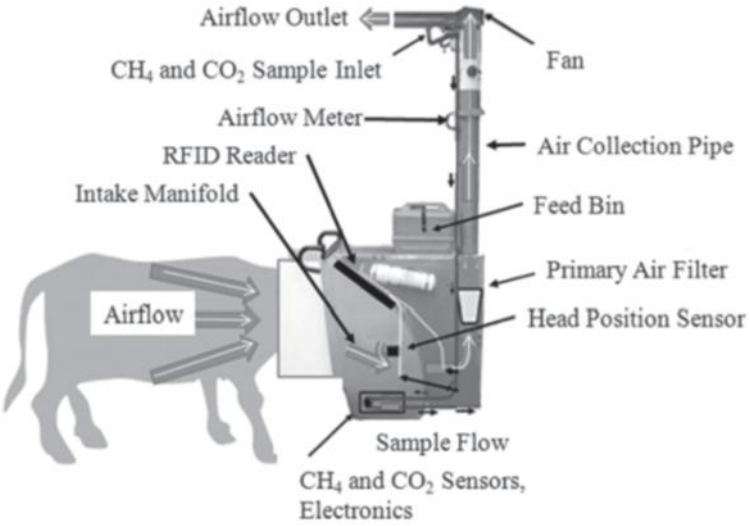

Figure 3. Layout of the GreenFeed system (C-Lock Inc., Rapid City, SD, USA). Sourced from Huhtanen et al. (2015).

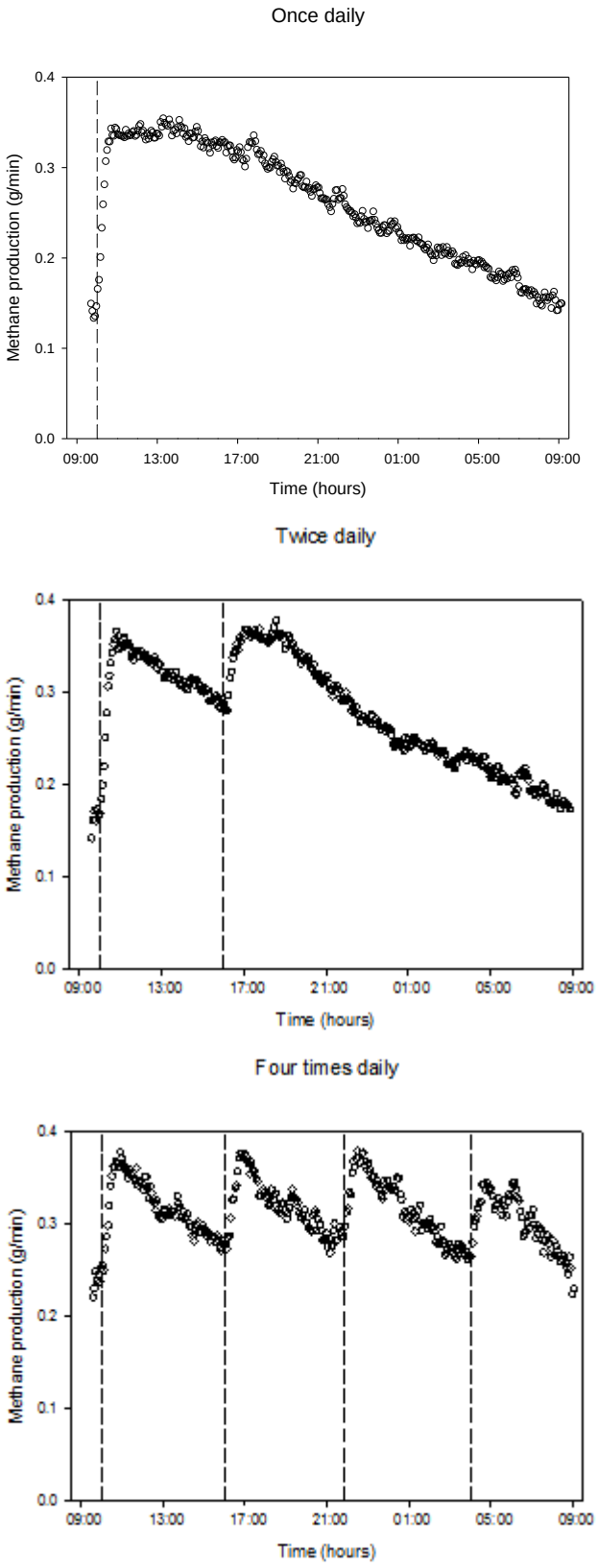

Figure 4. Diurnal pattern of methane emission (mean of three daily measurements for 4 animals) by lactating dairy cows fed the same total mixed ration once, twice, or four times daily. Dotted lines indicate the time of feeding. Sourced from Crompton et al. (2011).

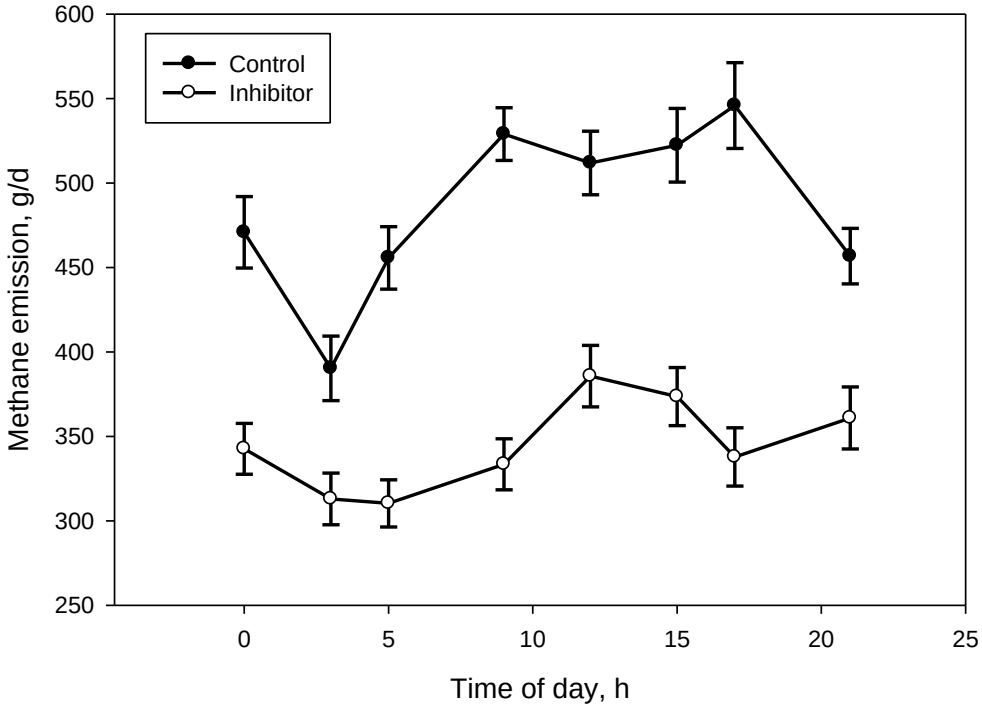

Figure 5. Methane emission by lactating dairy cows (n = 12 for control and n = 36 for treatment) fed a total mixed ration without (control) or with (inhibitor) 3-nitroxyproponal. Sourced from Hristov et al. (2015b).

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