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Interactions between microbial consortia in biofilms: a paradigm shift in rumen microbial ecology and enteric methane mitigation

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Abstract. Minimising enteric CH_4 emissions from ruminants is a current research priority because CH_4 contributes to global warming. The most effective mitigation strategy is to adjust the animal's diet to complement locally available feed resources so that optimal production is gained from a minimum of animals. This essay concentrates on a second strategy – the use of feed additives that are toxic to methanogens or that redirect H_2 (and electrons) to inhibit enteric CH_4 emissions from individual animals. Much of the published research in this area is contradictory and may be explained when the microbial ecology of the rumen is considered.

Rumen microbes mostly exist in organised consortia within biofilms composed of self-secreted extracellular polymeric substances attached to or within feed particles. In these biofilms, individual colonies are positioned to optimise their use of preferred intermediates from an overall process of organic matter fermentation that generates end-products the animal can utilise. Synthesis of CH_4 within biofilms prevents a rise in the partial pressure of H_2 (pH_2) to levels that inhibit bacterial dehydrogenases, and so reduce fermentation rate, feed intake and digestibility. In this context, hypotheses are advanced to explain changes in hydrogen disposal from the biofilms in the rumen resulting from use of anti-methanogenic feed additives as follows.

Nitrate acts as an alternative electron sink when it is reduced via NO_2^- to NH_3 and CH_4 synthesis is reduced. However, efficiency of CH_4 mitigation is always lower than that predicted and decreases as NO_3^- ingestion increases. Suggested reasons include (1) variable levels of absorption of NO_3^-or NO_2^- from the rumen and (2) increases in H_2 production. One suggestion is that NO_3^- reduction may lower pH_2 at the surface of biofilms, thereby creating an ecological niche for growth of syntrophic bacteria that oxidise propionate and/or butyrate to acetate with release of H_2.

Chlorinated hydrocarbons also inhibit CH_4 synthesis and increase H_2 and formate production by some rumen methanogens. Formate diffuses from the biofilm and is converted to HCO_3^- and H_2 in rumen fluid and is then excreted via the breath. Short-chain nitro-compounds inhibit both CH_4 and formate synthesis when added to ruminal fluid but have little or no effect in redirecting H_2 to other sinks, so the pH_2 within biofilms may increase to levels that support reductive acetogenesis. Biochar or activated charcoal may also alter biofilm activity and reduce net CH_4 synthesis; direct electron transfer between microbes within biofilms may also be involved. A final suggestion is that, during their sessile life stage, protozoa interact with biofilm communities and help maintain pH_2 in the biofilm, supporting methanogenesis.

Additional keywords: chlorinated hydrocarbons, direct interspecies electron transport, electron acceptors, formate, inter-bacterial distance, motility symbiosis, partial pressure hydrogen, reductive acetogenesis, role of protozoa, short-chain nitro-compounds, syntrophism, transparent exopolymer particles, viscotactic spirochete.

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Introduction

Methane (CH_4) is a potent greenhouse gas that has a high priority for mitigation because of its detrimental global warming potential and because, in combination with tropospheric ozone and carbon (C) black, it is a health hazard that could reduce the life expectancy of 3.1 billion people worldwide (UNEP 2011). CH_4 produced by ruminants is targeted as a significant and potentially mitigatable source of this greenhouse gas (Hristov *et al*. 2013).

Under normal feeding conditions, CH_4 production is an inescapable consequence of the fermentation of organic matter (OM) in the digestive tract of ruminant animals. Theoretically, the generation of CH_4 in the rumen can be decreased by the following factors (McAllister and Newbold 2008; Eckard *et al*. 2010; Morgavi *et al*. 2010; Cottle *et al*. 2011; Hristov *et al*. 2013):

(1) by promoting a shift in fermentation toward production of the more reduced volatile fatty acids (VFA), e.g. propionate;
(2) on nutrient deficient diets, which increase microbial growth efficiency (microbes are more reduced than the VFA end products and are therefore a sink for hydrogen (H_2)) by providing supplements of minerals and, where the crude protein content of the diet is low, a non-protein nitrogen (N) source;
(3) by addition of feed additives that inhibit methanogenesis (e.g. bromochloromethane, BCM) or have a high affinity for bioreduction (e.g. long-chain unsaturated fatty acids);
(4) immunisation against methanogens;
(5) defaunation of the rumen: because of e.g. physical association of methanogens in or on the surface of rumen protozoa;
(6) stimulation of the growth of bacteriophages that infect and lyse methanogens;
(7) supplementation of a diet with compounds that specifically promote the growth of bacteria and/or *Archaea* that use compounds such as nitrates and sulfates and have a higher affinity for H_2 than do methanogens; and
(8) by creating an environment in the rumen that encourages the growth of
  (i) reductive acetogenic microbes, and
  (ii) methanotrophic microbes.

Recently, attempts were made to create a new microbial habitat in the rumen by including biochar in the diet, to increase the inert surface area for biofilm formation that may allow close association of both methanotrophs and methanogens and increase anaerobic CH_4 oxidation (see Leng *et al*. 2012*a*) or improve overall microbial-growth efficiency (Leng *et al*. 2012*b*, 2012*c*; Liu *et al*. 2012). McAllister and Cheng (1996) proposed that methanogenesis cannot be eliminated without adverse effects on ruminant production, and there is a general view that the clearance of dissolved H_2 from the rumen by methanogens is critical for maintenance of a low partial pressure of H_2 at the sites of fermentation; this condition, in turn, is a pre-requisite for the regeneration of cofactors such as NADH, NADPH and reduced ferridoxins that are necessary for continuous glycolytic activity by the rumen microbial consortia. Syntrophic acetogenic bacteria grow in mixed culture with H_2-consuming bacteria such as methanogens. A simultaneous electron transfer from an organism fermenting OM to a H_2-consuming species (termed interspecies H_2 or electron transfer) is putatively essential for growth and metabolism. More particularly, recent developments have indicated that the crucial factor in releasing electrons from reduced cofactors (allowing the glycolytic pathway to function) is the partial pressure of H_2 in the biofilm matrix or in aggregate forms such as flocs in digesters (Thiele *et al*. 1988) associated with the plant particles (Wolin 1979; McAllister and Newbold 2008; Janssen 2010).

The production of CH_4 in the rumen can be reduced by more than 90% by direct inhibition using chlorinated hydrocarbons or CH_4 analogues added to feed (see e.g. McCrabb *et al*. 1997). Surprisingly, with this type of inhibition, there is no reduction in feed digestibility or production and the reduction in CH_4 release is accompanied by a concomitant stoichiometric production of H_2 (Mitsumori *et al*. 2012). If it is assumed that the H_2 is produced in fermentative sites in the biofilm, it is reasonable to expect an increased partial pressure of H_2 at these sites and, therefore, adverse effects on feed digestion and intake. The studies with the CH_4 analogue, BCM, have created a conundrum. If rumen microbial ecology can change to produce H_2, thereby maintaining fermentation efficiency and animal production, then the use of compounds toxic to methanogens or inhibitory for the pathways of CH_4 synthesis (such as BCM, chloroform or tannins), or even immunisation against methanogens, are not rational ways of ameliorating greenhouse-gas emissions because these strategies are likely to release H_2 in place of CH_4. When H_2 reaches the troposphere, it reacts with hydroxyl radicals and perturbs the distribution of CH_4 and ozone; its effect is that of a greenhouse gas with a global-warming potential (GWP) of 5.8 over a 100-year time horizon (Derwent *et al*. 2006). Because it takes 4 mol of H_2 to form 1 mol of CH_4 that has a GWP of 23, little is gained from mitigating 1 mol enteric CH_4 if the consequence is the release of 4 mol H_2 to the atmosphere. (This situation could change in the future if the GWP of CH_4 is assessed at a much higher level.)

In the following discussion, an attempt is made to rationalise the potential value of enteric CH_4 mitigation by inhibiting methanogens, and to suggest research priorities. However, the arguments developed depend on an understanding of the roles of biofilms in anaerobic rumen digestion and their importance is explored in the initial section of this review.

Rumen microbial ecology

In the past, the rumen has been viewed as a milieu of microorganisms (a microbial 'soup') which, as a unit, is highly effective in degrading feed resources to VFA, with the ATP generated being used to synthesise the microbial polymers required for cell growth (Annison and Lewis 1959). There has been a gradual change in this perception as the concept of anaerobic, microbial communities in discrete, organised and structured systems has become recognised as essential for the control of the complex hydrolytic and enzymatic breakdown of feed in the rumen (Cheng and Costerton 1980; Costerton *et al*. 1987; McAllister *et al*. 1994; Cheng *et al*. 1995; McAllister and Cheng 1996; Costerton 2007; Edwards *et al*. 2007). Environmental microbiologists have long recognised that

associated complex bacterial communities are responsible for driving all the major biogeochemical nutrient cycles within the earth's biosphere (C, sulfur (S) and N cycles) that maintain relative stability in the biosphere (Davey and O'Toole 2000). Until recently, the lack of methods for exploring these microbial communities *in situ* has hampered detailed analyses in the rumen. Traditionally, studies of rumen microbes were performed using organisms isolated from rumen fluid and cultured in roll tubes (Hungate 1966). Application of technologies that were independent of cultured microbes has shown that species diversity in the rumen has been vastly underestimated (Rappé and Giovannoni 2003; Edwards *et al*. 2008). As this technology evolves, it is being adapted to examine microbial communities in their natural habitat and is playing a major role in describing the rumen biome (Bath *et al*. 2013).

The importance of rumen microbial ecology

Over the past 50 years, rumen microbiologists have emphasised the need for attachment of bacteria to feed materials, to enable them to efficiently digest OM in the rumen (Cheng and Costerton 1980; Cheng *et al*. 1980). Measurements of the sites of bacterial ATP formation and their location in the rumen indicated that the majority of ATP was associated with plant particles (Forsberg and Lam 1977; Craig *et al*. 1987) and isotope-dilution studies using organisms labelled with ^{15}N suggested that 80–90% of the bacteria in the rumen are associated with particulate matter (Rodríguez *et al*. 2003). Krebs (1987) labelled bacteria with ^{35}S-sulfate and examined the movement of ^{35}S between the free-floating and particle-associated organisms and found that 80% of the microbes washed out of the rumen had been particle-associated and that bacteria moved between particles without entering the free-floating bacterial pool. It is now clear that particle-associated microbes play the most important role in rumen digestion (McAllister *et al*. 1994; Mayorga *et al*. 2007; Edwards *et al*. 2008). These microbes are found in associated consortia embedded in a biofilm matrix where end products produced by one colony are sequentially used by closely associated colonies.

Although compounds such as VFA, amino acids and ammonia (NH_3), and gases such as CH_4, H_2 and carbon dioxide (CO_2), diffuse into and out of the biofilm, these materials can be expected to be in higher concentrations within the biofilm matrix than in the external rumen fluid. Thus, the bicarbonate (HCO_3^-) that is reduced during methanogenesis will probably be mostly drawn from within the biofilm matrix (that has diffused in and/or been produced locally) rather than from a single homogenous HCO_3^- pool in rumen fluid. In this connection, Loughnan (1982) infused $H^{14}CO_3^-$ into the rumen of sheep and showed that the specific radioactivity of excreted CH_4 was less than 50% of that of the HCO_3^--C in the rumen fluid. This result would be expected if some of the C in CH_4 were derived from unlabelled HCO_3^- produced close to methanogenic colonies in the biofilms and the remainder came from HCO_3^- that diffused from the external rumen fluid via water channels in the biofilm structure. An alternative possibility is that formate produced during fermentation of OM or synthesised *in situ* could have supplied unlabelled C for methanogenesis (see later in text); however, when Loughnan (1982) infused ^{14}C-labelled formate into the rumen contents of the same sheep, formate-C made little contribution to CH_4. Notably, some methanogens produce formate and its production is greatly enhanced when CH_4 synthesis is inhibited by CH_4 analogues (Bleicher and Winter 1994). *In vitro*, addition of anthroquinone to rumen fluid resulted in the accumulation of formate (Asanuma *et al*. 1998)

It is generally accepted that attachment of rumen microbes to feed particles is essential in maintaining a high rate of solubilisation of feed OM. In general, however, ruminant nutritionists have not connected the consequences to the actual mechanisms of fermentative digestion that require several different species of microbes to act in concert. Perhaps the term 'interspecies electron transfer' has not conveyed the concept of the organised biofilm mode of degradation. Recently, Wang and Chen (2009) and Weimer *et al*. (2009) highlighted the stark differences between the efficiency of fermentative production of bioethanol based on cellulosic feed stock (by planktonic yeast cells) and the efficiency of rumen organisms when converting cellulosic biomass to short-chain VFA. Clearly, the rumen has also evolved highly efficient mechanisms for these processes (Wang and Chen 2009) that depend on organised sequential breakdown of the cellulosic biomass and involve numerous species of organisms (in particular, bacteria, fungi and protozoa).

The modes of breakdown of complex plant OM in the rumen were thoroughly reviewed by McAllister and his colleagues (McAllister *et al*. 1994) and were reviewed more generally by Costerton (2007) and Leng (2011). In the current presentation, emphasis is placed on the interspecies transfer of electrons that facilitates, and perhaps integrates, the fermentative processes. McAllister *et al*. (1994) emphasised the need for microbes to attach to feed particles, to initiate the consortia that then enzymatically solubilise the complex components and circumvent barriers that restrict access to the more fermentable OM substrates within plant particles.

The microbial colonies, encased in self-produced polymeric substances, both grow inward to access the internal fermentable materials, as well as access those on the surfaces of feed particles. Anaerobic fungi, in contrast, grow within the plant structures (Gordon and Phillips 1998). They produce sporangia that release zoospores that actively invade plant materials, particularly in the areas where damage to the waxy surface has occurred. Penetration of fungal mycelia through plant particles weakens the structures and promotes more rapid reduction of particle size and greater access for other organisms. The fungi are in close contact with the biofilm consortia or can be considered as an extension of the biofilm into the solid plant particles. They actively provide hydrolytic breakdown products and H_2 and/or formate that can be assimilated by the associated microbial colonies (Nagpal *et al*. 2009). For plant cell-wall degradation, such anaerobic fungi produce a wide range of hydrolytic enzymes such as cellulases, hemicellulases, proteases, amylases, feruloyl and p-coumaryl esterases, various disaccharidases, pectinases and exonucleases (Nagpal *et al*. 2009). Rumen fungi produce appreciable amounts of H_2 and, therefore, are advantaged by being close to methanogens; they also produce a range of hydrolytic end products that provide substrates for other associated bacterial colonies.

The initial colonisers that adhere to the surface of plant materials are bacteria. Primary colonisers attach to newly ingested forage particles, exude extracellular polymeric substances and then develop into biofilm colonies on perennial ryegrass leaf, for instance, in less than 1 h (Huws *et al.* 2013). Bacteria have evolved signalling mechanisms that enable them to communicate and co-ordinate their activities so that they can respond quickly to environmental changes (such as establishment of nearby bacteria or the presence of nutrients or toxins); they exhibit a wide range of interactive, multicellular behaviors such as dispersal, nutrient acquisition, biofilm formation and quorum sensing (West *et al.* 2006).

The assembly of non-motile cellulolytic bacteria such as *F. succinogenes* on a feed particle, usually close to where the waxy cuticle has been damaged, may be facilitated by motility symbiosis between the bacteria and spirochetes (Stanton and Canale-Parola 1980). These workers studied *Treponema bryantii*, a highly motile, viscotactic spirochete that grows on soluble sugars but is unable to utilise cellulose and is representative of one of seven morphological types of spirochetes found in the rumen (Stanton and Canale-Parola 1979). *T. bryantii* was shown to migrate through culture media *in vitro* and position itself near cellulosic fibres being degraded by *F. succinogenes* bacteria. These workers suggest passive motility of bacteria occurs due to close proximity between the spirochetes and the *F. succinogenes*, rather than actual attachment. The incessant movement of the spirochetes towards potential substrate appears to propel the cellulolytic bacteria towards their non-diffusible substrate in the feed particle (Stanton and Canale-Parola 1980). At the same time, the *T. bryantii* positions itself to access the soluble sugars released by the hydrolytic breakdown of the complex polysaccharides in the feed particle by *F.succinogenes* (Stanton and Canale-Parola 1980).

There is evidence that most aquatic ecosystems contain planktonic transparent exopolymer particles (TEPs) which may also be present in the rumen. These particles are organic microgels that are partly composed of polymers of fucose and rhamnose that are highly surface-active (Bar-Zeev*et al.* 2012) TEPs may originate from dissolved polymeric organic matter or from preformed biofilms and display most of the characteristics of developing biofilms, except they are not attached to a surface. However, they are extremely ‘sticky’ and, within minutes of exposure of solid particles to an aqueous medium, can start to adhere to solid surfaces and begin the process of biofilm development. Although TEPs have not been identified in the rumen, there is a high probability that they exist as sloughed biofilm fragments released from the surface of feed particles during rumination (Leng 2011). These fragments would be analogous to ‘protobiofilms’, i.e. TEPs that are colonised with microbes and can quickly form ‘hot spots’ of biofilm microbes on the surface of inert particles (Bar-Zeev *et al.* 2012).

After biofilms are established as outlined in Fig. 1, the initial colonisers then begin the process of hydrolysing complex structural compounds in solid materials. Next, apparently attracted by the solubilised materials, and probably also by signalling molecules (or inducer molecules) produced by other bacteria (Williams 2007), secondary colonisers are attracted and establish colonies by embedding themselves in the extracellular polymeric substances around the initial colonisers and grow by assimilating some of the intermediate products of bacterial hydrolysis (mainly simple sugars and other compounds such as peptides and amino acids). Further microbes with specific substrate requirements become associated as the biofilm grows. These bacteria are users of end products released by the microbes that initially establish sessile colonies and by the fungal biomass within the plant particles, and the biofilms grow as they become associated. The consortia that develop progressively degrade both complex and simple carbohydrates via the glycolytic pathways to VFA; the H_2 that is also produced is mostly incorporated into CH_4 by methanogenic *Archaea*. The extent of degradation of true protein within the biofilm consortia is not understood, but McAllister *et al*. (1994) suggested that this was the mode of utilisation of feed proteins (i.e. attached colonies). In addition, the fungi have high protease activity that may have a role in protein degradation, because the plant structural proteins increase the integrity of plant cell wall (Wallace and Joblin 1985). Proteins are either degraded via peptides to amino acids that are utilised in cell growth, or degraded further to organic acids and NH_3, presumably with interspecies transfer of N compounds playing a major role in microbial growth. Methanogenic colonies are always found in the biofilms attached to surfaces of solid substrates (Cheng *et al*. 1981); these methanogens are distributed within a cluster on the outer layers of the biofilm (Song *et al*. 2005). Biofilms with a high level of microbial digestive ability are always composed of complex multi-species layers of microbes or as separate but associated colonies (Stoodley *et al*. 2002).

A key feature of biofilm organisation is that the interspecies distances among colonies are small and metabolic end products of one species become substrates for nearby species until the final end products accumulate and diffuse into the external rumen fluid. Feedback inhibition by end products from one colony of organisms can affect other colonisers, so the ease of transfer of intermediates among colonies and the eventual diffusion to the bulk fluid regulates the breakdown of feed particles. The biofilm mode of fermentative degradation allows a greatly increased rate of OM breakdown as compared with that in planktonic communities that are not in organised consortia (de Bok *et al*. 2004; Wang and Chen 2009). Feedback from H_2 is particularly important because, if it were not removed, it would inhibit the re-oxidation of reduced cofactors produced in the fermentation pathways and restrict glycolysis and feed-degradation rate.

Syntrophism in biofilm communities and inter-bacterial distance

Syntrophism is used to describe the cooperation of two or more metabolically different bacteria that depend on each other to be able to degrade particular substrates and share the energy released for their maintenance and growth. The term was coined to describe the close cooperation between VFA-oxidising, fermenting bacteria and H_2-oxidising methanogens (McInerney *et al*. 1979). However, the benefits to colonies in close proximity to each other are not restricted to those involved in CH_4 formation; they apply universally where the end products of one microbial species are the substrates for another species.

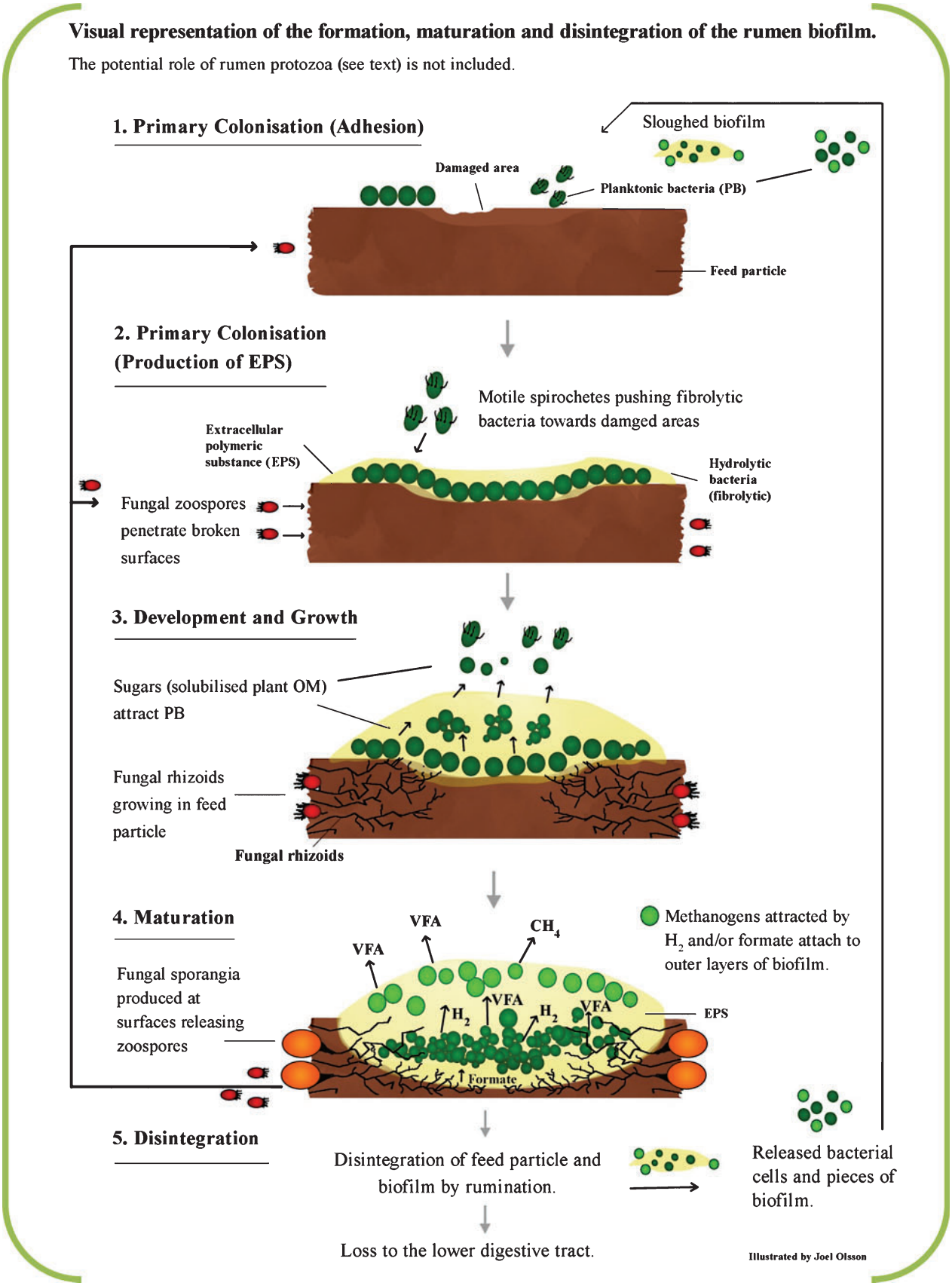

Fig. 1. Diagrammatic representation of the biofilm mode of digestion in the rumen.

The distance between bacterial syntrophs is critical in energy terms. Proximity can overcome energy barriers and make coupled growth possible. The relative proximity of organisms also allows reactions to proceed at rates unobtainable in mixed suspensions of planktonic cells (de Bok *et al*. 2004).

The transfer of a metabolite between microorganisms occurs by diffusion, as defined by Fick's law, which is described by the equation:

$$F_{h_2} = A \cdot D \cdot (C_1 - C_2)/d,$$

where F_{h_2} = flux of metabolite, D = diffusion coefficient in water, A = surface of producers, C = concentration of metabolite and d = distance between microorganisms (see Fig. 2 adapted from de Bok *et al*. 2004).

The effect of distance on the flux of interspecies electron carriers between producing and consuming organisms is shown in Fig. 2. Assuming a bacterium has a diameter of 2 µm, it can be calculated that, at densities 10^8, 10^9, 10^{10} and 10^{11} cells/mL, the inter-microbial distances in dispersed organisms are ~25, 10, 4 and 0.5 µm, respectively (Stams and Plugge 2009), as compared with distances in aggregates, flocs or biofilms of ~0.05 µm (de Bok *et al*. 2004). The interspecies distance may be even smaller in the case of *Archaea* that are appreciably smaller than bacteria. The need for consortia to bring microbes close to each other to facilitate H_2 transfer applies in all anaerobic ecosystems. It also applies to species that use direct interspecies electron transfer via pila (Malvankar and Lovley 2012) or via solid conducting surfaces such as activated charcoal

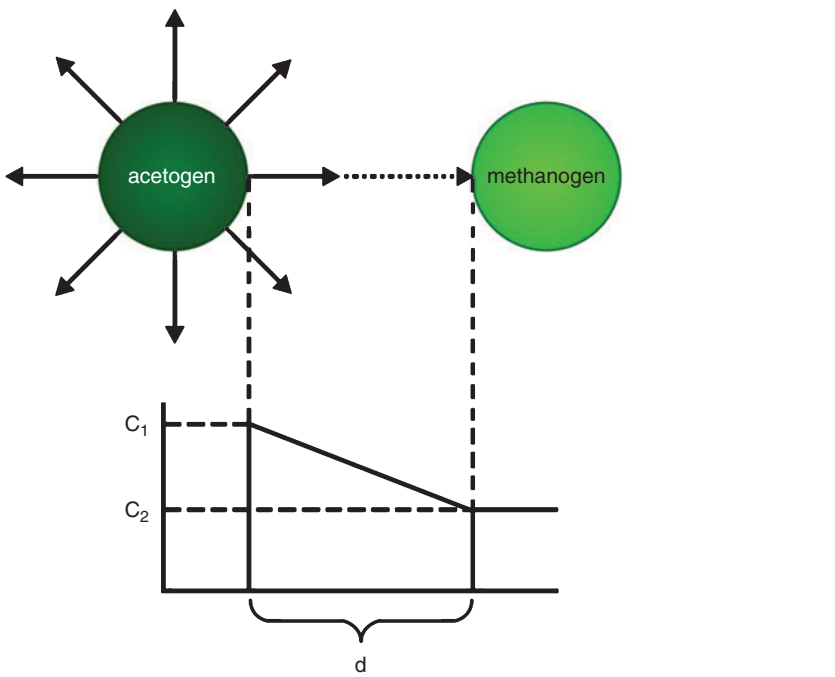

Fig. 2. Effect of diffusion distance on the inter-bacterial fluxes of hydrogen (H_2) for bacteria either in a suspension or aggregated distribution. In this example, the H_2 flux was calculated for cells with a 1-µm radius and a density of 10^9 cells/mL (adapted originally from Schink and Thauer 1988). The concentration of H_2 at the surface of the consuming bacteria is C, r is the radius of the bacteria, d is the distance between cells. The figure has been adapted from de Bok *et al*. (2004) and further information can be obtained from this source.

(Liu *et al*. 2012), or where microbial aggregates are electrically conductive, as has been demonstrated in waste water-treatment plants (Morita *et al*. 2011). In treatment plants, transfer of electrons occurs between fermenting bacteria and methanogens in biofilms containing colonies of *Geobacter* spp. (Reguera *et al*. 2005). Thus, it is possible that materials with charged particles such as biochar or activated charcoal or montmorilonite clay may facilitate both primary (hydrogenotrophic) and secondary (acetoclastic) fermentations by providing a large surface area as microbial habitat, and facilitating methanogenesis by electrical conductance between organisms (Leng *et al*. 2013).

Interspecies H_2 transfer

The term 'interspecies H_2 transfer' is often used to describe the transfer of H_2 from fermenting organisms to methanogenic *Archaea*. A low partial pressure of H_2 in the vicinity of actively fermenting organisms that contain hydrogenase enzymes enables the reduced cofactors to be re-oxidised. This releases electrons or H_2 which are quickly taken up by methanogens that use the H_2 to reduce HCO_3^- to CH_4. This is efficient because the fermenting microbial consortia are organised in self-produced biofilms with optimal interspecies localisation (McAllister *et al*. 1994). The closer the microbes that use H_2 are to those that produce it, the more rapid the rate of CH_4 production (de Bok *et al*. 2004). It appears that interspecies electron transfer from primary fermenting organisms that produce H_2 and VFA can be coupled to the reduction of several compounds including HCO_3^- (by methanogens), nitrate (by nitrate-reducing bacteria, NRB) and sulfate (by S-reducing bacteria, SRB).

Alternate electron acceptors include unsaturated fatty acids (Czerkawski *et al*. 1966), sulfate (Marty and Demeyer 1973) and nitrate (Allison and Macfarlane 1988). The presence of these acceptors can alter the microbial mix within the biofilm and divert electron flow away from methanogenesis. Hence, the inclusion of these salts in a diet seems to offer a logical means of lowering ruminal CH_4 production (Leng 2008). The competition for H_2 is affected by the Gibbs free-energy change of the reactions, so the reduction of nitrate (AG^o = –163 KJ/mol) and sulfate (AG^o = –152 KJ/mol) is thermodynamically more favourable than HCO_3^- reduction (AG^o = –130 KJ/mol); their higher affinity for H_2 gives NRB and SRB a further competitive advantage over methanogens (Oremland 1988), provided that the distances between colonies of SRB and NRB in the attached biofilm are similar to that of the competing methanogens. Essentially, the NRB and SRB must be concentrated in the biofilm matrix to outcompete the methanogens. The SRB and NRB occur naturally in the rumen (Coleman 1960; Hungate 1966; Howard and Hungate 1976; Cheng *et al*. 1988; Leng 2008) and experiments with sheep and cattle indicate that the population density of these species increases as the concentration of their respective electron acceptors in the ruminant diet increases (Alaboudi and Jones 1985; Hao *et al*. 2009). Which microbial species is the most successful in using the particular oxidised substrate (HCO_3^-, nitrate or sulfate) depends on the distance between the colonies of the organism involved in the interspecies transfer, the partial pressure of H_2 and the Gibbs free-energy change of the reaction. There is a substantial suppression of methanogenesis when dietary nitrate is reduced to ammonia in rumen fluid (see later discussions) and NRB colonies must take up a position in the biofilm that favours nitrate reduction, rather than sulfate or HCO_3^- reduction. Similarly, SRB must be favourably distributed when competing with methanogens and NRB, although many species of NRB will assume the role of SRB when nitrate is not available (Moura *et al*. 2007). In summary, the affinity of various substrates for H_2 varies directly with the Gibbs free-energy change of the relevant reaction. This suggests that interspecies distances among the NRB, SRB and methanogens in the rumen are similar and that these species are distributed within the biofilms associated with plant particles. In a planktonic cell culture and in the absence of a biofilm micro-environment, the H_2 partial pressure can probably exert little effect on fermentation rate, as H_2 is relatively insoluble and diffuses only slowly through water.

In waste-water treatment plants, the interspecies transfer of H_2 is self-organised within microbial syntrophs by their aggregation into methanogenic granules and/or flocs, which brings together two or more cooperating species (de Bok *et al*. 2004). This appears to happen in the rumen at times when the animal is given liquid feeds, for example when molasses is the main energy source (Rowe *et al*. 1979; Leng 2011) and where the quantity of feed particles provides minimal surface area for biofilm formation. However, it is likely that the colonies of microbes in biofilms are spatially more dispersed than in flocs, granules or other aggregates. The biofilm communities that form on surfaces of feed particles grow inward as cellulose and other plant structural components are hydrolysed but also form mushroom-like 'gels' that hold sessile colonies of inter-associated microbes (McAllister *et al*. 1994).

The NRB and SRB in waste water are found together with methanogens in biofilms where both nitrate and sulfate are abundant (Martínez Amador *et al*. 2011), but these species have not been examined in biofilms in rumen digesta. Interspecies H_2 transfer in microbial aggregates has been modelled and its relative importance is indicated by the calculated rates of reactions, which depend on the distance among the different species (Fig. 2 after de Bok *et al*. 2004).

Formate in interspecies H_2 transfer

Syntrophic interactions (the combined effect of two organisms in completing a chemical reaction) usually involve interspecies H_2 transfer, although formate may act as an alternative electron carrier. The H_2 and formate concentrations in syntrophic cultures are usually extremely low, and it is therefore difficult to determine which is the more important electron carrier. Many of the syntrophs involved are able to produce both H_2 and formate, and most of the methanogenic partners are able to oxidise both substrates. In addition, and of major importance, is that methanogens (which can metabolise both H_2 and formate) are usually able to reversibly produce formate from H_2 and HCO_3^-(Beaty and McInerney 1987). Rumen anaerobic fungi growing *in vitro* on cellulose (Bauchop and Montfort 1981) and wheat straw (Lowe *et al*. 1987) produced considerable amounts of formate, which would have no effect on the partial pressure of H_2 at the site of fermentative activity within the feed particle. This formate may diffuse towards the bulk fluid and

may be converted to H_2 and CO_2 or CH_4 by feed-associated methanogens, or by planktonic microbes in the fluid phase, to these same end products.

The first evidence that formate could be used as an electron carrier was obtained with microbes from a whey-treating reactor when the rate of CH_4 formation by syntrophic butyrate-degrading cultures could not be explained by interspecies H_2 transfer alone (Thiele *et al*. 1988; Boone *et al*. 1989). Using diffusion models based mainly on Fick's law, it was predicted that interspecies formate transfer could sustain an uptake of electrons for methanogenesis that was 100 times faster than was interspecies H_2 transfer. A similar modelling approach demonstrated the importance of formate in propionate-degrading and butyrate-degrading co-cultures (Dong and Stams 1995). Further evidence for formate transfer came from growth and biochemical studies; *Syntrophobacter fumaroxidans* grew well on propionate in co-culture with methanogens that could use both H_2 and formate, but no measurable growth was observed with methanogens that used only H_2 (Dong *et al*. 1994; Stams and Plugge 2009).

Recently, Felchner-Zwirello *et al*. (2013) measured inter-bacterial distances between the propionate degraders and methanogens in syntrophic associations within granules that increased in size with time after inoculation of the growth media. According to these authors, the two microbial types find each other (probably by quorum sensing) and the aggregates increase in size over time, while the interspecies distances decrease from 5.30 to 0.29 μm. At the same time, the maximum possible H_2 flux is increased from 1.1 to 10.3 nmol/mL.min. The results indicated that aggregation and reduction of the interspecies distance between inter-dependent microbes is highly advantageous in these complex ecosystems.

Recent studies have suggested that, in many anaerobic systems, formate is an important interspecies carrier of the H_2 that is used to produce CH_4 (Crable *et al*. 2011). In such systems, inhibition of methanogenesis with CH_4 analogues has demonstrated that methanogens that produce small amounts of formate in flocs increase formate production by a factor of 10 in the presence of the CH_4 inhibitor chloroform (Thiele and Zeikus 1988). In contrast, methanogens that used only HCO_3^- and H_2 did not produce any formate when inhibited with the same CH_4 analogue. More recent studies have shown that the synthesis of formate from H_2 and HCO_3^- by pure cultures of methanogens or complex methanogenic consortia was much increased when H_2 utilisation for CH_4 synthesis was inhibited with chloroform, ethanol or bromoethanesulfonic acid (Bleicher and Winter 1994).

In summary, methanogenesis in biofilms or other microbial aggregates is dependent on interspecies transfer of electrons, either via H_2 or formate. In both cases, only CH_4 is released to the bulk medium. When CH_4 analogues are used to inhibit methanogenesis, it is suggested that the inhibition results in the release of formate to the bulk fluid where it is converted to H_2 and CO_2. This would produce only a moderate increase in the partial pressure of H_2 in rumen fluid due to its relatively large volume, and any increase in the biofilm would be small. H_2, being relatively insoluble, would be excreted quickly via the rumen gas cap. The change of site of H_2 production would thus maintain the partial pressure of H_2 commensurate, with oxidation of NADH at the fermentation site on the feed particle. The interspecies transfer requires the methanogens to be closely associated with the fermentative organisms. In flocs, formate transfer appears to be 100 times more important than the transfer of H_2 (Thiele and Zeikus 1988). Formate, being more soluble than H_2, can produce higher concentrations of substrate at the surface of microbes but H_2 diffuses 30 times faster than does formate. On the basis of calculations of diffusion kinetics (Boone *et al*. 1989), formate would be the preferred electron transfer system in planktonic cultures where the carrier molecule has to diffuse over a relatively long aqueous path; transfer of H_2 would be more efficient in densely packed aggregates that dominate in anaerobic digesters. H_2 would probably also be a more efficient carrier in sediments and in other microbial biofilms. It seems the relatively rapid turnover of rumen contents (2–8%/h) requires growth of methanogens to be more rapid than in some other fermentation systems; faster growth necessitates a close relationship between hydrogenotrophic organisms and primary fermentation organisms including bacteria, protozoa and fungi.

Little or no research has been undertaken to evaluate the ability of rumen biofilm microbial consortia to produce and use formate. Although formate is produced as an end product of primary fermentation of cellulose to VFA by *F. succinogenes* (Suen *et al*. 2011), the rate of conversion of formate to H_2 in rumen biofilms is not known for any diet because methods have not been developed to measure formate fluxes within and from the biofilm matrix. If methanogenesis is inhibited, the fluxes will most probably be represented by the passage rates through the rumen-fluid pools (Hungate *et al*. 1970).

Aspects of rumen protozoan metabolism and potential for H_2 transfer between methanogens and protozoa

Rumen protozoa are mainly holotrich and entodiniomorphid ciliates (Williams 1986). The type and biomass of protozoa present depends on diet, feed intake and the feeding patterns. The roles played by protozoa in ruminant nutrition are enigmatic. Ruminants are able to survive and grow without the presence of protozoa in the rumen and, following defaunation, appear to have more dietary and microbial protein available for digestion (for reviews, see Bird *et al*. 1979; Eugène *et al*. 2004). Fauna-free ruminants also have higher microbial-growth efficiencies due to the higher net passage of microbial N to the lower tract, which is usually attributed to the absence of protozoal predation on bacteria (Williams 1986). Fauna-free ruminants also produce a higher proportion of propionate in the total VFA. As a consequence of the higher ratio of propionate to acetate production and the higher microbial cell outflow from the rumen (both of which are electron sinks), defaunated ruminants therefore tend to emit less enteric CH_4 (Morgavi *et al*. 2010).

The population density of protozoa in the bulk fluid of the rumen (the usual site of sampling), however, varies according to the daily feeding regimen. The entodiniomorphid population in rumen fluid decreases for up to 16 h after the animal ingests feed and then increases and returns to the pre-feeding level (Warner 1965). The holotrich population, consisting mainly of species of larger protozoa, e.g. *Isotricha* and *Dasytricha* that are 50–100 μm in diameter and predominant on diets high in

soluble carbohydrates (Valdez *et al*. 1977), declines for a period of 12–20 h after feeding (Williams 1986) but the numbers in rumen fluid return to pre-feeding levels within 4–6 h. The numbers of *Isotricha* spp. and *Dasytricha ruminantium* begin to increase in the fluid phase before (0.5–2 h) feeding (Williams 1986), apparently because they sense the impending availability of substrate. An approximately three-fold increase in numbers of *Dasytricha* and *Isotricha* spp. was observed in the 2-h post-feeding period in cattle given red clover (Clarke 1977) or sugarcane (Valdez *et al*. 1977). In animals fed more often than once a day, a similar rise and fall in numbers in rumen fluid occurs in the shorter time between meals, indicating that protozoal sequestration and re-entry into rumen fluid are related to changes in the presence of feed (Michalowski and Muszynski 1978).

Various hypotheses have been proposed to explain the post-feeding decrease in holotrich numbers and their subsequent return to pre-feeding levels in the fluid phase. The apparent disappearance of the large ciliate protozoa has been attributed to several factors, as summarised by Williams (1986) and listed below.

(1) *Increased dilution rate in the rumen associated with feed intake.*
(2) *Protozoal lysis as a consequence of an over-accumulation of storage polysaccharide.* This suggestion is unlikely to be true because protozoal populations return to the same densities in rumen fluid before, or shortly after, the next meal.
(3) *Settlement of the protozoa in the rumen when their density increases with engulfment of feed particles or the storage of starch-like materials*. This suggestion is not credible because protozoa have been found to attach to materials likely to be part of the mat that floats near the surface of the fluid of the rumen. The rumen is also continuously stirred by muscular contractions that ensure mixing. Nevertheless, large protozoa in rumen fluid do settle to the bottom of test tubes when left to stand.
(4) *Sequestration of the protozoa onto feed particles or onto the wall of the reticulo-rumen*. *Isotricha* spp., for example, have a specialised attachment organelle (Orpin and Hail 1983) that allows them to adhere to plant particles after feeding (Orpin and Letcher 1978). This attachment to feed particles may have evolved as a mechanism that permits protozoa to associate with other microbial consortia that are also attached to surfaces of, or within, feed particles in biofilms. Such an association suggests that protozoa are interacting, and possibly cross-feeding with the biofilm microbial consortia.

The sequestration theory was extended by Abe *et al*. (1981) to explain a four-fold increase in holotrich numbers in rumen fluid in the hour after feeding and the ensuing abrupt decline in numbers. Large numbers of holotrichs were observed to associate with the reticulum wall after an overnight fast (Abe *et al*. 1981). These workers proposed that the holotrichs sequester on the wall of the reticulum and then migrate into the rumen after new feed arrives. The migration into the rumen may represent a response to a chemical stimulus, or the contraction of the reticulum during feed ingestion or in anticipation of feeding. Glucose entering the rumen shortly after feeding has been shown to increase the protozoal density in rumen fluid, probably because holotrich protozoa migrate into the rumen from sequestration sites in the reticulum (Murphy *et al*. 1985).

Protozoa in rumen contents quickly collect methanogens (by engulfment or attachment) after the animal ingests feed, going from virtually symbiants to 10^{-4} per protozoan within 1–2 h (Ushida and Jouany 1996; Tokura *et al*. 1997). This suggests that H_2 produced by protozoa attracts methanogens (as the increase in numbers was too rapid to have been the result of cell division). An alternative possibility is that the protozoa sequester on to feed particle surfaces where they are close to, and can attract methanogens, probably from the outer layers of the biofilm matrix or the walls of the rumen and reticulum. These methanogens may be engulfed by the protozoa or voluntarily detach from the biofilm and re-attach to the protozoal hydrogenosomes where they form clusters. When soluble carbohydrate is freely available, it appears that protozoa quickly acquire enough methanogens to effectively maintain a low partial pressure of H_2 at the sites of conversion of polysaccharides to organic acids. It is also possible that, when attached to particles, protozoa align their hydrogenosomes so that there is a shorter distance between the sites of protozoal H_2 production and the organisms that can provide the most available, energetically favourable electron acceptor. This acceptor will usually be HCO_3^- or CO_2, but other compounds with higher affinity for electrons could be used when available. A further possibility that is worthy of exploration is that direct transfer of electrons may result where two organisms are in physical contact (Morita *et al*. 2011).

Is the reason for the cyclic patterns of appearance of protozoa in rumen fluid related to their need to seek out soluble carbohydrates and/or to reduce cytoplasmic H_2 partial pressure?

The attachment by protozoa to feed particles in the rumen is highly advantageous because this closely associates protozoa with both a continuous source of soluble substrates (largely sugars from hydrolysis of polysaccharides by fibrolytic bacteria) and the partial pressure of H_2 is kept at a low level by associated methanogens. It seems likely that protozoa attach to the surface of feed particles at sites where bacterial and fungal activities are highest, e.g. the partially digested areas where the initial bacterial and fungal colonisers of the biofilm have stripped and solubilised complex plant components, with production of simple sugars which are not used in their own metabolism. For instance, the genome of *F. succinogenes* (one of the most prevalent cellulolytic bacteria) encodes for several enzymes capable of degrading an array of polysaccharides (Suen *et al*. 2011). This species appears to use these enzymes to gain access to cellulose in plant particles by solubilising the compounds surrounding cellulose fibres, but appears to utilise only glucose, cellobiose and cellodextrins to obtain energy for maintenance and growth. It has incomplete pathways (enzymes are not present) for the utilisation of galactose, mannose, fructose and pentose sugars and it makes these monosaccharides available for use by other fermenting bacteria or protozoa. Protozoa are able to take advantage of

these monosaccharides and so are possibly attracted to the sites where fibrolytic activity is highest (Williams 1986), where they anchor themselves (Orpin and Letcher 1978). In addition, they would be close to the methanogenic colonies or have these methanogens attached to their external surface which would ensure they maintain partial pressures of H_2 at the low levels required to enable them to ferment the sugars produced by the hydrolytic bacteria nearby. It is also possible that protozoa with their often large population of associated methanogens (up to 10^{-4}/cell) provide a mobile depot for uptake of H_2 that can quickly move to the sites where fermentation rate is the highest and most H_2 is being produced; they would also be ideally placed to engulf and digest bacteria, obtaining amino acids which, in synchrony with the availability of ATP from fermentation of sugars, would enable them to grow and divide more quickly. By so doing, they may be expected to reduce the population densities of hydrolytic bacteria and perhaps even methanogenic *Archaea* at the sites of the highest cellulolytic activity. This hypothesis, however, is contrary to conclusions arising from the meta-analysis by Eugène *et al*. (2004) who concluded that fibre digestion is consistently lower in fauna-free than in faunated ruminants, while duodenal N flow rate, expressed as a ratio of N intake, is enhanced.

This apparent conflict may be resolved if protozoa associated with feed particles assist in supporting hydrolytic solubilisation of the complex polysaccharides by their high rate of removal of the soluble sugars. This would prevent feedback inhibition of hydrolysis of polysaccharides, in particular by *F. succinogenes*, but also possibly other dominant cellulolytic bacteria such as *Ruminococcus flavefaciens* and *R. albus*. In the absence of protozoa, the fibrolytic activity depends on hydrolysis and fermentation by bacterial and fungal populations and the removal of H_2 by methanogens. In contrast, when protozoa are abundant, they could potentially take up the soluble sugars produced by *F. succinogenes* more rapidly; if allowed to accumulate, these sugars might limit overall fibrolytic activity. Protozoa store these sugars as amylopectin, thereby avoiding the immediate production of H_2 from this source and reducing feedback inhibition of glycolysis by both monosaccharide accumulation and high rates of H_2 production. Overall, this would improve cell-wall carbohydrate digestibility as compared with that in fauna-free animals. The lower fibrolytic activity in the fauna-free rumen is compensated for by a higher net microbial growth (a consequence of less protozoal predation) and a resulting increase in the ratio of protein to energy in the substrate absorbed.

In summary, it is suggested that protozoa spend a considerable proportion of the day as sessile organisms attached to feed particles or the rumen or reticulum wall. When attached to particles, they are closer to the biofilm matrices and microbial colonies where particulate OM is being fermented and, ultimately, H_2, HCO_3^- and organic acids are produced. The protozoa scavenge some of the soluble intermediates and preferentially store them as glycogen-like materials, or utilise them for energy metabolism and growth. The protozoa may also benefit from a close association with the fermentative bacterial consortia where there is a high population of microbial cells facilitating predation. Additionally, they provide a dense mobile population of methanogens that can be attracted to sites where H_2 is being rapidly produced and, when feed is high in soluble sugars, they are attracted into the fluid phase, still carrying adherent methanogenic symbionts; this again provides a more dense mobile concentration of methanogens in the bulk fluid where fermentation of sugar is rapid. Protozoa that detach from feed particles are likely to have more adherent methanogens when they have been associated for long periods with biofilm matrices on feed particles. This explains the observation that methanogens increase in numbers on the planktonic protozoa at too high a rate to be a result of growth. When the soluble sugars in rumen fluid are exhausted, the protozoa move to sites on particles where structural carbohydrates are being more slowly mobilised during the breakdown of plant OM and where the biofilm-embedded methanogens maintain a low partial pressure of H_2. At the same time, they have a source of available protein from the particle-associated bacteria that are mostly hydrolytic microbes.

If the above hypotheses prove to be correct, the explanation for how the rumen adapts to the absence of protozoa and returns to pre-defaunation methanogenesis (Bird *et al*. 2008; Hegarty *et al*. 2008) resides in a simple increase in the hydrolytic bacteria and methanogenic communities in the biofilms attached to plant particles in the fauna-free rumen, with an increase in *Ruminococcus* species at the expense of *F. succinogenes* facilitated by potentially lower levels of those sugars that are not utilised by the latter organism. This argument is supported to some extent by the work of Mosoni *et al*.(2011) who showed that the abundance of cellulolytic bacteria and methanogens was higher in sheep during a long-term (2 years), fauna-free period than it was in faunated sheep. Recognition of (1) the potential roles of biofilm-associated microbes in facilitating fermentation (by maintaining partial pressures of H_2 low in the immediate environment of the microbial consortia) and (2) the apparent plasticity in the end-product production by methanogens are discussed in the following sections, in relation to ongoing research aimed at enteric CH_4 mitigation.

Methane-mitigation strategies and consequencies

Several reviewers have discussed potential mechanisms for reducing enteric CH_4 emissions from ruminant animals (for a comprehensive coverage, see Hristov *et al*. (2013). In general, the most effective way to reduce CH_4 release per unit of production is to reduce the amount of feed an animal used to produce a unit of product. This is best achieved by feeding highly digestible diets with no nutritional deficiencies. Ideally, the diets will be based on cereal grain, thereby allowing the animal to produce to its genetic potential. However, for economic reasons, feed ingredients generally need to be locally available and, in the majority of countries and, in particular, in those considered to be developing countries, the available ingredients will be by-products of plant production (Preston and Leng 1987). Under these conditions, the priority is to optimise production efficiency by management practices. For diets based on local biomass, this will generally be achieved by pre-treatment of feed staples to increase their digestibility and the provision of balanced supplementation (Preston and Leng 1987; Leng 1991, 2004), together with good management practices that minimise any ill-thrift syndromes (Leng 2005).

Mitigation of enteric CH_4 per animal or per unit of digestible feed intake – the priorities

By applying these simple nutritional and management principles, the improvement in utilisation of cereal straw by ruminants that can be achieved has been well demonstrated. In India, milk production, which is largely from cows fed straw or other poor-quality forages, has been markedly improved by the application of good feed management (Banerjee 1994). In the northern wheatbelt of China, cattle growth rates on straw, treated to enhance digestibility and with strategic supplementation, approached 0.9 kg/day, which is 50–75% of the growth rate that could be achieved with similar animals fed grain-based feedlot diets (Dolberg and Finlayson 1995; Cungen *et al*. 1999). At these growth rates, the numbers of animals that can be fattened on the same quantity of untreated straw is increased by 10–13-fold, with a concomitant large decrease in the amount of CH_4 per kg of liveweight or per kg of animal protein produced (see Fig. 3 from Klieve and Ouwerkerk 2007).

Optimising productivity per unit of feed intake is by far the most important approach to lowering the world's enteric CH_4 production from ruminants because most of ruminants exist under poor nutritional conditions (Steinfeld *et al*. 2006). The primary strategies are relatively well established (Preston and Leng 1987) but their implementation is limited by logistic problems. The next priority is to incorporate more 'sophisticated' approaches that may reduce CH_4 production from individual animals with no detriment to production levels. The direct approach to mitigating rumen CH_4 production has been referred to above and will not be discussed further here; the following discussion will focus on manipulation of rumen function to mitigate CH_4 production. However, as the majority of recent research in this area has been undertaken on animals given diets based on high-quality feed resources that will become more expensive in the future, the practicality of rumen-centred approaches should always be critically assessed. Grains, for example, may be more efficiently used in the future for human consumption, or to produce pig and poultry meats. As the demand for animal proteins increases with increasing wealth and population, the demand for ruminant feed has to be met by using locally produced biomass and locally available supplements (Leng 2004; Devendra and Leng 2011).

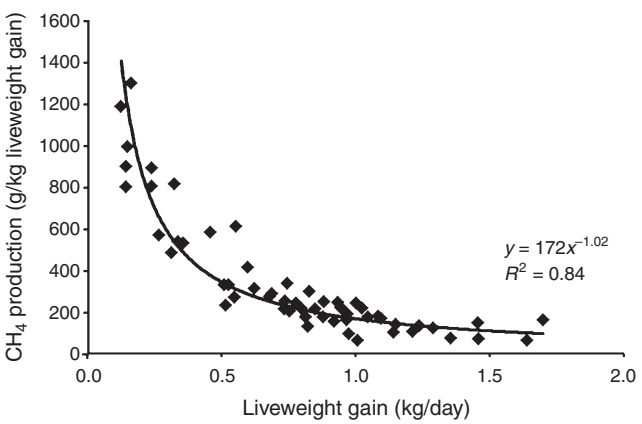

Fig. 3. The relationship between liveweight gain of cattle and enteric methane production per kilogram of gain (Klieve and Ouwerkerk 2007).

The effects of methanogen inhibitors on fermentative metabolism of OM

Methane analogues, including BCM, are potent inhibitors of methanogenesis (Bauchop 1967). Addition of BCM to rumen contents *in vitro*, or to the rumen directly, strongly inhibited CH_4 production (McCrabb *et al*. 1997; Goel *et al*. 2009; Mitsumori *et al*. 2012). The most successful compounds tested *in vivo* have been the chlorinated hydrocarbons, including BCM, 2-bromoethane sulfonate (BES), chloroform and cyclodextrin. Inclusion of any of these compounds in the diets of sheep, goats and cattle has reduced CH_4 production by 50–100% (Immig *et al*. 1996; Lila *et al*. 2004; Knight *et al*. 2011; Mitsumori *et al*. 2012). Notably, when Sawyer *et al*. (1974) added increasing concentrations of BCM to the diet of lambs for 105 days, CH_4 excretion was substantially reduced, with no effects on feed intake, digestibility, molar proportions of VFA in ruminal fluid, or on animal growth and production. In a series of experiments with Brahman-cross steers, Tomkins *et al*. (2009) reported a 93% reduction in CH_4 production when BCM was included in the diet at 0.3 g/100 kg BW. There were no treatment differences in daily liveweight gain, feed intake, feed efficiency and carcass quality. In a study in which goats were given 0.3 g BCM/100 kg BW for 10 weeks, Abecia *et al*. (2012) reported a 33% reduction in CH_4 production per unit of DM intake and an increase in the molar proportion of rumen propionate in the total VFA of nearly 40%. These workers observed a highly significant 36% increase in milk yield, with no difference in DM intake, which was attributed to a higher proportion of propionate in the ruminal VFA production. The increase in propionate proportions in the VFA was largely due to a reduction in branched-chain fatty-acid production. An alternative explanation for the increase in milk yield is that there was a concomitant increase in escape protein from the rumen. This suggestion stems from the fact that branched-chain VFA are formed largely from the fermentation of feed protein, but it would be necessary for the goats to be given a diet such that the protein to energy ratio in the substrates absorbed limited the efficiency of milk formation.

Adaptation to the chemicals occurred in some studies and the CH_4 mitigation effect became lower with time (Johnson *et al*. 1972; Immig *et al*. 1996). However, the effect of BCM appeared to be persistent (Sawyer *et al*. 1974; Tomkins *et al*. 2009; Abecia *et al*. 2012). Recently, Knight *et al*. (2011) found that there was an immediate lowering of rumen CH_4 production in dry cows given chloroform in their diet and the effect persisted for up to 42 days. However, CH_4 production gradually increased to 62% of the pre-treatment levels over this period, indicating that there was some adaptation to chloroform by the rumen ecosystem. When dietary BCM concentration was increased stepwise every 8 days, from zero to 5 g/100 kg liveweight in the diet of sheep, CH_4 production was almost completely inhibited (by 91%), with no effect on diet digestibility (Mitsumori *et al*. 2012). A concomitant decrease in the population of H_2-sensitive cellulose-digesting bacteria (*Ruminococcus* spp.) was observed with an increase in numbers of *F. succinogenes*, a cellulolytic bacterium that produces formate. The alteration to the microbial population occurred both *in vivo* (Mitsumori *et al*. 2012) and *in vitro* (Goel *et al*. 2009).

Mitsumori *et al*. (2012) concluded that the inhibition of methanogenesis was accounted for by the release of H_2, which they predicted but not actually measured (being outside the limits of detection, given the relatively high gas-flow rate through their calorimeters). It seems reasonable, however, in the light of the research discussed above, to accept that methanogenesis was almost completely suppressed, with H_2 gas being released in its place. This observation appears to be contrary to the often-repeated concept that, at high partial pressures of H_2 in the rumen, the oxidation of reduced cofactors generated in the fermentative pathways will inhibit the rates of fermentation of feed materials. This enigma may be resolved if formate is produced by the biofilm consortia, and by fungi in particular (Lowe *et al*. 1987), and is then released into the external fluid and converted to H_2 and CO_2 by enzymes produced by the planktonic bacteria (see *Formate in interspecies H_2 transfer*, earlier in the paper). This concept is further discussed in the next section, in relation to the apparent differences in action on rumen gas production when CH_4 analogues or short-chain nitro-compounds are used to mitigate CH_4 production.

Inhibition of methanogenesis with short-chain nitro-compounds as compared with CH_4 analogues

Studies by Anderson and colleagues have shown that short-chain nitro-compounds such as nitroethane, 2 nitroethanol, 2-nitro-1-propanol and 3-nitro-1-propionic acid, dimethyl-2-nitroglutarate and 2-nitro-methyl-propionate inhibit ruminal CH_4 production *in vitro* (for references, see Anderson *et al*. 2010) and nitroethane and 2-nitro-1-propanol have been shown to reduce CH_4-producing activity *in vivo* (Anderson *et al*. 2006; Gutierrez-Bañuelos *et al*. 2007; Brown *et al*. 2011). These nitro-compounds inhibit both CH_4 and formate synthesis (Anderson *et al*. 2008). Inhibition of methanogenesis is accompanied by increased formate production when BCM (a coenzyme M inhibitor) and other CH_4 analogues are incubated with methanogens or complex consortia of methanogens (Thiele and Zeikus 1988; Bleicher and Winter 1994). In contrast to the majority of inhibitors, these nitro-compounds do not bring about marked changes in the molar proportions of VFA produced by the mixed microbial population (Bleicher and Winter 1994; Anderson *et al*. 2003; Brown *et al*. 2011). Any H_2 released by reversal of cofactor reduction does not yet have an identified sink other than that attributable to reduction of the nitro-compound itself, and on the basis of stoichiometry, this could account for only a small fraction of the H_2 removed (Božic *et al*. 2009). It appears that some other unidentified H_2 sink is being utilised, because feed intake and digestibility are relatively unaffected. Anderson *et al*. (2010) discussed the possibility that the H_2 is used by *Denitrobacterium detoxificans*, an obligate, non-fermentative, anaerobic bacterium that conserves energy via respiration. Alternatively, reductive acetogens (that are present in measurable numbers in the rumen) may be responsible for the H_2 removal. However, at the partial pressures of H_2 maintained in biofilm matrices, these bacteria would probably be outcompeted by methanogens because they have a 10- to 40-fold lower H_2 threshold than do acetogens (Greening and Leedle 1989; Breznak and Blum 1991). Nevertheless, even under 'normal' feeding conditions, acetogens appear to be present in the rumen in significant numbers and this suggests that they can obtain energy without reducing HCO_3^- (Le Van *et al*. 1998). *Balautia producta*, *Eubacterium limosum* and *Acetitomaculum ruminis* are chemolithoautotrophic acetogenic bacteria that have been isolated from the bovine rumen (Boccazzi and Patterson 2013) but are not considered to be the primary H_2-consuming organisms because their numbers are consistently lower than those of methanogens. Many organisms that oxidise H_2 to acetate can also use sugars, and this may be the principal role of these organisms in the rumen.

The factors dictating whether reductive acetogenesis or methanogenesis will predominate in anaerobic environments are not yet fully understood. It appears possible that when nitro-compounds are incubated with rumen fluid, H_2 not accounted for may enter reductive acetogenesis. For example, Pinder and Patterson (2012) showed that an acetogen isolated from rumen contents displayed cellular growth in two phases (diauxie) when incubated with glucose under a gas phase of H_2/CO_2 (80 : 20). Acetate, formate and H_2 were detected during growth on glucose, but only acetate was detected during later growth on H_2 and HCO_3^-. This acetogen would be well suited to a medium receiving intermittent inputs, where sugars that become available after each input are rapidly removed by fermentation and then the organisms switch to utilising H_2 as an alternative source of energy.

The effects of short-chain nitro-compounds on CH_4 production were evaluated *in vitro* in batch cultures of rumen fluid (Anderson *et al*. 2010). After incubation at 39°C for 24 h under 100% CO_2 in ruminal-fluid cultures containing nitro-compounds, the CH_4 production was reduced to 8% of that in control cultures, whereas total VFA production was greater than in the control incubations. Addition of nitroethane appeared to be particularly active in this respect, because acetate production rate was markedly increased (from 41 to 87 mol/L over 24 h) and, theoretically, only 50% of the increase (21 mol/L) was attributable to possible degradation of the nitroethane. The simultaneous increase in propionate and butyrate production in batch cultures containing nitroethane relative to controls also accounted for some of the decrease in CH_4 production. The authors cautiously stated that it was 'attractive' to speculate that, in these cultures and particularly within the nitroethane incubation, conditions may have been conducive to reduction of HCO_3^- to acetate via acetogenesis. An alternative explanation is that the results were an artefact of the incubation technique such that control fermentations were inhibited. For example, CH_4 and small amounts of H_2 would have accumulated in the head space in the control cultures and increased the partial pressure of CH_4 and H_2 in solution. This could inhibit the removal of CH_4 and H_2 from biofilms and so inhibit the amounts of OM fermented, whereas much less gases would have accumulated in the cultures containing nitro-compounds. Head space gas pressures could also lower the amounts of VFA produced by the control incubations. Further research is needed in this area.

It seems possible that, when CH_4 and formate synthesis are both inhibited, acetogenesis rapidly takes over the role of maintaining H_2 partial pressure below the level that would inhibit the regeneration of cofactors needed to maintain the glycolytic breakdown of both simple and complex

carbohydrates in the rumen. This is consistent with the property of self-generation in biofilms of anaerobic ecosystems that have evolved to adapt to changing conditions and to protect the intrinsic conditions needed for bacterial growth and survival and to maintain custom-made communities that can respond to nutrient opportunities (Costerton 2007). As Costerton (2007) observed 'if the particles in an anaerobic digester consolidate their structure, so that a methanogenic core is surrounded by concentric layers of heterotrophs with graded H_2 tolerance', we must be open to the notion that other microbial communities undergo dynamic restructuring.

Nitro-compounds and chlorinated hydrocarbons reduce CH_4 emissions in different ways. As discussed above, it appears likely that CH_4 analogues cause methanogens in the rumen biofilm to switch to formate synthesis (Bleicher and Winter 1994) and this provides a potential explanation for the results obtained in the studies of Mitsumori *et al*. (2012). Production of formate and its diffusion from the biofilm would reduce the H_2 concentration in those organisms that have hydrogenase activity; the formate would leave the biofilm and be then diluted in the large volume of rumen fluid where it can be converted to H_2 and CO_2 by dispersed bacteria. As formate breakdown occurs at a significant distance from the sites of fermentative activity, the H_2 partial pressure in the biofilm would be kept below a level that would inhibit oxidation of NADH, and H_2 rather than CH_4 would be excreted in the eructated gases, cf. studies of the effects of feeding BCM to sheep (Mitsumori *et al*. 2012). Nitro-compounds, however, appear to block both methanogenesis and formate synthesis, thus allowing the partial pressure of H_2 in colonies in the biofilm matrix to initially increase to levels sufficient to promote the growth of acetogens. Further H_2 accumulation in the biofilm would then be prevented by reductive acetogenesis. Supporting the above concept, Anderson *et al*. (2010) showed there was only a small increase in H_2 production in rumen fluid incubated with nitro-compounds as compared with control incubations. When CH_4 inhibition was in response to the presence of BCM, H_2 production appeared stochiometrically to match the decrease in CH_4 production (Mitsumori *et al*. 2012).

Bleicher and Winter (1994) showed that most methanogens were able to produce formate, particularly when fermentative activity and H_2 production were high, but if the partial pressure of H_2 declined, they produced H_2 and CO_2 so as to maintain formate for CH_4 production. In the light of results on formate synthesis from H_2 and HCO_3^- and its re-utilisation by all formate-utilising methanogens, Bleicher and Winter (1994) argued that the concept of interspecies formate transfer proposed by Thiele and Zeikus (1988) should be reconsidered. An alternative explanation as to why formate is produced is that its synthesis pathway has evolved to enable methanogens to outcompete acetogens for H_2. That is, in complex ecosystems with excess H_2, formate synthesis by methanogens may serve as a means of disposing of surplus reducing power that would, if allowed to increase at the site of fermentative activity, promote reductive acetogenesis at the expense of methanogenesis.

Some of the differences in the literature concerning the relative reduction of methanogenesis and H_2 production almost certainly arise from comparisons of *in vitro* and *in vivo* gaseous exchanges. In the intact animal, the rumen gaseous environment is controlled by the production rates of H_2 and CH_4 and their solubilities. *In vivo*, both are removed rapidly from the gas space by eructation and so they do not accumulate in rumen fluid to any extent. The partial pressure of H_2 in the extracellular polymeric substances in the biofilm will be elevated as compared to that in the rumen fluid. In incubations *in vitro*, if the gases are allowed to accumulate in the head space, the partial pressure of H_2 in the incubation fluid may in turn affect the partial pressure of H_2 in the biofilm; for this reason, *in vitro* experiments may not replicate *in vivo* conditions where H_2 is removed by eructation. This effect may be particularly significant where the pressure in the gas space in the incubation vessels is allowed to increase throughout the incubation period. As Bleicher and Winter (1994) pointed out, it is not the H_2 concentration in the gas phase, but the H_2 concentration in the vicinity of the microorganisms that is crucial for formate and CH_4 generation; the same reasoning must apply to reductive acetogenesis.

The potentially high formate concentration in the biofilm relative to rumen fluid was demonstrated by Hungate *et al*. (1970). A dialysis bag containing a buffered salt mix was placed in the rumen for 1 h and the fluid was analysed for formate and compared with the formate concentration in whole-rumen contents. The concentration in the whole-rumen contents was 1000 times that of the dialysate, whereas the concentrations of the acetate, propionate and butyrate were the same at both sites. The formate in the non-fluid contents was attributed to the formate contained in microorganisms, but it is possible that it more accurately represented both intracellular formate and formate in the biofilm matrices. It is potentially possible that formate is retained in microbes, or its diffusion through the biofilm to the external medium is slowed by either chemical or physical binding to the extracellular polymeric substances. Such a mechanism would be advantageous in the potential control of partial pressure of H_2 by methanogens, especially if they can switch on formate production to lower the H_2 pressure at the surface of the biofilm matrix, or increase it by activating formate dehydrogenase. Bleicher and Winter (1994) also argued that formate generation by methanogens is a means for disposal of surplus H_2, which can be reversed when the reducing power is lowered. Stewart (2003) summarised diffusion in biofilms in the following four points:

(1) diffusion is the predominant solute transport process within cell clusters,
(2) the time scale for diffusive equilibration of a non-reacting solute will range from a fraction of a second to tens of minutes in most biofilm systems,
(3) diffusion limitation readily leads to gradients in the concentration of reacting solutes and, hence, to gradients in physiology, and
(4) water channels can carry solutes into or out of the interior of a biofilm, but they do not guarantee access to the middle of cell clusters.

Nitro-compounds such as nitroethane inhibit ruminal methanogenesis by as much as 90% *in vitro* (Anderson *et al*. 2003) and by more than 43% *in vivo* (Anderson *et al*. 2006), via inhibition of formate and H_2 oxidation (Anderson *et al*. 2008). If rumen protozoa depend on associated methanogens to enable

reduced cofactors to be re-oxidised, then protozoal populations should be reduced in the presence of short chain nitro-compounds. In spite of this, there appear to be no reports of the effects of these compounds on ruminal protozoa.

The ruminal effects of promoting NRB and/or SRB by supplementation with alternative electron acceptors (nitrate or sulfate) on methanogenesis, and potential consequences for the microbial ecology

Ruminants given low-protein diets are able to use non-protein N in the rumen, usually in the form of urea or NH_3, to stimulate fermentative digestion and feed intake (see Preston and Leng1987). It has been recognised for some time that nitrate salts can be used to replace urea because both are degraded to NH_3, which is a principal source of N for microbial protein synthesis and growth. In addition, the chemical reduction of nitrate and/or sulfate to NH_3 and/or H_2S in the rumen provides alternative electron sinks and animals given nitrate in the diet have lower CH_4 emissions. Depending on the basal diet, there will always be a balance between the amount of nitrate required to satisfy the fermentable-N requirements of the ruminal biota and the potential reduction in CH_4 production that can be achieved. In addition, the response in CH_4 emission to increases in ingested nitrate is curvilinear. One of the first reports of this effect was that of Sophea and Preston (2011); when nitrate progressively replaced urea in a diet given to goats, the apparent effectiveness of the nitrate in reducing CH_4 decreased (Fig. 4). The reduction in CH_4 production was assessed by the method of Madsen *et al*. (2010), which uses the lowering in the ratio of CH_4 to CO_2 in breath as an index of the proportional reduction in CH_4 release. The response to increased nitrate intake was curvilinear, reaching 60% reduction when all the urea-N was replaced by nitrate-N in the diet, i.e. CH_4 mitigation per unit of nitrate decreased with increasing nitrate supply. Without knowing the CH_4 production rate, however, the absolute reduction in CH_4 production rate cannot be calculated.

Studies by Hulshof *et al*. (2012) in cattle in which nitrate progressively replaced dietary urea also showed a progressive decline in the actual CH_4 mitigation compared with the theoretical reduction; the latter was based on the stoichiometric prediction that 100 g of dietary nitrate reduced to NH_3 should lower CH_4 emissions by 25.8 g (assuming that all of the added nitrate was converted to ammonia in the rumen and the resulting reducing equivalents were used solely for CH_4 mitigation). The available data on this ratio (termed here 'fractional CH_4 reduction') from reported studies where nitrate has replaced urea in ruminant diets have been summarised by (van Zijderveld 2011; see Fig. 5).

There was a negative correlation between the amount of nitrate given per kilogram of metabolic bodyweight and the fractional CH_4 reduction. The apparently inefficient use of nitrate has been suggested to originate from differences among animal species or incomplete nitrate reduction in the rumen (van Zijderveld 2011) or from re-direction of VFA production toward propionate production in the rumen. This difference in CH_4 mitigation by dietary nitrate was attributed to the production of more reduced end products (e.g. propionate or microbial cells) in the rumen in

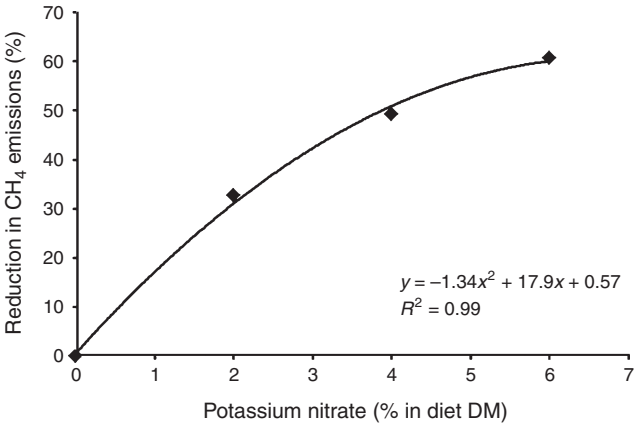

Fig. 4. The reduction (%) in methane production as potassium nitrate replaced urea in a diet offered to goats (Sophea and Preston 2011).

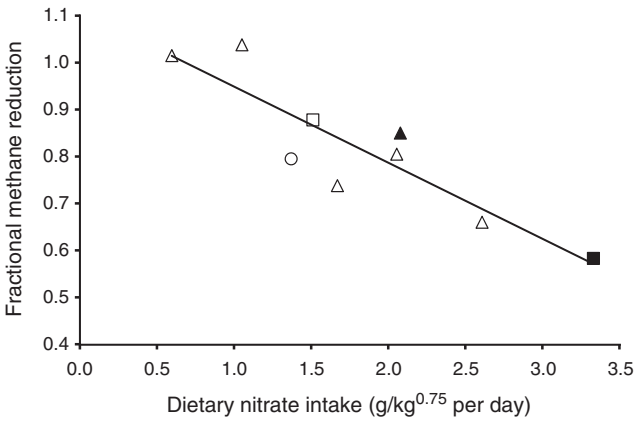

Fig. 5. Apparent efficiency of methane reduction or fractional reduction in methane emission in response to increasing intakes of nitrate fed to various ruminant species (van Zijderveld 2011).

dairy cows fed high-concentrate diets (van Zijderveld *et al*. 2011) than in sheep and beef cattle on much more fibrous feeds (Nolan *et al*. 2010; van Zijderveld *et al*. 2010). However, in the studies reported by van Zijderveld (2011) in cattle ingesting the same basal ration, the apparent fractional CH_4 reduction decreased with increasing dietary nitrate supply, confirming the results of Sophea and Preston (2011).

The potential explanation for the apparently low efficiency of CH_4 mitigation by dietary nitrate based on changes in metabolism of VFA in the rumen (syntrophic butyrate and propionate oxidation to acetate) is not straight forward. There appear to be at least three major (or combination) reasons that bring about the apparently inefficient fractional CH_4 reduction as nitrate concentrations in a diet are increased, as follows:

- nitrate or nitrite produced in the rumen is absorbed and excreted in the urine,
- nitrate alters the microbial ecology of the rumen and stimulates additional H_2 production when compared with urea as the fermentable N source, and
- nitrate stimulates formate production by methanogens which diffuses into the bulk fluid and is converted to H_2 that is removed by eructation.

If the first is correct, then nitrate and/or nitrite excretion in the urine could result in the release of nitrous oxides to the atmosphere, which carries high greenhouse-gas implications that could offset the benefits of the reduction in CH_4 production. This would be a major impediment to the acceptance of nitrate supplementation of ruminants as a means of mitigating greenhouse-gas production. At the highest reported level of intake of nitrate, which was by dairy cows, the fractional CH_4 reduction from added nitrate was 59%. If 41% of the nitrate had not been reduced to NH_3, a deficiency of rumen-degradable N could have been expected to reduce feed intake and digestibility and lower milk yield. However, this did not occur. An alternative explanation is that replacing urea with nitrate resulted in a change in microbial communities in the rumen, leading to changes in the production of acetate relative to propionate and butyrate, and an increase in H_2 production. When nitrate replaced urea in the rumen, there was also a tendency for an increased microbial cell yield (cells also represent an electron sink) (Nolan *et al*. 2010). Increased acetate relative to propionate production may result from a channelling of more carbohydrate through pyruvate and acetyl CoA in the fermentative pathways, or it could be a result of acetogenic oxidation of butyrate and propionate by the boosted nitrate-reducing capacity of rumen contents and increased populations of NRB (Alaboudi and Jones 1985) and SRB. The latter, from animal and human large intestine, have been shown to oxidise propionate and butyrate to acetate (Gibson 1990).

In anaerobic environments rich in OM, nitrate stimulates the growth of syntrophic organisms through changes in the partial pressures of H_2 within the particle-associated biofilm consortia. It is argued above that the reduction of nitrate to NH_3 occurs in the biofilm communities associated with feed OM present in the rumen. As shown by Bleicher and Winter (1994), for many pure cultures of methanogens and for a complex sewage-sludge culture, some formate was formed as an intermediate during growth on H_2 and CO_2. High concentrations of formate were formed from H_2 and HCO_3^- when conditions for methanogenesis were impaired by the presence of CH_4 analogues such as bromoethanesulfonic acid or chloroform, or an elevated redox potential in co-cultures with nitrate reducers. The formation of formate rather than H_2 under such circumstances would allow the production of small amounts of H_2, e.g. when inclusion of nitrate in the diet of sheep lowered CH_4 production substantially (van Zijderveld *et al.* 2010).

The potential for syntrophic oxidation of butyrate and propionate in the rumen in nitrate/sulfate-supplemented ruminants

Syntrophism is essential in CH_4 production, which involves an interaction between H_2 and formate-producing microbes with H_2 and formate-using partners. The Gibbs free-energy changes involved in syntrophic metabolism are very low, i.e. close to the minimum free-energy change needed to sustain microbial growth. In single cultures, the oxidation of butyrate to acetate and H_2 is energetically unfavourable. However, when methanogens are co-cultured with bacteria capable of butyrate oxidation, methanogenesis significantly lowers the concentration of H_2 (down to 10^{-5} atm or ~1 Pa) and thereby shifts the equilibrium of the butyrate oxidation reaction to non-standard conditions. The concentration of one product is lowered and the reaction is shifted towards net energetically favourable conditions (for butyrate oxidation: $\Delta G^{\circ\prime} = +48.2$ kJ/mol, but $\Delta G' = -8.9$ kJ/mol at 10^{-5} atm H_2). The higher affinity of SRB or NRB for H_2 can lower the partial pressure of H_2 at the site of butyrate oxidation more rapidly, and to a greater extent in the biofilm matrix (Lovley and Godwin 1988), potentially increasing the growth rates of butyrate-oxidising bacteria. Methanogens appear to be unable to use H_2 below partial pressures of 6.5 Pa (Lovley 1985). However, the threshold partial pressures of several methanogens have recently been shown to vary from 1 to 4.7 Pa and the most dominant methanogen in the rumen (*Methanobrevibacter* spp.) was reported to have a H_2 threshold of 4.7 Pa (Kim 2012).

Butyrate-oxidising organisms similar to *Syntrophomonas wolfei* have been isolated from the rumen (McInerney *et al*. 1981) and have been shown to oxidise butyrate to acetate when H_2 partial pressures are maintained at extremely low levels by co-culture with methanogens (Lovley and Godwin 1988). The same species is also known to oxidise propionate to acetate. The situation is complicated further by the fact that syntrophic propionate-oxidising bacteria also appear to be able to reduce sulfate (Schink 1997) and perhaps nitrate (Moura *et al*. 2007). Nitrate, having a much higher affinity than CO_2 for electron capture, may maintain the H_2 partial pressure low enough to enable these organisms to proliferate and this could explain the lowered net production of butyrate when nitrate replaces urea in diets (Farra and Satter 1971) (see later in the text). These organisms also obtain ATP for growth from these reactions (Schink 1997), which would increase the net microbial growth in the rumen. It has been calculated that the rate of OM degradation in H_2-syntrophic co-cultures is dependent on the efficiency with which H_2-consuming organisms can grow when concentrations of H_2 are low (Cord-Ruwisch *et al*. 1988). In the light of these observations, the terminal electron acceptor in anaerobic systems may be the limiting factor for the rate of substrate oxidation. This fits with the observation that syntrophic co-cultures grow more rapidly with sulfate reducers than with methanogens as H_2 scavengers (McInerney *et al*. 1981), and therefore growth of *S. wolfei* with nitrate should be higher than that with sulfate or HCO_3^- as terminal H_2 acceptors. In support of this concept, Lovley and Godwin (1988) showed that the H_2 concentrations associated with the specified predominant terminal electron-accepting reactions in bottom sediments of a variety of surface-water environments were as follows: methanogenesis 7–10 nM; sulfate reduction, 1.5 nM and nitrate reduction, <0.05 nM.

The most successful hydrogenotrophic species in any anaerobic system is the one that keeps the H_2 partial pressure below the level that is necessary to allow H_2 uptake by competitors and so SRB should have lower H_2 threshold levels than do methanogenic bacteria. In fact, it has been demonstrated that thresholds of H_2 oxidation were about one order of magnitude lower in sediments that contained sulfate as well as bicarbonate as electron acceptors (Lovley *et al*. 1982). In addition, the threshold levels of H_2 for nitrate reduction are extremely low (Cord-Ruwisch *et al*. 1988; Kim 2012), indicating that nitrate would establish the most favourable

growth conditions for syntrophic metabolism of butyrate or propionate to acetate.

Calculating the potential additional H_2 production as a result of syntrophic metabolism in the rumen

The NRB (which are probably also capable of reducing S) are able to utilise several organic compounds and produce H_2 and C intermediates for the synthesis of cells. Two electron donating reactions are shown below. These reactions could explain the changes in VFA production patterns in rumen fluid that occur when nitrate is introduced into the diet (Farra and Satter 1971). Stimulation of the growth of the syntrophic microbes, with concomitant increase in H_2 could also explain a lower apparent efficiency of CH_4 mitigation when dietary nitrate is reduced to NH_3.

$$\text{Propionate}\rightarrow\text{acetate}: CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + H^+ + HCO_3^- + 3H_2,$$

$$\text{butyrate}\rightarrow\text{acetate}: CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2.$$

On the basis of stoichiometry, each 100 g of nitrate reduced to ammonia in the rumen would lower CH_4 production by 25.8 g and, if ~40% of the nitrate escaped reduction in the rumen (van Zijderveld *et al*. 2011), then CH_4 production would be lowered by 10.3 g CH_4 or 0.645 mol CH_4. Four mol of H_2 are required to produce 1 mol of CH_4, so an additional H_2 production of 2.58 mol would account for the apparent inefficiency in CH_4 mitigation. The 2.58 mol of H_2 unaccounted for could be produced by the conversion of 0.86 mol of propionate, or 1.29 mol of butyrate to acetate, occurring in response to changes in the microbial ecology as a result of the presence of nitrate.

From measurement of VFA production rates in the rumen of beef cattle fed diets based on ground corn, Sharp *et al*. (1982) found that the net production of acetate was 34 mol/day, of propionate 16 mol/day and of butyrate 6.3 mol/day. In the CH_4-mitigation studies discussed above, the intake of of DM from the total mixed ration by dairy cows was three times the feed intake of the beef cattle (19 kg/day versus 6.2 kg/day). Assuming a roughly three-fold VFA production in the rumen of dairy cows as compared with the beef animals and an intake of 400 g nitrate/day by the dairy cows, then the amount of propionate needed to be converted to acetate to account for the actual reduction in CH_4 production relative to that calculated is ~6–7% of production. If, however, butyrate oxidation was the only pathway affected by nitrate, then 29% of the butyrate would need to be converted to acetate. These calculations are being made here only so as to gauge the magnitude of the changes that may occur to the production of rumen metabolites and, therefore, the feasibility for this being a result of the change in the microbial ecology discussed above when switching from urea to nitrate as the fermentable N source in a diet.

From a review of seven publications, where inter-conversions of VFA in the rumen have been calculated from isotope-dilution studies, 0.45–15.4% of acetate was produced directly from propionate and 1–21% of the acetate was directly produced from butyrate in the rumen (Sharp *et al*. 1982). These data provide evidence for syntrophic oxidation of propionate and butyrate (Schink 1997) to occur to a limited extent under a variety of feeding conditions. None of these studies included nitrate as a component of the diet; however, the direct utilisation of these VFA could have been attributed to the activity of SRB/NRB that can utilise a diverse range of substrates (Muyzer and Stams 2008).

Farra and Satter (1971) showed that the ratio of acetate in total VFA in rumen fluid increased after adaptation of dairy cows to a nitrate-based diet. Following adaptation to nitrate in a feed (4% of DM intake), the percentages (mol/100 mol total VFA) of these VFA changed as follows: for acetate from 62.3% to 80.2% (increased by 22.3%); propionate from 19.6% to 14.7% (decreased by 25%); and butyrate from 16.1% to 5.0% (decreased by 69%). A lower level of nitrate (2% of DM intake, which was similar to that fed by van Zijderveld *et al*. 2011) increased acetate (mol %) from 51.5% to 65.7% and decreased propionate (mol %) from 33.6% to 20.9% without altering butyrate proportions. Nolan *et al*. (2010) reported a similar response in VFA proportions in rumen fluid when sheep fed oaten hay in equal meals each hour were given nitrate to replace urea in the diet, i.e. the acetate to propionate ratio increased markedly (from 3.22 to 4.28) and butyrate proportions were reduced. It appears that the effect of nitrate on VFA production increases with increasing feed intake. There would therefore be markedly different responses to dietary nitrate depending on the experimental feeding strategies; in addition, the effects in animals fed at frequent intervals could be quite different from those fed once daily. In the research of van Zijderveld *et al.* (2010), the VFA concentrations and proportions were not altered when urea N was quantitatively replaced by nitrate N but the samples analysed were collected 24 h after the meal was offered. The results may be misleading because Farra and Satter (1971) observed an increase in the acetate to propionate ratio in total VFA and a fairly rapid fall in butyrate proportions in the rumen fluid of cows, following ingestion of feed containing nitrate, but both measures returned to 'normal' after the nitrate was apparently fully reduced to NH_3. Hulshof *et al*. (2012) studied cattle fed nitrate in a sugarcane–maize silage diet and found that dietary nitrate lowered CH_4 production by 32% at an apparent mitigation efficiency of 87%. These workers reported a small increase in the proportion of acetate relative to propionate but no effect on the butyrate percentage (mol/100 mol) in the total VFA in rumen fluid.

Clearly, the diet must have considerable influence on the microbial ecology of the rumen and on the response when urea is replaced with nitrate as a fermentable N source. However, it does appear that any change in the microbial ecology leads to higher levels of H_2 production, and this, in turn, increases the requirement for electron acceptors. In the past, a change in the proportions of VFA has been attributed to whether carbohydrate fermentation is directed into the more or less reduced VFA. Generally, metabolism of VFA is associated with a relatively low energy yield (ATP) and slow growth of organisms, which is more similar to sludge fermentations. The relatively high liquid turnover rate in the rumen (2–8%/h) often prevents their establishment in the rumen of most secondary fermentative (acetoclastic) organisms because of their slow

growth rate. Nevertheless, it is emphasised here that NRB appear to have growth rates high enough to enable them to maintain high population densities in the rumen. Dwyer *et al*. (1988) described an organism termed NASF-2 which is a strictly anaerobic, non-spore-forming, acetogenic, H_2-reducing, butyrate-oxidising bacterium that resembles *S. wolfei*. When given optimum conditions (i.e. a low partial pressure of H_2), this organism grew exponentially and had a doubling time of 10 h, which would be compatible with substantial growth in the rumen.

Potential mechanism to overcome H_2 accumulation and inhibition of hydrogenase activity

Formate production as a potential mechanism to overcome H_2 inhibition of hydrogenase activity

Formate synthesis may play an important role in the control of H_2 partial pressure in rumen digesta. In most studies in which attempts have been made to mitigate enteric CH_4, formate production has not been monitored. Normally, the partial pressure of H_2 in methanogenic biofilms is relatively low because the H_2 is used immediately for CH_4 production and so there is minimal production of formate. However, when the methanogens are partially inhibited, formate may be produced and released into the bulk rumen fluid as reported for flocs in biodigesters (Thiele *et al*. 1988). Planktonic organisms that have a formate dehydrogenase would then rapidly convert the formate to H_2 and CO_2 (Doetsch *et al*. 1953). Hungate *et al*. (1970) demonstrated that the amount of formate in rumen contents was 1000-fold higher than that in particle-free rumen fluid (a dialysate of rumen digesta), even though the total amount present was still extremely small. This could indicate that most of the formate is present in biofilms. When methanogenesis is inhibited, formate may be produced in the rumen microbial consortia associated with digesta particles, thereby ensuring a low H_2 concentration that would not inhibit bacterial hydrogenase activity; accordingly, renewal of functional coenzymes in the fermentative organisms closely attached to and within the biofilm matrix would still be possible. This hypothesis is supported by research with *R. flavefaciens* (Shi *et al*. 1997), indicating that, at high growth rates, this species produces formate by reducing HCO_3^-; at lower growth rates, H_2 is formed via hydrogenase. Similarly, when reducing equivalents are in higher concentration, the other dominant cellulolytic species (*R. albus*) also reduces HCO_3^- to formate (Miller and Wolin 1973; Asanuma *et al*. 1990). Formate production by anaerobic fungi could also be involved (Lowe *et al*. 1987). In fungi, a shift in fermentation toward formate production seemingly maintains low H_2 partial pressures. The available evidence suggests that reducing equivalents may be balanced through formate or H_2 production, without affecting the yields of the major C-containing fermentation end products. In the rumen digesta, methanogenesis or HCO_3^- reduction to formate are both systems that compete for available H_2. A small proportion of both formate and H_2 will normally diffuse into the external rumen fluid where formate is converted back to CO_2 and H_2. Both gases may be excreted together with CH_4 via the gas cap in the rumen.

A similar scenario occurred in flocs from a whey-processing digester (Thiele and Zeikus 1988). Formate production by digester contents or purified digester flocs was dependent on HCO_3^- and either ethanol or lactate, but not H_2, as an electron donor. During syntrophic methanogenesis, flocs were the primary site for formate production via ethanol-dependent HCO_3^- reduction. Floc preparations accumulated formate, reaching concentrations four-fold higher than digester contents. The formate was generated from reduction of HCO_3^-, as the formate production continued when methanogenesis was inhibited by chloroform and the primary site for formate cleavage to CO_2 and H_2 was the dispersed flora. More than 90% of the syntrophic conversion of ethanol to CH_4 by mixed cultures containing mainly *Desulfovibrio vulgaris* and *Methanobacterium formicicum* was mediated via interspecies formate transfer and less than10% was mediated via interspecies H_2 transfer. Mixed consortia of sewage sludge or pure cultures of methanogens (both H_2 and formate utilising) generated some formate, even at high partial pressures of H_2 (Bleicher and Winter 1994). When partial pressures of H_2 decreased, the formate was taken up again and converted to CH_4. If methanogenesis was inhibited by BES, methanogens with the ability to use formate for methanogenesis produced formate from H_2 and HCO_3^-. No formate was excreted by methanogens that could use only H_2 and HCO_3^-.

The conundrum presented by the lack of effect of high H_2 production rates in the rumen on feed utilisation when CH_4 analogues are administered can be explained if the methanogenic *Archaea* are capable of synthesising formate and using it as the carrier for interspecies transfer of H_2 when methanogenesis is inhibited. When H_2 partial pressures are relatively high, formate-producing *Archaea* may become more dominant and prevent further increases in the partial pressure of H_2. In support of this hypothesis, methanogenic bacteria with coccobacillus morphology – similar to *Methanobrevibacter ruminantium* isolated from bovine rumen fluid – grew rapidly and metabolised formate extremely quickly (Lovley *et al*. 1984). However, recent research has indicated that, even though the short-chain nitro-compounds are inhibitory to methanogens, formate is not always produced (Anderson *et al*. 2008).

A curious effect when feeding BCM was that, even when CH_4 was reduced by 91%, protozoal numbers in the rumen were not affected (Mitsumori *et al*. 2012). As noted previously, rumen protozoa share a symbiotic relationship with methanogens and participate in interspecies H_2 transfer; this transfer provides methanogens with the H_2 they need to reduce HCO_3^- to CH_4 and thereby continue to function. It has been estimated that the methanogens associated, both intracellularly and extracellularly, with the ciliate protozoa are responsible for 9–37% of the CH_4 production in the rumen. However, the dependency of protozoa on methanogens to re-oxidise NADH and allow glycolysis to continue must be questionable, unless the methanogenic symbionts also produce formate (Hook *et al*. 2010). If rumen methanogens produce formate that is subsequently converted to H_2 and CO_2 that are eructated by the animal, then direct inhibition of CH_4 is an inappropriate way to mitigate unwanted gas emissions from ruminants because H_2 is just as potent a greenhouse gas as CH_4.

Reductive acetogenesis

Creating conditions to support the growth of reductive acetogens is a further strategy for maintaining a low partial pressure of H_2 at the fermentative sites in the rumen digesta (Joblin 1999). However, in the rumen, methanogens usually outcompete acetogens for H_2. The usual reason put forward for this is that the reduction of CO_2 to acetate is thermodynamically less favourable than the reduction of HCO_3^- to CH_4 (McAllister and Newbold 2008). In this connection, however, it is interesting to note that CH_4 is not produced in the fermentative areas in the gut of macropods (Kempton *et al*. 1976) where methanogenesis is apparently replaced by reductive acetogenesis (Ouwerkerk *et al*. 2009).

Acetogens appear to be present in the rumen in numbers similar to or slightly lower than those of methanogens (Leedle and Greening 1988) and are present in higher numbers in gnobiotic lambs (Le Van *et al*. 1998; Fonty *et al*. 2007). Also, acetogens are capable of interspecies H_2 transfer when in co-culture with *R. albus* (Miller 1995). However, the system seems not to support acetogens in the presence of methanogens (Fonty *et al*. 2007). Growth of acetogens in the rumen is probably limited by their inability to establish in the biofilm consortia close enough to the site of H_2 production, to enable them to compete effectively for H_2 at the partial pressure maintained by the methanogens. However, in the presence of chlorobromoethane, H_2 is produced in amounts that should induce an increase in the partial pressure of H_2, without stimulation of reductive acetogenesis. In all probability, acetogens are unable to replace methanogens, numbers being kept low by a combination of Gibbs free-energy change (leaving them relatively less competitive) and their need to associate more closely with the acetogenic fermenting microbes to have any chance of competing. Close proximity to acetate-producing organisms would induce a higher acetate concentration at the site in the biofilm and this would result in feedback inhibition when acetate is produced by HCO_3^- reduction.

A detailed understanding of how kangaroos support acetate synthesis from H_2 and HCO_3^- in their forestomach would enhance the possibility that this pathway might be introduced effectively in the rumen. There appears to be little information as to the extent of biofilm formation in the tubiform foregut of kangaroos but the uni-directional propulsion of feed through the forestomach may restrict the potential for inoculation of feed particles with hydrolytic and syntrophic organisms. In ruminants, inoculation of feed particles may be largely through rumination (Leng 2011). Although contraction and some regurgitation (termed *merycism*) occurs in the forestomach of macropods, this is a sporadic event (Hume 1982) and inoculation of feed with microbes may be facilitated by the blind sac – a pouch formed at the junction of the oesophagus and the forestomach. This pouch may provide a reservoir of microbes similar to that in the vermiform appendix of the large bowl of humans. This appendix is now believed to be a reservoir of microbes that form colonies with adherent biofilms in the bowel following recovery after their collapse under, for instance, antibiotic treatment (Bollinger *et al*. 2007).

It seems possible that acetogenesis rather than methanogenesis is promoted when the establishment of biofilms or the inoculation of digesta is slow and new feed is poorly mixed with residual contents. There is also the possibility that stomach anatomy is important in determining the microbial ecology of the forestomach of ruminants compared with kangaroos. The rumen has evolved to quickly expel gases produced in fermentation. Gases produced collect rapidly in the gas cap or dome and the strong mixing contractions of the rumen result in frequent eructation with gases either entering the mouth directly, or being drawn into the lungs before being expelled in breath (Dougherty 1968).

The solubilities of CH_4 and H_2 in water at 37°C are ~0.016 g/kg and 0.0014 g/kg, respectively. Thus, if methanogenesis occurred in the kangaroo forestomach, in the absence of a mechanism such as eructation, it might quickly increase the CH_4 concentration in the biofilm consortia and the bulk fluid. This in turn could initially stimulate H_2 production, with a concomitant increase in partial pressures to a level compatible with establishment of reductive acetogenesis. Such interplay between the concentrations of gases could be responsible for maintaining a reductive acetogenic population that generates less net-gas production. When methanogenic *Archaea* are inhibited by CH_4 analogues, formate is produced (Bleicher and Winter 1994) but is quickly converted to CO_2 and H_2 by fermenting organisms. Thus, if formate were produced in the kangaroo forestomach, it would not necessarily reduce the partial pressure of gases. It, therefore, seems that acetate synthesis may offer the only means of relieving the partial pressure of H_2 within the biofilm. The biofilm mode of fermentation of OM appears to be very delicately balanced by the partial pressures of gases capable of end-product inhibition, but this interplay appears to be crucial for the mitigation of CH_4. Further discussion of the potential for reductive acetogenesis to be bolstered in the presence of nitro-compounds is provided in *Inhibition of methanogenesis with short-chain nitro-compounds as compared with CH_4 analogues*, earlier in the paper.

Methanotrophic activities

Methane emissions from biological systems represent a balance between production by methanogenic *Archaea* and oxidation by methanotrophic microorganisms. CH_4 oxidation has been reported in both aerobic and anaerobic environments (Hanson and Hanson 1996). Stocks and McCleskey (1964) isolated CH_4-oxidising bacteria from the rumen of steers that were similar to methanotrophic anaerobes isolated from soil and water and Mitsumori *et al*. (2002) demonstrated that methanotrophs were present in both rumen fluid and in biofilm attached to the rumen wall. However, studies using an artificial rumen indicated that an insignificant amount of the CH_4 flux was anaerobically oxidised by a reversal of methanogenesis, with sulfate as the terminal electron acceptor (Kajikawa and Newbold 2003; Kajikawa *et al*. 2003).

Recent studies have demonstrated that the application of biochar to soils supporting rice production lowered CH_4 release (Liu *et al*. 2011) and this was a result of increased numbers of methanotrophic proteobacteria. Biochar

amendment greatly increased the ratio of methanotrophic to methanogenic abundances in paddy soils (Feng *et al*. 2012). The possibility of increasing methanotrophic activity in the rumen in a similar manner led to a hypothesis that increasing microbial habit with material such as biochar, which has a large surface area to weight ratio, might reduce the net rate of CH_4 production (Leng *et al*. 2012*a*). To test this hypothesis, biochar was added to an *in vitro* incubation of rumen fluid; the presence of biochar resulted in a 15% reduction in CH_4 release (Hansen *et al*. 2012; Leng *et al*. 2012*a*, 2012*b*, 2012*c*). Biochar added to diets of cattle also decreased their CH_4 emissions and, at the same time, increased the efficiency of liveweight gain (Leng *et al*. 2012*b*; Sophal *et al*. 2013). The following question is raised by the research with biochar: does the relatively large surface area and highly porous structure of biochar provide a favourable habitat for the organisms involved in a methanogenic–methanotrophic interaction, increasing the potential for anaerobic CH_4 oxidation? Recently, research revealed a further property of biochar that may be important in this context; it seems that electrical conductivity of biochar surfaces may facilitate direct electron transfer among syntrophic organisms (S. Cheng, A. E. Rotaru, N. S. Malvankar, F. Liu, K. Nevin, D. R. Lovley, pers. comm.).

Brunauer–Emmett–Teller (BET) surface area is a measure of the ability of a material to absorb gases and also of its accessible surface area for microbial attachment. Biochars often have BET surface areas of 2–40 m^2/g but biochars with much greater surface areas may be produced by particular production technologies. The use of biochar and/or activated charcoal in ruminant diets has been shown to mitigate enteric CH_4 (Leng *et al*. 2012*a*, 2012*b*, 2012*c*). A concentrated research effort is needed to refine the mode of action.

Direct interspecies electron transfer

Direct interspecies electron transfer (DIET) may be a more effective mechanism for interspecies electron exchange under anaerobic conditions than is indirect transfer via reduced molecules such as H_2 and formate. Improved rates of CH_4 production in biodigesters after inoculation with activated charcoal have been shown to result from a more rapid exchange of electrons between bacteria and methanogenic *Archaea* attached to the surface of charcoal by conduction across its surface (Liu *et al*. 2012). The demonstration that charcoals enable direct electron transfer suggests that stimulation of metabolism in methanogenic digesters may be attributed, at least in part, to better interspecies electrical connections than those forged biologically. Biochar appears to have similar properties in promoting direct electron transfer (S. Cheng, A. E. Rotaru, N. S. Malvankar, F. Liu, K. Nevin, D. R. Lovley, pers. comm.), but in the rumen it is suspected that biochar surfaces provide habitat for more efficient and rapid microbial growth that may also favour a closer relationship between methanotrophs and methanogens. DIET was found to be an important process for interspecies electron exchange in multi-species aggregates from a methanogenic digester in which *Geobacter* and *Methanosaeta* spp. predominated (Morita *et al*. 2011).

Conclusions

The solubilisation of plant OM within biofilms in the rumen has bestowed numerous advantages on the ruminant animal; in particular, it provides a highly efficient mode of digestion of structural components of plants, yielding nutrients the animal can absorb (organic acids) or digest (microbial cells) to meet their nutrient requirements.

That biofilms provide a micro-environment with opportunities for important interactions between microbes is apparently often not taken into account in studies aimed at mitigating enteric CH_4. An in-depth understanding of microbial ecology is a valuable asset when attempting to manipulate anaerobic microbial ecosystems, and ongoing research of biofilms in the ruminant digestive system is a high-priority research area. Biofilm communities in the rumen appear to be self-organising and they adapt to changes in the animal's diet or other perturbations. The resilience of these microbial structures and their ability to elicit changes in either the composition of colonies, or the end products excreted, suggests that simply surveying for substances with anti-methanogenic properties may not be rewarding. The accidental discovery that chlorinated hydrocarbons inhibit methanogenesis made by Bauchop (1967) has led to many subsequent studies aimed at inhibiting methanogenesis in the rumen by feeding CH_4 analogues. Other workers have surveyed a large number of CH_4 inhibitors from natural sources.

The direct inhibition of methanogenesis by blocking metabolic pathways (e.g. using CH_4 analogues or nitro-compounds) is probably impractical. Research, particularly into the biochemistry of waste-water treatment, has shown that when the methanogenic pathway of H_2 uptake is inhibited, the *Archaea* switch to produce formate from H_2 and CO_2. Mitsumori *et al*. (2012) fed BCM to sheep and this inhibited CH_4 production by over 90%, but with a concomitant increase in the production of H_2. Unfortunately, the H_2 emissions from the animals would have the same greenhouse consequences as do CH_4 emissions. The consequences of inhibiting methanogenesis with BCM and perhaps many other natural feed ingredients can be explained as follows: the rumen *Archaea* adjust by producing formate, thereby maintaining the required low partial pressure of H_2 within the biofilm matrix. The formate diffuses to the external rumen fluid where it is reconverted to H_2 and CO_2 by the planktonic microbes. Because H_2 solubility in water is low, H_2 is quickly released from the rumen fluid into the rumen gas cap. The best evidence for this hypothesis was provided by Bleicher and Winter (1994) who demonstrated that methanogenic consortia from sludge, and also those methanogens in culture that utilise formate as a means of interspecies H_2 transfer, become net producers of formate from H_2 and CO_2 when methanogenic pathways are blocked. Importantly, the conversion of formate to H_2 and CO_2 is a reversible process in methanogens that possess enzymes capable of both HCO_3^- reduction and oxidation (Crable *et al*. 2011).

Nitro-compounds have been shown to inhibit CH_4 and formate synthesis and yet have only minor effects on other aspects of digestion. It is argued above that the biofilm consortia have evolved several survival and growth strategies, including the ability to recruit different microbial species to

adjust to changing nutrient availabilities, threats from toxic compounds and other changes in the local environment. From the above discussion, it appears that the partial pressure of H_2 is a component of mechanisms that control the microbial diversity within the biofilm and therefore the extent and composition of fermentative end products. In particular, maintaining an appropriately low H_2 partial pressure at the site of fermentative activity is a critical strategy; depending on the H_2 partial pressure, the electron acceptors may be CH_4, formate or acetate or, in the presence of nitrate or sulfate, NH_3 and H_2S, respectively.

If the direct chemical inhibition of CH_4 production results in the methanogens using formate as an electron sink, which is then released and metabolised to H_2 and CO_2 in ruminal fluid, there is little to be gained by survey research for natural or synthetic CH_4 inhibitors unless their effects on formate or H_2 production are also tested. The ability of methanogens to produce formate provides greater justification for investigations into the potential role of dietary substances that act as alternative electron acceptors, such as nitrate and sulfate. As well as acting as electron acceptors, the presence of these substances may also be responsible for major changes to the microbial ecosystem and so further research is required to optimise their benefits. Concepts such as improving the microbial habitat or stimulating direct electron transfer through electrical conductance, e.g. by including biochars in the diet, may be applicable and may increase the efficiency of microbial growth. A focussed research effort to better understand the role of rumen protozoa and ways by which they interact with the methanogens is also a priority.

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