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Importance of hydrogenotrophic, aceticlastic and methylotrophic methanogenesis for methane production in terrestrial, aquatic and other anoxic environments: A mini review

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ABSTRACT

Microbial methanogenesis is a major source of the greenhouse gas methane (CH_4). It is the final step in the anaerobic degradation of organic matter when inorganic electron acceptors such as nitrate, ferric iron, or sulfate have been depleted. Knowledge of this degradation pathway is important for the creation of mechanistic models, prediction of future CH_4 emission scenarios, and development of mitigation strategies. In most anoxic environments, CH_4 is produced from either acetate (aceticlastic methanogenesis) or hydrogen (H_2) plus carbon dioxide (CO_2) (hydrogenotrophic methanogenesis). Hydrogen can be replaced by other CO_2-type methanogenesis, using formate, carbon monoxide (CO), or alcohols as substrates. The ratio of these two pathways is tightly constrained by the stoichiometry of conversion processes. If the degradation of organic matter is complete (*e.g.*, degradation of straw in rice paddies), then fermentation eventually results in production of acetate and H_2 at a ratio of $> 67\%$ aceticlastic and $< 33\%$ hydrogenotrophic methanogensis. However, acetate production can be favored when heterotrophic or chemolithotrophic acetogenesis is enhanced, and H_2 production can be favored when syntrophic acetate oxidation is enhanced. This typically occurs at low and elevated temperatures, respectively. Thus, temperature can strongly influence the methanogenic pathway, which may range from 100% aceticlastic methanogenesis at low temperatures to 100% hydrogenotrophic methanogenesis at high temperatures. However, if the degradation of organic matter is not complete (*e.g.*, degradation of soil organic matter), the stoichiometry of fermentation is not tightly constrained, resulting, for example, in the preferential production of H_2, followed by hydrogenotrophic methanogenesis. Preferential production of CH_4 by either aceticlastic or hydrogenotrophic methanogenesis can also happen if one of the methanogenic substrates is not consumed by methanogens but is, instead, accumulated, volatilized, or utilized otherwise. Methylotrophic methanogens, which can use methanol as a substrate, are widespread, but it is unlikely that methanol is produced in similar quantities as acetate, CO_2, and H_2. Methylotrophic methanogenesis is important in saline environments, where compatible solutes are degraded to methyl compounds (trimethyl amine and dimethyl sulfide) and then serve as non-competitive substrates, while acetate and hydrogen are degraded by non-methanogenic processes, *e.g.*, sulfate reduction.

Key Words: electron acceptor, fermentation, methanogenic pathway, organic matter degradation, pH, soil microbial community, temperature, Wood-Ljungdahl pathway

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INTRODUCTION

Methanogenesis is the final step in the anaerobic microbial degradation of organic matter, yielding methane (CH_4) and carbon dioxide (CO_2) as the end products. Anaerobic microbial methane production (AMMP) is the only process that allows complete mineralization of organic matter in the absence of inorganic oxidants such as oxygen (O_2), nitrate, sulfate, ferric iron, *etc.* In the Precambrian, it was the almost exclusive process of recycling dead biomass (Holland *et al.*, 1986), and even in our contemporary oxygenated biosphere, it still accounts for about 1%–2% of the total carbon cycle (Ehhalt, 1979). Nowadays, in the terrestrial biosphere, AMMP occurs in aquatic environments, such as wetlands, flooded rice fields, and lake sediments. In addition, it operates in the guts of many (but not all) animals (Hackstein *et al.*, 1996) and in man-made systems like anaerobic digestors and landfills. These environments and process sites account for more than 60% of the global CH_4 budget, which amounts to about 500–600 Tg year^{-1} (Conrad, 2009). In principle, AMMP may occur wherever degradable organic matter exists under anoxic (no O_2) conditions.

Operation of AMMP requires a complex community of different microorganisms consisting of various taxonomic groups that fulfill the following functions

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(Zehnder, 1978; Zinder, 1993; Conrad, 1999; Schink and Stams, 2013): 1) hydrolysis of polymeric organic matter (*e.g.*, polysaccharides, proteins, and lipids) to monomers, followed by fermentation of the monomers to simple compounds, usually short-chain fatty acids (including acetate and formate), alcohols, hydrogen (H_2), and CO_2; 2) methanogenic conversion of acetate to CH_4 and CO_2; 3) methanogenic conversion of H_2 and CO_2 to CH_4; 4) syntrophic conversion of short-chain fatty acids (including acetate) and alcohols to acetate, CO_2, and H_2; and 5) fermentation of monomers to acetate only (heterotrophic acetogenesis) or conversion of H_2 and CO_2 to acetate (chemolithotrophic acetogenesis) (Fig. 1a). Functions 1, 4, and 5 are usually accomplished by bacteria, while functions 2 and 3 are accomplished by archaea. Functions 2 and 3 are termed aceticlastic and hydrogenotrophic methanogenesis, respectively. The ratio at which these two methanogenic functions contribute to total CH_4 production depends on the relative net production rates of the two CH_4 precursors, namely acetate and H_2. Carbon dioxide, which is also required for hydrogenotrophic methanogenesis, is usually not a limiting substrate in most anoxic environments. The relative turnover rates of acetate and H_2 and the resulting ratio of aceticlastic and hydrogenotrophic methanogenesis are complex and depend on many different factors in the environment. These relative turnover rates are the subject of the present mini review. In addition, the role of methylotrophic methanogenesis will be discussed.

Why is it important to know the relative contribution of hydrogenotrophic and aceticlastic methanogenesis, *i.e.*, the methanogenic pathway? Besides pure scholastic curiosity, it is a necessity to understand all the details of the complex CH_4 production pathway to be able to create mechanistic models. Such models are required for predictions of future CH_4 emission rates, including the emission of CH_4 carbon isotopes (Walter and Heimann, 2000; Van Bodegom and Scholten, 2001; Vavilin, 2012; Bridgham *et al.*, 2013; Xu *et al.*, 2015). Atmospheric isotope budgets are helpful constraints for quantifying the global CH_4 cycle and the

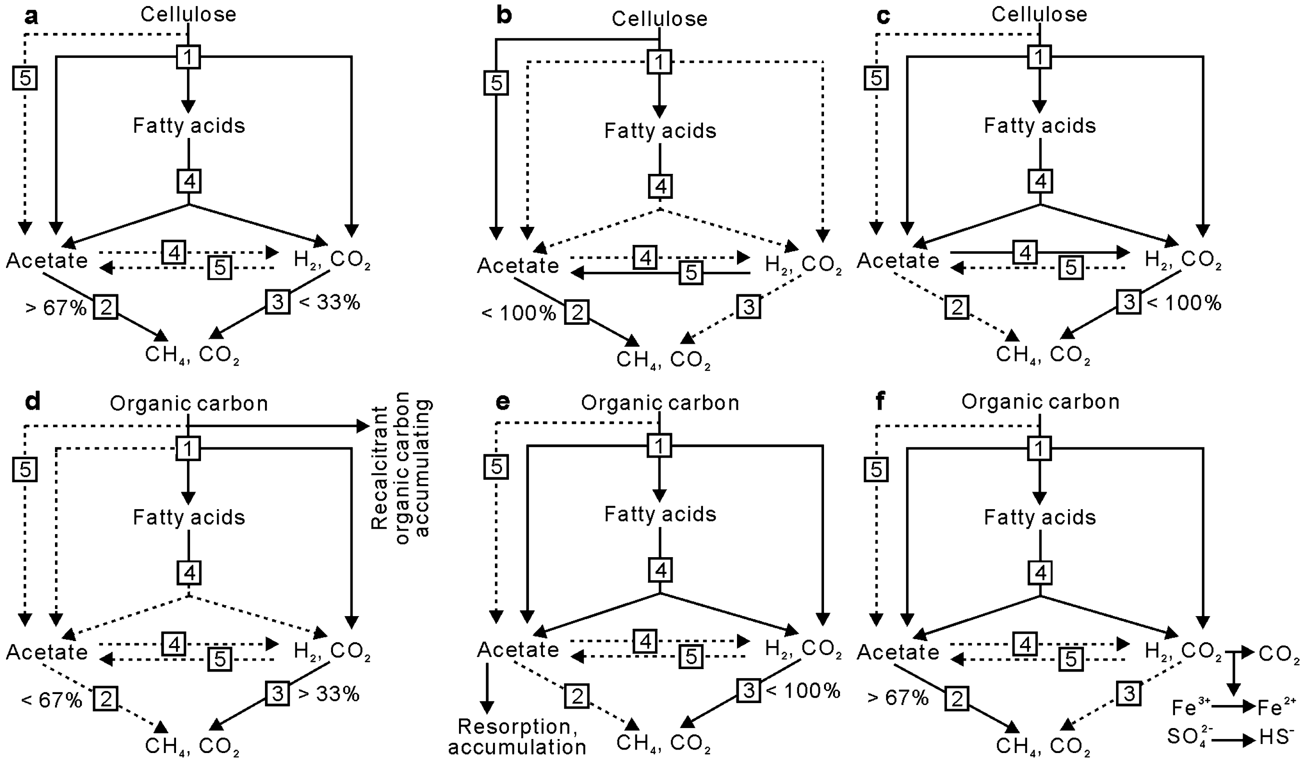

Fig. 1 Schemes of anaerobic microbial methane (CH_4) production (AMMP) from organic matter (*e.g.*, cellulose) involving the following functions (number in squares): hydrolysis of polymeric organic matter to monomers, followed by fermentation of the monomers to short-chain fatty acids, hydrogen (H_2), and carbon dioxide (CO_2) (1); methanogenic conversion of acetate to CH_4 and CO_2 (2); methanogenic conversion of H_2 and CO_2 to CH_4 (3); syntrophic conversion of short-chain fatty acids to acetate, CO_2, and H_2, or of acetate to CO_2 and H_2 (4); and fermentation of monomers to acetate only (heterotrophic acetogenesis) or conversion of H_2 and CO_2 to acetate (chemolithotrophic acetogenesis) (5). Organic matter degradation schemes include: complete degradation with only negligible acetogenesis (function 5) (a); complete degradation with acetogenesis (function 5) as main fermentation process (b); complete degradation with aceticlastic methanogenesis (function 2) being largely replaced by syntrophic acetate oxidation (function 4) (c); incomplete degradation of organic matter (d); complete degradation with accumulation of acetate (e); and complete degradation with hydrogenotrophic methanogenesis (function 3) being outcompeted by iron reduction, sulfate reduction, *etc.* (f).

carbon cycle in general (Tyler *et al.*, 2007; Brownlow *et al.*, 2017). Knowledge regarding the process details is also required for the development of suitable mitigation strategies, since individual reactions may serve as targets to reduce CH_4 emission without compromising other important ecosystem functions. A good example is the successful reduction of CH_4 production in rumen fermentation without compromising animal health (Hristov *et al.*, 2015; Duin *et al.*, 2016).

THEORETICAL CONSTRAINTS

The complete anaerobic degradation of organic matter to CH_4 plus CO_2 is constrained by the nature of the organic material and by stoichiometry. Polysaccharides (*e.g.*, cellulose and xylan) are arguably the most important class of dead plant biomass. Polysaccharides are relatively easily degraded by AMMP. For example, 80%–90% of the rice straw that is added to flooded paddy soils is degraded within the first growth season (Neue and Scharpenseel, 1987).

If, for example, we assume the complete degradation of cellulose by AMMP, we arrive at the following stoichiometric equations:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1)$$

$$2CH_3COOH \rightarrow 2CH_4 + 2CO_2 \quad (2)$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (3)$$

$$C_6H_{12}O_6 \rightarrow 3CH_4 + 3CO_2 \quad (4)$$

Note that Eq. 1 encompasses all possible hydrolysis and fermentation reactions (function 1) and also all possible syntrophic conversions (function 4). However, it assumes that acetogenesis (function 5) is negligible (Fig. 1a). From these stoichiometric equations (Eqs. 1–4) one can conclude that complete degradation of cellulose by AMMP results in equal amounts of CH_4 and CO_2, and that, of the three CH_4 produced in total, two are derived from aceticlastic methanogenesis (67%) and one is derived from hydrogenotrophic methanogenesis (33%).

Furthermore, if we assume that fermentation of cellulose occurs mainly by acetogenesis (function 5), with other fermentation processes (functions 1 and 4) being negligible, we obtain the following equations:

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH \quad (5)$$

$$3CH_3COOH \rightarrow 3CH_4 + 3CO_2 \quad (2)$$

$$C_6H_{12}O_6 \rightarrow 3CH_4 + 3CO_2 \quad (4)$$

We notice that under these conditions, CH_4 is exclusively (100%) produced by aceticlastic methanogenesis (Fig. 1b).

It is also possible that AMMP is almost exclusively hydrogenotrophic, if organic matter is degraded to H_2 plus CO_2, and no acetate is produced. However, such degradation is only weakly exergonic under standard conditions. The conversion of glucose to six CO_2 and twelve H_2 only gives a $\Delta G^{o'} = -25$ kJ. Such process is thermodynamically equivalent to the operation of syntrophic acetate oxidation (Eq. 6) converting the initially produced acetate to H_2 plus CO_2 (Fig. 1c):

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1)$$

$$2CH_3COOH + 4H_2O \rightarrow 4CO_2 + 8H_2 \quad (6)$$

$$12H_2 + 3CO_2 \rightarrow 3CH_4 + 6H_2O \quad (3)$$

$$C_6H_{12}O_6 \rightarrow 3CH_4 + 3CO_2 \quad (4)$$

Hence, complete cellulose degradation by AMMP may occur *via* all combinations of hydrogenotrophic plus aceticlastic methanogenesis.

Since syntrophic acetate oxidation is probably comparatively rare in nature (but, see next section), degradation of cellulose, and organic matter in general, seems to predominantly occur by aceticlastic methanogenesis. Indeed, early studies on anaerobic digestion have found AMMP operating at a ratio of about 67% aceticlastic and 33% hydrogenotrophic methanogenesis (Smith and Mah, 1966). Such a ratio has also been found during early studies of anoxic rice field soil (Takai, 1970) and lake sediments (Cappenberg and Prins, 1974; Winfrey and Zeikus, 1979). Therefore, this pattern in AMMP has been considered the norm (Fig. 1a). However, in a former review (Conrad, 1999), I have pointed out that exceptions to the general pattern of AMMP do exist. During the last 20 years, many more studies have been done, in particular analyses of stable isotopes and molecular analyses of microbial communities; so, a new mini review on the regulation of methanogenic pathways during AMMP is warranted.

REGULATION BY TEMPERATURE

The relative contribution of hydrogenotrophic methanogenesis has been found to decrease with decreasing temperature, both in lake sediment (Schulz and Conrad, 1996; Glissman *et al.*, 2005) and rice field soil (Fey and Conrad, 2000). This effect is expressed in the physiological range of about 4–40 °C. Thus, it has been found that H_2-consuming chemolithotrophic acetogenic bacteria outcompete methanogens at low temperatures (Conrad *et al.*, 1989; Nozhevnikova *et al.*, 1994; Fu *et al.*, 2018). Hence, acetate production is stimulated at low temperatures in methanogenic environments such as rice field soil or lake sediments, while hydrogenotrophic methanogens are suppressed in these conditions (Nozhevnikova *et al.*, 2007). This happens despite the fact that hydrogenotrophic methanogenesis is thermodynamically more favorable than chemolithotrophic acetogenesis (Conrad and Wetter, 1990; Kotsyurbenko *et al.*, 2001). The reason why acetogenic bacteria seem to be more psychrotolerant than methanogenic archaea is unknown, but one might speculate that it is caused by the different membrane structures of bacteria (ester lipids) when compared with that of archaea (ether lipids) (Valentine, 2007). At low temperatures, ester lipids can be more flexible than ether lipids, which is probably advantageous for membrane function. Further research is necessary to understand and quantify the role of acetogenesis in AMMP. A second season for the increase in aceticlastic methanogenesis with decreasing temperature is the thermodynamics of H_2 production. Production of H_2 by syntrophic fatty acid oxidation (function 4) becomes increasingly less exergonic when temperature decreases, resulting in less H_2 production (Chin and Conrad, 1995). Both adaptation of acetogens and thermodynamics of syntrophic fatty acid oxidation may result in relatively more production of acetate than H_2, and thus, enhance aceticlastic methanogenesis (Fig. 1b).

The microbial community structures responsible for AMMP are quite complex and diverse. There are differences in the various methanogenic environments, such as rice field soil, lake sediments, acidic peat, animal guts, anaerobic digestors, landfills, saline environments, *etc.* Detailed descriptions of the archaeal and bacterial sequences and operational taxonomic units are out of scope for this mini review. Briefly, the community of methanogenic archaea usually contains both putatively hydrogenotrophic taxa (*e.g.*, Methanomicrobiales, Methanobacteriales, Methanocellales, and Methanosarcinales) and putatively aceticlastic taxa (*e.g.*, Methanosarcinaceae and Methanotrichaceae (formerly Methanosaetaceae; Oren, 2014)); thus, both hydrogenotrophic and aceticlastic methanogenesis are possible. However, an important exception is methanogenesis at elevated temperatures (40–50 °C). Rice field soils and other anoxic environments with methanogenic activity are frequently able to produce CH_4 under moderately thermophilic conditions (Schulz *et al.*, 1997; Fey *et al.*, 2001). There are soils that contain moderately thermophilic aceticlastic methanogens (Wu *et al.*, 2006). In such a soil, AMMP does not change with temperature increase above 40 °C (Liu *et al.*, 2018) and both aceticlastic and hydrogenotrophic methanogenesis operate at the common ratio (Fig. 1a). However, there are other soils in which populations of aceticlastic methanogens vanish when they are exposed to elevated temperatures. These soils produce CH_4 exclusively by hydrogenotrophic methanogenesis (Conrad *et al.*, 2009; Liu *et al.*, 2018). This is possible since thermophilic syntrophic acetate oxidizers convert the initially produced acetate to H_2 plus CO_2, followed by hydrogenotrophic methanogenesis (Liu and Conrad, 2010). Syntrophic acetate oxidation is thermodynamically endergonic under standard conditions, but becomes increasingly less endergonic if temperature increases (Lee and Zinder, 1988). Syntrophic acetate oxidation represents the only possibility to remove intermittently accumulating acetate, which otherwise could not be degraded (Fig. 1c). The syntrophic acetate oxidizers were identified as Thermoanaerobacteraceae, and possibly, also as Heliobacteriaceae (Liu and Conrad, 2010; Liu *et al.*, 2018; Peng *et al.*, 2018).

In principle, such syntrophic acetate oxidizers may also operate under mesophilic conditions, especially since mesophilic syntrophic species (*e.g.*, *Clostridium ultunense* and *Syntrophaceticus schinkii*) have been isolated (Schnürer *et al.*, 1996; Westerholm *et al.*, 2010). Indeed, the contribution of acetate oxidizers to AMMP has been found in some environments with low or moderate temperatures (Nüsslein *et al.*, 2001; Chauhan and Ogram, 2006; Vavilin *et al.*, 2017). The importance of syntrophic acetate oxidizers is obvious if aceticlastic methanogens (*e.g.*, Methanotrichaceae) are lacking (Nüsslein *et al.*, 2001). However, if a methanogenic environment does contain putatively aceticlastic methanogens (which is common for most environments under mesophilic conditions), it is hard to understand why syntrophic acetate oxidation, instead of aceticlastic methanogenesis, should be operating. One reason may be that the existing aceticlastic methanogens are inhibited by environmental factors. For example, elevated concentrations of ammonia (Schnürer *et al.*, 1999; Zhang *et al.*, 2014; Müller *et al.*, 2016), phosphate (Conrad *et al.*, 2000; Chin *et al.*, 2004), or acetate (Petersen and Ahring, 1991; Karakashev *et al.*, 2006) can represent such factors, which inhibit the extant populations of aceticlastic methanogens. Then, acetate would no longer be degraded, or would be incompletely degraded, unless syntrophic acetate oxidizers are active (Fig. 1c). However, our knowledge of syntrophic acetate oxidation is still very scarce.

REGULATION BY QUALITY OF ORGANIC MATTER

When cellulose is degraded by AMMP, CH_4 is produced by $> 67\%$ aceticlastic and $< 33\%$ hydrogenotrophic methanogenesis (Fig. 1a). However, if cellulose is replaced by chitin, which is an important structural material in fungi and arthropods, there is an additional acetate produced from each monomer (*i.e.*, *N*-acetylglucosamine). Then, the contribution of aceticlastic methanogenesis increases to 75% (or 100% in case fermentation is consummated by acetogenesis).

By contrast, the relative contribution of hydrogenotrophic methanogenesis increases if the organic material is more reduced than saccharides, *e.g.*, lipids. For simplicity, let us assume the degradation of an alkane hydrocarbon, *e.g.*, hexane, by AMMP:

$$C_6H_{14} + 6H_2O \rightarrow 3CH_3COOH + 7H_2 \qquad (5)$$

$$3CH_3COOH \rightarrow 3CH_4 + 3CO_2 \qquad (2)$$

$$7H_2 + 1.75CO_2 \rightarrow 1.75CH_4 + 3.5H_2O \qquad (3)$$

$$C_6H_{14} + 2.5H_2O \rightarrow 4.75CH_4 + 1.25CO_2 \qquad (6)$$

In the degradation of hexane, compared to cellulose, AMMP would result in the production of much more CH_4 than CO_2. However, AMMP would still be dominated (67%) by aceticlastic methanogenesis. The stoichiometries above shows that lipids or other reduced organic compounds would only marginally affect the methanogenic pathways, which would still be close to that shown for cellulose degradation in Fig. 1a. Hence, it is reasonable to conclude that CH_4 production occurs predominantly ($> 67\%$) by aceticlastic methanogenesis, provided the original organic material is degraded to completion and syntrophic acetate oxidation is negligible.

Note, however, that the emphasis has so far been on the complete degradation of organic matter. Contrarily, if we assume that organic matter is not completely degraded, stoichiometries may be completely different. Simple aromatic compounds, such as phenylacetate, may be incompletely degraded, yielding benzoate, CO_2, and H_2, but yielding no acetate (Harwood *et al.*, 1998):

$$C_8H_7O_2^- + 2H_2O \rightarrow C_7H_5O_2^- + CO_2 + 3H_2 \qquad (7)$$

$$3H_2 + 0.75CO_2 \rightarrow 0.75CH_4 + 1.5H_2O \qquad (3)$$

$$C_8H_7O_2^- + 0.5H_2O \rightarrow C_7H_5O_2^- + 0.75CH_4 + 0.25CO_2 \qquad (8)$$

Such degradation process produces CH_4 exclusively by hydrogenotrophic methanogenesis. Phenylacetate (and phenylpropionate) is indeed a minor product of AMMP from rice straw (Glissmann *et al.*, 2005) and peat (Kotsyurbenko *et al.*, 2004). More importantly, there are plenty of other complex organic substances in soil or sediment that might possibly be degraded in a similar way, thereby greatly enhancing the contribution of hydrogenotrophic methanogenesis in AMMP (Fig. 1d). Such processes likely explain the observation that CH_4 production in many lake sediments occurs by the contribution of $> 50\%$ hydrogenotrophic methanogenesis (Conrad *et al.*, 2010, 2011; Ji *et al.*, 2016). Lake sediments or accumulating peat layers are likely sites for such processes, since they are formed by sedimentation processes and are not considerably disturbed. There, only the most labile part of the freshly settled organic matter is completely degraded, while the less labile one accumulates in sediment layers that increase in age with depth (Fig. 1d). Hence, deeper layers of organic matter are increasingly recalcitrant for degradation and support increasingly less CH_4 production, albeit with an increasing contribution of hydrogenotrophic methanogenesis (Chan *et al.*, 2005; Conrad *et al.*, 2010; Hodgkins *et al.*, 2014; Vaughn *et al.*, 2016; Liu *et al.*, 2017).

On the contrary, rice field soils are regularly ploughed and fresh input of rice straw is degraded by 80%–90% during the first season (Neue and Scharpenseel, 1987). In such environments, most of the AMMP occurs with the normal mixture of aceticlastic and hydrogenotrophic methanogenesis (Fig. 1a). Interestingly, even under these conditions, the relative contribution of hydrogenotrophic methanogenesis increases when most (70%) of the rice straw has been degraded, indicating that increased recalcitrance of organic matter results in increased contribution of hydrogenotrophic methanogenesis (Ji *et al.*, 2018a).

Methanogenic archaeal communities of environments with incomplete conversion of organic substances and enhanced hydrogenotrophic methanogenesis generally consist of both hydrogenotrophic and aceticlastic methanogenic archaea, albeit hydrogenotrophic ones may be enhanced relatively to the aceticlastic ones (Chan *et al.*, 2005; Mondav *et al.*, 2014; Liu *et al.*, 2017). However, the coexistence of both indicates that the regulation of the pathway of methanogenesis mainly occurs by flux control rather than by control of the functional methanogenic populations.

ALTERNATIVE FATES OF CH_4 PRECURSORS

If AMMP proceeds as theoretically expected (compare Fig. 1a), the ratio of hydrogenotrophic and aceticlastic methanogenesis may change if either H_2 or acetate are consumed or otherwise diverted before they are converted by methanogens (Fig. 1e). This typically happens in animal guts, where acetate (and other short-chain fatty acids) is resorbed as a food source, with methanogens only using H_2 as CH_4 precursor (Hungate, 1967). Similarly, acetate may be consumed in microbial or algal mats as a carbon source, making the contribution of hydrogenotrophic methanogenesis increase (Sandbeck and Ward, 1981). Furthermore, H_2 production and hydrogenotrophic methanogenesis may be stimulated by light-driven processes (Lee *et al.*, 2014). On the contrary, H_2 may be lost from the environment thus enhancing the contribution of aceticlastic methanogenesis. Since H_2 is a gas with low solubility, it may easily evade from the habitat, especially if it is produced close to the habitat-atmosphere interface, such as in microbial mats (Hoffmann *et al.*, 2015), or ventilated by ebullition or plant gas vascular systems (Schütz *et al.*, 1988).

The ratio of hydrogenotrophic and aceticlastic methanogenesis may also be affected by temporal changes in ecosystem functions, such as reported in some northern peatlands. There, CH_4 is predominantly produced by hydrogenotrophic methanogenesis and acetate is not consumed at all, accumulating during the season with a high water table (Duddleston *et al.*, 2002) (Fig. 1e). This is due to a lack of aceticlastic methanogens (Rooney-Varga *et al.*, 2007). However, when the water table drops at the end of the season, allowing O_2 to penetrate into the formerly methanogenic peat layers, acetate is oxidized by microorganisms using O_2 or ferric iron as oxidants (Duddleston *et al.*, 2002). Such temporal changes in environmental conditions may occur in many different ecosystems, *e.g.*, upon water management in rice fields (Krüger *et al.*, 2002; Zhang *et al.*, 2013); thus, alternative fates of CH_4 precursors can change the pathway of CH_4 production.

In particular, it may be important to consider the temporary or local effects of inorganic oxidants (O_2, nitrate, sulfate, ferric iron, *etc.*), which allow the oxidation of methanogenic substrates to CO_2 instead of its reduction to CH_4 (Fig. 1f). Such oxidation reactions may be selective for either H_2 (such as in Fig. 1f) or acetate, and thus, specifically suppress hydrogenotrophic or aceticlastic methanogenesis (Achtnich *et al.*, 1995; Klüber and Conrad, 1998; Conrad, 1999; Scheid *et al.*, 2003). While inorganic oxidants are normally unavailable if methanogenic environments have been under reducing conditions for several days or weeks, they can be relevant electron acceptors in environments with local or temporal input of O_2, for example, as provided by plant ventilation or water table fluctuations (Beckmann and Lloyd, 2001; Knorr and Blodau, 2009). Inorganic oxidants (*e.g.*, sulfate) can be plentiful in marine and other saline environments, in which AMMP is a niche process compared to microbial sulfate reduction (Mountfort *et al.*, 1980; Crill and Martens, 1986). There, AMMP may be restricted to the degradation of compatible solutes coupled to methylotrophic methanogenesis (see below).

REGULATION BY pH

For the complete degradation of organic matter in the absence of inorganic oxidants, AMMP can work at any pH. The theoretical constraints for complete degradation are the same as outlined above. However, it is feasible that degradation of organic matter is incomplete at pH values that largely deviate from neutrality. The coincidence between accumulating peat layers and acid pH values is obvious. Therefore, it has been argued that imbalances in microbial fermentation processes may contribute to acidification (Zeikus, 1983). Indeed anaerobic H_2 turnover was found to decrease with decreasing pH in lake sediments (Goodwin *et al.*, 1988), indicating that hydrogenotrophic methanogenesis may become increasingly limited by H_2. Although aceticlastic methanogenesis sometimes is the almost exclusive CH_4-production process in acidic lake sediments (Phelps and Zeikus, 1984; Goodwin and Zeikus, 1987), this may also be explained by acetogenic fermentation (Fig. 1b). In fact, most studies found the existence of both hydrogenotrophic and aceticlastic methanogenic archaea in sediments of acidic lakes, rivers, peat mires, or soils (Chan *et al.*, 2002; Basiliko *et al.*, 2003; Horn *et al.*, 2003; Kotsyurbenko *et al.*, 2004; Cadillo-Quiroz *et al.*, 2006; Metje and Frenzel, 2007; Sanz *et al.*, 2011; Barbier *et al.*, 2012; Liebner *et al.*, 2015). However, aceticlastic methanogens may be absent or inactive in some peat environments, resulting in the accumulation of acetate (Duddleston *et al.*, 2002; Metje and Frenzel, 2005). With these exceptions, AMMP in acidic environments is not different from AMMP in neutral ones. In particular, hydrogenotrophic methanogenesis is apparently not impeded by low pH. In methanogenic rice field soils, CH_4 production decreases if soil pH is artificially decreased (Wang *et al.*, 1993; Jugsujinda *et al.*, 1995). It is well known that CH_4 production in acid sulfate soils is strongly suppressed, albeit it is not clear whether this suppression is mainly due to low pH or high concentrations of sulfate and ferric iron, which may serve as alternative electron acceptors in the anaerobic degradation of organic matter (Yao *et al.*, 1999).

Methanogenesis can be found in the alkaline environments, which usually are halo-alkaline (Oremland *et al.*, 1982; Antony *et al.*, 2012; Sorokin *et al.*, 2015a). The active methanogens apparently belong to the group of methylotrophic methanogens, which are commonly found in saline environments and use the so-called "non-competitive" methanogenic substrates, such as methanol, methylamines, and methyl sulfides (see section "Role of methylotrophic methanogenesis"). However, hydrogenotrophic alkaliphilic methanogens have also been isolated (Sorokin *et al.*, 2015b).

In summary, there is presently no evidence that pH by itself regulates the relative contribution of hydrogenotrophic *vs.* aceticlastic methanogenesis in terrestrial environments. It is probably rather the presence of alternative oxidants (*e.g.*, sulfate in acid precipitation (Gauci *et al.*, 2008)) that affects methanogenesis.

OTHER CO_2-TYPE METHANOGENESIS

The so-called hydrogenotrophic pathway does not necessarily depend on the provision of H_2 as an electron donor. Many hydrogenotrophic methanogens can utilize formate, carbon monoxide (CO), or even alcohols (*e.g.*, ethanol, 2-propanol, *etc.*) as substrates (Daniels *et al.*, 1977; Widdel, 1986). For example, CH_4 is produced from ethanol and CO_2:

$$2CH_3CH_2OH + CO_2 \rightarrow 2CH_3COOH + CH_4 \tag{9}$$

Ethanol (and other alcohols) is only partially oxidized, resulting in the production of acetate, which is subsequently utilized by aceticlastic methanogens, while the reducing equivalents are used to reduce CO_2 to CH_4 (Fig. 2a). Formate and CO are oxidized to CO_2, while the electrons are used to reduce CO_2 to CH_4. Therefore, all these physiological types can be summarized as CO_2-type methanogens, contrasting acetate-type methanogens and methylated C1 compounds-type methanogens (Liu and Whitman, 2008). For example, ethanol and 2-propanol can be important methanogenic substrates in peat (Metje and Frenzel, 2005) and pond sediments (Dalcin Martins *et al.*, 2017). It is not easy to distinguish direct utilization by an alcohol-oxidizing methanogen from syntrophic alcohol oxidation coupled to hydrogenotrophic methanogenesis (Fig. 2a). For the differentiation of the hydrogenotrophic methanogenic pathway from aceticlastic and methylotrophic pathways, it does not matter how CO_2 is reduced, since alcohols (or formate and CO) function like H_2 as normal intermediates in fermentative AMMP, and are summarily included in Eq. 1. Also, for the differentiation of hydrogenotrophic and aceticlastic methanogenic pathways it does not matter whether the methanogens consume the fermentatively produced H_2 in dissolved form, by interspecies-H_2-transfer (or interspecies-formate-transfer), or by direct interspecies electron transfer (Bryant *et al.*, 1967; Conrad *et al.*, 1985; Thiele and Zeikus, 1988; Rotaru *et al.*, 2012).

Another intriguing idea would be the existence of glycolytic methanogens, *i.e.*, a methanogenic species which could utilize glucose as substrate and convert it to CH_4 and CO_2, as shown in Eq. 4. Thus, the microbial community accomplishing AMMP would be replaced by only one organism, which would do everything (Fig. 2b). This idea is presently pure speculation, but it is not completely unlikely since some methanogens can metabolize glycogen, which they produce as storage material. For example, *Methanosarcina acetivorans* displays vigorous gluconeogenesis and glycolysis (Santiago-Martínez *et al.*, 2016). Metatranscriptome analyses of methanogenic paddy soils showed that Methanosarcinaceae transcribe glucosyl hydrolases involved in polymer breakdown (Peng *et al.*, 2018). Hence, why should species of methanogens not

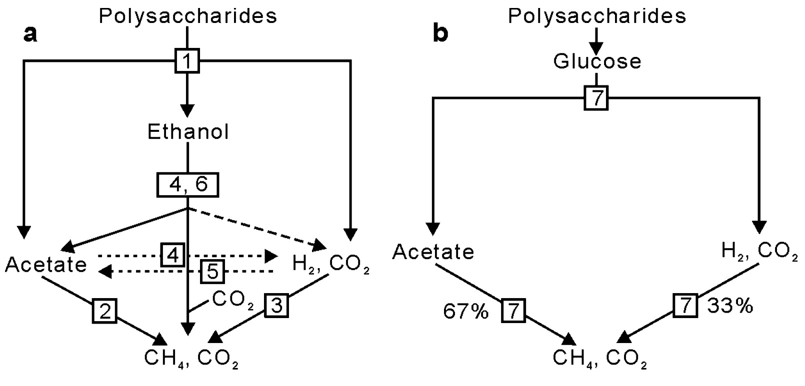

Fig. 2 Schemes of anaerobic microbial methane production (AMMP) from organic matter (*e.g.*, polysaccharides) involving the following additional functions (number in squares): methanogenic conversion of alcohols (function 6) (a) and methanogenesis from glycolysis (function 7) (b). See Fig. 1 for the detailed descriptions of functions 1–5.

be able to utilize glucose or polysaccharides entirely without sharing the energy content with other members of a complex microbial community? Theory suggests that microbes can optimize either rate or yield of adenosine triphosphate (ATP) production (Pfeiffer *et al.*, 2001). While high ATP yields would favor complete degradation (glucose to CH_4), high ATP rates are achieved by reducing the length of the pathway (*e.g.*, acetate to CH_4). Our procedures for enrichment and isolation of microbes are all selecting for fast-growing species. However, slow-growing species with optimized ATP yields may be found in biofilms or aggregates (Kreft, 2004). Such microbes may not have yet been isolated because of technical reasons. The completely nitrifying bacteria (Comammox), which have recently been isolated and described (Kits *et al.*, 2017), may be considered as an analogue for putatively glycolytic methanogens. Both accomplish complete conversion of a substrate, which is normally converted by a chain of processes divided between several different microbes. However, the existence of putatively glycolytic methanogens would result in the same stoichiometry of polysaccharide degradation as shown in Eqs. 1–4, albeit being catalyzed in one organism instead of a community (Fig. 2b). The percentages of hydrogenotrophic and aceticlastic methanogenesis (33% and 67%, respectively) would be the same, since glycolysis would eventually result in the production of two acetyl-coenzyme A (CoA) and four hydrogen equivalents, analogous to Eq. 1, and these glycolytic products would be converted by the methanogenic machinery to CH_4, analogous to Eqs. 2 and 3.

ROLE OF METHYLOTROPHIC METHANOGENESIS

Whereas aceticlastic and hydrogenotrophic methanogens are required for AMMP in almost all anoxic methanogenic environments, the role of methylotrophic methanogenesis is not completely clear. Methylotrophic methanogens all belong to the family Methanosarcinaceae, except the genus *Methanosphaera*, which belongs to the order Methanobacteriales (Liu and Whitman, 2008). Methylotrophic methanogenesis is the conversion of a methyl compound to CH_4 plus CO_2 (*e.g.*, methanol, which is disproportionated to CH_4 and CO_2):

$$4CH_3OH \rightarrow CO_2 + 2H_2O + 3CH_4 \tag{10}$$

Methanol is a product of pectin degradation and can be degraded by methanogens (Schink and Zeikus, 1980). However, pectin turnover in lake sediments is slow (Schink and Zeikus, 1982), and methanol has been found to be a marginal methanogenic precursor (Lovley and Klug, 1983). The same is true for anoxic rice field soils (Conrad and Claus, 2005). Since small amounts of methanol may also be produced from xylan (Rosell and Svensson, 1975) and lignin (Ander *et al.*, 1985), methanol-driven methanogenesis cannot be completely dismissed (Fig. 3a). However, it may primarily be important in special environments, such as methanogenic wetwood, where wood pectin is degraded by AMMP (Schink *et al.*, 1981). Peat soils from the Zoige wetlands were also claimed to produce CH_4 mainly from methanol (Jiang *et al.*, 2010). However, even if pectin (or xylan) is the major polymer driving methanogenesis, AMMP would not only result in the production of methanol, but also in the production of monosaccharides (*e.g.*, gluconic acid and galacturonic acid) that would be further degraded to acetate, CO_2, and H_2. Each of these sugar acid molecules would result in the production of two molecules of acetate, which are methanogenically converted to two molecules of CH_4.

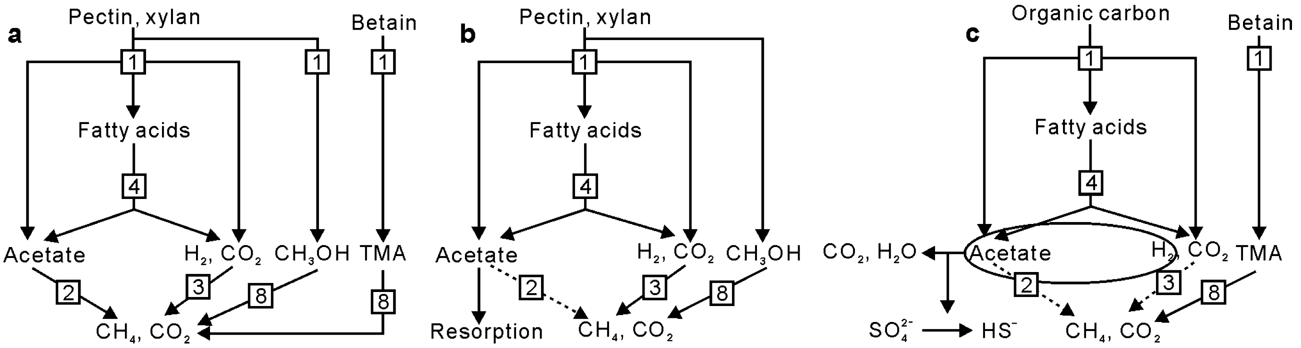

Fig. 3 Schemes of anaerobic microbial methane (CH_4) production (AMMP) from organic matter (*e.g.*, pectin and xylan) involving methylotrophic methanogenesis (function 8): general degradation scheme involving CH_4 production from methanol and trimethylamine (TMA) (a); methanol-dependent methanogenesis and resorption of acetate (*e.g.*, in gut systems) (b); and TMA-dependent methanogenesis and predominant utilization of acetate and H_2 by sulfate reduction (c). See Fig. 1 for the detailed descriptions of functions 1–4.

Hence, pectin would stimulate methanogenesis from acetate more than from methanol (Fig. 3a).

More recently, methanol-degrading methanogens of the order Methanomassiliicoccales have been described (Dridi *et al.*, 2012; Paul *et al.*, 2012). These methanogens reduce methanol with H_2:

$$CH_3OH + H_2 \rightarrow CH_4 + H_2O \tag{11}$$

These methanogens occur in animal gut systems, but also have frequently been detected in other anoxic environments such as fens (Söllinger*et al.*, 2016), mangrove sediments (Li *et al.*, 2016), digesters (Wilkins *et al.*, 2015), and anoxic paddy soils (Ji *et al.*, 2018b). Methanomassiliicoccales are probably even more widespread, since they have only recently been added to sequence databases and probably were previously recorded as Thermoplasmatales-related Euryarchaeota. Their role in carbon flux is presently unknown. In gut systems, they may play an important role since acetate is reabsorbed, thus rendering methanol a relatively important methanogenic precursor (Fig. 3b). However, the relative contribution of methylotrophic *vs.* hydrogenotrophic methanogenesis in gut systems is presently not known (Söllinger *et al.*, 2018). The genus *Methanosphaera* can also reduce methanol with H_2 to CH_4 (Liu and Whitman, 2008). Recently, genomic information indicates that this type of physiology may also occur in several archaeal candidate taxa, *e.g.*, *Verstraetearchaeota* (Vanwonterghem *et al.*, 2016).

Methylamines, trimethylamine (TMA) in particular, may be important methanogenic substrates in saline environments, where biota produces glycine betaine as an osmolyte. Betaine is fermented yielding TMA, which is a methanogenic substrate (Hippe *et al.*, 1979; King, 1984a). Since TMA seems to be a substrate that is better degraded by methanogens than by sulfate reducers, TMA is a so-called non-competitive methanogenic substrate (Oremland and Polcin, 1982; King, 1984b). Thus, it was found that methylotrophic methanogenesis is responsible for much of the CH_4 production in anoxic marine environments, despite the presence of active sulfate reducers. These sulfate reducers were successfully competing for H_2 and acetate, but not for methyl compounds like TMA, which was exclusively degraded by methanogens (Fig. 3c). Similarly, CH_4 production seems to be caused by methylotrophic methanogens in many saline environments, *e.g.*, saline lake sediments on the Tibetan Plateau (Liu *et al.*, 2016), hypersaline microbial mats (Smith *et al.*, 2008), and other hypersaline environments (Oren, 1990; Zhuang *et al.*, 2016; Sorokin *et al.*, 2017).

Methanethiol and dimethylsulfide can also serve as methanogenic substrates (Zinder and Brock, 1978; Ni and Boone, 1991; Lomans *et al.*, 1999). Like methylamines, these methylsulfides are produced from the osmolyte dimethyl sulfoniopropionate (Kiene and Visscher, 1987). Dimethyl sulfide may serve a similar role as trimethylamine. However, degradation of dimethyl sulfide happens not only in marine (Van der Maarel and Hansen, 1997; Zhuang *et al.*, 2018) but also in freshwater environments (Lomans *et al.*, 1997).

CONCLUSIONS AND OUTLOOK

Anaerobic degradation of organic matter by soil microbial communities eventually results in the production of CH_4 by aceticlastic and hydrogenotrophic methanogenesis. These two major pathways of microbial CH_4 production may contribute different percentages ranging from zero to 100%. The extent of their contributions depends on whether the available organic matter is degraded almost completely (such as straw in paddy fields) or is only partially degraded (such as soil or sediment organic matter). The complete degradation of organic matter is tightly constrained by the stoichiometry of fermentation reactions, which result in the production of acetate and H_2 at a ratio that consequently gives rise to CH_4 production from $> 67\%$ aceticlastic and $< 33\%$ hydrogenotrophic methanogenesis. This ratio is similar for various organic substrates, provided they are completely degraded, and the fermentation products are exclusively consumed by fermentation and methanogenesis, and are not accumulated, absorbed, volatilized, or consumed by oxidation reactions coupled with the reduction of inorganic electron acceptors such as ferric iron or sulfate. However, since environmental conditions affect the thermodynamics and kinetics of these individual reactions, and also affect population dynamics and the structuring of the entire microbial community, fermentation reactions can be pushed into production of surplus acetate or H_2. Thus, temperature can control the methanogenic pathways by influencing the thermodynamics of fermentative production of H_2 and acetate, and by affecting the composition of the microbial communities. While acetogenesis followed by aceticlastic methanogenesis is favored at low temperatures, syntrophic acetate oxidation followed by hydrogenotrophic methanogenesis is favored at high temperatures.

It is remarkable that temperature control of the methanogenic pathways primarily depends on the activity of acetate-producing and acetate-oxidizing bacteria, which thus play a key role in the regulation of AMMP. The group of acetate-producing microorganisms is termed acetogenic or homoacetogenic bacteria, since they produce acetate as virtually the sole fermentation product using the Wood-Ljungdahl pathway (Drake, 1994). Those, which produce acetate chemolithotrophically from H_2 and CO_2 (at least some of them), can reverse the direction of the process if certain thermodynamic conditions are met, and then oxidize acetate to H_2 plus CO_2 (Zinder, 1994). Under such conditions, they act as syntrophic acetate-oxidizers. Hence, the control of the methanogenic pathways depends to a large extent on the activity of the acetogens/acetotrophs operating the Wood-Ljungdahl pathway. Unfortunately, this conclusion is primarily based on our knowledge of the regulation of the relative contribution of aceticlastic *vs.* hydrogenotrophic methanogenic pathways, which represents indirect evidence. Acetogenic bacteria are widespread in nature and can display remarkable activity, such as in the litter layer of forest soils (Küsel and Drake, 1994), the gut of termites (Breznak and Switzer, 1986), or the deep biosphere (Lever, 2012). However, the *in situ* quantification of their contribution to the anaerobic degradation of organic matter (*e.g.*, AMMP) is very difficult and has so far not been achieved unequivocally (Hoehler *et al.*, 1999; Liu and Conrad, 2011; Hädrich *et al.*, 2012; Fu *et al.*, 2018). Understanding the ecology of acetogens/acetotrophs is certainly an important objective for future research.

The role of methylotrophic methanogenesis in nature is also an interesting research question. Methylotrophic methanogenesis is probably important in highly saline environments, where biota form large amounts of compatible solutes that are anaerobically degraded to methyl compounds. Although this conclusion has never been questioned, there are only a few studies quantifying the carbon flow under *in situ* conditions (King, 1984a).

The role of methanol-dependent methanogenesis under field conditions is completely unclear. Recent studies indicate a remarkable diversity of potentially methanol-reducing methanogens across several archaeal classes (Evans *et al.*, 2015; Nobu *et al.*, 2016; Vanwonterghem *et al.*, 2016). However, to be important for carbon flow, production rates of methanol must be comparable to those of H_2 and acetate to allow a significant contribution to the total CH_4 production. Since concentrations of methanol are usually below the detection limit, methanol is not likely to play a major role in CH_4 production. However, there are only very few studies addressing methylotrophic methanogenesis contribution to total CH_4 production in non-extreme soil and aquatic environments (Lovley and Klug, 1983; Conrad and Claus, 2005).

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