

Review Article

Dissolved Microbial Methane in the Deep Crystalline Crust Fluids—Current Knowledge and Future Prospects

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Received 30 March 2022; Accepted 5 October 2022; Published 14 October 2022

Academic Editor: Mohammad Sarmadivaleh

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Methane is a powerful greenhouse gas, of which most is produced by microorganisms in a process called methanogenesis. One environment where methanogenic microorganisms occur is the deep biosphere. The deep biosphere environment comprises a variety of ecosystem settings; marine habitats such as seafloor sediments, rock pore volumes within seafloor basalts, and terrestrial settings such as sedimentary rocks and crystalline bedrock fracture networks. Microbial methane formed in these environments influence the biological, chemical, and geological cycles of the upper crust, and may seep out of the deep into the atmosphere. This review focuses on the process of microbial methanogenesis and methane oxidation in the relatively underexplored deep crystalline-bedrock hosted subsurface, as several works in recent years have shown that microbial production and consumption occur in this energy-poor rock-fracture-hosted environment. These recent findings are summarized along with techniques to study the source and origins of methane in the terrestrial crust. Future prospects for exploration of these processes are proposed to combine geochemical and microbial techniques to determine whether microbial methanogenesis is a ubiquitous phenomenon in the crystalline crust across space and time. This will aid in determining whether microbial methane in the globally vast deep rock-hosted biosphere environment is a significant contributor to the global methane reservoir.

1. Introduction

Methane is a powerful greenhouse gas, and its concentrations in the atmosphere have been increasing over the past decade [1]. Because it has a greater warming potential than CO₂, it is the second most important greenhouse gas and accounts for 14 ± 4% of greenhouse gas-induced global warming on Earth [1, 2]. To mitigate anthropogenic climate change and to make as precise models as possible, it is important to understand both anthropogenic and natural sources and sinks of methane. Various natural sources emit methane into the atmosphere, mainly wetlands, but also agriculture, oceans, freshwater system, and geological sources, including the deep continental subsurface [3]. Methane from these geological sources is formed by various abiotic or microbially mediated chemical reactions in the crust and can be released through natural seepage or leakage

from geothermal wells to the atmosphere. In the Earth's subsurface, microbial methane is formed through methanogenesis by methanogenic archaea living in the pore spaces and fractures. Methanogens, methanotrophs, and microbial methane have been detected at several continental sites globally, including in Fennoscandian Shield, Canadian Shield, South Africa, and Japan [4–7]. The fluid within the deep Precambrian crystalline crust in which microbiota reside [8] and in which microbial methane is detected [7] has been proposed to be up to 1.5 billion years old based on radiometric noble gas dating [9]. All this together suggests that deep terrestrial microbial methane formation might be widespread on Earth through space and time. However, the determination of microbial methane from the deep subsurface is complicated due to its inaccessibility and costs of sampling and drilling campaigns, and hence, observations are few of hydrocarbons in these systems in general.

The topic of methane origin in the continental bedrock has previously been reviewed by Kietäväinen and Purkamo [10] and by Kotelnikova [11]. However since then, new findings have been made regarding the archaeal phylogeny and metabolisms which support generic classifications of methane sources [12]. Additionally, refinement of clumped isotope methodology provides discrimination of methane origins [13, 14]. Finally, for the deep biosphere of crystalline rocks in particular, discoveries of anaerobic oxidation of methane at deep-seated sulfate methane transition zones in the fracture networks [15–19] and new observations of community diversity and syntrophic relationships have been reported [20, 21]. Co-occurrence of methanogenesis-related signatures with fossilized remains of anaerobic fungi further suggests a potential symbiotic relationship between fungi and methanogens [22], although such a relationship is yet to be confirmed by microbial ecology evidence. Co-occurrence and positive correlation of sulfate reducing bacteria and fungi have been documented in the Outokumpu Deep Drill Hole, eastern Finland [23]. Culture-independent genomics have further expanded our knowledge about archaeal phylogeny and the occurrence of methanogenesis [24], with the current diversity of archaea capable of methanogenesis is much larger than previously known, with methanogens occurring in several archaeal phylogenetic lineages [25]. Methanogenic lineages that are phylogenetically distant from previously known lineages are being recognized from advances in genomic sequencing. The occurrence of methanogenesis even outside of the Euryarchaeota implies a methanogenic ancestor for all Archaea [26, 27]. The importance of viral predation exerting top-down control on microbial communities in the terrestrial deep biosphere, also suggests that the predation of viruses on the microbial communities may provide an important resource of organic carbon to the deep ecosystems [28]. In addition, recent investigations of biosignatures in secondary mineral coatings and fossilized microbial remains in deep crystalline bedrock fractures of the Fennoscandian shield have revealed that microbial methanogenesis and anaerobic oxidation of methane have been widespread processes in the deep subsurface over hundreds of millions of years [22, 29–31].

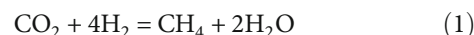
Taking the recent advances into account, we aim to give a condensed overview on the current knowledge of formation processes of methane in the crystalline crust, particularly on the process of microbial methanogenesis, its diagnostic characteristics and concluding with a brief discussion and perspective on the current limitations and future outlooks.

2. Methane in the Crystalline Crust

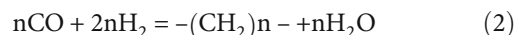
In the deep subsurface, methane is either produced by microbial processes, by thermogenic degradation of organic matter, or abiotically through various mechanisms such as magmatic CH_4 derived from the mantle and water-rock or gas-rock reactions. Of these processes, thermogenic methane is considered to make the bulk of the global natural gas resources, with microbial contribution accounting for 20%

[32], whereas a globally significant abiogenic source of hydrocarbons has been ruled out [33].

2.1. Abiotic Methane. Inorganic synthesis of CH_4 occurs predominantly via gas-water-rock reactions. Natural analogues of Fischer-Tropsch Type (FTT) reactions are important mechanisms for abiotic CH_4 production, especially in the presence of ultramafic rocks undergoing serpentinization [34]. Serpentinization refers to an alteration process of low-silicic ultramafic rocks, rich in olivine or pyroxene: ferrous iron from olivine and pyroxene is oxidized, resulting in precipitation of ferric iron in magnetite and other ferric minerals, producing H_2 and decreasing the activity of water, thereby providing favorable conditions for the reduction of CO_2 to CH_4 such as the Sabatier reaction:



which produces methane by one step, but can also include production of long-chain hydrocarbons:



The molecular H_2 needed for the FTT synthesis (Reactions 1 and 2) can also be used by autotrophic microbes.

FTT reactions can occur at low temperatures of $<50^\circ\text{C}$ [35], however experimental studies show that substantial kinetic barriers exist below temperatures of 200°C for the formation of abiotic CH_4 during serpentinization of olivine [36]. The FTT reactions are catalyzed by transition metals, mostly Ni, Fe, and Cr, which are the most abundant transition metals in ultramafic rocks (e.g., [37]), thus these reactions mostly occur at the rock-gas interface. Despite kinetic barriers, because FTT synthesis of CH_4 can occur at low temperatures, it is possible that it occurs widely in the crystalline crust, even at shallow depths [34, 38, 39].

2.2. Thermogenic Methane. Thermogenic CH_4 is produced through the break-up of organic matter at high temperature and large depth, and produces a series of hydrocarbons, from longer to shorter chains. It is thought to be the most important source of methane in sedimentary systems [32]. However, in the crystalline bedrock, thermogenesis might be a less prevalent source of methane production than abiotic and microbial production, resulting from the scarcity of potential organic matter [40]. Nevertheless, the crystalline bedrock can sometimes host organic carbon (such as bitumen/seep oil) within fracture networks [41, 42]. Migration of organic matter from organic-rich source rock into to fractured crystalline basement during burial heating has been suggested in localities such as the Siljan impact structure in Sweden [43] and the Bergslagen area [22, 31] of the Fennoscandian shield. The bitumen and seep oil are associated with thermogenic methane but can also provide a ready energy source for microbial activity [30, 41].

2.3. Microbial Methane. The microbial formation of CH_4 is mostly conducted by archaea of Euryarchaeota lineages. These methanogens produce CH_4 through three main metabolic pathways: a hydrogenotrophic pathway in which

methanogens that utilize CO_2 and H_2 as their energy source, or methylotrophic or acetotrophic pathways which utilize organic carbon molecules like formate, methanol, methylamines methylotrophic, and/or acetate as substrate. Microbial methanogenesis was previously believed to be exclusive to the domain of archaea and a strictly anaerobic process [44], but recent evidence of cyanobacteria producing methane contradicts these previous views [45]. The clade of methanogenic archaea consists of the orders Methanopyrales, Methanebacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, Methanocellales, and Methanomassiliococcales [27]. The orders of Methanosarcinales and Methanoplasmatales comprise taxa that can utilize CO_2 or other substrates than CO_2 and are therefore both organotrophic and chemolithoautotrophic taxa. The other orders mainly consist of obligate chemolithoautotrophic (CO_2 reducing) species, barring a few exceptions [46]. In the continental deep subsurface Methanobacteriales and Methanosarcinales are most common, but overall distribution is site-specific [4, 47, 48].

Microbial methanogenesis occurs most commonly at anoxic conditions at redox levels $\text{Eh} < -200 \text{ mV}$ [49], but is also possible in oxic conditions by cyanobacteria [45]. In the CO_2 -reduction pathway (Reaction 1), H_2 is oxidized, and CO_2 is reduced to methane. In the acetoclastic pathway (Reaction 3), acetate is cleaved with the methyl group reduced to CH_4 and carbonyl group oxidized to CO_2 . Coenzyme M (HS-CoM) is common in both pathways, as it catalyzes the last step in methanogenesis [27].



CO_2 is fixed by methanogens using the reductive acetyl CoA pathway, which involves Ni and Fe dependent enzymes, such as acetyl CoA-synthase. Intermediates such as CO and HCOO^- are produced in this pathway. The ability to metabolize H_2 is through the enzymes [FeFe]- or [NiFe]-hydrogenase [50, 51]. Methanogens with cytochromes (redox-active proteins) occur within the order of Methanosarcinales, have a much higher growth yield using the CO_2 -reduction pathway [44].

2.4. Energy Sources for Microbial Methanogenesis. The production of microbial methane through CO_2 -reduction pathway requires available CO_2 and low sulphate concentrations [11]. If sulphate concentrations are too high, sulphate reducing bacteria (SRB) are thought to outcompete methanogens for the available H_2 and carbon substrates, as CO_2 -reduction is a thermodynamically more favourable reaction, though active methanogenesis can occur alongside sulphate reduction [52, 53]. H_2 and CO_2 in the deep biosphere are made available to methanogens through multiple processes. At low H_2 concentrations, $\text{pH} < 7$, and temperatures $> 15^\circ\text{C}$, acetogenic bacteria convert acetic acid into H_2 and CO_2 , which can then be used by methanogens utilizing CO_2 -reduction [54]. At high H_2 concentration, high pH and low temperature, acetogenic bacteria preferentially synthesize acetic acid [44]. The CO_2 and H_2 can also come from abiotic sources. H_2 originates mainly from hydrolysis of water by

ferrous iron under reduced conditions [11], such as during serpentinization, but also through the dissociation of water molecules during radioactive decay (radiolysis, e.g. [55]), or from mechanochemical processes in fault zones (e.g. [56]). As a result, high H_2 concentrations are observed in the deep subsurface [57], but at certain sites where relatively young freshwater has infiltrated and serpentinized rock is absent, H_2 concentrations can be very low [58, 59]. Another suggested source of H_2 for methanogenesis comes from fungi [22, 60]. Anaerobic fungi can degrade refractory material, and the presence of fungi has been found in multiple sites across the deep Fennoscandian bedrock [61–63]. Fossilized remnants of intergrown fungal hyphae with substantially ^{13}C -enriched calcite suggest a close syntrophic relationship between anaerobic fungi and methanogens in the deep biosphere [22]. Fungi may thus contribute substrates for microbial methanogenesis in deep continental bedrock by degrading organic matter.

In addition to the CO_2 -reduction pathway, methanogens can use organic compounds for methane production. This organic material can be produced in abiotic reactions in Earth's crust [64], and used by heterotrophs, producing CO_2 (Reaction 4), which can be used again by hydrogenotrophic methanogens. At shallow depths, descending dissolved organic carbon provides energy to heterotrophic metabolisms, but this influence declines with depth, and the communities are changed accordingly [65, 66]. At great depths, organic carbon is commonly scarce or only available as a refractory material in the crystalline crust [67], in addition to being inaccessible in some areas due to low permeability [47]. Dissolved inorganic carbon is found in the form of bicarbonate within crystalline rock fracture aquifers, in which the concentrations are low as well [40]. Clostridia have been found side by side with methanogens, suggesting a syntrophic relation where Clostridia supplies carbon through their fermentation products to methanogenic microorganisms [10]. Daly et al. [28] found that viral predation exerts top-down control on shale microbial communities in the terrestrial deep biosphere. The findings also suggest that the predation of viruses on the microbial communities may also provide an important resource of organic carbon to the deep ecosystems [28].

Secondary produced methane is another common formation pathway and occurs when petroleum and other thermogenic products in a reservoir are biodegraded [68, 69]. The production of secondary microbial gas is referred to as methanogenic hydrocarbon degradation and occurs through a diverse microbial consortium. First, acetogenic bacteria decompose refractory organic matter to acetate and H_2 . Then, the acetate is converted to CO_2 and H_2 through syntrophic acetate oxidation (Reaction 3). These products can then be used by methanogens using CO_2 reduction, resulting in CH_4 (Reaction 1). Another possibility is that the acetate is cleaved into methane and CO_2 (Reaction 4) by acetoclastic methanogenesis [32]. In oil reservoirs, acetoclastic methanogenesis seems subordinate and most methanogenesis (>80%) results from carbonate reduction pathway with H_2 [70].

2.5. Environmental Conditions of Microbial Methanogenesis.

Literature data indicates that methanogens occur ubiquitously in the continental subsurface (e.g. [11, 40, 47]). Continental subsurface findings of methanogenic and microbial activity exceed depths of 3 km [67, 71]. In the Fennoscandian shield, microbial methane is more commonly found at depths shallower than 1.5 km [72]. Warr et al. [7] found isotopic evidence for microbial methane at depth of 2.4 in the Canadian Shield at Kidd Creek Mine, situated within a 2.7 Ga Volcanogenic Massive Sulphide deposit. At Outokumpu deep drill hole, methanogens were less diverse metabolically at depths below 1.3 km than at shallower depths, hydrogenotrophic methanogenesis were found more common as well at these depths [10, 73]. Although the study sites are still quite few, these findings indicate methanogens are widespread, though the conditions and constraints of microbial methanogenesis are still somewhat elusive.

The constraints on anaerobic crude oil biodegradation in the deep subsurface, which produces secondary microbial gases, have been extensively studied in sedimentary rock systems [32, 68, 70, 74]. The maximum temperature limit for the process seems to be 90°C, as in-reservoir petroleum biodegradation ceases above this temperature [70, 75]. Salinity effects on methanogenic oil biodegradation depends on the pathway, as the carbonate reduction pathway has higher salinity tolerance (175 g/l) than other pathways [68]. So far, no inorganic nutrient limitations have been found, and it seems the main limiting factor is carbon availability [68]. These limitations found in petroleum reservoirs might be applicable to all deep life, even though theoretically the deepest extent of the biosphere is at 122°C, or up to 16-23 km below surface in the Fennoscandian Shield of Russia [76]. Realistically, at such high temperatures, the concomitant metabolic rate would be too high to sustain the energy-limited environment of the deep biosphere [75], although cell growth has been reported at 122°C by *Methanopyrus kandleri* [77]. However, characteristics such as pH or temperature have not shown to be a determining factor in the microbial contribution in CH₄ cycle [40], but findings in the Fennoscandian Shield suggest that the amount and isotopic composition of methane could be controlled by lithology [72]. Within the Fennoscandian Shield, no depth dependency was found and C₁/C₂₊ varied from site to site. Kietäväinen et al. [72] found that metasedimentary rocks, particularly graphite-bearing rocks, typically contain high amounts of methane and have C₁/C₂₊ ratios of > 100.

2.6. Isotopic Signature of Microbial Methane. The stable isotopic composition of C and H in CH₄ and substrates can be diagnostic of its origin. Kinetic fractionation, such as microbial metabolism, changes the relative abundance of isotopes in the product compared to the source. The isotopic composition is given by the ratio between ¹³C and ¹²C of the sample compared to a standard reference, such as V-PDB (Vienna Pee Dee Belemnite):

$$\delta^{13}C = \left[\frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{reference}} - 1 \right] \cdot 1000. \quad (4)$$

As microbes selectively prefer ¹²C over ¹³C, this fractionation effect results in a low (i.e., depleted) δ¹³C of the product. Therefore, CH₄ with δ¹³C of -50‰ or less is generally considered diagnostic for microbial origin. Thermogenic CH₄ is generally ¹³C-enriched compared to microbial CH₄, although there is overlap [12, 49]. Abiotic methane is typically even more enriched in ¹³C, but does have a large range in δ¹³C [39].

However, the isotopic composition of the product (CH₄) depends on many factors, including fractionation during the production of microbial methane, temperature, and metabolic rate [78], as well as the substrate type and mixing of different pathways or CH₄ types. Within the Fennoscandian Shield in Finland Kietäväinen et al. [72] suggested that methane of metasedimentary origin characterized by δ¹³C of -40 to -20‰ V-PDB, was produced at low temperatures by microbial methanogenesis and/or abiotic reactions from ancient organic carbon. In the Siljan impact structure, there are samples from crystalline bedrock/sedimentary rock interface showing δ¹³C down to -65‰ V-PDB, suggesting a dominantly microbial fractionation [43], and at Kidd Creek Mine δ¹³C down to -42‰ V-PDB, suggesting a mixture of microbial and abiotic methane [7]. Fractionation resulting from alteration through oxidation and potential secondary methane formation after the formation of CH₄ can alter the isotopic composition as well. Oxidation of CH₄ causes an enrichment in ¹³C and ²H in the residual methane [79]. The many different factors influencing isotopic fractionation complicate straightforward determination of the origin of methane based on isotopic composition alone.

2.7. Kinetic Effects δ¹³C. The magnitude of C isotope fractionation differs for the different metabolic pathways of microbial methanogenesis. Generally, the fractionation is larger for CO₂ reduction pathway than the acetoclastic pathway, evidenced by multiple methanogenic culture experiments [80]. The preference for lighter isotopes by methanogens results in isotopic enrichment of the residual substrate. Subsequently, if the substrate is not replenished, methane gradually becomes more ¹³C-enriched in a closed system as the methanogens are forced to use the increasingly heavier residual substrate. Thus, in cases of substrate depletion, methane isotopic compositions approach those of the original substrate, resulting in a very small fractionation effect. The temperature could also have an effect, with a decreasing fractionation effect with increasing temperature [81, 82]. This can possibly be explained by an increased metabolic rate due to the higher temperature and therefore the substrate concentration decreases faster.

A similar effect is seen during methane oxidation, where lighter isotopes are preferentially oxidized over heavier, which enriches the δ¹³C and δ²H of the residual CH₄. Organisms that perform anaerobic methane oxidation (AOM) preferentially oxidize isotopically lighter CH₄, leaving methane enriched in ¹³C, and the dissolved inorganic carbon (DIC) isotopically lighter [83]. The methane metabolic pathway is mediated by the Wood-Ljungdahl pathway,

for both AOM and methanogenesis. The isotopic fractionation arises from the enzymatic reaction within the pathway, though it has been suggested that isotopic equilibration can occur [84].

The isotopic fractionation can also be seen in carbonate minerals: DIC is used in methanogenesis as a carbon source for carbonate reduction, and the residual DIC pool is enriched in ^{13}C . When carbonate precipitates, the resulting calcite is enriched in ^{13}C , reflecting the methanogenesis [85]. This process has resulted in calcite as isotopically heavy as +36.5‰ V-PDB [30]. Methane oxidation on the other hand, leaves ^{13}C depleted carbonate, which precipitates as ^{13}C -depleted calcites [29, 86]. Other pathways, such as acetoclastic methanogens, would not directly affect the DIC composition.

2.8. Kinetic Effects $\delta^2\text{H}$. The H_2 source for methanogenesis can be H_2O , organic matter or molecular H_2 . As the sources differ, H_2 fractionation between the CO_2 reduction and acetate fermentation pathways is also different (e.g., [49]). The concentration of H_2 also seems to be of importance, and high partial pressure of H_2 causes depleted $\delta^2\text{H}$ values in CH_4 [87, 88]. The high partial pressure of H_2 decreases fractionation between CO_2 and CH_4 , possibly due to limited reversibility of methanogenesis, or inhibition of some methanogenic species [44, 78]. This may have been the case in deeper sourced samples from the Outokumpu deep drill hole where H_2 is abundant and CH_4 depleted in ^2H [72].

2.9. Identification of CH_4 Origin. The source of hydrocarbons including CH_4 (C_1) can be interpreted using genetic diagrams of isotopic signatures and the composition, such as $\delta^{13}\text{C} - \text{C}_1$ vs. $\text{C}_1/(\text{C}_2 + \text{C}_3)$, $\delta^{13}\text{C} - \text{C}_1$ vs $\delta^2\text{H} - \text{C}_1$ and $\delta^{13}\text{C} - \text{C}_1$ vs. $\delta^{13}\text{C} - \text{CO}_2$ [79, 89, 90]. These empirical diagrams have overlapping fields but can be particularly useful separating carbonate reduction pathway methanogenesis derived gas, abiotic gas, or secondary microbial gases (Figure 1(a)).

Microbial methane is generally more depleted in ^{13}C than abiotic, whereas abiotic methane is more associated with higher hydrocarbons (e.g., [91]). However, the maturity of the gas and other secondary processes can affect the composition of the natural gas, making it difficult to interpret its source. Milkov and Etiope [12] revised the three main genetic natural gas diagrams based on a large global dataset of hydrocarbon-containing natural gases. Each category (abiotic, primary, and secondary microbial and thermogenic) was defined by a certain set of characteristics. Primary microbial gases only contain C_1 , C_2 , and C_3 , with C_1 present in much higher amounts, thus being characterized by a high $\text{C}_1/(\text{C}_2 + \text{C}_3)$ ratio along with a strongly depleted $\delta^{13}\text{C}$ value [92]. Thermogenic gases contain C_1 to C_5 and a semilinear relationship of $\delta^{13}\text{C}_n$ values versus $1/n$. As the majority of microbially generated hydrocarbon gas is CH_4 , the ratio between C_1 and C_{2+} can be used to separate microbial from thermogenic gas ($\text{C}_1/\text{C}_{2+} > 1000$). When combined with isotopic composition, it can give further indication of the formation mechanism. Particularly, microbial gas may overlap in the discrimination diagram with early mature thermo-

genic gas, which has lower $\delta^{13}\text{C}$ values and higher C_1/C_{2+} ratio than late mature thermogenic. Secondary microbial gases are specifically identified by a $\delta^{13}\text{C} - \text{CO}_2$ value exceeding +2‰, C_3 enriched in ^{13}C and increased $i - \text{C}_4/n - \text{C}_4$ ratio [32, 74, 93]. This definition does partly overlap with primary microbial carbonate reduction field. Abiotic gases are characterized by a specific combined isotopic signature of C and H in methane, decreasing ^{13}C content in longer chained alkanes and high H_2 concentration [94]. Furthermore, abiotic gases are characterized by an inverse trend for $\delta^{13}\text{C}_n$ values versus $1/n$, opposite to the trend characteristic for thermogenic gases. The presence of He or other noble gasses can indicate whether the natural gas is mantle-derived, and therefore abiotic in origin [39]. The geological setting, habitat, and presence of oil should also be considered in the interpretation of origin. Signs of oxidation and biodegradation include extreme enrichment of C and H in heavy isotopes [95].

2.10. Clumped Isotopes. Multiply substituted isotopologues, also called clumped isotopes, are molecules containing more than one heavy isotope. For methane this refers to mostly $^{13}\text{CH}_3\text{D}$ and $^{12}\text{CH}_2\text{D}_2$, as these are the most common isotopologues [102]. Distribution of isotopes of methane is dependent on temperature, and the heavier isotopes (^{13}C and D) are less likely to be clumped together at higher temperatures [103]. Deviation of multiply substituted isotopologues compared to a purely stochastic distribution of all isotopes is dependent on temperature. Measuring the isotopologues of methane can thus be used as a geothermometer [102]. This only is true when the system is in thermodynamic equilibrium, which would require independent constraints on the environmental temperatures to verify that the system is in equilibrium. If these conditions are met, the clumped isotope composition can be used to understand the origin of methane, as thermogenic methane generally yields a higher formation temperature than microbial methane [100]. This is especially of value in systems where the gas may have migrated from its original source (Figure 1(b)).

In a crystalline rock environment, clumped methane isotopes have been so far used in the Canadian Shield, where values of $\Delta^{13}\text{CH}_3\text{D}$ of 3.5 – 5.8‰ and of $\Delta^{13}\text{CH}_2\text{D}_2$ 12.1 – 18‰ were associated with microbial methane mixed with abiotic methane in crystalline bedrock at depths of 2.4 km in Kidd Creek Mine, Canada [7]. These values indicate microbial reprocessing through reversibility during methanogenesis or AOM, as the values are close to isotopic equilibrium [13, 101, 104]. Stolper et al. [100] proposed that isotopic equilibrium could depend on the reversibility of the enzyme of the methanogens. Slower growth rates show more reversibility in their enzymes, resulting in methane that is in clumped-isotopic equilibrium [105]. AOM could alter the methane isotopologues, by reprocessing microbial methane yielding clumped isotopes values close to thermodynamic equilibrium [106]. However, microbial methane can yield low clumped isotope values (e.g., $\Delta^{13}\text{CH}_3\text{D} < 0\text{‰}$) due to nonequilibrium isotope effects related to methanogenesis [14, 98]. These kinetic isotope effect have been

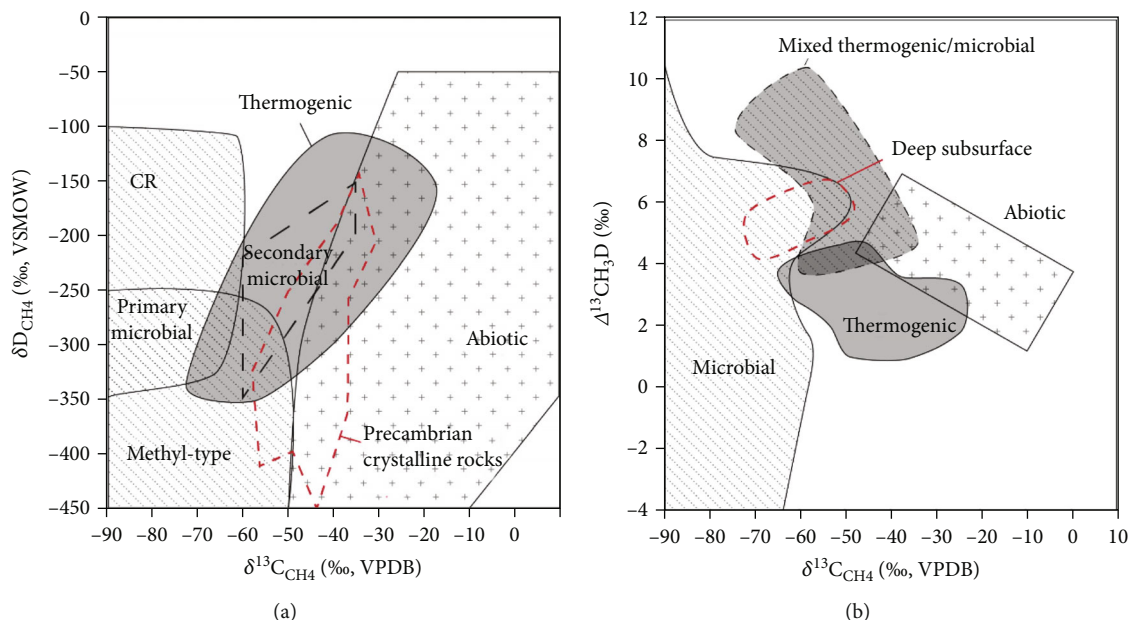


FIGURE 1: Genetic diagrams showing composition fields for methane origins (a) $\delta^{13}\text{C}$ vs δD based on Milkov and Etiope [12]; Etiope and Schoell [96]; (b) $\delta^{13}\text{C}$ vs clumped isotopes, fields based on the figure of Douglas et al. [97]. Environmental origin field for “Deep subsurface” indicated only for microbial samples. Data from [13, 14, 97–101].

shown to vary widely in microbial methane produced in Arctic lake sediments [98]. This further shows the utility of multiply substituted isotopologues in identifying the sources of methane.

2.11. Microbiology and Biomarkers. In addition to analysing the gas composition and isotopic signatures to find the source of methane, the use of other proxies such as organic molecules (often referred to as biomarkers) can aid in determining whether it is possibly microbially formed or not. Biomarkers are chemical compounds that are specific to a group of organisms or type of environment and can be used to determine the archaeal and bacterial composition, pathway, and cycling of carbon. In combination with compound specific isotope analysis, the isotopic composition of these biomarkers can be more specific in elucidating the biomarker source. Archaeal biomarkers indicative of methanogenesis or anaerobic methanotrophs (ANME) were reviewed by Niemann and Elvert [107]. These included archaeol and hydroxyarchaeol, pentamethyleicosane and different isoprenoidal glycerol dialkyl glycerol tetraethers (GDGTs), as well as crocetane. In combination with a ^{13}C -depleted $\delta^{13}\text{C}$ signal, which is typically depleted from -40 to -70‰ compared to the source methane of these lipids, AOM communities can be detected [107]. Additionally, the different ANME groups ANME-1, ANME-2, and ANME-3 have characteristic lipid profiles, which can be distinguished based on the amount and ratio of GDGTs and archaeol, or relative amounts of archaeol and associated bacterial lipid biomarkers. Lipid profiles of ANME-1 archaea are strongly dominated by diglycosidic GDGT (2-Gly-GDGT) [107, 108]. Fatty acids in combination with their compound specific $\delta^{13}\text{C}$ signature can also be used to indicate presence of bac-

terial sulphate reduction [31]. The use of biomarkers can be valuable in detecting metabolic pathways in the deep subsurface, however, as biomarkers are rarely specific to just one group of organisms, it is essential to combine biomarker analysis with additional proxies.

Metagenomic and metatranscriptomic studies can help detect the presence of methanogens in a more detailed way than biomarkers can, by revealing metabolic potentials present in the deep groundwater. For instance, the use of metagenomics has provided evidence for autotrophic carbon fixation by methanogens in the deep biosphere of Outokumpu [10]. The use of metatranscriptomics elucidated the active metabolic processes in the deep subsurface and consequently the syntrophic interactions between SRB, ANME, and methanogens [21]. Key marker genes for methanogenesis can be detected by PCR assay. The same gene is also a marker for AOM. Gene sequences coding for the alpha subunit of methyl-coenzyme M-reductase (*mcrA*) are used to study diversity of ANME populations. *pmoA* marker genes have been used to detect methanotrophic activity in Olkiluoto, Finland and Witwatersrand, South Africa [15, 109]. So far, methanogen marker genes have not been studied comprehensively in the deep continental subsurface.

3. Limitations and Future Outlooks

As the deep biosphere is mostly low in biomass, risk of contamination of the already low cell numbers is high when doing microbial investigation into methanogens. As these studies often take place in predrilled holes, as an economical solution, the drilling itself introduces potential for microbial contamination [110]. As another source of contamination, Purkamo et al. [71] found that most contaminants in their

sequencing dataset originated from nucleic acid extraction kits. Furthermore, the method of filtering for collection of samples for biomass can introduce a bias towards larger cells [71]. In low nutrient environments, it might be advantageous for organisms to have ultra-small cell size ($<0.2\ \mu\text{m}$, [111]), as this increases the area/volume ratio. Biomass collection through filtering creates the impression of a microbial community dominated by larger cells, often heterotrophs, and low proportion of ultrasmall cells. Thus, smaller autotrophic cells are lost during biomass collection, skewing the results towards a high proportion of heterotrophs [112].

Sampling the deep subsurface groundwater under in situ pressure poses its own difficulties. Gases dissolved in the groundwater, specifically gases with low solubility such as H_2 and He can escape when the fluids are brought to the surface and the pressure decreases. However, taking pressurized samples means only a restricted sample volume can be obtained, and gas separation from the fluid sample is laborious [48]. Furthermore, the findings reported so far vary considerably in concentration of methane for instance, and representativity and extrapolation of these findings is uncertain. To analyse the composition and isotopic signature of gas a sufficient volume is required, which causes that samples with small gas volumes fully analysed, leading to a potential bias where only locations with large gas volumes are reported.

The origin of gases in Precambrian Shields is largely unknown, as methane from crystalline bedrock environments was considered to be “geothermal or hydrothermal” methane, adopted after the genetic diagram of Whiticar [49] (also see Figure 1(a)). As this refers to the environmental setting, not its genetic origin, the origin of crystalline methane still requires further addressing [39]. Due to mixing of methane from different sources, determining the fraction and origin of the different sources requires extensive analysis of gas compositions and environmental parameters.

The presence of methanogenic archaea, indicating ongoing microbial methanogenesis, have been detected at sites of relatively ^{13}C -enriched CH_4 , complicating determining the origin of methane ([40] and references therein). This can reflect multiple causes: a mixture of abiotic and microbial methane with a small contribution of microbial methane, or a microbial source with a heavily enriched starting substrate (which is not always known), or a substrate limitation that causes less isotopic fractionation. These issues complicate the determination of microbial methane contribution to the methane pool in the vast continental subsurface realm, and hence to the global carbon cycle.

Apart from how much methane is produced microbially in the crystalline crust, the amount of methane consumption by microbes poses another gap in knowledge. Progress has been made regarding the knowledge of the environmental setting of AOM [15, 17–19, 29] and of rates of AOM in deep subsurface granitic environments, finding AOM rate of $3.7\ \text{nMyr}^{-1}$ in incubation experiments [16]. Methane that would otherwise be released to the atmosphere is being consumed and possibly completely recycled by other microorganisms, such as ANME and other methane oxidizers

[113]. However, whether this methane is released through natural seepage or in pulses and how much subsurface methane is released to the atmosphere, if any, is not known.

Microbial methanogenesis could be widespread in the crystalline crust, though its exact contribution is not yet defined. Recent findings suggest a link between lithology and methane, either due to higher carbon content in the sedimentary rocks or a lithological control on microbiology. The ubiquitous occurrence of methanogenic archaea does suggest presence of microbial methane in the terrestrial subsurface. The determination of microbial methane is complicated by processes obscuring its source, such as mixing of different types of methane, secondary oxidation, and substrate limitation. Furthermore, microbial methane itself can have different diagnostic characteristics as both heterotrophic and autotrophic microbes are involved in the methane cycle, highlighting the need for a multiproxy approach in the interpretation of methane sources. Combining the composition and the isotopic signature of the natural gas, sequencing and biomarker proxies, can help the understanding of CH_4 formation in the deep crystalline subsurface. Taken together, there is an urgent need to expand and more comprehensively explore the processes of microbial methane formation and oxidation in the crystalline bedrock fracture networks.

Data Availability

Review article, no primary data involved.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

We acknowledge the following grants, Swedish Research Council (contracts 2017-05186 and 2021-04365 to H.D.), Formas (contracts 2020-01577 to H.D.), Crafoord Foundation, (contract 20210524).

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