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CH_4-consuming microorganisms and the formation of carbonate crusts at cold seeps

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Abstract

To understand the role played by microorganisms in the formation of cold seep carbonates, we conducted an integrated microbial, mineralogical and organic geochemical study of methane-related authigenic carbonate crusts formed on eastern Mediterranean mud volcanoes. We show that supersaturation with respect to carbonate minerals is induced by microbial anaerobic oxidation of methane. Combined lipid biomarker analysis and 16S rRNA gene surveys identified a highly diversified methane-consuming archaeal community possibly comprising novel species, implying that the anaerobic oxidation of methane is phylogenetically widespread and directly implicating these organisms in the process of crust precipitation. Moreover, pore-water sulphate gradients produced by co-occurring methane-based sulphate reduction exert the main control on aragonite versus magnesian calcite precipitation. We propose that this may be the dominant mode of carbonate crust formation at cold seeps world-wide, in agreement with aquatic chemistry predictions and explaining carbonate mineralogy.

Keywords: cold seeps; carbonates; oxidation; anaerobic environment; DNA; lipids; biomarkers

1. Introduction

Authigenic carbonate crusts formed at seafloor cold seeps are a sink for methane carbon migrating from depth or released from shallow gas hydrates [1,2], and thus partly regulate ocean–atmosphere carbon fluxes. Although the association of methane seepage and oxidation with carbonate crust formation is easily established on the basis

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of carbonate ^{13}C depletion, a number of questions regarding carbonate crust formation remain unanswered: the methane-oxidising microorganisms have not been identified and the relative importance of aerobic and anaerobic processes has to be elucidated. Furthermore, a better understanding of how microbial processes affect dissolved inorganic carbon and carbonate–mineral equilibria is needed and controls on authigenic carbonate mineralogy have to be understood.

Growing evidence is being provided by molecular biogeochemical [3–5] and phylogenetic [6,7] investigations that anaerobic oxidation of methane, which accounts for most of the methane consumption in marine sediments, is carried out by prokaryotic consortia composed of methanotrophic archaea and sulphate-reducing bacteria. Thiel et al. [8] and Peckmann et al. [9] report on cold seep carbonates that contain such molecular indicators, suggesting that the anaerobic oxidation of methane may play an important role in cold seep carbonate formation. However, the microbial community structure of carbonate crusts has not been described so far and the phylogenetic diversity and spatial variability of the microorganisms involved in their formation are largely unknown. To investigate these aspects, we combined mineralogical and stable isotope studies of authigenic carbonates recovered from eastern Mediterranean mud volcanoes with lipid biomarker analysis, compound-specific carbon isotope measurements and 16S rRNA gene surveys.

2. Submersible dives and samples

Eastern Mediterranean mud volcanoes form when tectonically over-pressured and methane-charged mud is extruded at the seafloor and redeposited as mud breccia [10]. Seven such structures at depths between 1600 and 2000 m were explored with the *Nautile* submersible during the Medinaut cruise of the R/V *Nadir* in 1998. Their central parts actively release methane to the bottom waters and are covered by carbonate crust pavements up to several decimetres thick [10] (Fig. 1a). These form by precipitation of aragonite and lesser quantities of high-Mg calcite and dolomite in the shallow pore-water of mud breccia deposits [11]. Two crust samples, collected via the *Nautile* submersible from the Napoli mud volcano (MN16BT2; 33°43.548, 24°41.036; 1945 m

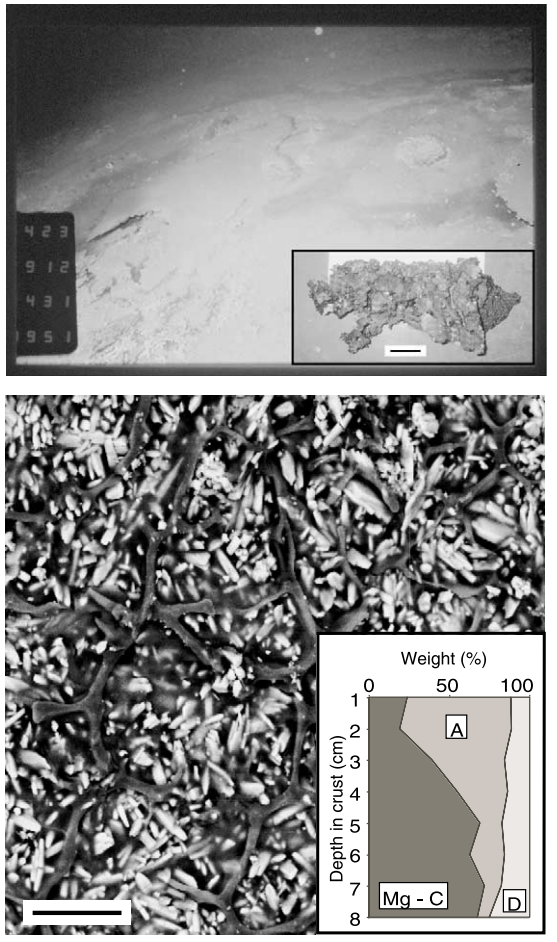

Fig. 1. In situ, macroscopical, microscopical and mineralogical characteristics of carbonate crusts. (a) Seafloor photograph taken from the Napoli mud volcano during a *Nautile* submersible dive showing carbonate crust pavements covering decametric portions of the seafloor. Inset: macroscopic aspect of a carbonate crust sampled from the Amsterdam mud volcano (scale bar is 5 cm). (b) Scanning electron microscope photograph of organic matter associated with authigenic aragonite crystals in crust MN16BT2 (scale bar is 25 μm). Inset: relative abundance of authigenic carbonate minerals in a vertical transect of crust MN13BT4. Authigenic aragonite (A) prevails in the top 3 cm of the crusts and is substituted by authigenic high-Mg calcite (Mg-C) in the bottom part of the crust. Dolomite (D) accounts for less than 20% of the authigenic carbonates.

water depth) and from the Amsterdam mud volcano (MN13BT4; 35°19.859, 30°16.528; 2034 m water depth), were investigated.

3. Methods

3.1. Mineralogy and stable isotope composition of carbonates

The mineralogy of the carbonate crusts was determined by X-ray diffraction of dried and ground subsamples of carbonate crusts. Authigenic (high-Mg) calcite was distinguished from pelagic (low-Mg calcite) on the basis of *d* values of the (104) diffraction peak which are equal to approximately 2.998 Å and 3.028 Å, respectively [11]. The oxygen and carbon stable isotope compositions of aragonite and high-Mg calcite were measured using a specific procedure to account for the presence of authigenic dolomite and calcite of pelagic origin in the crusts as explained in Aloisi et al. [11]. The carbon and oxygen isotopic compositions are expressed against VPDB.

3.2. Lipid analysis

Lipid extraction, separation and analysis by gas chromatography (GC), GC-mass spectrometry (MS), GC-isotope ratio monitoring MS and high-performance liquid chromatography (HPLC)-MS were conducted as reported previously [3,12]. $\delta^{13}C$ values are expressed against VPDB after correction for the addition of carbon during derivatisation and have an error of less than $\pm 1.0‰$.

3.3. 16S rRNA gene sequencing

Total DNA was isolated from dried and ground crust samples using a Tris/EDTA/SDS extraction buffer (0.1 M Tris–HCl/0.5 M EDTA/1% SDS, pH = 8.0) and 0.12 M phosphate washing buffer (pH = 8.0). DNA was extracted with a phenol–chloroform extraction and precipitated with ethanol. DNA was cleaned by gel filtration. The total amount of DNA was measured spectrophotometrically. Archaeal 16S ribosomal RNA genes were amplified from the total chromosomal DNA pool by using a slightly modified S-D-Arch-0021-a-S-20 forward primer (5′-TTCCGGTTGATCCTGCCGGA-3′) in combination with the S-D-Arch-0958-a-A-19 reversed primer (5′-YCCGGCGTTGAMTCCAATT-3′), yielding PCR products of ca. 950 bp. After gel purification PCR products were cloned with a pCRII plasmid vector kit. Two archaeal 16S rDNA libraries were constructed yielding 41 positive clones. Insert containing clones (10–15 for each library) were selected and partial sequences were determined (ca. 600 bp) on an ABI 310 Automated Sequencer. Partial 16S rDNA sequences were submitted to GenBank and BLAST was used to determine the closest relatives. Sequences were aligned using the multiple alignment method in the ClustalW computer program. Evolutionary distances were corrected [13], and a phylogenetic tree was constructed by neighbour-joining analysis with TreeconW.

4. Mineralogy and stable isotope composition

Aragonite and high-Mg calcite cements account for up to 82% of the crusts in weight. The carbonate cements are depleted in ^{13}C ($\delta^{13}C$ values of MN16BT2 and MN13BT4 are $-28.9‰$ and $-24.8‰$, respectively) indicating that methane is a major source for the carbonate carbon. They are also up to $2.2‰$ enriched in ^{18}O ($\delta^{18}O$ values of MN16BT2 and MN13BT4 are $5.1‰$ and $3.5‰$, respectively) compared to expected values for aragonite and high-Mg calcite precipitating from modern eastern Mediterranean bottom waters [11]. This is likely due to the decomposition of gas hydrates that are widespread in the shallow sediments of eastern Mediterranean mud volcanoes [10] and/or the seepage of ^{18}O-rich brines on the Napoli mud volcano. Other, deeper, sources of ^{18}O-rich fluids, however, cannot be excluded.

5. Microbial community structure

Scanning electron microscope observations revealed organic matter within the authigenic car-

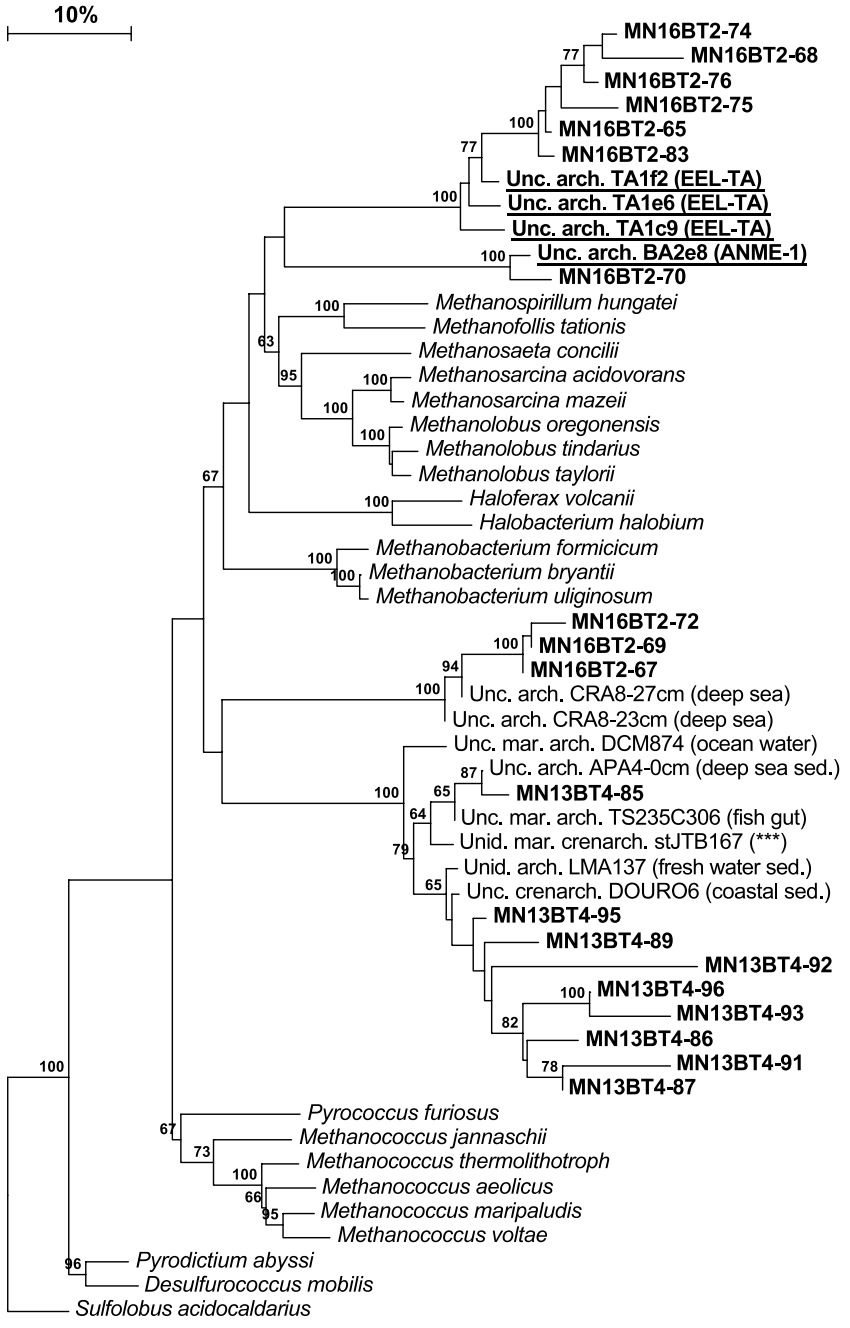

Fig. 2. Phylogenetic tree of archaeal rDNA sequences obtained from carbonate crusts. Known sequences of methanogenic orders (*Methanococcales*, *Methanobacteriales*, *Methanomicrobiales* and *Methanosarcinales*) are shown as well as (unidentified) archaeal sequences obtained from deep sea sediments (deep sea), coastal lagoon sediments (coastal sed.), intestinal tract of pelagic fish (fish gut), fresh water lake sediments (fresh water sed.), ocean water samples (ocean water) and a deep sea cold seep sediment sample from the Japan Trench (***). *Sulfolobus acidocaldarius*, *Desulfurococcus mobilis* and *Pyrodictium abyssi* were used as outgroups. The scale bar represents the estimated evolutionary distance (average of 10 substitutions per 100 nucleotide positions).

bonate mineral matrix (Fig. 1b) that could partly derive from microorganisms involved in the formation of such minerals. To identify these microorganisms, DNA was extracted from the carbonate; this is the first time DNA has been obtained from cold seep carbonate crusts and allowed the microbial community structure to be directly evaluated. Bacterial 16S rRNA gene surveys failed to identify sequences related to aerobic methanotrophic bacteria and suggest that, if present, aerobic methanotrophs play a minor role in methane oxidation and carbonate crust formation. Archaeal 16S rRNA gene surveys identified a great diversity of archaeal lineages, none of which is directly related to known methanogens (Fig. 2). In crust MN16BT2, three archaeal clusters are present, two of which are related to archaea previously identified in cold seep sediments (EEL-TA and ANME-1 clusters) [6], while a third is comprised of novel, previously uncharacterised archaeal sequences. In crust MN13BT4, 10 new archaeal sequences comprising several lineages were identified. All archaeal clusters in crust MN13BT4 are distinct from those of crust MN16BT2 and have not been previously reported for any setting. The closest matches to these lineages are archaeal sequences obtained from deep sea sediments or gut intestines of pelagic fish [14,15].

Phylogenetic studies of cold seep [6,7] and normal marine [16] sediments have identified a potentially diverse methane-oxidising archaeal community. The direct involvement of one group of archaea (ANME-2 group) in this process has been proved by fluorescence in situ hybridisation (FISH) [17] and by coupling FISH with secondary ion MS [18]. Sequences belonging to the ANME-2 group, however, are absent from the samples we studied. Here, the anaerobic oxidation of methane is likely carried out by archaea related to the ANME-1 group in crust MN16BT2 and, possibly, to the newly identified groups in both crusts. This possibility, that will be discussed later, would confirm and expand previous assumptions [3,5,7] that the archaeal community capable of oxidising methane anaerobically is phylogenetically diverse.

In crust MN16BT2, archaea co-occur with previously uncharacterised chemoorganotrophic sulphate-reducing bacteria belonging to the delta proteobacteria group which account for 25% of the sequenced bacterial clones (data not shown). In contrast, the bacterial gene survey failed to detect any related sequences in crust MN13BT4 reflecting either the absence (or low abundance) of these organisms in this crust or the degradation of bacterial DNA.

6. Lipid biomarker analysis

Lipids were analysed to further evaluate the nature of the microbial communities in the crusts. The lipid extracts of both crusts lack biomarkers diagnostic of aerobic methanotrophic bacteria [19,20], consistent with the results of the DNA analyses. Instead, they contain a highly diverse set of archaeal biomarkers [12,21] including diphytanylglycerol diethers (archaeol and *sn*-2- and *sn*-3-hydroxyarchaeol), glycerol dibiphytanylglycerol tetraethers, as well as saturated and unsaturated C_{20} (crocetane) and C_{25} (PMI) irregular isoprenoids (Fig. 3). These are the most abundant compounds in both crusts, and in MN16BT4 archaeal lipids are several orders of magnitude more abundant than pelagic-derived compounds. This suggests that the abundant organic material observed in the crusts (Fig. 1b) is indeed of prokaryotic origin. However, the distributions of the archaeal biomarkers differ significantly between the two crusts (Fig. 3), likely reflecting two distinct archaeal communities and reaffirming the DNA analyses. All archaeal biomarkers are highly depleted in ^{13}C ($-96.0 < \delta^{13}C‰$ vs PDB < -66.1) indicating assimilation of ^{13}C-depleted methane by the source organisms. This confirms that, in agreement with recent reports [3,5,8], archaea are essential components of the anaerobic methane-oxidising consortium.

Other major lipid constituents are two novel series of dialkyl glycerol diethers characterised by either an anteiso pentadecyl moiety (series I) or an 11,12-methylenehexadecyl moiety (series II) ether bound at the *sn*-2 position of the glycerol group [22] (Fig. 3). Alkyl glycerol monoethers (and to a lesser extent alkyl glycerol diethers), which are structurally different from the ones identified in the present study, were previously

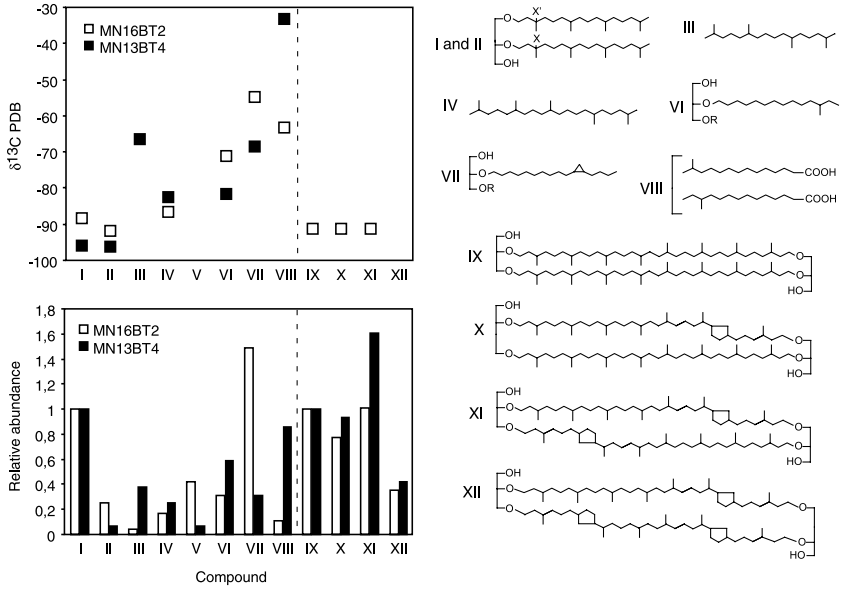

Fig. 3. $\delta^{13}C$ values and relative abundances of major biomarkers in carbonate crusts. Relative abundances are normalised to the concentration of archaeol (I: 4.7 μg g^{-1} in sample MN16BT2 and 0.27 μg g^{-1} in sample MN13BT4) for I–VIII and to caldarchaeol (IX) for IX–XII. Selected structures are shown: I, archaeol (X, X′ = H); II, *sn*-2- or *sn*-3-hydroxyarchaeol (X or X′ = OH); III, crocetane; IV, pentamethylicoesane (PMI); V, unsaturated PMI's; VI, series 1 dialkyl glycerol diethers; VII, series 2 dialkyl glycerol diethers; VIII, iso- and anteiso-C_{15} and C_{17} (not shown) fatty acids; IX, caldarchaeol; X, tetraether with one cyclopentane ring; XI, tetraether with two cyclopentane rings; XII, tetraether with three cyclopentane rings. In both series of dialkyl glycerol diethers R = C_{14}–C_{17} alkyl unit (either *n*-alkyl or alkylcyclohexyl). $\delta^{13}C$ values of compounds IX–XII were not measured; reported values instead represent the carbon isotopic compositions of the acyclic, monocyclic and dicyclic biphytanes released by HI/LiAlH4 cleavage of intact tetraethers.

described from a cold seep setting [5]. Both alkyl glycerol mono- and diethers have been tentatively ascribed to previously uncharacterised sulphate-reducing bacteria [5,22]. In the present study, this is consistent with the predominance of these compounds in crust MN16BT2 in which new sulphate-reducing bacterial lineages were identified. The presence of the dialkyl glycerol diethers in crust MN13BT4, where no such lineages were found, could reflect the markedly lower abundance of such organisms at that site; indeed these diethers are over an order of magnitude less abundant than in MN16BT2. The $\delta^{13}C$ values of both series I and II alkyl diethers ($-83.6 < \delta^{13}C$‰ PDB < -51.9) are significantly lower than expected for normal marine biomarkers, indicating that the source organisms utilise carbon at least partially derived from ^{13}C-depleted methane (Fig. 3). The presence of sulphate reducers that incorporate methane-derived carbon in both crusts is further supported by the presence of ^{13}C-depleted iso- and anteiso-C_{15} and -C_{17} fatty acids [23] (Fig. 3). These compounds, similarly isotopically depleted, were also found in an extensive study of the surrounding seep sediments [3], where, in contrast, the dialkyl glycerol diethers are generally absent. Consequently, if the source organisms of the dialkyl glycerol diethers are sulphate-reducing bacteria, they belong to different species than those present in nearby seep sediments. Their predominance in crusts suggests that they may play an important role in authigenic carbonate precipitation.

7. Discussion

In sediments, DNA degrades more rapidly than lipids do. Thus, archaeal lipids and archaeal 16S rRNA genes co-occurring in a carbonate crust

may not originate from the same organisms, if the methane flux supporting the archaeal methanotrophic communities is no longer active and the carbonate crust has aged prior to sampling.

Preferential preservation of lipids compared to 16S rRNA genes in crust MN16BT2 seems improbable, though. Both the 16S rRNA sequences (the ANME-1 group) and the ^{13}C-depleted archaeal biomarkers in this crust dominate and have been previously ascribed with confidence to methanotrophic archaea. In crust MN13BT4, instead, only novel archaeal sequences have been found and it is possible that some (or all) of these sequences are unrelated to methanotrophic archaea. Should this be the case, however, the presence of archaea not directly assimilating methane would likely have diluted the isotopic signature of methanotrophic archaeal-derived biomarkers. On the contrary, all archaeal-derived lipids in this crust are strongly depleted in ^{13}C. In fact, archaeol and hydroxyarchaeol are more depleted in crust MN13BT4 than in the nearby unconsolidated sediments [3] or in crust MN16BT2 (Fig. 3), arguing against dilution of the archaeal lipids by a non-methanotrophic archaeal community. It is thus plausible that some of the novel sequences in crust MN13BT4 may belong to previously uncharacterised methane-consuming archaea.

These newly discovered archaea could be either facultative or obligate methanotrophs. The former is consistent with the proposal that some methanogenic archaea can assimilate, rather than produce, methane in a process known as reverse methanogenesis [3,24]. In crust MN16BT2, this would account for the high relative abundance and ^{13}C depletion of hydroxyarchaeol and unsaturated PMI, biomarkers that are particularly diagnostic for methanogens [25–27]. It is also consistent with the presence of sulphate-reducing bacteria that would consume H_2 and keep reverse methanogenesis thermodynamically favourable [24]. However, since no sequences closely related to known methanogens were detected, this requires that some or all of the novel archaeal clusters represent new clades of methanogens. Alternatively, the novel archaea could be obligate methanotrophs. Although such organisms have never been isolated, their existence has been proposed [6]. Alternatively, both facultative and obligate methanotrophs are present in the crusts and the phylogenetic and archaeal lipid diversity reflects their presence in different proportions.

Previous studies – including those involving related mud volcano sediments – clearly show that anaerobic methane-oxidising archaea co-exist with sulphate-reducing bacteria [3,17]. The differences in the relative abundances of lipids diagnostic for sulphate-reducing bacteria in our samples suggest that the sulphate reducers also could be phylogenetically diverse.

It is clear that microorganisms – particularly anaerobic methane-oxidising archaea and in some cases sulphate-reducing bacteria – are intimately associated with carbonate crust precipitation. This association is likely due to microbially mediated reactions that affect dissolved inorganic carbon equilibria and carbonate mineral stability. Methane oxidation in an aerobic environment produces CO_2 and decreases pH, favouring dissolution of carbonates rather than their precipitation. In the studied crusts, however, microorganisms have oxidised methane anaerobically, a process that increases carbonate alkalinity and favours the precipitation of authigenic carbonates [1]:

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$$

Moreover, co-occurring sulphate reduction likely affects the carbonate mineral phase. In crust MN13BT4, authigenic aragonite and high-Mg calcite concentrations show opposite vertical trends (Fig. 1b, inset). Aragonite is dominant in the topmost part of the crust (up to 65% of the authigenic carbonates) and is substituted by high-Mg calcite in its lower part. Detailed observations with scanning electron microscopy and thin section analysis do not show evidence of aragonite re-crystallisation into Mg calcite. Instead, these trends are consistent with the mineralogy being controlled by a gradient of dissolved sulphate, one of the most important inhibitors of high-Mg calcite precipitation [28]. Pore-water geochemical studies have shown that the anaerobic oxidation of methane in marine sediments is coupled to sulphate reduction [29–31], and in eastern Mediter-

ranean mud volcano sediments, these processes result in complete sulphate depletion at a depth of 15 cm (unpublished results of the Medinaut investigation). Here, for the first time, mineralogy-independent diagenetic indicators show that aragonite can be the predominant authigenic carbonate phase in an anaerobic cold seep environment provided that sulphate is sufficiently abundant to inhibit precipitation of high-Mg calcite. Thus, previous conclusions that aragonite in cold seep carbonate crusts is an indicator of aerobic oxidation of methane [32,33] might not be valid.

8. Implications and conclusions

Recent studies show that gas hydrate destabilisation following bottom water temperature increase affected past global climate [34] and could be an important aspect of future climate change. Authigenic carbonates formed at cold seeps are both archives of ancient methane release events as well as carbon sinks that potentially mitigate the climatic impact of such events. The microbial organisms and processes we describe provide new insights into the interpretation of methane release from marine sediments. They reveal that active microbial consortia are involved with crust precipitation, that these consortia are supported by methane oxidation, and that both crust formation and methane oxidation occur under anoxic conditions. Thus, methane-derived carbon at cold seeps could be partly sequestered at or below the sediment–water interface. Moreover, lipid and phylogenetic analyses reveal variability in both the archaeal and bacterial components of the microbial consortia, suggesting the organisms responsible are more diverse than previously thought and could be widespread.

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