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Carbon stable isotope patterns of cyclic terpenoids: A comparison of cultured alkaliphilic aerobic methanotrophic bacteria and methane-seep environments

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ABSTRACT

Aerobic methanotrophic bacteria are known to synthesize a variety of cyclic terpenoids which are typified by ^{13}C-depleted, methane-derived carbon. This peculiarity facilitates identification of methanotroph biomarkers in natural samples. However, the current biomarker database does not always allow biomarker patterns of marine samples to be assigned to the different types of aerobic methanotrophs. To overcome this shortcoming, the carbon stable isotope composition of cyclic terpenoids of two strains of the Type I methanotroph genus *Methylomicrobium* was analyzed. Other than aerobic methanotrophs used for biomarker studies in the past, these two strains deriving from soda lake environments are able to tolerate the conditions typifying marine environments including high alkalinity and salinity. The cyclic terpenoid inventory of the two strains comprises 4-methyl steroids, 3-methyl- and desmethyl bacteriohopanepolyols (aminotetrol and aminotriol), and tetrahymanol, all of which are ^{13}C-depleted. The average carbon isotope fractionation between methane and the respective lipid ($\Delta\delta^{13}C_{terpenoid-methane}$) is found to be −25‰ for *M. kenyense* and −16‰ for *M. alcaliphilum*. These data shed new light on the previously reported compound and carbon stable isotope patterns of cyclic terpenoids from methane-seep environments. Particularly, ^{13}C-depleted tetrahymanol and gammacerane are reinterpreted as biomarkers of aerobic methanotrophic bacteria based on their occurrence in methane-seep deposits in association with other biomarkers of aerobic methanotrophs. The use of $\delta^{13}C$ values of anaerobic methane-oxidizing archaea (ANME) lipids for the reconstruction of the isotopic composition of parent methane allows us to calculate the $\Delta\delta^{13}C_{terpenoid-methane}$ even for ancient seep environments. With this calculation, Type I and Type II methanotrophs can be discriminated, representing a new approach to better characterize past methanotrophy at seeps and possibly other marine environments.

1. Introduction

Methane is the simplest alkane and among the most abundant organic compounds in the atmosphere. Due to its radiative forcing, it is considered a major contributor to global warming (Myhre et al., 2013). Understanding the biogeochemistry of methane and the processes leading to its formation and consumption are consequently of major interest (Deppenmeier, 2002). Natural sources of methane are rice paddies, cattle, thawing of permafrost, as well as marine cold seeps (Reeburgh, 2007; Lupascu et al., 2014). Marine methane seepage occurs in a variety of geologic settings, including active and passive continental margins (Suess, 2010). Methane at marine seeps can be biogenic, thermogenic, or of a mixed source. Even though seeping methane can reach the atmosphere, a larger fraction of it is already microbially oxidized in the sediment before reaching the overlaying water column and atmosphere (Ciais et al., 2013).

The major biogeochemical process removing methane at marine seeps is the sulfate-driven anaerobic oxidation of methane (AOM; Boetius et al., 2000). As methane-rich fluids move upward through sulfate-rich pore water, methane is oxidized under anoxic conditions by a consortium of anaerobic methane-oxidizing archaea (ANME) and sulfate-reducing bacteria (Hinrichs et al., 1999; Hinrichs and Boetius, 2002; Orphan et al., 2002; Birgel et al., 2006a; Niemann et al., 2006). Signatures of the AOM community are typically preserved in authigenic carbonates that form at seeps as a consequence of an increase in alkalinity caused by AOM (Peckmann and Thiel, 2004). Although AOM is the predomi-

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nant biogeochemical process at seeps, aerobic methane oxidation (MOx) performed by methanotrophic bacteria contributes to different degrees to local methane consumption (Birgel et al., 2006a; Niemann et al., 2006; Lösekann et al., 2007; Tavormina et al., 2008).

Molecular fossils (i.e., lipid biomarkers) of the microorganisms involved in AOM are typically preserved in seep carbonates as old as the Paleozoic, revealing information on the affiliations of microorganisms (Peckmann et al., 1999; Thiel et al., 1999; Birgel et al., 2008b). Biomarkers of both AOM-consortia and MOx, especially bacteriohopanepolyols (BHPs) for the latter, have been identified in sediments, authigenic carbonates, and mussel gills from recent seeps (e.g., Birgel et al., 2011; Kellermann et al., 2012; Himmler et al., 2015). In ancient seep deposits, BHPs have not been identified to date, but BHP degradation products including hopanoic acids and hopanes, together with tetrahymanol or gammacerane, and 4-methyl steranes (lanostanes) have been reported (Peckmann et al., 1999, 2004; Birgel et al., 2006b; Sandy et al., 2012; Natalicchio et al., 2015). The capacity of aerobic methanotrophic bacteria to synthesize a variety of cyclic triterpenoids – a group of lipids among the oldest and most ubiquitous compounds on Earth (Taylor, 1984) – facilitates their identification in the rock record. The biosynthesis of cyclic triterpenoids in aerobic methanotrophs is controlled by various cyclases, which catalyze the transformation of the acyclic triterpenoid squalene ($C_{30}H_{50}$) either to pentacyclic (i.e., chiefly hopanoids) triterpenoids (by squalene-hopene cyclase) or tetracyclic (i.e., steroids) triterpenoids (by oxidosqualene cyclase) (Welander et al., 2010; Wei et al., 2016). Among the pentacyclic triterpenoids, various BHPs have been reported to occur in some bacteria and are commonly accompanied by the C_{30} hopanoids diploptene and diplopterol (Rohmer et al., 1984; Talbot and Farrimond, 2007).

Aerobic methanotrophic bacteria utilize methane as a sole carbon and energy source. The oxidation of methane by methanotrophs is catalyzed by enzymes known as methane monooxygenases (MMO), which occur, depending on the methanotroph species, either in a particulate membrane-bound form (pMMO) or a cytoplasmic soluble form (sMMO) (Hanson and Hanson, 1996; Bowman, 2006). Aerobic methanotrophs are classified into three groups based on the carbon assimilation pathway they use (i.e., Type I, Type II, Type X; Hanson and Hanson, 1996). The lipid biomarker inventories of Type I and II methanotrophs have been detailed by Talbot et al. (2001). These authors reported characteristic BHP patterns for Type I (dominated by aminopentol) and Type II methanotrophs (dominated by aminotetrol). Type I methanotrophs are known to show great versatility in terms of adaptation to different environmental conditions and occur in terrestrial, aquatic, and marine ecosystems, whereas Type II methanotrophs are apparently less versatile and are believed to be restricted to terrestrial habitats (Knief, 2015). However, Birgel et al. (2011) and Himmler et al. (2015) reported BHP patterns of seep carbonates that rather point to Type II methanotrophs as source organisms. In contrast, the occurrence of 4-methyl steroids in some ancient (e.g., Peckmann et al., 1999, 2004; Birgel and Peckmann, 2008) and modern seeps (Elvert and Niemann, 2008; Bouloubassi et al., 2009) has been interpreted to reflect the former presence of Type I methanotrophs, because Type I and X methanotrophs are unique in synthesizing steroids among Bacteria (e.g., Bouvier et al., 1976; Schouten et al., 2000).

The putative occurrence of Type II methanotrophs at marine seeps is not the only inconsistency between reported biomarker patterns of cultures of aerobic methanotrophs and environmental samples. Rush et al. (2016) demonstrated that some Type I methanotrophs rather produce a BHP pattern typified by the dominance of aminotriol, while aminopentol, which is considered specific to Type I methanotrophs according to culture studies (e.g., Neunlist and Rohmer, 1985; Talbot et al., 2001), is absent. In methane-affected sediments and carbonates, aminotetrol and aminotriol are most common, but aminopentol has not been reported (Birgel et al., 2011; Himmler et al., 2015). In fact, the only known marine sediments with abundant aminopentol are characterized by high riverine input; in this case aminopentol is rather derived from soil methanotrophs (Wagner et al., 2014; Spencer-Jones et al., 2015).

The difficulties in assigning molecular fossils of aerobic methanotrophs from seep environments to the three types of methanotrophic bacteria do not end with molecular patterns but continue with compound-specific carbon stable isotope patterns. The different types of aerobic methanotrophs show significant variations in the $\delta^{13}C$ values of lipids and the fractionation between carbon source and respective lipid (Summons et al., 1994; Jahnke et al., 1999). Compound-specific isotope compositions provide insights into the origin of the carbon assimilated by microorganisms and can be used to constrain microbial populations and biosynthetic pathways (Hayes et al., 1990). Because the carbon source and predominant microbial populations are dependent on environmental conditions, carbon isotopic compositions can provide information on the biochemical processes occurring in the environment (Hayes, 1993). The carbon isotopic fractionation between methane and methanotroph biomass depends on the isotopic composition of the substrate (i.e., methane), the assimilation pathway, and methane and oxygen availability (Summons et al., 1994). For biomarker applications on geologic material, the carbon assimilation pathway is particularly relevant, yet difficult to identify. Biomarkers from ancient seep limestones assigned to methanotrophic bacteria are commonly highly depleted in ^{13}C with values as low as $-100‰$ (e.g., 3-methyl-anhydrobacteriopane tetrol; Birgel and Peckmann, 2008), suggesting significant fractionation relative to the carbon source.

Type I and X methanotrophs terpenoids are typically more ^{13}C-depleted than Type II terpenoids. The fractionation between methane and terpenoids for Type X methanotrophs is approximately $-23‰$ on average, ranging from $-31‰$ to $-18‰$ (cf., Jahnke et al., 1999). Type II terpenoids are less ^{13}C-depleted and fractionation is smaller, ranging from $-12‰$ to $10‰$ (avg.: $-1‰$; Jahnke et al., 1999). Due to ^{13}C depletion of thermogenic and, particularly, biogenic methane, low $\delta^{13}C$ values of terpenoids from environmental samples can be interpreted as biomarkers of aerobic methanotrophic bacteria (Collister et al., 1992; Himmler et al., 2015). Unfortunately, only very few and exclusively non-marine strains have been used for carbon isotope studies of aerobic methanotrophic bacteria in the past (Summons et al., 1994; Jahnke et al., 1999). As a consequence of this, it is uncertain whether or not the range of carbon isotope fractionations among Type I and Type II methanotrophs is as diverse as their BHP patterns.

Here, two alkaliphilic strains of Type I methanotrophs (*Methylomicrobium kenyense* and *Methylomicrobium alcaliphilum*) were selected for a carbon stable isotope study, since BHP patterns of the two strains are similar to the patterns reported for recent seep carbonates. *Methylomicrobium kenyense* and *Methylomicrobium alcaliphilum* are non-marine strains, but tolerate high alkalinities and salinities, which are conditions under which seep carbonates are forming (see Section 4.1). A comparison of the results of our culture experiments with environmental data from 13 seep deposits is provided to better understand the range of compound-specific isotope compositions of various terpenoids from methane-seep environments and to differentiate between Type I and II methanotrophs. We also provide new biomarker data for three seep deposits (Alaminos Canyon, Gulf of Mexico; Makran convergent margin, offshore Pakistan; Marmorito, Miocene, Italy). The 13 modern and ancient methane-seep deposits represent fully marine settings where both AOM and MOx occurred. Known stable isotope fractionations between methane and AOM-biomarkers are used to estimate the isotopic composition of the respective methane sources. The latter is done to assess if fractionation between methane and MOx biomarkers may allow to identify the type of aerobic methanotrophs that dwelled at seeps.

2. Material and methods

2.1. Cultures of aerobic methanotrophs

Methylomicrobium kenyense and *Methylomicrobium alcaliphilum* are both Type I methanotrophs. The strains were isolated from surface sediments of highly alkaline soda lakes in Kenya and Russia, respectively (Kalyuzhnaya et al., 2008) and were obtained for this study from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ No. 19305 and 19304, respectively). The biomarker inventories of *M. kenyense* and *M. alcaliphilum* were previously described by Banta et al. (2015) and Rush et al. (2016). The cultivation of both strains was done at the Center for Applied Geosciences at the University of Tübingen. Strains were grown in serum bottles using a high salt NMS medium (nitrate mineral salts), containing 1.5% NaCl, 0.1% KNO_3, 0.1% $MgSO_4$, 0.02% $CaCl_2$ and trace elements. The gas phase to liquid ratio was 10:1. The pH was adjusted to 9.1 and cultures were incubated at 28 °C and shaken at 200 rpm. The initial gas-mixing ratio was adjusted to methane:air (1:1, v/v), representing a O_2:CH_4 ratio of 0.21. The conditions of the batch culture provided a closed system, where new nutrients were not added, and waste products were not removed. Only new methane was supplied daily to the experiments to compensate for methane consumption. Cells were harvested by centrifugation as they entered the stationary phase and freeze-dried for posterior analyses. The growth stage was determined by measuring the optical density at 600 nm (OD600).

2.2. Lipid extraction

Freeze-dried bacterial cells of *M. kenyense* and *M. alcaliphilum* as well as carbonate samples from three methane seep deposits (Alaminos Canyon, Makran convergent margin, Marmorito) available in house (Peckmann et al., 1999; Birgel et al., 2011; Himmler et al., 2015), were extracted and analyzed for their lipid biomarker contents. The samples were extracted with a mixture of dichloromethane/methanol (3:1, v/v) by ultrasonication until the solvents became colorless. An aliquot of the total lipid extract (TLE) was hydrolyzed with 6% KOH in methanol to cleave ester-bond lipids. The neutral lipids were separated by aminopropyl-bonded silica gel column chromatography into fractions using a sequence of solvents with increasing polarity: (1) hydrocarbons were eluted with *n*-hexane; (2) ketones/esters with *n*-hexane/dichloromethane (3:1, v/v); and (3) alcohols with dichloromethane/acetone (9:1, v/v). Alcohols were analyzed as their trimethylsilyl ethers (TMS-derivatives) by reaction with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) in dichloromethane and pyridine (Birgel et al., 2006b). A second aliquot of TLE was acetylated by reaction with acetic anhydride/pyridine (1:1, v/v) for analyses of BHPs.

2.3. Biomarker analysis

All fractions were analyzed via gas chromatography mass spectrometry (GC–MS) using an Agilent 7890 A GC system coupled to an Agilent 5975 C inert MSD mass spectrometer at the University of Vienna. Compounds were separated using a 30 m HP-5 MS UI fused silica capillary column (0.25 mm i.d., 0.25 µm film thickness) and He as a carrier gas. The GC temperature program was: 60 °C (1 min) to 150 °C at 10 °C/min, then 150 °C to 325 °C at 4 °C/min, 35 min isothermal. The identification by GC–MS was based on GC retention times and comparison of mass spectra with published data. Internal standards with known concentrations were added prior to extraction for quantitation. Acetylated aliquots of TLE were analyzed by means of liquid chromatography–mass spectrometry (LC–MS) for identification and quantitation of BHPs, as described in Rush et al. (2016). The quantification was done using 5α-cholestane as internal standard on the GC–FID for aminotriol and was correlated with non GC-amenable aminotetrol on the HPLC–MS (see Eickhoff et al., 2013 for details). For stable carbon isotope measurements of BHPs, an aliquot of underivatized TLE was treated with periodic acid and subsequently with sodium borohydride to cleave the C–C bonds between neighboring polyols and convert the BHPs in GC-amenable hopanols (Rohmer et al., 1984). The periodic acid cleavage procedure yield C_{32} 17β(H),21β(H)-hopanol (bishomohopanol) from tetrafunctionalised BHPs (e.g., aminotriol and bacteriohopanetetrol), C_{31} 17β(H),21β(H)-hopanol (homohopanol) from pentafunctionalised BHPs (e.g., aminotetrol and bacteriohopanepentol), and C_{30} 17β(H),21β(H)-hopanol (hopanol) from hexafunctionalised BHPs (e.g., aminopentol). Finally, the hopanol products were derivatized with BSTFA and pyridine as described for the alcohols (see Section 2.2).

The identified sterols, hopanoids, and hopanol products of BHPs, were analyzed for their compound-specific isotope compositions on a gas chromatograph (Agilent 6890) coupled with a Thermo Finnigan Combustion III interface to a Finnigan Delta Plus XL isotope ratio monitoring-mass spectrometer (GC–IRM–MS) at the University of Hamburg. The GC conditions were identical to those above for GC–MS analyses. The $\delta^{13}C$ values have been corrected for the addition of carbon during preparation of TMS-derivatives. Each measurement was calibrated using several pulses of carbon dioxide with known isotopic composition at the beginning of the run. Instrument precision was checked with a mixture of *n*-alkanes (C_{15} to C_{29}) of known isotopic composition. The carbon isotope ratios are expressed as $\delta^{13}C$ values relative to the V-PDB standard. Standard deviation was less than 0.8‰.

2.4. Stable carbon isotopic composition of methane

The stable carbon isotopic signature of the methane supplied to the strains *M. kenyense* and *M. alcaliphilum* (−46‰ vs V-PDB) was determined by means of GC–IRM-MS using a Thermo Fisher Scientific Trace GC connected to a Thermo Fisher Scientific MAT253 Isotope ratio mass spectrometer at the University of Duisburg-Essen.

3. Results and compilation of previous data

Results presented here are a combination of new data and published data. First, we present the carbon stable isotope values of lipids of cultured Type I methanotrophs *M. kenyense* and *M. alcaliphilum*. Second, carbon stable isotope values of lipids from ancient and modern methane-seep deposits (13 sites in total), as well as data for Type II (*Methylosinus trichosporium*) and Type X (*Methylococcus capsulatus*) methanotrophs are compiled from published work. We also provide some new data for three out of the 13 seep deposits (Alaminos Canyon, Gulf of Mexico; Makran convergent margin, offshore Pakistan; Marmorito, Miocene, Italy).

3.1. Biomarker patterns of Methylomicrobium kenyense and M. alcaliphilum

Contents of cyclic triterpenoids of the Type I methanotrophs *M. kenyense* and *M. alcaliphilum* are provided in Table 1 along with corresponding compound-specific $\delta^{13}C$ values. In cultures of both species, various 4-methyl sterols, (3-methyl) tetrahymanol, and

Table 1
Contents and $\delta^{13}C$ values of lipid biomarkers from cultures of aerobic methanotrophs.

Compound	*M. alcaliphilum* (n = 3)				*M. kenyense* (n = 3)			
	Content (µg/g)	$\delta^{13}C$ (‰) V-PDB	$\Delta\delta^{13}C^{*}$ (‰)	ε^{*} (‰)	Content (µg/g)	$\delta^{13}C$ (‰) V-PDB	$\Delta\delta^{13}C^{*}$ (‰)	ε^{*} (‰)
Squalene	n.d.	n.d.			356	−73	−27	
Diploptene	19	tr			257	−71	−25	
Hop-21-ene	10	tr			33	tr		
3-Me-diploptene	n.d.	n.d.			22	tr		
3-Me-hop-21-ene	n.d.	n.d.			33	tr		
4α-Methylcholest-8(14)-en-3β-ol	n.d.	n.d.			522	tr		
4α-Methylcholesta-8(14),24-dien-3β-ol	n.d.	n.d.			179	tr		
4,4-Dimethylcholest-8(14)-en-3β-ol	500	tr			1332	−75	−29	31
4,4-Dimethylcholesta-8(14),24-dien-3β-ol	tr	tr			246	tr		
Diplopterol	85	tr			2934	−74	−28	30
Tetrahymanol	2376	−65	−19	20	291	tr		
3-Me-diplopterol	164	tr			380	−77	−31	34
3-Me-tetrahymanol	869	−62	−16	17	tr	tr		
C_{31} 17β(H),21β(H)-hopanol**	346	−62	−16	17	71	−67	−21	23
unsaturated C_{32} 17β(H),21β(H)-hopanol**	132	tr			n.d.	n.d.		
C_{32} 17β(H),21β(H)-hopanol**	2189	−63	−17	18	459	−66	−20	21
3-Me-C_{32} 17β(H),21β(H)-hopanol**	723	−60	−14	15	228	−65	−19	20
Sum lipids of aerobic methanotrophy	7413				7343			
Average $\delta^{13}C$ value		−62	−16			−71	−25	
Average ε value				17				26
3-Me-diplopterol/diplopterol	1.9				0.1			
3-Me-tetrahymanol/tetrahymanol	0.4				0			
3-Me-BHP/BHP	0.3				0.5			

n.d. = not detected; tr = traces.
* Values calculated relative to methane source. $\delta^{13}C_{methane}$ = −46‰ vs V-PDB (n = 5).
** Products of periodic acid cleavage procedure; C_{31} 17β(H),21β(H)-hopanol (homohopanol) is derived from aminotetrol, and C_{32} 17β(H),21β(H)-hopanol (bishomohopanol) and 3-Me-C_{32} 17β(H),21β(H)-hopanol (3-methylbishomohopanol) are derived from aminotriol and 3-methyl aminotriol, respectively.

(3-methyl) BHPs were present, whereas squalene and (3-methyl) C_{30} hopanes (diploptene, hop-21-ene) were detected in significant amounts only in *M. kenyense* strains. Tetrahymanol and BHPs are the most abundant compounds in cultures of *M. alcaliphilum*, with an average relative abundance of 44% for the former and 45% for the latter of all cyclic triterpenoids. In cultures of *M. kenyense*, 4-methyl sterols and C_{30} hopanols (diplopterol and 3-Me-diplopterol) predominate, with relative amounts of 32% and 45%, respectively (Fig. 1a). Sterols with one and two methylations at the C-4 position of ring A were found in cultures of *M. kenyense*, while only sterols with two methylations at this position were present in *M. alcaliphilum*, more specifically 4,4-dimethylcholest-8(14)-en-3β-ol and 4,4-dimethylcholesta-8(14),24-dien-3β-ol. The former compound is also the most abundant sterol in *M. kenyense* (Fig. 1b). The full range of BHPs is not shown, since BHP patterns of the two strains were previously published in Rush et al. (2016). The BHP inventories obtained in this and in other studies (Banta et al., 2015; Rush et al., 2016) include aminotriol (bishomohopanol after periodic acid cleavage) as the most abundant BHP in both species, with 29% in *M. alcaliphilum* and 7% in *M. kenyense* (Fig. 1c), followed by minor contributions of 3-methyl aminotriol (3-methylbishomohopanol after periodic acid cleavage) and aminotetrol (homohopanol after periodic acid cleavage).

In general, the proportion of 3-methyl compounds is low relative to their desmethyl homologues (3-Me-diplopterol/diplopterol, 3-Me-tetrahymanol/tetrahymanol, and 3-Me-BHP/BHP < 1), except for diplopterol in *M. alcaliphilum*, where the 3-methyl homologues are more abundant than the desmethyl homologues (3-Me-diplopterol/diplopterol > 1). The terpenoids of both methanotrophs are strongly ^{13}C-depleted with $\delta^{13}C$ values ranging from − 65‰ to − 60‰ for *M. alcaliphilum* and −77‰ and −65‰ for *M. kenyense* (values relative to V-PDB; Table 1; carbon isotopic composition of single experiments is provided in Supplementary Table S1). The $\delta^{13}C$ values of aminotetrol and aminotriol, measured as their cleavage products homohopanol and bishomohopanol, respectively, range between −63‰ to −60‰ for *M. alcaliphilum*, while BHPs produced by *M. kenyense* are more depleted with respect to the methane source, with ^{13}C values ranging from −67‰ to −65‰. Sterols with two methylations at position C-4 in *M. kenyense* are among the most ^{13}C-depleted cyclic terpenoids (−75‰) for this strain. Unfortunately, contents of 4-methyl sterols in *M. kenyense* were too low to obtain isotope values. In *M. kenyense* cultures, the carbon isotope fractionation ($\Delta\delta^{13}C$) between methane and terpenoids is −25‰ on average, overall ranging from −29‰ (4,4-dimethylcholest-8(14)-en-3β-ol) to −19‰ (3-Me-aminotriol; measured as 3-methylbishomohopanol); while in *M. alcaliphilum*, the average $\Delta\delta^{13}C_{terpenoids-methane}$ is −16‰, ranging from −19‰ (tetrahymanol) to −14‰ (3-Me-aminotriol; measured as 3-methylbishomohopanol). Isotopic fractionation between methane and lipids is also expressed in terms of the ratio of isotopes using the epsilon (ε) notation (Hayes, 1993).

$$\varepsilon = \left(\propto_{A/B} - 1\right) \times 10^{3} \quad (1)$$

$$\text{where } \propto_{A/B} = R_A/R_B = (1000 + \delta_A)/(1000 + \delta_B) \quad (2)$$

$\propto_{A/B}$ is the fractionation factor, δ_A is the isotopic composition of substrate (methane), and δ_B is the isotopic composition of the respective lipid.

The differences in fractionations expressed as ε (17 for *M. alcaliphilum* and 26 for *M. kenyense*) and Δ (as absolute values) are only minor (Table 1). The ε values provide information on the nature and the environment of bacterial strains, while Δ values reflect isotopic shifts associated with the reworking of organic matter prior to burial and trophic structure, i.e., secondary isotopic fractionations (Hayes, 1993), which makes Δ values more suitable to express carbon isotopic fractionation observed for the various seep sites studied herein. For the above reasons, we have chosen the $\Delta\delta^{13}C$ notation throughout the discussion for both, culture experiments and methane-seep deposits.

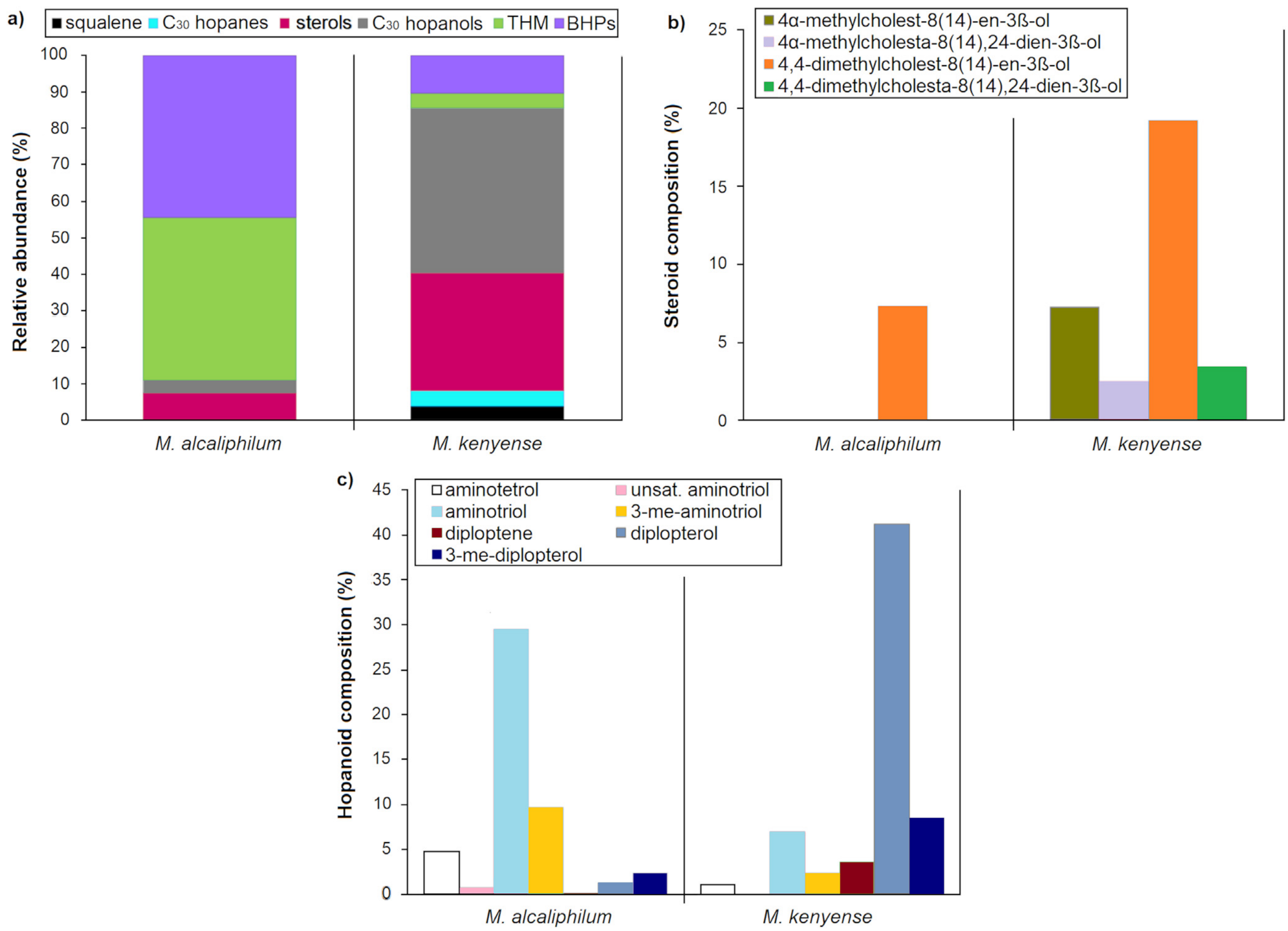

Fig. 1. Relative contents (in relation to all extracted lipids) of: (a) lipid biomarkers, (b) 4-methylated sterols and (c) hopanoids in *M. alcaliphilum* and *M. kenyense*; TMH = tetrahymanol; BHP = bacteriohopanepolyols. Note: BHPs in 1(c) were measured as products of periodic acid cleavage products (aminotetrol yielded homohopanol, aminotriol yielded all bishomohopanols, and 3-Me-aminotriol yielded 3-Me-bishomohopanol). See also Table 1 for details.

3.2. Biomarker patterns and compound-specific $\delta^{13}C$ values of aerobic methanotrophs in modern and ancient seep deposits

For a comparison with the cyclic triterpenoid inventories of *M. kenyense* and *M. alcaliphilum*, data from samples of 13 seep provinces were examined, including modern sediments (Haakon Mosby: Niemann et al., 2006; Elvert and Niemann, 2008), subrecent carbonates (Alaminos Canyon 645: Birgel et al., 2011; oxygenated zone of the Makran convergent margin: Himmler et al., 2015), mussel symbionts (*Bathymodiolus brooksi* and *Bathymodiolus childressi*; Jahnke et al., 1995; Kellermann et al., 2012), and various ancient seep deposits spanning in age from Miocene to Jurassic (Marmorito: Peckmann et al., 1999; Pietralunga: Peckmann et al., 2004; Tepee Buttes: Birgel et al., 2006a; Cold Fork of Cottonwood Creek, Wilbur Springs and Paskenta: Birgel et al., 2006b; Zizin: Sandy et al., 2012; Buje: Natalicchio et al., 2015). For all deposits, information on carbon isotope compositions of MOx biomarkers and ANME biomarkers is provided in Table 2. For comparison, this table also includes compound-specific $\delta^{13}C$ values previously obtained from cultures of Type X (*Methylococcus capsulatus*) and II (*Methylosinus trichosporium*) methanotrophs (Jahnke et al., 1999). For Type X and II methanotrophs, values are calculated considering the expression of the soluble methane monooxygenase enzyme (sMMO), expressed under copper-limited conditions, considering that these strains require low levels of copper for growth (Hanson and Hanson, 1996). Molecular probe studies have also shown that sMMO may be the prevalent enzyme form in a wide range of natural environments (McDonald et al., 1995).

3.2.1. 4-Methyl steroids

4-Methyl sterols are possibly the most specific biomarkers of aerobic methanotrophic bacteria, because no other organisms are known to synthesize these compounds (Wei et al., 2016). Their characteristic ^{13}C depletion provides further evidence of methane consumption. Seep environments yielded 4-methyl sterols with average $\delta^{13}C$ values of −75‰ for sediments of the Haakon Mosby mud volcano (Elvert and Niemann, 2008) and mussel symbionts of *Bathymodiolus childressi* and *B. brooksi* with values of −44‰ and −60‰, respectively (Kellermann et al., 2012). ^{13}C-depleted 4-methyl sterols were also reported for sediments of Ace Lake, Antarctica (Coolen et al., 2008) and in surface sediments from the REGAB pockmark, an active seep area on the Angola-Congo margin, accompanied by ^{13}C-depleted diploptene, also indicative of aerobic methanotrophy (Bouloubassi et al., 2009). In modern seep carbonates, 4-methyl sterols were only reported for Alaminos Canyon of the Gulf of Mexico with a $\delta^{13}C$ value of −57‰ (Birgel et al., 2011). Even though 4-methyl sterols seem to be scarce in seep carbonates, they have been found in traces in a Miocene seep carbonate (Marmorito, Italy), but unfortunately $\delta^{13}C$ values were not obtained due to too low contents (Peckmann et al., 1999). Other than for the Marmorito limestone, 4-methyl sterols have

Table 2
Average compound-specific $\delta^{13}C$ values (‰ V-PDB) of lipids from four species of aerobic methanotrophs bacteria, two mussel symbionts, one sediment sample from Haakon Mosby (H.M.), two sub-recent carbonates (A.C. = Alaminos Canyon 645, Makr = Makran) and eight ancient seep carbonates; Pietr = Pietralunga (Miocene), Marm = Marmorito (Miocene), Buje (Eocene), Tepee Buttes (Late Cretaceous), C.C. = Cold Fork of Cottonwood Creek (Early Cretaceous), W.S. = Wilbur Springs (Early Cretaceous), Zizin (Early Cretaceous), PSK = Paskenta (Late Jurassic); tr = traces.

	Type I/X			Type II	Sub-recent to recent methane-seep deposits					Ancient methane-seep deposits (n = 1)							
	M. alcaliphilum	*M. kenyense*	*M. capsulatus*^	*M. trichosporium*^	*B. childressi*	*B. brooksi*	H.M.	A.C. (n = 3)	Makr (n = 5)	Pietr	Marm	Buje	Tepee Buttes	C.C.	W.S.	Zizin	PSK
Aerobic methanotrophs (MOx)																	
Tetrahymanol	−65	tr						−59	−72	−87[g]	−92						
3-Me-THM	−62	tr															
Gammacerane										−81[g]		tr[l]		−68[m]	tr[m]	−90[n##]	tr[m]
Lanostane										−73[g,h]		−46[l]					
Nor-lanostane										−86[g,h]		−47[l]				tr[n]	
4-Methyl sterol*					−44[b]	−60[b]	−75[c]	−57[e]			tr[j]						
4,4-Dimethylsterol**	tr	−75															
Diplopterol	tr	−74					−75[c]	−53[e]	−70[f]								
3-Me-diplopterol		−77															
Hop-17(21)-ene										−32[g##]	−85[h]	−64[l]					
C_{32} 17β(H),21β(H)-hopanol								−59	−78[f]	tr[h]	−47[h##]						
C_{32} 17β(H),21β(H)-hopanoic acid								−57[e]	−82[f]	−74[g,h]	−75[h]						
3-Me-C_{32} 17β(H),21β(H)-hopanoic acid										tr[h]	−100[h]		−43[k##]				
Anhydrobacteriohopanetetrol										tr[h]	−42[h##]						
3-Me-anhydro bacteriohopanetetrol											−100[h]						
C_{30} 17β(H),21β(H)-hopanol***			−58[a]														
3-Me-C_{30} 17β(H),21β(H)-hopanol***			−54[a]														
C_{31} 17β(H),21β(H)-hopanol***	−62	−67		−37[a]	−51[b]			−58[e]	−75[f]								
3-Me-C_{31} 17β(H),21β(H)-hopanol***			−51[a]														
C_{32} 17β(H),21β(H)-hopanol***	−63	−66		−37[a]		−56[b]		−49[e]	−70								
3-Me-C_{32} 17β(H),21β(H)-hopanol***	−60	−65															
Hopane													−49 [h##]	−55 [m]	−64 [m]	−60[n]	−60 [m]
Homohopanes													−44 [h##]	−65 [m]	−65 [m]		tr[m]
C_{34}-Secohexahydrobenzohopane													−109 [k]				
MOx average	−62	−71	−56	−37	−48	−58	−75	−56	−75	−80	−90	−52	−109	−63	−65	−60	−60
Anaerobic methanotrophs (AOM)																	
Crocetane/phytane								−83[e+]	−106[f]	−50[g,h+]	−33[h#]	−98[l]	−82[h]	−85[m]	−98[m]	−86[n]	−47[m+]
Pentamethylicosane (PMI)							−105[d]	−92[e]	−120[f]	−105[g,h]	−82[i]	−111[l]	−118[h]	−94[m]	−101[m]	−61[n#]	−122[m]
Biphytane											−94[h]	−109[l]	−102[h]	−81[m]	−105[m]	−92[n]	−101[m]
Acyclic biphytanic diacid										−110[i]	−96[i]						
Monocyclic biphytanic diacid										−110[i]	−109[i]						
Bicyclic biphytanic diacid										−109[i]	−115[i]						
Archaeol							−98[d]	−107[e]	−119[f]	−52[h#]	−90[i]						
sn2-hydroxyarchaeol							−107[d]	−108[e]	−118[f]	−108[h]	tr[h]						
AOM average							−103	−102	−116	−108	−98	−106	−101	−87	−101	−89	−112

[a] Jahnke et al. (1999).
[b] Kellermann et al. (2012).
[c] Elvert and Niemann (2008).
[d] Niemann and Elvert (2008).
[e] Birgel et al. (2011).
[f] Himmler et al. (2015).
[g] Peckmann et al. (2004).
[h] Birgel and Peckmann (2008).
[i] Birgel et al. (2008a).
[j] Peckmann et al. (1999).

[k] Birgel et al. (2006a).
[l] Natalicchio et al. (2015).
[m] Birgel et al. (2006b).
[n] Sandy et al. (2012).
* Comprise 4-α-methylcholest-8(14)-en-3β-ol and 4-α-methylcholesta-8(14),24-dien-3α-ol.
** Comprises 4,4-dimethylcholest-8(14)-en-3β-ol and 4,4-dimethylcholesta-8(14),24-dien-3β-ol.
*** Products of periodic acid cleavage procedure; C_{30} 17β(H),21β(H)-hopanol (hopanol) and 3-me-C_{30} 17β(H),21β(H)-hopanol (3-methyl hopanol) are derived from aminopentol and 3-methyl aminopentol, respectively; C_{31} 17β(H),21β(H)-hopanol (homohopanol) and 3-Me-C_{31} 17β(H),21β(H)-hopanol (3-methylhomohopanol) are derived from aminotetrol and 3-methyl aminotetrol, respectively; and C_{32} 17β(H),21β(H)-hopanol (bishomohopanol) and 3-Me-C_{32} 17β(H),21β(H)-hopanol (3-methylbishomohopanol) are derived from aminotriol and 3-methyl aminotriol, respectively.
\+ Values excluded (coelution with phytane).
\# Values excluded (mixed with non-AOM sources).
\#\# Values excluded (non-MOx sources).
^ values for expression of sMMO; *M. capsulatus* was grown at 37 °C and high methane concentration; M. *trichosporium* was grown at 30 °C and high and low methane and carbon dioxide concentrations.

not been reported from ancient seep deposits to date. Instead, 4-methyl and 4,4-dimethyl steranes, so-called lanostane and nor-lanostane have been found with isotope values ranging from −86‰ to −73‰ in a Miocene seep carbonate (Pietralunga, Italy) and with values from −47‰ to −46‰ in an Eocene seep carbonate (Buje, Croatia). Lanostanes are considered to represent diagenetic products of C-4 monomethylated and C-4 dimethylated sterols; when found in seep deposits, they are typically accompanied by ^{13}C-depleted hopanoids (Peckmann et al., 2004; Birgel and Peckmann, 2008; Natalicchio et al., 2015). Lanostanes were also detected in Cretaceous seep carbonates from Zizin, Romania, but their contents were too low to measure isotopic compositions. Since the occurrence of 4-methyl sterols in methane-seep deposits seems to be limited to young deposits, the preservation potential of these compounds is apparently low. Yet, their derivatives (i.e., lanostanes) enable recognition of aerobic methanotrophy in ancient seep environments.

3.2.2. Hopanoids and 3-methyl hopanoids

Although hopanoids, including BHPs and geohopanoids, have commonly been encountered in modern and ancient seep carbonates, it is often unclear if they were synthesized by methanotrophic bacteria. Specific hopanoids including aminotetrol and 3-methyl hopanoids are thought to be produced mainly by Type I methanotrophs; in particular when exhibiting significant ^{13}C depletion, such compounds can be assigned to aerobic methanotrophic bacteria (Welander and Summons, 2012). Aminotetrol and aminotriol have been found in methane-seep carbonates from the oxygenated zone of the Makran accretionary wedge, exhibiting δ^{13}C values as low as −75‰ (Himmler et al., 2015) and −70‰, respectively. A similar pattern was found for seep carbonates from Alaminos Canyon of the Gulf of Mexico, but there aminotetrol (−58‰) and aminotriol (−49‰) were less ^{13}C-depleted. Similarly, aminotetrol (δ^{13}C: −51‰) and aminotriol (trace amounts) have been observed in the gills of *B. childressi* and only aminotriol (δ^{13}C: −56‰) in *B. brooksi*. Although the δ^{13}C values of BHPs from Alaminos Canyon carbonates and bacterial symbionts are not as low as cyclic terpenoids in some methane-seep deposits, the values are still in accordance with isotope fractionation determined for methanotrophic cultures in this and previous studies (Jahnke et al., 1999). Even less ^{13}C-depleted BHPs (−41‰) supposedly produced by aerobic methanotrophs have been found in recent marine sediments of the Congo deep-sea fan (Talbot et al., 2014). However, in this case accompanying evidence indicated a terrestrial source of BHPs. Sediments of permanently stratified Ace Lake yielded a suite of ^{13}C-depleted BHPs (−73‰ to −31‰; Coolen et al., 2008), some of which were interpreted to have been derived from Type I methanotrophs.

BHPs are absent in methane-seep carbonates older than the Neogene or are present in trace amounts only. They tend to be transformed during diagenesis and burial (Sinninghe Damsté et al., 1995). Nevertheless, anhydrobacteriohopanetetrol (anhydroBHT), which is considered to represent a diagenetic product of bacteriohopanetetrol (BHT) via dehydration of the side chain (Bednarczyk et al., 2005; Saito and Suzuki, 2007), and its 3-methyl homologue were found in a Miocene seep limestone (Marmorito limestone), revealing δ^{13}C values of −42‰ and −100‰, respectively (Birgel and Peckmann, 2008). From the same deposit, C_{32} hopanoic acid, 3-Me C_{32} hopanoic acid, and C_{32} hopanol were reported (−75‰, −100‰ and −47‰, respectively). These compounds are considered to represent oxidative degradation products of BHPs, specifically those of tetrafunctionalised BHPs such as aminotriol and BHT (Innes et al., 1997; Farrimond et al., 2002). Although, anhydroBHT and C_{32} hopanol may principally derive from aerobic methanotrophs, their only moderate ^{13}C depletion in comparison to other methanotroph biomarkers found in the

Marmorito limestone, did not allow assignment to this group of bacteria. In contrast, ^{13}C-depleted hopanoic acids (−74‰) as well as minute amounts of a 3-methyl homologue (no δ^{13}C data) from another Miocene seep limestone (Pietralunga) have been assigned to aerobic methanotrophic bacteria based on the low δ^{13}C values of hopanoic acids (Peckmann et al., 2004; Birgel and Peckmann, 2008).

In Cretaceous and Jurassic seep carbonates from California only hopanoids without C-3 methylations, yet with low δ^{13}C values from −65‰ to −55‰ were found. Since no 4-methyl sterols or their degradation products were found in the Californian carbonates, the biomarkers have not been considered as unequivocal evidence of aerobic methanotrophy since these compounds could have been derived from anaerobic bacteria too (Birgel et al., 2006b). Cretaceous seep limestones from the Western Interior Seaway yielded a series of unusual C_{34} 8,14-secohexahydrobenzohopanes with extremely low δ^{13}C values (averaging −109‰; Table 2) and C_{35} 8,14-secohexahydrobenzohopanes (no δ^{13}C data; Birgel et al., 2006a). These compounds have previously only been reported from oils and sediments of evaporitic, carbonate-rich, and anoxic environments (Connan and Dessort, 1987). The C_{35} 8,14-secohopanes are believed to derive from BHT via a sequence of dehydration and cyclisation reactions, with subsequent degradation of the side chain involved in the formation of the lower homologs (Hussler et al., 1984).

3.2.3. Tetrahymanol and gammacerane

Tetrahymanol has been reported to occur in many seep carbonates where also other biomarkers of aerobic methanotrophic bacteria have been recognized (Peckmann et al., 1999, 2004; Birgel et al., 2011; Himmler et al., 2015). Gammacerane, the diagenetic product of tetrahymanol, has also been found in a number of seep deposits (Peckmann et al., 2004; Birgel et al., 2006b; Sandy et al., 2012; Natalicchio et al., 2015). For all sites, tetrahymanol and gammacerane are strongly ^{13}C-depleted, exhibiting δ^{13}C values similar to the associated hopanoids and sterols (Table 2). With the recognition of the presence of tetrahymanol in aerobic methanotrophic bacteria (Banta et al., 2015), the occurrence of tetrahymanol and gammacerane at seeps and in ancient seep deposits needs to be re-evaluated (see Section 4).

4. Discussion

4.1. Cyclic triterpenoids of Type I methanotrophs and their carbon stable isotope composition

The Type I methanotrophs *M. kenyense* and *M. alcaliphilum* are non-marine strains. They are, however, of interest for the study of marine environments, because they are adapted to environmental conditions that are close to those found at marine methane seeps (high alkalinity, high salinity; see Kalyuzhnaya et al., 2008). This makes them good candidates for the reconstruction of environmental conditions – normal marine or higher salinities and high alkalinity – under which carbonate minerals precipitate at methane seeps. The analysis of cultures of the two strains revealed the presence of aminotetrol, aminotriol, and 3-Me-aminotriol (Table 1), as well as the absence of aminopentol – typically the most abundant BHP in Type I methanotrophs (Neunlist and Rohmer, 1985). It has previously been assumed that aminotriol and aminotetrol are predominantly synthesized by Type II methanotrophs (e.g., *Methylosinus trichosporium*), while BHP distributions in Type I and X methanotrophs had been found to be dominated by aminopentol or 3-Me-aminopentol with minor contributions of aminotetrol, 3-Me-aminotetrol, and aminotriol (Talbot et al., 2001); as for example reported for *Methylococcus capsulatus* (Type X; Jahnke et al., 1999). Talbot et al. (2001) had already found aminotriol and aminotetrol in the Type I methanotroph *Methylomicrobium album*. However, at that time, the results were attributed to a contamination. More recently, Banta et al. (2015) and Rush et al. (2016) studied the lipid inventories of *M. alcaliphilum* and *M. kenyense*, revealing a BHP distribution more typical of Type II methanotrophs. The samples from Rush et al. (2016) are identical to the samples studied herein. The capability of Type I methanotrophs to produce aminotriol and aminotetrol is of importance to interpret some lipid signatures found in environmental samples, since Type I methanotrophs are consequently possible source organisms when similar biomarker patterns are found in marine environments including methane-seep deposits (cf. Birgel et al., 2011; Kellermann et al., 2012; Himmler et al., 2015).

The sterol inventories of *M. alcaliphilum* and *kenyense* comprise 4,4-dimethyl sterols, confirming the ability of *M. alcaliphilum* (cf. Banta et al., 2015) to produce 4,4-dimethyl sterols with and without unsaturation at the C-24 position. The inventory of sterols of *M. kenyense* also includes 4-methyl sterols (Table 1). To date, the only species known to produce these sterols in significant amounts other than the strains studied herein are *Methylococcus capsulatus* (Bouvier et al., 1976), *Methylosphaera hansonii* (Schouten et al., 2000), and *Methylobacter whittenbury* (Wei et al., 2016). Jahnke and Nichols (1986) identified 4α-methyl sterol as the predominant sterol of Type X *M. capsulatus*, and 4,4-dimethyl sterol as the less abundant sterol. However, at low oxygen concentrations, the production of 4,4-dimethyl sterols was found to increase with respect to the total amount of sterols (Jahnke and Nichols, 1986). The authors explained this observation with a higher oxygen requirement for the second demethylation step of lanosterol. This suggests that the production of 4,4-dimethyl sterols as the most abundant sterols in our cultures of *M. kenyense* and *M. alcaliphilum* might reflect oxygen limitation. Oxygen limitation may indeed have occurred during the experiments, since oxygen was not replenished. The findings of Banta et al. (2015) and our findings help to expand the knowledge on sterol production in aerobic methanotrophs and allow for a better interpretation of biomarker signatures found in sediments and rocks.

Another interesting outcome of the experiments is the production of tetrahymanol by *M. kenyense* and *M. alcaliphilum* (Table 1). Tetrahymanol synthesis by *M. alcaliphilum* has already been reported by Banta et al. (2015). Tetrahymanol and its degradation product gammacerane are pentacyclic triterpenoids commonly used as biomarkers for water column stratification (Sinninghe Damsté et al., 1995). In modern methane-seep deposits (Werne et al., 2002; Birgel et al., 2011; Himmler et al., 2015) and at ancient methane-seep carbonates (Sandy et al., 2012) tetrahymanol and gammacerane have been found to show similar δ^{13}C values to the ANME and MOx biomarkers. Since aerobic methanotrophic bacteria had not been known to produce tetrahymanol at the time of these studies, it had been suggested these molecular fossils represent input from ciliates. Ciliates are known to inhabit oxic-anoxic interfaces, feeding on bacteria (Werne et al., 2002). Low δ^{13}C values of tetrahymanol from seep environments were consequently previously explained by ciliates feeding on methanotrophic bacteria or archaea or AOM-associated sulfate-reducing bacteria, inheriting the isotopic fingerprint of the seep-dwelling prokaryotes. With the recognition of aerobic methanotrophic bacteria as producers of tetrahymanol (Banta et al., 2015; this study), findings of ^{13}C-depleted tetrahymanol in seep environments need to be re-evaluated. Many of the reported occurrences of tetrahymanol and its diagenetic product gammacerane are likely to reflect input from aerobic methanotrophs (e.g., Peckmann et al., 2004; Birgel et al., 2006b, 2011).

The cyclic terpenoids synthesized by *M. kenyense* and *M. alcaliphilum* are strongly ^{13}C-depleted, averaging −69‰ and −62‰, respectively (Table 2). Carbon isotopic fractionation from substrate to lipid for cultures of *M. kenyense* ($\Delta\delta^{13}$C: −25‰; Table 3) and *M. alcaliphilum* ($\Delta\delta^{13}$C: −16‰; Table 3) is similar to that of the Type X methanotroph *M. capsulatus* ($\Delta\delta^{13}$C: −21‰; Jahnke et al., 1999). Fractionation for Type II *M. trichosporium* ($\Delta\delta^{13}$C: −2‰) is considerably lower than for Type I and X methanotrophs, varying between −8‰ under high methane conditions and +5‰ at low methane availability (Jahnke et al., 1999). Interestingly, the expression of pMMO (favored under high copper conditions) in both Type X and II methanotrophs seems to produce stronger fractionation; *M. capsulatus* exhibits offsets of up to −31‰ and *M. trichosporium* up to −12‰ at high methane concentrations (Jahnke et al., 1999). At low methane concentrations, *M. trichosporium* expressing pMMO produces cyclic terpenoids that are ^{13}C-enriched relative to the methane source ($\Delta\delta^{13}$C: +10‰; Jahnke et al., 1999).

Various types of aerobic methanotrophs use different carbon assimilation pathways. Type I methanotrophs (Gammaproteobacteria) utilize ribulose monophosphate (RuMP) as primary pathway for formaldehyde assimilation, while Type II methanotrophs (Alphaproteobacteria) use the serine pathway. Type X methanotrophs (Gammaproteobacteria) assimilate formaldehyde using the RuMP pathway like Type I methanotrophs, but also possess enzymes of the serine pathway (Taylor et al., 1981; Hanson and Hanson, 1996; Chistoserdova et al., 2009). The different assimilation pathways result in differences in carbon isotopic fractionation relative to the substrate (i.e., methane), with Type I and X methanotrophs showing greater fractionation (Jahnke et al., 1999).

Despite *M. kenyense* and *M. alcaliphilum* strains being phylogenetically closely related (Banta et al., 2015), there are significant differences in the cyclic terpenoid distribution between the strains. *M. alcaliphilum* is dominated by tetrahymanol and BHPs, while 4-methyl sterols and C_{30} hopanols predominate in *M. kenyense*. The differences in distribution may be related to the presence and length of specific proteins involved in the biosynthesis of these compounds (e.g., Pearson et al., 2007; Banta et al., 2015; Wei et al., 2016). Even though the lipid inventories, especially the BHP patterns, seem to vary among Type I methanotrophic bacteria, their $\Delta\delta^{13}$C values appear to be more constant and are consequently a useful tool for differentiation. With tools being developed that allow for the reconstruction of the carbon stable isotope composition of methane in ancient and inactive subrecent seep environments using ANME lipids (Himmler et al., 2015), δ^{13}C values provide a new approach to distinguish biomarkers of Type I and Type II methanotrophs.

4.2. Comparison of carbon isotope fractionation patterns

4.2.1. Cultures and methane-seep deposits

The culture experiments conducted herein revealed an average $\Delta\delta^{13}C_{terpenoids\text{-}methane}$ of −25‰ with an overall range from −31‰ to −19‰ for *M. kenyense*, and an average $\Delta\delta^{13}C_{terpenoids\text{-}methane}$ of −16‰ with an overall range from −19‰ to −14‰ for *M. alcaliphilum*. Culture experiments of Jahnke et al. (1999) yielded fractionations corresponding to $\Delta\delta^{13}C_{terpenoids\text{-}methane}$ of −25‰ to −16‰ (avg.: −21‰, for sMMO; Table 3) for Type X *M. capsulatus*, and of −8‰ (high methane) to +5‰ (low methane) (avg.: −2‰, for sMMO; Table 3) for Type II *M. trichosporium*. The same authors showed that fractionation associated with pMMO is higher, varying from −31‰ to −27‰ for *M. capsulatus* and −12‰ (high methane) to +10‰ (low methane) for *M. trichosporium*. The differences in isotopic fractionation ($\Delta\delta^{13}$C) between terpenoids and the methane source are the result of different carbon assimilation pathways and can therefore be used to differentiate between Type I/X and II methanotrophs.

BHPs and 4-methyl sterols extracted from the gills of *B. childressi* and *B. brooksi* revealed the presence of methanotrophic symbionts (Kellermann et al., 2012). Based on the average δ^{13}C value of −58‰ of these terpenoid biomarkers, the $\Delta\delta^{13}C_{terpenoids\text{-}methane}$ for the *B. brooksi* symbionts was −13‰ given a δ^{13}C value of −45‰ of seeping methane (Table 3; Lanoil et al., 2001). Although the high abundance of aminotriol agreed with Type II methanotrophs in that case, the $\Delta\delta^{13}C_{terpenoids\text{-}methane}$ values for the *B. brooksi* symbiont lipids rather pointed to Type I/X methanotrophs. Such interpretation is consistent with the new finding that some strains of Type I methanotrophs, such as *M. kenyense* and *M. alcaliphilum*, also produce tetrafunctionalised BHPs, and, therefore, cannot be excluded as possible source organisms. In addition, comparative 16S rRNA sequence analysis confirmed that the gill symbionts in the Gulf of Mexico bathymodiolin bivalves are exclusively Type I/X methanotrophs (Duperron et al., 2007). In the case of the *B. childressi* symbionts, the $\Delta\delta^{13}C_{terpenoids\text{-}methane}$ value was found to only amount to −3‰ (Kellermann et al., 2012). Such isotope offset suggested the dominance of Type II methanotrophs, an inference supported by the abundance of aminotetrol and the lower amounts of aminotriol, which is typical of Alphaproteobacteria. The fatty acid profile of *B. childressi* was found to contain abundant $C_{16:1\omega8}$ and significant amounts of $C_{18:1\omega8}$ (Kellermann et al., 2012), compounds typical of Type I and Type II methanotrophs, respectively (Bowman, 2006). However, the 16S rRNA analysis of Duperron et al. (2007) contradicted the hypothesis of a presence of Type II methanotrophic symbionts in *B. childressi*, yielding only sequences of Gammaproteobacteria. Based on such contradictory evidence, the presence of both types of methanotrophs in the *B. childressi* symbionts should not be ruled out at this point.

Abundant 4-methyl sterols found in the sediments of Haakon Mosby Mud Volcano yielded a $\Delta\delta^{13}C_{terpenoids\text{-}methane}$ of −14‰ (Table 3), agreeing with the dominance of Type I/X methanotrophs. A suite of 4-methyl sterols and hopanoids from recent seep carbonates of Alaminos Canyon revealed a $\Delta\delta^{13}C_{terpenoids\text{-}methane}$ of 0‰ (Table 3), falling in the range of values diagnostic for Type II methanotrophs. In the latter case, however, evidence was contradictory since the presence of 4-methyl sterols together with BHPs more typical of Gammaproteobacteria disagree with Type II methanotrophs as source organisms. Another possible source in this case are Type X methanotrophs, which are capable to assimilate small amounts of CO_2 using the ribulose bisphosphate carboxylase oxygenase (Rubisco) pathway and possibly the serine pathway (Taylor et al., 1981; Baxter et al., 2002; Chistoserdova et al., 2009), which can result in different carbon isotope fractionation (Summons et al., 1994). In the case of seep carbonates from the oxygenated zone of the Makran accretionary prism, cyclic triterpenoids yielded a $\Delta\delta^{13}C_{terpenoids\text{-}methane}$ of −8‰ (Table 3; Himmler et al., 2015), suggesting the dominance of Type II methanotrophs. Interestingly, the BHP patterns of Makran and Alaminos Canyon seep carbonates resembled those of *M. kenyense* and *M. alcaliphilum*. Moreover, in case of Alaminos Canyon seep carbonates, the presence of 3β-methyl hopanoids, 4-methyl sterols and steroids, and tetrahymanol is strong evidence for Type I methanotrophs, particularly because type I methanotrophs are the only organisms known to synthesize 4-methyl sterols (Bouvier et al., 1976; Schouten et al., 2000; Wei et al., 2016). Another aspect that needs to be considered is the circumstance that Type II methanotrophs have never been discovered in the marine realm to date (Knief, 2015). Although the chain of arguments is anything but straightforward and the $\Delta\delta^{13}C_{terpenoids\text{-}methane}$ of the Makran seep carbonates points to Type II methanotrophs, it seems more likely that Type I methanotrophs are the BHP source even in this case. We might just still be lacking the appropriate cultures of Type I methanotrophs with little carbon isotope fractionation involved in terpenoid synthesis. Taken together, current evidence suggests that Type I methanotrophs are exclusively responsible for aerobic methane oxidation at marine methane seeps.

Table 3
Offset of $\delta^{13}C$ values ($\Delta\delta^{13}C$) between methane and lipid biomarkers; H.M. = Haakon Mosby, A.C. = Alaminos Canyon 645, Makr = Makran, Pietr = Pietralunga (Miocene), Marm = Marmorito (Miocene), Buje (Eocene), Tepee Buttes (Late Cretaceous), C.C. = Cold Fork of Cottonwood Creek (Early Cretaceous), W.S. = Wilbur Springs (Early Cretaceous), Zizin (Early Cretaceous), PSK = Paskenta (Late Jurassic).

	Type I/X			Type II	Recent methane-seep deposits					Ancient methane-seep deposits							
	M. alcaliphilum	*M. kenyense*	[a]*M. capsulatus*^	[a]*M. trichosporium*^	[b]*B. childressi*	[b]*B. brooksi*	[c]H.M.	[d]A.C.	[e]Makr	[f,g]Pietr	[g,h]Marm	[j]Buje	[i]Tepee Buttes	[k]C.C.	[k]W.S.	[l]Zizin	[k]PSK
$\delta^{13}C_{methane\ (measured)}$	−46	−46	−35	−35	−45**	−45**	−61	−56	−67								
$\delta^{13}C_{methane\ (ANME)}$*								−52	−66	−58	−68 to −58	−76	−71	−47 to −37	−61 to −51	−59	−72 to −62
$\Delta\delta^{13}C_{ANME-methane\ (measured)}$								−46	−49								
$\Delta\delta^{13}C_{ANME-methane}$*								−50	−50	−50	−40 to −30	−30	−30	−50 to −40	−50 to −40	−30	−50 to −40
$\Delta\delta^{13}C_{terpenoid-methane\ (measured)}$	−16	−25	−21	−2	−3	−13	−14	0	−8								
$\Delta\delta^{13}C_{terpenoid-methane}$*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.			−22	−32 to −22	24	−38	−26 to −16	−14 to −4	−1	2 to 12
$\delta^{13}C_{lipid\ biomarkers}$																	
$\delta^{13}C_{ANME}$ (avg.)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−103	−102	−116	−108	−98	−106	−101	−87	−101	−89	−112
$\delta^{13}C_{terpenoids}$ (avg.)	−62	−71	−56	−37	−48	−58	−75	−56	−75	−80	−90	−52	−109	−63	−65	−60	−60
ANME							ANME-3	ANME-2	ANME-2 ANME-1	ANME-2	ANME-1 ANME-2	ANME-1	ANME-1	ANME-2 ANME-1	ANME-2 ANME-1	ANME-1	ANME-2 ANME-1
Aerobic methanotrophy	Type I	Type I	Type X	Type II		Type I	Type I			Type I/X	Type I/X	?	Type I/X	Type I/X			

[a] Jahnke et al. (1999).
[b] Kellermann et al. (2012).
[c] Elvert and Niemann (2008);
[d] Birgel et al. (2011).
[e] Himmler et al. (2015).
[f] Peckmann et al. (2004).
[g] Birgel and Peckmann (2008);
[h] Peckmann et al. (1999).
[i] Birgel et al. (2006a).
[j] Natalicchio et al. (2015).
[k] Birgel et al. (2006b).
[l] Sandy et al. (2012).
^ Values for expression of sMMO.
* $\delta^{13}C_{methane}$ values were back-calculated using $\delta^{13}C$ values of AOM biomarkers ($\Delta\delta^{13}C_{ANME-methane}$) (Niemann and Elvert, 2008). For deposits with mixed ANME-1 and ANME-2 communities (Marmorito, Cold Fork of Cottonwood Creek, Wilbur Springs, and Paskenta), a range of $\Delta\delta^{13}C_{ANME-methane}$ values between −40‰ (average $\Delta\delta^{13}C$ of ANME-1 and ANME-2 biomarkers) and the $\Delta\delta^{13}C$ of the predominant ANME community (underlined) was used for calculations.
** Lanoil et al. (2001).

4.2.2. How to constrain the carbon isotope composition of methane at ancient seeps

A comparison of δ^{13}C values of ANME biomarkers and the respective methane sources ($\Delta\delta^{13}C_{ANME\text{-}methane}$) originally put forward by Niemann and Elvert (2008) has been applied to sediments, mats, and carbonates of modern seeps to develop a proxy for $\delta^{13}C_{methane}$ values when seeping methane is not available anymore (Himmler et al., 2015). It has been found that the isotopic offset ($\Delta\delta^{13}C_{ANME\text{-}methane}$) is approximately −50‰ in ANME-2 dominated systems and approximately −30‰ in ANME-1 dominated systems (Niemann and Elvert, 2008). For the Alaminos Canyon carbonates, which contained mostly lipids typical of the ANME-2/*DSS* consortium, a $\Delta\delta^{13}C_{ANME\text{-}methane}$ of −50‰ can be expected, resulting in an estimate for the $\delta^{13}C_{methane}$ value of −52‰. These values are close to the measured $\delta^{13}C_{methane}$ value (−56‰) and the correspondingly calculated $\Delta\delta^{13}C_{ANME\text{-}methane}$ (−46‰) for this site, supporting the applicability of the approach. Based on the wide range of carbon isotope fractionation during terpenoid synthesis in aerobic methanotrophic bacteria, such an approach to constrain the isotopic composition of parent methane cannot be easily adopted for MOx biomarkers. However, if one estimates the isotopic composition of parent methane with ANME biomarkers, $\Delta\delta^{13}C_{terpenoids\text{-}methane}$ will help with the assignment of terpenoid biomarkers to either Type I methanotrophs or Type II methanotrophs (Table 3; Fig. 2). For some of the sites, especially some of the ancient sites, an exclusive assignment to ANME-1 or ANME-2 is not possible. For those samples, which show signatures of both types of ANMEs, we report ranges rather than single values (see Table 3).

The Miocene Pietralunga seep deposit contains abundant *sn2*-hydroxyarchaeol (δ^{13}C: −108‰, Table 2), suggesting the dominance of ANME-2/*DSS* and resulting in an estimated $\delta^{13}C_{methane}$ value of −58‰. Using the average Pietralunga $\delta^{13}C_{terpenoid}$ value of −80‰, the $\Delta\delta^{13}C_{terpenoids\text{-}methane}$ was −22‰ and suggests the dominance of Type I/X methanotrophs at the Miocene seep. Such interpretation is supported by the occurrence of abundant 4-methyl steranes and accessory 3-methyl hopanoic acids (Table 3; Peckmann et al., 2004; Birgel and Peckmann, 2008), both only known to be produced by Type I/X methanotrophs. For the Marmorito seep limestone, the low abundance of *sn2*-hydroxyarchaeol and crocetane indicate the dominance of ANME-1 over ANME-2, and assuming that ANME-1/*DSS* predominated, a range of $\delta^{13}C_{methane}$ values between −68 and −58‰ and a $\Delta\delta^{13}C_{terpenoids\text{-}methane}$ value between −32‰ and −22‰ is calculated. Such high fractionation agrees with the presence of a 4-methyl sterol and 3-methyl BHP (Peckmann et al., 1999; Birgel and Peckmann, 2008) and, accordingly, the dominance of Type I/X methanotrophs. Similarly, the presence of 4-methyl steranes in the Buje seep deposit points to Type I methanotrophic bacteria (Table 3; Natalicchio et al., 2015). However, the calculated average $\Delta\delta^{13}C_{terpenoids\text{-}methane}$ value of +24‰ is not in accord with isotope fractionation in any aerobic methanotroph reported to date. Based on such uncertainty, the affiliation of the Buje aerobic methanotrophs cannot be constrained.

The absence of crocetane and the abundance of ^{13}C-depleted phytane and phytanic acid (Table 2) – most likely originating from cleavage of archaeol – in the Cretaceous Tepee Buttes point towards the dominance of ANME-1. Based on the correspondingly calculated $\delta^{13}C_{methane}$ value of −71‰ and the resultant $\Delta\delta^{13}C_{terpenoids\text{-}methane}$ of −38‰, Type I methanotrophs apparently produced the precursor lipids of a suite of uncommon C_{34} and C_{35}8,14-secohexahydrobenzohopanes. Interestingly, as for the Marmorito limestone, such large $\Delta\delta^{13}C_{terpenoid\text{-}methane}$ values exceed all fractionation that has been observed in culture to date (Fig. 2). The Cretaceous seep carbonates of Cold Fork of Cottonwood Creek and Wilbur Springs and the Jurassic seep carbonates of Paskenta contain ^{13}C-depleted PMI, crocetane, and only small amounts of biphytanes (Table 2). The presence of crocetane is diagnostic for ANME-2 archaea, while biphytane, derived from GDGTs, is indicative of ANME-1. Assuming a predominance of ANME-2

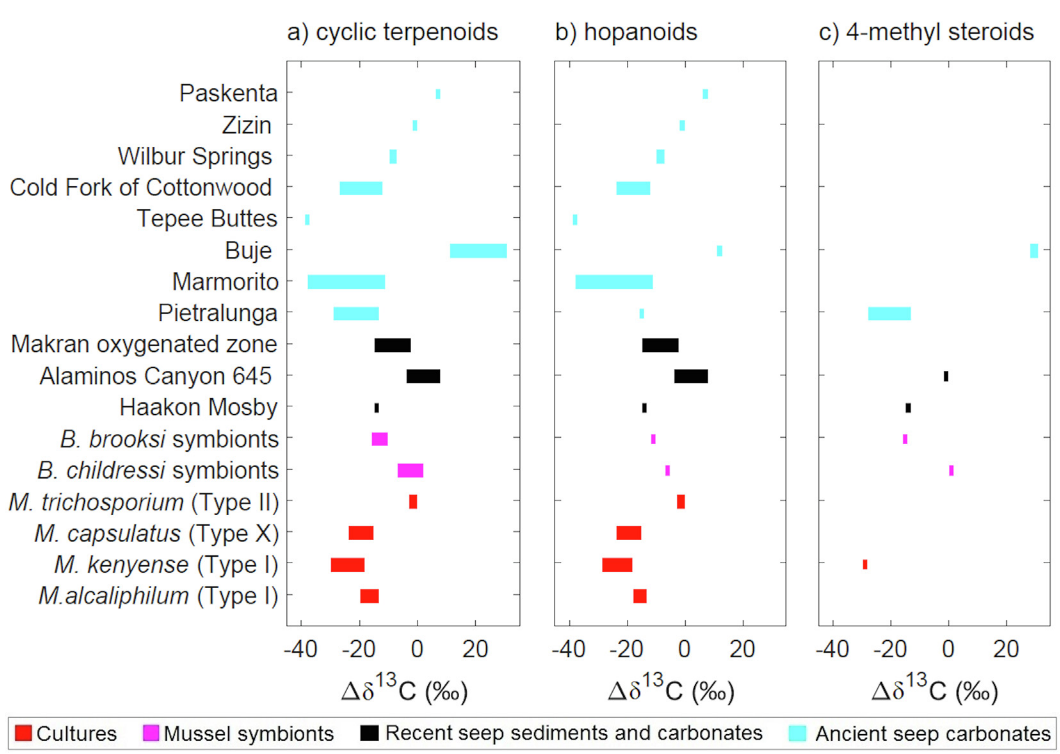

Fig. 2. Offset of δ^{13}C values ($\Delta\delta^{13}$C) between methane and terpenoid biomarkers of aerobic methanotrophs in cultures and seep deposits. See Table 1 for isotope values of compounds.

over ANME-1, $\delta^{13}C_{methane}$ values ranging from −47‰ to −37‰, −61‰ to −51‰, and −72‰ to −62‰, as well as $\Delta\delta^{13}C_{terpenoids-methane}$ values from −26‰ to −16‰, −14‰ to −4‰, and +2‰ to +12‰, are obtained for Cold Fork of Cottonwood Creek, Wilbur Springs, and Paskenta, respectively. According to these calculations, that are admittedly based on the uncertain assumption of an ANME-2 dominance, the former presence of Type I/X methanotrophs can be inferred for Cold Fork of Cottonwood Creek, while for Wilbur Springs and Paskenta the isotope signatures are again rather in accord with Type II methanotrophs. Unfortunately, diagnostic lipids that would help with such assignment are absent. Lastly, ^{13}C-depleted phytane ($\delta^{13}C$: −86‰, Table 2) and high contents of biphytane ($\delta^{13}C$: −92‰) in the Cretaceous Zizin seep carbonates most likely represent a predominant input from ANME-1. Based on this assumption, a $\delta^{13}C_{methane}$ value of −59‰ and a $\Delta\delta^{13}C_{terpenoids-methane}$ value of −1‰ would indicate Type II methanotrophs, but the occurrence of trace amounts of nor-lanostanes (Table 3; Sandy et al., 2012) rather point to Type I methanotrophs, very similar to the inconclusive patterns found for the Alaminos Canyon seep carbonates of the Gulf of Mexico.

It is commonly assumed that all marine strains of aerobic methanotrophic bacteria are Type I methanotrophs (Knief, 2015). Fractionation patterns derived from cultured aerobic methanotrophs and compound-specific $\delta^{13}C$ values of MOx biomarkers from marine methane seeps suggest that fractionation in the course of lipid synthesis can be stronger among Type I methanotrophs in the environment under certain environmental conditions than what is known from culture experiments. The $\Delta\delta^{13}C_{terpenoids-methane}$ values calculated here vary widely from seep locality to seep locality, also suggesting a strong dependence of the degree of fractionation on local environmental conditions. For most seep provinces, biomarker evidence agrees with the presence of Type I/X methanotrophs, whereas for some examples the calculated isotope fractionation seems at odds with this interpretation (Fig. 2). Interestingly, the fractionation calculated for the cyclic terpenoids of the Tepee Buttes seep deposits exceeds all fractionation documented for cultures. Our study indicates that terpenoid biomarkers and their carbon stable isotope patterns have great potential to constrain the affiliation of aerobic methanotrophs that dwelled in ancient environments. But it also becomes obvious that more work on cultures and environmental samples is needed to use the full potential of this approach.

5. Conclusions

The suite of cyclic terpenoids of the Type I methanotrophs *Methylomicrobium kenyense* and *Methylomicrobium alcaliphilum* comprises 4-methyl sterols, C_{30} hopanols, tetrahymanol, and BHPs, namely aminotetrol and aminotriol (cf. Banta et al., 2015), which are strongly depleted in ^{13}C. The average carbon stable isotope fractionation relative to the methane source in *M. kenyense* and *M. alcaliphilum* are −25‰ and −16‰, respectively, considerably higher than isotope fractionation in Type II methanotrophs. Aerobic methanotrophs are likely source organisms of ^{13}C-depleted tetrahymanol and its degradation product ^{13}C-depleted gammacerane when present along with other biomarkers of aerobic methanotrophs, as discussed for examples from methane-seep environments. 4-Methyl sterols represent reliable biomarkers of Type I aerobic methanotrophs in young sediments and sedimentary rocks. However, the preservation potential of these compounds is apparently low. Lanostanes are interpreted to represent derivatives of 4-methyl sterols and are sometimes preserved in ancient methane-seep deposits. BHPs occur in recent and sub-recent samples, but are absent in older rocks, where only their degradation products such as anhydroBHT and secohexahydrobenzohopanes are found. Some ancient seep deposits have been shown to contain 3-methyl hopanoids, allowing Type I methanotrophs to be traced into the rock record. The biomarker data base for aerobic methanotrophy reveals great variability of $\delta^{13}C$ patterns. While ANME biomarkers can be used to calculate the $\delta^{13}C$ values of the methane source, this approach is hampered for MOx biomarkers by the observed great variability of $\delta^{13}C_{terpenoid}$ values. Fractionation between methane and terpenoids of aerobic methanotrophs seems to vary greatly as a function of environmental conditions, and in some instances the extent of fractionation cannot be used to unequivocally discriminate Type I and Type II methanotrophs.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.orggeochem.2019.103940.

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