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## Rare bacteriohopanepolyols as markers for an autotrophic, intra-aerobic methanotroph

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### ABSTRACT

Bacteriohopanepolyols (BHPs) and their diagenetic products, hopanoids, are of great interest for their potential as biomarker lipids in both present day environments as well as in the geological record. Specific structural features such as methylation of the A-ring, and number and type of functional groups of C<sub>35</sub> BHPs have been held characteristic for certain groups of organisms. Here we investigated the potential presence of BHPs in the unusual anaerobic methanotroph *Methylomirabilis oxyfera* and another *Methylomirabilis* sp. Although *M. oxyfera* thrives in anoxic settings, it uses internally produced molecular oxygen (from nitrite) for the oxidation of methane. We found that *Methylomirabilis* spp. synthesizes bacteriohopanehexol (BHP-hexol), -pentol, and -tetrol, and 3-methyl derivatives of each as major BHPs. None of the C-35 amino-BHPs that are more commonly observed in methanotrophs were detected. Our findings provide the first ever account of a 3-methyl-BHP-hexol, and only the second known source organism for BHP-hexol after *Alicyclobacillus acidoterrestris*. As the genes required for C3-methylation seem to be exclusively present in microorganisms with an aerobic metabolism, the abundant presence of 3-methyl-BHPs in *Methylomirabilis* spp. denotes for the first time the potential production of 3-methyl hopanoids in anoxic environments. Furthermore, with <sup>13</sup>C-labeling experiments we show that *M. oxyfera* does not assimilate methane-carbon, but rather C from bicarbonate/CO<sub>2</sub>, into its BHPs. This implies that methanotroph-derived hopanoids do not necessarily exhibit a strongly depleted carbon isotopic signature, which is commonly anticipated with methanotrophy. This may have implications for the interpretation of the presence of hopanoids and their isotopic signature in the paleorecord.

## 1. INTRODUCTION

Hopanoids are widely distributed in the environment (Ourisson and Albrecht, 1992; Ourisson et al., 1979), in both recent and ancient sediments dating back as far as the Proterozoic (Brocks et al., 2005). Hopanoids are derived from biosynthetically produced C<sub>30</sub> and the extended C<sub>35</sub> hopanepolyols, which are commonly named bacteriohopanepolyols (BHPs), as bacteria are their only known source organisms. Both intact BHPs and their diagenetic products are used as molecular biomarkers as certain properties, such as the structure of the extended side chain of the intact BHPs or methylation of the hopanoid A-ring, have been associated with distinct groups of bacteria or specific metabolic processes. Hopanoids in general were once interpreted as evidence for oxygenated conditions, as there had been few reports of hopanoids in anaerobic organisms. This idea changed with the observation of hopanoid biosynthesis in several facultative anaerobes and fermentative bacteria (e.g. Bringer et al., 1985; Llopiz et al., 1992; Neunlist et al., 1985; Neunlist and Rohmer, 1985a), and more recently also in anaerobic cultures of anammox bacteria (planctomycetes) (Sinninghe Damsté et al., 2004), *Geobacter* species (Eickhoff et al., 2013; Fischer et al., 2005; Hartner et al., 2005), and sulfate reducing bacteria (SRB) of the genus *Desulfovibrio* (Blumenberg et al., 2006, 2009a, 2012).

Methylation at the C2 or C3 position of the A-ring of hopanoids has been thought to be characteristic for specific bacteria. C2-methylated BHPs are commonly found in cyanobacteria, for which they were initially held as specific biomarker (Summons et al., 1999), but were later found to also be produced by the anoxygenic phototroph *Rhodospseudomonas palustris* under strictly anaerobic conditions (Rashby et al., 2007), and the biosynthetic genes required for methylation at the C2-position were found to be present in various alpha-proteobacteria and acidobacteria as well (Ricci et al., 2013; Welander et al., 2010). C3-methylation was first described in acetic acid bacteria (Rohmer and Ourisson, 1976) and subsequently in aerobic methanotrophs (Zundel and Rohmer, 1985a,b). A recent study of the taxonomic distribution of the genes encoding the (potential for) C3-methylation showed that they are widely distributed among different taxa, although their occurrence seems to be restricted to bacteria with an aerobic metabolism (Welander and Summons, 2012). This implies that the presence of C3-methylation cannot be ascribed specifically to methanotrophic activity, at least not without supporting evidence from carbon isotope studies (e.g. Jahnke et al., 1999; Summons et al., 1994), but may indeed remain a biomarker for aerobic respiration.

Structural features of the side chain of BHPs have also been associated with certain organisms. Specifically, BHPs with penta- and hexafunctionalised side chains incorporating an amine at C-35 are thought to be characteristic for methanotrophic bacteria. The pentafunctionalised 35-aminohopane-31,32,33,34-tetrol (aminotetrol IIIc; see Fig. 1 for structures) is found in most methanotrophs from the alpha- and gammaproteobacteria (Talbot and Farrimond, 2007), in very small amounts in *Methylacidiphilum fumariolicum* SolV (a recently reported methanotroph from the phylum Verrucomicrobia) (van Winden et al., 2012), and in low levels in some SRB (Blumenberg et al. 2006, 2009a, 2012). For the hexafunctionalised 35-aminohopane-30,31,32,33,34-pentol (aminopentol IVc), the gamma-proteobacterial (also known as type I) methanotrophs are thus far the only known source organisms (Talbot and Farrimond, 2007), except for one report of trace levels in SRB (Blumenberg et al., 2012).

Here we investigated the presence and composition of BHPs in the unusual methanotroph *Candidatus* "Methylomirabilis oxyfera". Unlike most described methanotrophic bacteria, which are aerobic proteobacteria, *M. oxyfera* belongs to the NC10 phylum and lives under anoxic conditions, while it internally produces its own oxygen from nitrite (Ettwig et al., 2010). Previous analyses indicated that *M. oxyfera* has the genomic potential to produce 3-methyl hopanoids (Welander and Summons, 2012), but actual production has yet to be confirmed. As BHPs have been specifically used as biomarkers for methanotrophs (e.g. Berndmeyer et al., 2013; Coolen et al., 2008; van Winden et al., 2012; Zhu et al., 2010; Talbot et al., 2014; Wagner et al., 2014), it is interesting to determine whether *M. oxyfera* is also

capable of synthesizing BHPs and could form a potential source of BHPs, including 3-methyl BHPs, previously associated with aerobic metabolism, in anoxic environments.

## 2. MATERIALS AND METHODS

### 2.1 Material

Biomass was obtained from four enrichment cultures capable of nitrite-dependent methane oxidation that had been enriched from three different environments in the Netherlands, as previously described in Kool *et al.* (2012). Two enrichment cultures of *M. oxyfera* originated from sediment from a freshwater ditch in the 'Ooijpolder' (Ooij-1 and Ooij-2) (Ettwig *et al.*, 2009, 2010), the third enrichment culture of *M. oxyfera* was derived from sludge from a wastewater treatment plant in Lieshout (Luesken *et al.*, 2011). The last enrichment culture was obtained from the peatland 'Brunsummerheide' (BRH) and constitutes another species within the genus *Methylomirabilis* (Zhu *et al.*, 2012). The cultures were enriched and maintained in anoxic sequencing batch reactors under constant temperature conditions, as described previously (Ettwig *et al.*, 2009; Luesken *et al.*, 2011; Zhu *et al.*, 2012), and all enrichment cultures oxidized methane anaerobically with nitrite at the point of sampling at a relatively constant level of activity.

Biomass of enrichment culture Ooij-1 was used for incubation experiments with  $^{13}\text{C}$ -labeled substrates as described elsewhere (Rasigraf *et al.*, 2014). Briefly, *M. oxyfera* biomass was incubated with either  $^{13}\text{C}$ -labeled  $\text{CH}_4$  or  $^{13}\text{C}$ -labeled bicarbonate (99 atom%). The  $^{13}\text{C}$ - $\text{CH}_4$  treatment also contained (unlabelled) bicarbonate, and the treatment with  $^{13}\text{C}$ -bicarbonate also received  $\text{CH}_4$  as energy source. A control was included with  $^{13}\text{C}$ -bicarbonate without  $\text{CH}_4$ , and as a reference biomass was also incubated with unlabelled  $\text{CH}_4$  and unlabelled bicarbonate. Two experimental replicates were included for each treatment. Methane and nitrite consumption were monitored over the course of the incubation (24 h) and were similar for all experiments (Rasigraf *et al.*, 2014). The C isotopic enrichments of *M. oxyfera* hopanoids in the labelling treatments were compared to the C isotope signature of the reference.

In addition to the enrichment culture biomass, environmental samples from a soil profile were investigated. These samples were taken from a peatland (Brunsummerheide; BRH), where the most recent enrichment culture originated from. A soil profile was sampled from 51 to 102 cm depth, divided into nine sections (i.e. 51-60 cm, 5 cm increments from 60 to 95 cm, 95-102 cm) (Zhu *et al.*, 2012), spanning the zone from no to peak anaerobic methanotrophic activity.

### 2.2 Hopanoid analyses

#### 2.2.1 Conversion of BHPs to hopanols

Total lipid extracts (TLE) were obtained by sonication (x4) of freeze dried biomass (ca. 0.5 mg) with ca. 1 mL DCM:MeOH (2:1 v/v). After each sonication the mixture was centrifuged and the supernatants were collected and dried over  $\text{Na}_2\text{SO}_4$  and further under a continuous nitrogen flow. The dried TLE were treated with periodic acid and subsequently with sodium borohydride to convert BHPs to more readily analysable terminal alcohols by cleaving the C-C bonds of neighbouring polyols (Rohmer *et al.*, 1984). Periodic acid (30 mg  $\text{H}_5\text{IO}_6$ ) was added to the extract in 1 mL tetrahydrofuran:bidistilled water (8:1, v/v) and the mixture was stirred for 1 h at room temperature. The extract was washed with DCM (x3), dried over a  $\text{Na}_2\text{SO}_4$  and further under  $\text{N}_2$ . Sodium borohydride (20 mg  $\text{NaBH}_4$  in 1 ml MeOH) was then added to the dried extract and the mixture was stirred for 1 h at room temperature. Subsequently 1 mL 200  $\mu\text{M}$   $\text{KH}_2\text{PO}_4$  was added to the mixture and it was washed three times with DCM, dried over a  $\text{Na}_2\text{SO}_4$  and evaporated under  $\text{N}_2$ . The resulting extracts were methylated ( $\text{BF}_3$ -MeOH), and separated over aluminium oxide into a DCM ('apolar') and a DCM:MeOH (1:1) ('polar') fraction. The polar fractions, containing the BHP periodic acid degradation products, were silylated with  $\text{N}_3\text{O}$ -bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine (1:1 v/v) at 60°C for 20 min and dissolved in ethyl acetate.

#### 2.2.2 GC, GC-MS, and GC-IRMS analysis

Polar fractions were analysed by gas chromatography-mass spectrometry (GC-MS; Thermo Trace GC Ultra coupled to a Thermo Trace DSQ). The GC was equipped with a fused silica column (25 m x 0.32 mm i.d., film thickness 0.1  $\mu\text{m}$ ) coated with CP Sil-5CB with helium as a carrier gas. Samples were injected on-column at 70 °C, temperature was increased at 20 °C/min to 130 °C, raised further at 4 °C/min to 320 °C, followed by an isothermal hold for 20 min. Hopanoids were identified by comparison with known mass spectra.

The samples of the BRH peat profile were additionally analysed by GC-MS with a Selective Ion Monitoring (SIM) method specifically set-up to sensitively scan for the presence of the hopanoids identified in the biomass of *Methylomirabilis* spp. This SIM method exclusively scanned for the  $m/z$  191, 205, 189, 203, 217, 279, 293, and 307, as these were characteristic fragment ions of the designated compounds (as indicated in Fig. 2).

For analyses of the stable carbon isotope content of the generated hopanols, silylated polar fractions were analysed by GC coupled to isotope ratio mass spectrometry (GC-IRMS; Agilent 6890 coupled to a Thermo DeltaV IRMS) (in duplicate) as described previously (Schouten et al., 1998). Raw data were corrected for the (known)  $\delta^{13}\text{C}$  content of the (three) methyl groups added during silylation of the polar compounds.

### 2.2.3 Extraction of intact BHPs

Freeze dried biomass was extracted using a modified Bligh and Dyer technique as described by Schouten *et al.* (2008). In short, the samples were extracted by sonication in a solvent mixture containing phosphate buffer, MeOH, and DCM (0.8:2:1, v:v:v). After sonication (10 min) DCM and phosphate buffer were added to reach a ratio of phosphate buffer:MeOH:DCM of 0.9:1:1 (v:v:v). After centrifugation the DCM layer was collected and the residue was re-extracted twice using the same procedure. The combined DCM layers were dried under a continuous nitrogen flow.

All lipid extracts were acetylated with acetic anhydride and pyridine (0.5 mL, 1:1 v/v) for 1 h at 50°C and left at room temperature overnight, after which the solvent was removed under a continuous  $\text{N}_2$  flow. The acetylated extract was dissolved in methanol:propan-2-ol (3:2 v/v) and filtered over 0.2  $\mu\text{m}$  PTFE filters prior to HPLC-MS analysis.

### 2.2.4 HPLC-MS analysis

Reversed-phase HPLC analysis of the acetylated BHPs was carried out using a slightly modified method to that described previously (Cooke et al., 2008) using a Surveyor HPLC system (Thermo Finnigan, Hemel Hempstead, UK) equipped with a Phenomenex (Macclesfield, UK) Gemini  $\text{C}_{18}$  5  $\mu\text{m}$  HPLC column (150 mm x 3 mm i.d.) and a Phenomenex Security Guard<sup>TM</sup> column of the same material. Separation over the column was obtained with a flow rate of 0.5 ml/min at 30 °C with a solvent gradient profile starting at 90% A and 10% B (0-3 min), to 59% A, 1% B and 40% C at 25 min, then isocratic to 40 min, then returning to starting conditions over 5 min and stabilizing for 15 min (with A = MeOH, B = water, C = propan-2-ol; all HPLC-grade). For HPLC-MS, a Thermo Finnigan LCQ ion trap MS instrument equipped with an atmospheric pressure chemical ionization (APCI) source operated in positive ion mode was used. HPLC-MS was performed in data-dependent mode with two scan events: SCAN 1 over the whole spectrum ( $m/z$  300–1300) and with SCAN 2, a data-dependent  $\text{MS}^2$  spectrum recorded for the most abundant ion from SCAN 1. Most intact BHPs were identified by comparison of the mass spectra to known BHP mass spectra (Talbot et al., 2003a, b). BHPs were quantified relative to the total amount of BHPs, assuming similar response factors for the various non-amine containing BHPs.

For the environmental samples, a customized method was additionally used to selectively detect the BHPs present in *Methylomirabilis* spp. enrichment cultures. This selective scanning method was set to specifically scan for  $\text{MS}^2$  spectra (SCAN 2) of ions in SCAN 1 of  $m/z$  655, 669, 713, 727, 771, and 785, characteristic for BHP-tetrol (IIa), 3-methyl BHP-tetrol (IIb), BHP-pentol (IIIa), 3-methyl BHP-pentol (IIIb), BHP-hexol (IVa), and 3-methyl BHP-hexol (IVb), respectively.

### 3. RESULTS

#### 3.1 Hopanoids in enrichment cultures of *Methylomirabilis* spp.

Biomass of four enrichment cultures of the anaerobic methanotroph *Methylomirabilis* spp. were analysed for the presence of hopanoids by periodic acid treatment and by direct analysis with LC-MS. These cultures had been enriched from three different freshwater systems in the Netherlands, and had showed steady methane-oxidizing and nitrite-reducing activity for at least several months. Periodic acid treatment of biomass extracts converts relatively complex BHPs (II-IV) to more readily analysable terminal hopanols (Ia-Ih), and indeed revealed the presence of hopanoids in our extracts of enrichment cultures of *Methylomirabilis* spp.. A representative mass chromatogram of the polar fraction after periodic acid treatment of the extract of biomass of enrichment culture Ooij-2 is shown in Fig. 2. Three major hopanoids were identified by GC-MS as the TMS derivatives of C<sub>30</sub>, C<sub>31</sub> and C<sub>32</sub> regular hopanols (Ia, Ic, Ie) and as hopanols with an additional methyl group at the A-ring (Ib, Id, If). Two of the methylated hopanols (Ib and I) co-eluted with the C<sub>31</sub> and C<sub>32</sub> regular hopanols Ic and Ie (Fig. 2). Based on their relative retention times, the methylated hopanoids were identified as 3-methyl hopanols, since 2-methyl hopanols would elute earlier, i.e. with retention times similar to their non-methyl counterparts (Summons and Jahnke, 1992). The profiles of hopanols obtained by periodic acid treatment of the extracts of enrichment cultures Ooij-1, WWTP and BRH were similar to that of Ooij-2, all showing the presence of C<sub>30</sub>, C<sub>31</sub> and C<sub>32</sub> hopanoids and their 3-methyl derivatives (Fig. 3). In BRH, the relative abundance of the C<sub>32</sub> hopanol Ie was somewhat higher compared to the other enrichment cultures.

HPLC-MS analysis of BHPs in the biomass of the *Methylomirabilis* spp. enrichment cultures revealed the presence of bacteriohopanetetrol (BHT, IIa), bacteriohopanepentol (BHP-pentol, IIIa), bacteriohopanehexol (BHP-hexol, IVa), and the 3-methyl derivatives of all three BHPs as the major BHPs (Iib, IIIb, and IVb, respectively) (Fig. 4). BHT IIa is a commonly observed BHP and its characteristic fragmentation pattern has been described in detail (Talbot et al., 2003a). BHP-pentol IIIa and -hexol IVa are less common but fragment in a similar way (Fig. 4), losing one or two additional acetyl groups (acetylated hydroxyl-groups), supporting the interpretation of these BHPs as such (Talbot et al., 2003a). The mass spectra of the corresponding 3-methyl derivatives Iib, IIIb, and IVb are characterized by the fragment ion of m/z 205 instead of 191, and the series of fragments resulting from a characteristic neutral loss of 206 instead of 192 Da (as a result from cleavage in ring-C between C-9 and 11 and C-8 and 14) (Talbot et al., 2003a), and a parent ion ( $[M+H-CH_3COOH]^+$  for non-N-containing BHPs), whose m/z value is 14 units higher than that of the non-methyl BHPs (Fig. 4). The identification of the location of the additional methyl group at the C3 position (rather than C2) is tentative and based on retention time: 2-methyl BHPs would elute substantially earlier than the 3-methyl BHPs Iib, IIIb and IVb, which elute slightly later than the non-methyl BHPs IIa, IIIa, and IVa (Fig. 4). BHP-hexols IVa and IVb make up 50 to 80 % of the total BHPs of each enrichment culture and 20 to 50 % of all BHPs are methylated at the C3 position (Fig. 5). Overall, the distribution of BHPs in Ooij-1, Ooij-2 and WWTP are similar, whilst enrichment culture BRH contains a relatively large proportion of BHP-hexol IVa (Fig. 5). Overall these results match well with the recovered compounds identified after the periodic acid treatment, since C<sub>30</sub>, C<sub>31</sub>, and C<sub>32</sub> regular hopanols and their 3-methyl derivatives (Ia-h) are the anticipated degradation products of hexa-, penta-, and tetrafunctionalised BHPs. Only in BRH there is a discrepancy, where the relative abundance of BHT IIa is markedly lower than the abundance of its anticipated product after periodic acid treatment, i.e. the C<sub>32</sub> hopanol Ie (cf. Figs. 3 and 5). However, we did also identify two other commonly occurring tetrafunctionalised BHPs in this sample that were not detected in the other enrichment cultures, i.e. BHP-aminotriol Iic and BHT cyclitol ether Iif, which would also contribute to the C<sub>32</sub> hopanol product Ie. The enrichment culture BRH is of all enrichment cultures the least enriched (about 70% for BRH versus 80% for the others), containing a larger side bacterial community than the other enrichment cultures. Moreover, the strain dominating this enrichment culture constitutes another species within the genus *Methylomirabilis*, whilst the other three are considered to contain the same species, *M. oxyfera*.

### 3.2 Stable carbon isotope labelling experiment

The Ooij-1 enrichment culture of *M. oxyfera* was incubated with  $^{13}\text{C}$ -labelled  $\text{CH}_4$  and/or bicarbonate to identify the prime carbon source of the *M. oxyfera* hopanoids. Stable carbon isotope analyses cannot be carried out on the LC-amenable BHPs, so  $\delta^{13}\text{C}$  values were determined on the (GC-amenable) hopanol products after periodic acid degradation. Because of co-elution of the regular and 3-methyl hopanols (Fig. 2), isotopic signatures could not be distinguished for all individual hopanols. The isotope values reported are those of hopanol (Ia) and the integrated values of the coeluting homohopanol (Ic) and 3-methyl hopanol (Ic), and bishomohopanol (Ie) and 3-methyl homohopanol (Id). The analyses showed that the incubations with  $^{13}\text{C}$ -enriched  $\text{CH}_4$  resulted in only little enrichment of hopanols ( $\Delta\delta^{13}\text{C}$  of 6.4, 1.8 and 10.8 ‰, respectively; Fig. 6). However, when the enrichment culture was supplied with  $^{13}\text{C}$ -bicarbonate (and unlabelled  $\text{CH}_4$ ), considerably more incorporation of the  $^{13}\text{C}$ -label into hopanoids of *M. oxyfera* was observed ( $\Delta\delta^{13}\text{C}$  of 40.2, 8.6 and 25.0 ‰, respectively; Fig. 6). In the absence of  $\text{CH}_4$ , no incorporation from  $^{13}\text{C}$ -bicarbonate was observed (data not shown).

### 3.3 Environmental detection of *Methylomirabilis* spp. BHPs

We investigated a soil profile (51 to 102 cm) from the Brunssummerheide peatland for the presence of the hopanoids found in *Methylomirabilis* spp. enrichment cultures. In this section of the soil profile a counter gradient of methane and nitrate was observed, coinciding with a peak in abundance of *Methylomirabilis* spp.-like bacteria as measured by quantitative polymerase chain reaction (qPCR) and an *Methylomirabilis* spp.-specific fatty acid (Kool et al., 2012; Zhu et al., 2012). All horizons of the soil profile contained  $\text{C}_{30}$ ,  $\text{C}_{31}$  and  $\text{C}_{32}$  regular hopanols (Ia, Ic, and Ie) after periodic acid treatment. Bishomohopanol Ie was most abundant;  $\text{C}_{30}$  and  $\text{C}_{31}$  regular hopanols (Ia and Ic) were present in small amounts, and elevated relative abundance of the  $\text{C}_{30}$  hopanol Ia was observed in the 80 to 100 cm soil horizons (Fig. 7). The presence of 3-methyl hopanols could not be identified with certainty with the full scan GC-MS analyses. However, when SIM (see methods) was applied, the presence of (low amounts of) 3-methyl derivatives of the  $\text{C}_{30}$ ,  $\text{C}_{31}$  and  $\text{C}_{32}$  hopanols (Ib, Id, If) was confirmed.

The LC-MS BHP analyses revealed the presence of several BHPs in the peat soil samples other than those present in *Methylomirabilis* spp. enrichment cultures. Particularly, BHP-aminotriol Iic was abundant throughout the profile, matching the high recovery of  $\text{C}_{32}$  hopanol Ie after periodic acid treatment (Fig. 7). The prominent presence of BHP-aminotriol interfered with the (conclusive) identification of BHP-pentol IIIa, as BHP-aminotriol Iic has a retention time close to BHP-pentol and a parent ion (protonated molecule) of  $m/z$  of 714, where BHP-pentol gives a characteristic major fragment of  $m/z$  713 (parent ion minus the loss of one acetyl group) (e.g. Talbot et al., 2003a). Using a selective scanning method, the presence of BHP-pentol remained hard to verify as the specified  $\text{MS}^2$  spectra of  $m/z$  713 primarily derived from an actual parent ion of  $m/z$  714 (and additional fragment ions) of BHP-aminotriol. For the analyses of the LC-MS amenable BHPs, we further specifically focused on the detection of BHP-hexol IVa and its 3-methyl derivative (IVb), as these appear to be most characteristic for *Methylomirabilis* spp.. Data from the full scan (SCAN 1) suggested the possible presence of BHP-hexol IVa and 3-methyl BHP-hexol IVb by the detection of peaks in the  $m/z$  771 and 785 mass chromatograms in several of the samples of the Brunssummerheide profile, namely all those between 70 and 95 cm. Retention times of these compounds corresponded with their expected retention times. However, due to the low relative abundance no  $\text{MS}^2$  spectra could be obtained to verify their identity with the normal HPLC method. With a selective scanning method (see methods), however, we were able to acquire the essential  $\text{MS}^2$  spectra of these compounds in a few samples, which matched with the spectra obtained from the enrichment cultures. The presence of BHP-hexol IVa could be confirmed in the 80-85 cm and 85-90 cm horizons of the soil profile, which matches with the depth where the highest relative abundance of  $\text{C}_{30}$  hopanol Ia after periodic acid degradation was observed (Fig. 7). An  $\text{MS}^2$  spectrum, confirming the presence of the 3-methyl BHP-hexol IVb could only be obtained from one horizon (85-90 cm).

## 4. DISCUSSION

### 4.1 BHP composition of *Methylomirabilis* spp.

Our data suggest that the methanotrophic bacterium *Methylomirabilis* sp. synthesizes distinct BHPs. Since *Methylomirabilis* spp. have not been obtained as pure cultures and were consequently grown in enrichment cultures, which contained 20-30% of other bacteria, it cannot be fully excluded that these other bacteria are producing the BHPs. However, this possibility is deemed rather unlikely since all other bacterial species represent only insignificant amounts of the total enrichment culture (Ettwig et al., 2010) and are unlikely to contribute a substantial part of the lipids. Furthermore, the labeling experiment revealed that the hopanoids showed the same labeling pattern as the *Methylomirabilis* spp.-specific fatty acids (cf. Rasigraf et al., 2014). Some less specific fatty acids (e.g. C<sub>18:0</sub>; Rasigraf et al., 2014) showed a completely different labeling pattern and these fatty acids are thought to be derived from the co-existing bacteria in the enrichment culture (Rasigraf et al., 2014).

The dominance of hexa- and pentafunctionalised BHPs and BHPs with an additional methyl group at the C<sub>3</sub> position in *Methylomirabilis* spp. is highly unusual and characteristic. Tetrafunctionalised BHPs are by far the most common and abundant of all BHPs in both culture and environmental samples (Talbot et al., 2008). Also in anaerobic bacteria, the BHPs produced seem to be primarily tetrafunctionalised, mainly in the form of BHT (IIa) (Eickhoff et al., 2013; Fischer et al., 2005; Sinnighe Damsté et al., 2004), or with three hydroxyl-groups and an alternative group at the C-35 position such as an amine (IIc), cyclitol ether II(f), glucosamine (IIe), or glycoside (Blumenberg et al., 2006; Eickhoff et al., 2013; Hermans et al., 1991; Llopiz et al., 1992; Neunlist et al., 1985; Rashby et al., 2007). The only exceptions to our knowledge are the reported minor amounts of aminotetrol (IIIc) in some SRB of the genus *Desulfovibrio* (Blumenberg et al., 2006), and the C<sub>31</sub> and C<sub>30</sub> hopanols produced by periodic acid treatment from *Geobacter sulfurreducens* (Fischer et al., 2005) that suggest the presence of (small amounts of) penta- and hexafunctionalised BHPs as precursors, although the presence of these structure was not confirmed via LC/MS analysis of samples of the same organism (Eickhoff et al., 2013). Overall, the high abundance of penta- and hexafunctionalised BHPs found here in the anaerobic methanotroph *Methylomirabilis* sp. is exceptional as other methanotrophs that synthesize hexa- or pentafunctionalised BHPs in high abundance produce these as aminopentol (IVc) and aminotetrol (IIIc). We did observe some minor (tetrafunctionalized) amino BHPs in the enrichment culture BRH, but not in any of the other enrichment cultures. It is therefore likely that these amino BHPs are produced by the side-community in the enrichment cultures, while *Methylomirabilis* spp. only synthesizes non-amino-BHPs.

Known hexafunctionalised BHPs are the hexol (IVa), aminopentol (IVc) and its C<sub>3</sub>-methyl derivative (IVd), and hexol cyclitol ether: BHP-hexol and the hexol-cyclitol ether are only occasionally found in the environment (e.g. Cooke et al., 2008; Handley et al., 2010; Talbot and Farrimond, 2007; Berndmeyer et al., 2013; Talbot et al., 2014; Wagner et al., 2014). BHP-aminopentol IVc and the methylated derivative IVd is thus far associated with type I methanotrophs (Cvejic et al., 2000; Neunlist and Rohmer, 1985b; Talbot et al., 2001; van Winden et al., 2012) with one additional report of extremely low trace levels in one SRB (Blumenberg et al., 2012). Whilst aminopentol IVc is reported quite frequently in environmental studies (e.g. Talbot and Farrimond 2007; Coolen et al., 2008; Talbot et al., 2008; Zhu et al., 2010; van Winden et al., 2012; Wagner et al., 2014), the C<sub>3</sub>-methyl derivative IVd has only been reported, together with the non-methylated form, in a small number of settings including one lake sediment (Talbot and Farrimond, 2007), a geothermal silica sinter (Gibson et al., 2008) and the water column of the Black Sea (Blumenberg et al., 2007). *Methylomirabilis* spp. belong to the NC10 phylum, which together with *A. acidoterrestris* (Rezanka et al., 2011) (phylum: Firmicutes) comprise the only two known sources of hexafunctionalised BHPs outside the proteobacteria, and the only known source organisms for BHP-hexol IVa.

The pentafunctionalised BHP-pentol IIIa is not exclusive to *Methylomirabilis* spp.. Cyanobacteria have previously been reported to be able to produce this BHP (Bisseret et al., 1985;; Zhao et al., 1996),



and it was observed in the thermophilic bacterium *Alicyclobacillus acidoterrestris* (Rezanka et al., 2011), but it may in specific environments still serve as an additional biomarker for *Methylomirabilis* spp.. Other pentafunctionalised BHPs are aminotetrol (IIIc) and composite BHPs with a more complex group other than OH or NH<sub>2</sub> attached at the terminal position of the side chain. BHP-aminotetrol IIIc is considered characteristic for methanotrophs, although it is also found at low levels in certain (anaerobic) SRB (Blumenberg et al., 2006, 2009, 2012). Composite pentafunctionalised BHPs have been reported with a carbapseudopentose or cyclitol ether group at the C35 position (e.g. Talbot et al., 2008). The carbapseudopentose-form is rare and no source organism is known for it, although the tetrafunctionalised carbapseudopentose was identified in an enrichment culture of a cyanobacterium (Talbot et al., 2008). In the environment, the pentafunctionalised compounds have thus far only been detected in samples from Antarctica (Talbot et al., 2008). The pentafunctionalised BHP cyclitol ether is more common in the environment, and has been found in the nitrogen fixing *Azotobacter vinelandii* (Vilcheze et al., 1994), in an *Acetobacter* sp. (Hermann et al., 1996), in *Frateuria aurantia* (Joyeux et al., 2004), and in trace amounts in the cyanobacterium *Chlorogloeopsis* sp. LA (Talbot et al., 2003b). *Methylomirabilis* spp. constitutes one of the few organisms that are known to synthesize BHP-pentol IIIa, and forms an additional example of pentafunctionalised BHPs in methanotrophic organisms.

3-Methyl BHPs can, according to genetic analyses, only be produced by organisms with an aerobic metabolism (Welander and Summons, 2012). *M. oxyfera* employs an aerobic metabolism but produces the required oxygen itself internally, while it thrives in anoxic environments (Ettwig et al., 2010). Although the potential for *M. oxyfera* to synthesize 3-methyl-BHPs was already indicated by its genomic composition (Welander and Summons, 2012), the demonstrated presence of these hopanoids in this study of *Methylomirabilis* spp. enrichment cultures now directly shows that 3-methyl hopanoids can also be produced in anoxic settings. Most remarkable is the discovery of 3-methyl HP-hexol IVb, which to our knowledge has never been reported previously. This 3-methyl BHP-hexol provides a new biomarker for *Methylomirabilis* spp., and possibly other similar intra-aerobic methanotrophs.

#### 4.2 Biomarkers for *Methylomirabilis* spp. in the environment

We propose that the distinct BHP composition of *Methylomirabilis* spp. may be used to identify the presence of this bacterium in the environment. Both BHP-hexol IVa and the 3-methyl-BHP-hexol IVb could be detected in the field samples from Brunssummerheide, where genomic and fatty acid analyses had previously verified the presence of *Methylomirabilis* spp. (Kool et al., 2012; Zhu et al., 2012). This demonstrates the potential for these unusual BHPs as diagnostic biomarker for *Methylomirabilis* spp. and possibly related species.

*Methylomirabilis* spp. also produces the less characteristic BHP-pentol but, unfortunately, the detection of this BHP in our environmental samples was inconclusive. Positive identification of BHP-pentol in environmental samples where aminotriol is abundant is obscured by co-elution and the fact that the base peaks of these BHPS (i.e. the protonated molecular ion of BHP-aminotriol and the fragment ion formed by loss of one acetyl group for BHP-pentol) differ by only 1 mass unit. A possible solution to this may be to analyse the BHPs in their non-acetylated form, in which BHP-aminotriol and BHP-pentol will not show the overlapping m/z values of their key characteristic (parent or fragment) ion of 714 and 713 (when acetylated). Nevertheless, using a similar method analysing acetylated BHPs, previous studies have been able to identify BHP-pentol in the environment, where co-occurrence of BHP-aminotriol apparently did not constrain the identification of BHP-pentol (Blumenberg et al., 2009b; Pancost et al., 2005; Sáenz et al., 2011). Though the main known source organisms for BHP-pentol, cyanobacteria (Zhao et al., 1996), live under oxic conditions, BHP-pentol has also been observed in oxygen-limited environments and may thus originate from *Methylomirabilis* spp. or related organisms. Pancost et al. (2005) reported (low quantities of) BHP-pentol in carbonate crusts in the Gulf of Mexico, Blumenberg et al. (2009b) reported BHP-pentol in Black Sea sediments (2009b), and Sáenz et al. (2011) reported its presence in the oxygen minimum zones (OMZs) of the Arabian Sea, the Peru margin, and the Cariaco basin below the photic zone. However, relating these compounds to anoxic or oxygen limiting conditions remains ambiguous, as

the BHPs may not have been produced *in situ* but higher up in the water column under oxygenated conditions.

#### 4.3 Stable carbon isotope composition of *M. oxyfera* BHPs and implications for the geological record

In addition to the structural composition of BHPs and lipids in general, their carbon stable isotope content can provide a useful tool to identify the occurrence of methanotrophs (e.g. Blumenberg et al., 2004; Coolen et al., 2008; Jahnke et al., 1999; Summons et al., 1994). Especially for the less diagnostic diagenetic products of BHPs, stable carbon isotope analyses serve as complementary tool to confirm a methanotrophic origin of these compounds. Biogenic methane is typically substantially depleted in  $^{13}\text{C}$ , and this distinct low  $\delta^{13}\text{C}$  signature of  $\text{CH}_4$  is generally reflected in the biomass and lipids of those organisms that consume  $\text{CH}_4$ . However, a wide range in depletion of the C isotope composition of BHPs and geohopanoids related to methanotrophy has been reported, from  $\delta^{13}\text{C}$  values as low as -90‰ to as high as -30‰ (e.g. Birgel et al., 2006, 2011; Burhan et al., 2002; Coolen et al., 2008; Elvert et al., 2000; Hinrichs, 2001; Talbot et al., 2014; Thiel et al., 2003; van Winden et al., 2010). Our carbon isotope labelling experiments showed that *M. oxyfera* incorporated carbon from  $^{13}\text{C}$ -labeled bicarbonate into its hopanoids, while it incorporated little  $^{13}\text{C}$  from  $^{13}\text{C}$ -labeled  $\text{CH}_4$  (Fig. 6). These results are in good agreement with earlier results showing that bicarbonate/ $\text{CO}_2$  is the main actual carbon source for *M. oxyfera* cell material and fatty acids (Rasigraf et al., 2014). Autotrophy has recently also been shown to be more common than previously thought in other methanotrophs. Although no isotope experiments were conducted, the verrucomicrobial methanotroph *Methylacidiphilum fumariolicum* SolV was shown to oxidize carbon autotrophically (Khadem et al., 2011), and to also produce hopanoids, primarily aminotriol IIc (van Winden et al., 2012).

Overall, this implies that hopanoids that originate from methanotrophic organisms do not necessarily show a distinct depleted  $^{13}\text{C}$  isotopic signature, which is commonly anticipated with methanotrophy. Such ‘less-depleted’ signals were for example reported in a *Sphagnum* peat system where hopenes and tetrafunctionalized hopanoids, interpreted as part of the predominant methanotroph lipids, displayed a carbon isotope content of -31‰ to -38‰ (van Winden et al., 2010). This may have implications for interpreting the sedimentary record as the observation of non-depleted hopanoids in ancient sediment would not necessarily rule out past methanotroph activity, as hopanoids can also be synthesized by autotrophic methanotrophs that assimilate carbon from  $\text{CO}_2$  instead of  $\text{CH}_4$ , which thereby do not convey the distinct depleted carbon isotopic signature from  $\text{CH}_4$ . However, in such settings DIC is typically also depleted due to production of  $\text{CO}_2$  from isotopically depleted organic matter or methane and, consequently, hopanoids produced by autotrophic methanotrophs may still have a relatively depleted isotope signature in these environment.

## 5. CONCLUSIONS

We investigated the BHP composition of the intra-aerobic methanotroph *M. oxyfera*. Our main findings are that *M. oxyfera* synthesizes characteristic BHPs, including 3-methyl BHPs, which provides the first report of the potential production of 3-methyl hopanoids in anoxic environments. Further, based on carbon isotope labelling experiments, we showed that methanotroph-derived hopanoids were produced from DIC and not from methane. Hopanoids produced by *Methylomirabilis* spp. do, therefore, not necessarily exhibit a strongly depleted carbon isotopic signature, which is commonly anticipated for methanotrophs. This may have implications for the interpretation of the presence of hopanoids and their isotopic signature in the paleorecord.

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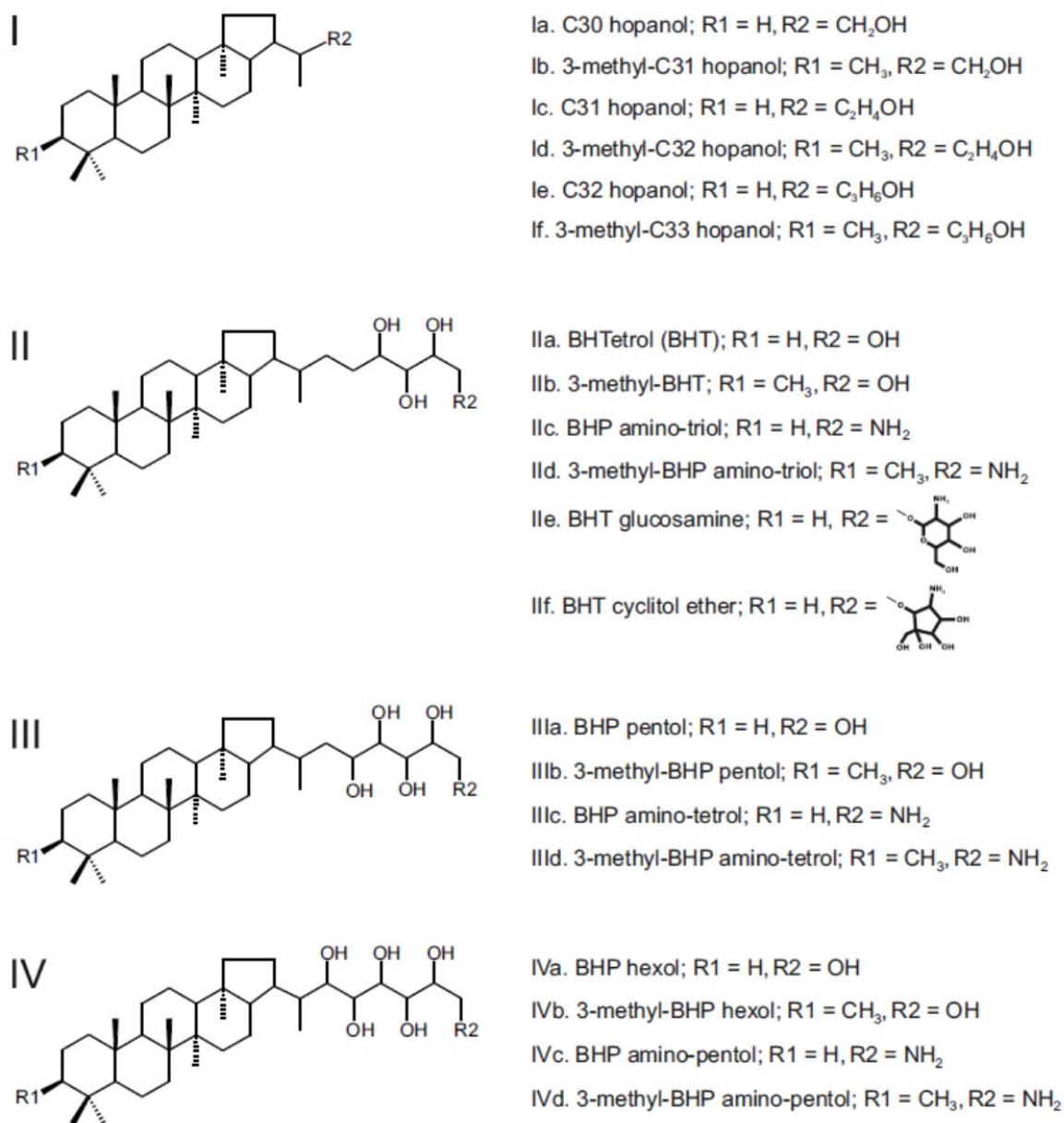


Fig. 1. Structures of various hopanoids, including tetra-, penta-, and hexafunctionalized bacteriohopanepolyols.



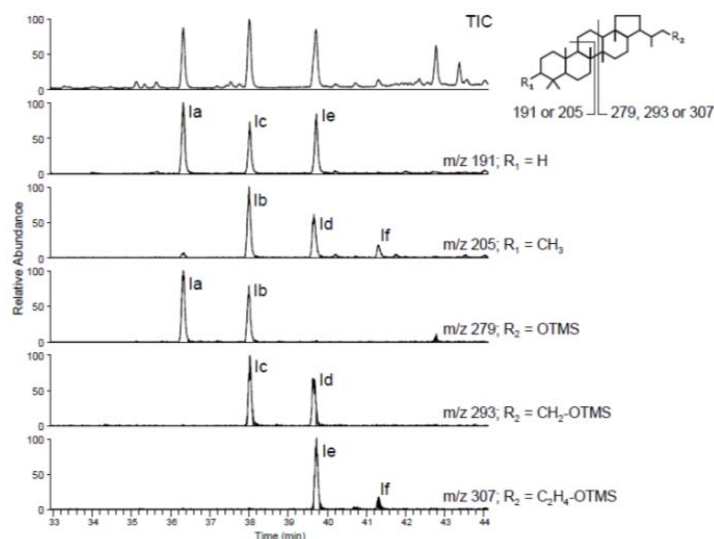


Fig. 2. Partial TIC and mass chromatograms (GC-MS) of the polar fraction after periodic acid treatment of the extract of *M. oxyfera* (enrichment culture WWTP). Characteristic fragments of  $m/z$  191 and 205 are indicative of the hopanoids without and with additional methylation at the A-ring, respectively. Fragments of  $m/z$  279, 293, and 307 identify the length of the (TMS-derivatized) side chains. Numbers refer to structures depicted in Fig. 1.

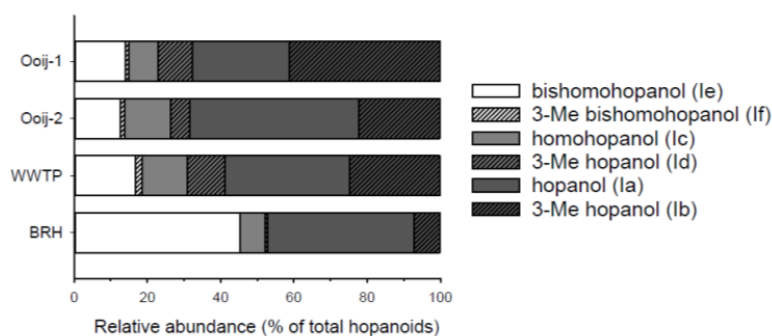


Fig. 3. Relative abundance of the  $C_{30}$ ,  $C_{31}$ ,  $C_{32}$  regular hopanols Ia, Ic, and Ie, and their 3-methyl derivatives Ib, Id, and If (all analysed as TMS derivatives) in the product mixtures of periodic acid degradation of the four studied *Methylospirillum* spp. enrichment cultures. Numbers refer to structures depicted in Fig. 1.

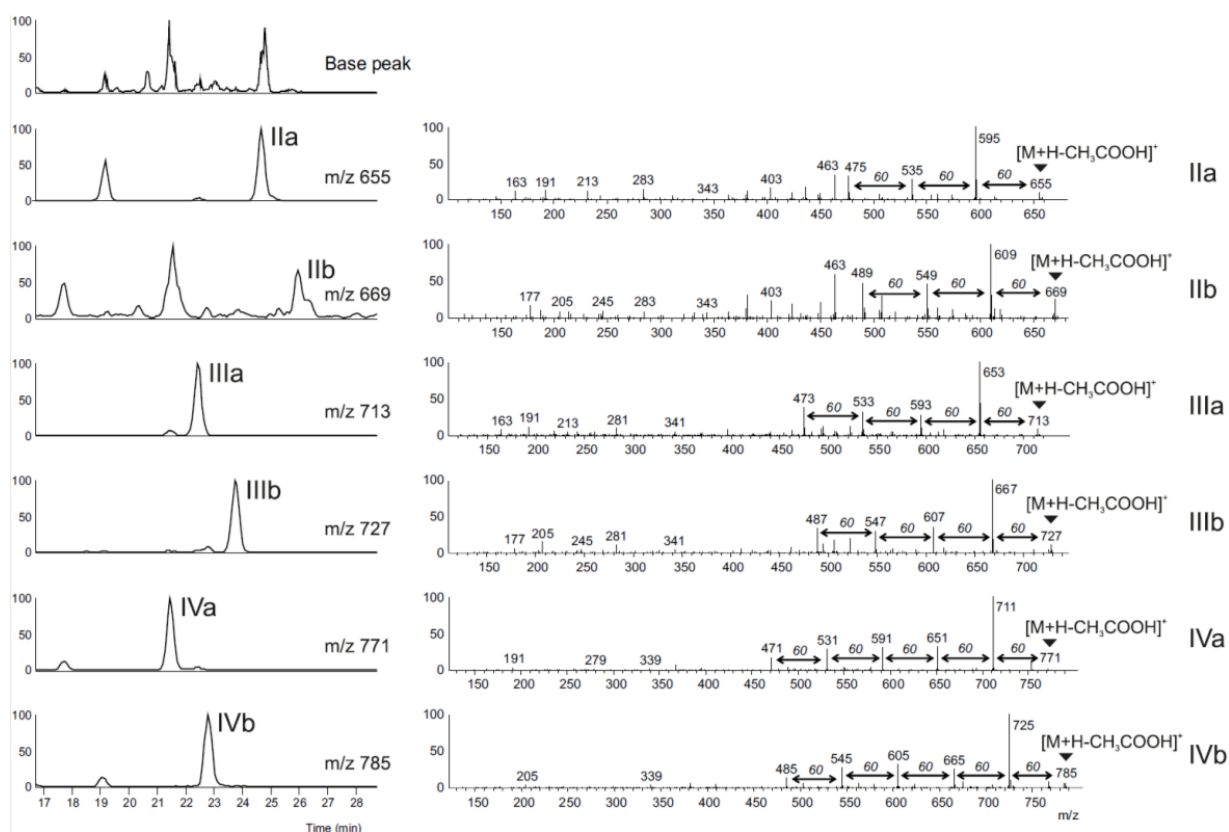


Fig. 4. Partial base peak chromatogram (BPC) and specific mass chromatograms showing the distribution of BHPs in enrichment culture Ooij-2 as revealed LC-APCI-MS analysis, including characteristic APCI MS<sup>2</sup> spectra of all six hopanepolyols (analysed as their acetyl-derivatives). Sequential loss of four, five and six acetyl groups is typical for the BHP-tetrol, -pentol and -hexol, respectively. Further fragmentation results in loss of 192 or 206, and a product with  $m/z$  191 or 205, for the BHPs without and with an additional methyl group at the A-ring, respectively. Numbers refer to structures depicted in Fig. 1.

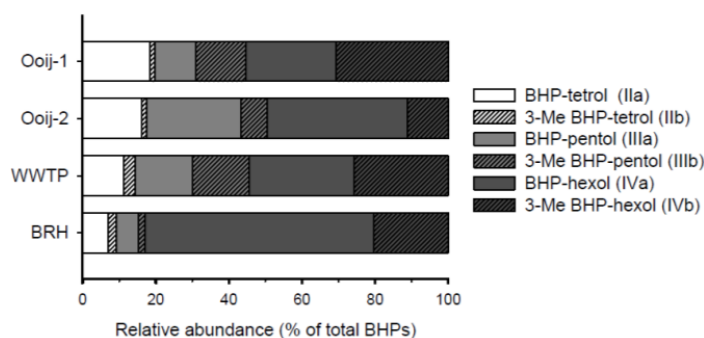


Fig. 5. The relative abundance of the different BHPs as percentage of the total BHPs, derived from LC-MS data. Equivalent response factors for all BHPs were assumed as standards are not available. Numbers refer to structures depicted in Fig. 1.

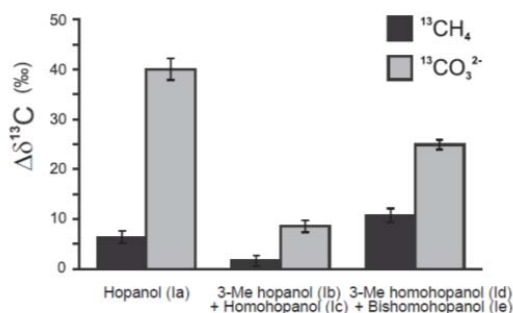


Fig. 6.  $^{13}\text{C}$  enrichment of *M. oxyfera* hopanols (analysed as TMS derivatives) generated by periodic acid degradation of enrichment culture Ooij-1 after incubation with  $^{13}\text{C}$ -labeled  $\text{CH}_4$  or bicarbonate. Due to co-elution of the hopanols, isotopic signatures cannot be distinguished for all individual hopanoids. The abundance of the 3-methyl bishomohopanol If was too low to accurately determine its stable carbon isotopic composition. Error bars indicate the standard error, based on duplicate analyses of two experimental replicates. Numbers refer to structures depicted in Fig. 1.

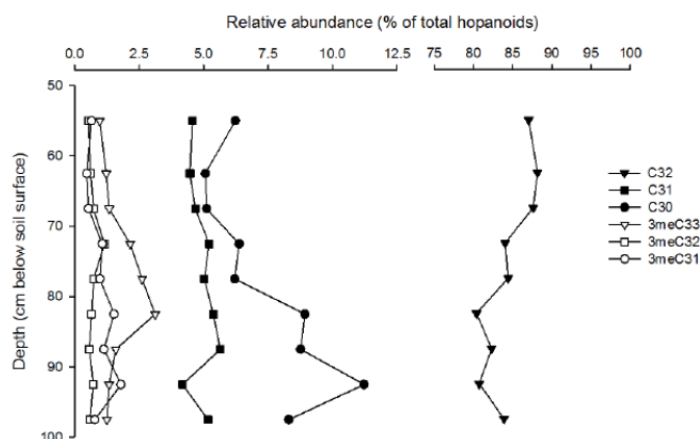


Fig. 7. Relative abundance of  $\text{C}_{30}$ ,  $\text{C}_{31}$  and  $\text{C}_{32}$  hopanols and their 3-methyl derivatives in the Brunssummerheide peat profile. Hopanols were quantified by GC-SIM-MS analyses of samples after periodic acid degradation. Numbers refer to structures depicted in Fig. 1.