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Variability in aerobic methane oxidation over the past 1.2 Myrs recorded in microbial biomarker signatures from Congo fan sediments

Helen M. Talbot a,*, Luke Handley a, Charlotte L. Spencer-Jones a, Bienvenu Jean Dinga b, Enno Schefuß c, Paul J. Mann d, John R. Poulsen e, Robert G.M. Spencer f, Jose N. Wabakanghanzi g, Thomas Wagner a

a School of Civil Engineering and Geosciences, Drummond Building, Newcastle University, Newcastle upon Tyne, NE1 7RU, UK
b Groupe de Recherche en Sciences Exactes et Naturelles (GRSEN/DGRST), Ministère de la Recherche Scientifique, Brazzaville, Congo
c MARUM – Center for Marine Environmental Sciences, University of Bremen, D-28359 Bremen, Germany
d Northumbria University, Department of Geography, Newcastle Upon Tyne, UK
e Nicholas School of the Environment, Duke University, P.O. Box 90328, Durham, NC 27708, USA
f Woods Hole Research Center, 149 Woods Hole Road, Falmouth, MA 02540, USA
g Department of Soil Physics and Hydrology, Congo Atomic Energy Commission, P.O. Box 868, Kinshasa XI, Democratic Republic of the Congo

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Abstract

Methane (CH₄) is a strong greenhouse gas known to have perturbed global climate in the past, especially when released in large quantities over short time periods from continental or marine sources. It is therefore crucial to understand and, if possible, quantify the individual and combined response of these variable methane sources to natural climate variability. However, past changes in the stability of greenhouse gas reservoirs remain uncertain and poorly constrained by geological evidence. Here, we present a record from the Congo fan of a highly specific bacteriohopanepolyol (BHP) biomarker for aerobic methane oxidation (AMO), 35-aminobacteriohopane-30,31,32,33,34-pentol (aminopentol), that identifies discrete periods of increased AMO as far back as 1.2 Ma. Fluctuations in the concentration of aminopentol, and other 35-aminoBHPs, follow a pattern that correlates with late Quaternary glacial-interglacial climate cycles, with highest concentrations during warm periods. We discuss possible sources of aminopentol, and the methane consumed by the precursor methanotrophs, within the context of the Congo River setting, including supply of methane oxidation markers from terrestrial watersheds and/or marine sources (gas hydrate and/or deep subsurface gas reservoir). Compound-specific carbon isotope values of δ²⁹C/C₀²⁹C and δ⁴₀C/C₀⁴₀C for BHPs in ODP 1075 and strong similarities between the BHP signature of the core and surface sediments from the Congo estuary and floodplain wetlands from the interior of the Congo River Basin, support a methanotrophic and likely terrigenous origin of the 35-aminoBHPs found in the fan sediments. This new evidence supports a causal connection between marine sediment BHP records of tropical deep sea fans and wetland settings in the feeding river catchments, and thus tropical continental hydrology. Further research is needed to better constrain the different sources and pathways of methane emission. However, this study identifies the large potential of aminoBHPs, in particular aminopentol, to trace and, once better calibrated and understood, quantify past methane sources and fluxes from terrestrial and potentially also marine sources.

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1. INTRODUCTION

Methane (CH$_4$) is a potent greenhouse gas (GHG) estimated to currently contribute about 20% to total global atmospheric radiative forcing (IPCC, 2007). However, the different pathways of biochemical cycling of methane, which exert a primary control on atmospheric and marine CH$_4$ concentrations through its production and microbiological consumption, remain poorly constrained. Methane emissions from natural sources are significant, although there are also large uncertainties (28–43%; Kroeger et al., 2011 and references therein). For example, in the terrestrial realm wetlands are the largest natural source of methane, estimated to account for about 70% of natural emissions and 40% of total emissions, with other main natural sources being termites (13%), oceans (6%) and a range of others (9%; Wuebbles and Hayhoe, 2002).

Furthermore, changes in the strength of tropical methane sources and sinks (i.e. wetlands, atmospheric oxidation), have been shown to exert a significant control on the atmospheric methane budget over the last 800,000 years (e.g. Blunier et al., 1995; Loulergue et al., 2008; Singarayer et al., 2011). Such changes were modulated by fluctuations in the hydrological cycle and the extent of periglacial wetlands (Loulergue et al., 2008).

Methane production is also substantial in the marine realm (Reeburgh, 2007), although under modern climate conditions up to 90% of methane produced in aquatic environments is believed to be oxidised before it reaches the atmosphere, resulting in a significantly reduced contribution to global GHG emissions. However, the efficiency of this marine filter may not have been constant over time: proportionally more marine-derived methane may have escaped from marine sediments and reached the atmosphere, thus contributing to global greenhouse forcing, at least transiently during periods of rapid climate change. The wide range of relationships and feedbacks between marine microbiologically-mediated turnover of methane, the Ocean-Climate System and biogeochemical cycles are poorly constrained and further elucidation of the underlying drivers of such processes is critical to improve reconstructions of past climate drivers.

To date, research into the marine microbial oxidation of CH$_4$ has largely focussed on the anaerobic oxidation of methane (AOM), as this process may remove as much methane in shallow sediments as is oxidised aerobically in terrestrial environments and the atmosphere (Boetius and Suess, 2004 and references therein). However, aerobic methane oxidation (AMO) has been proposed as playing another key role by acting to remove methane from the water column and in turn limiting emissions to the atmosphere (Valentine et al., 2001; Abril et al., 2007). Despite its potential importance in regulating marine methane fluxes, the role of marine AMO in global methane and carbon cycling, as well as the occurrence and importance of this process in the past, has received far less attention and remains poorly constrained (e.g. Valentine, 2011).

The biochemical cycling of methane, both on land and in the ocean, is believed to have been an important component of the global carbon cycle since the Archaean (e.g. Kasting, 2005; Eigenbrode et al., 2008) and is assumed to have perturbed global climate in the past, especially when released in large quantities over short time periods (Dickens et al., 1995; Norris and Roehl, 1999; Dickens, 2003; Kennett et al., 2003; Kemp et al., 2005; Wagner et al., 2007, 2008; Etope et al., 2008; Zeebe et al., 2009; De Conto et al., 2012). In addition to these punctuated perturbations in the past, ice-core records spanning the last 800,000 years reveal direct and periodic fluctuations in atmospheric methane concentrations, with higher values during interglacials (e.g. Loulergue et al., 2008). As such, understanding methane cycling and how microbial methane cycling and metastable CH$_4$ reservoirs drive or respond to changes in climate is crucial, as is the fate of methane once released from such reservoirs. To that end it is necessary to identify and develop diagnostic markers (proxies), which can be applied to the sedimentary archive.

The main scope of this study is to present a novel biomarker concept to investigate microbial AMO in marine sediments and its links with tropical continental hydrology and climate. For this purpose we use the sediment section covering the past 1.2 Ma from Ocean Drilling Program (ODP) Site 1075, located on the lower Congo fan. This setting is well-documented for methane seepage (Gay et al., 2007) as well as for having a significant but variable terrigenous contribution that has been closely linked to orbitally-driven variations in monsoonal forcing and tropical African climate (Schneider et al., 1997), modulated by sea surface temperature variations (Schefuß et al., 2003, 2005). These features make the Congo Fan well suited to test a novel biomarker proxy for AMO in the sedimentary record, and discuss how possible variations in such biomarker concentrations are linked with Quaternary climate cycles and possible marine and terrestrial sources.

2. BIOHOPANOID BIOMARKERS FOR RECONSTRUCTING METHANE CYCLING IN THE PAST

2.1. Biological sources and lipid composition

Aerobic methane oxidising bacteria of the Phylum proteobacteria are divided into two groups with members of the gammaproteobacteria collectively known as Type I and the alphaproteobacteria, Type II (e.g. Hanson and Hanson, 1996). These organisms produce a range of biomarkers including common C$_{30}$ hopanoids diploptene (I; see appendix) and diplopteroi (II) which are biosynthesised by all hopanoid producers, not just methanotrophs (e.g. Rohmer et al., 1984). Methanotrophs also produce a group of distinctive extended hopanoids called bacteriohopanepolys (BHPs) with an amine functionality at the terminal C-35 positions and 3, 4 or 5 additional hydroxyls (e.g. Cvejic et al., 2000; van Winden et al., 2012a).

The hexafunctionalised BHP 35-aminobacteriophepane-30,31,32,33,34-pentol (III, aminopentol) is considered specific to Type I aerobic methane oxidising bacteria based on culture studies (e.g. Neunlist and Rohmer, 1985a; Zhou et al., 1991; Cvejic et al., 2000; Talbot et al., 2001; van Winden et al., 2012a). Two other aminoBHPs are
commonly reported including the pentafunctionalised 35-amino
carboxihopane-31,32,33,34-tetrol (V; aminotetrol), produced by most Type I and II methanotrophs (Neunlist and Rohmer, 1985a,b; Cvejic et al., 2000; Talbot et al., 2001) with the exception of the Type II genera Methylocella (van Winden et al., 2012a,b). Finally the tetrafunctionalised 35-aminothexahopane-32,33,34,35-tetrol (IV; aminotriol) is synthesised by all Type II and some Type I methanotrophs (e.g. Neunlist and Rohmer, 1985b; Cvejic et al., 2000; Talbot et al., 2001; van Winden et al., 2012a); however, it is also produced by a wide variety of other organisms (e.g. Talbot and Farrimond, 2007 and references therein).

Some Type I methanotrophs also produce a group of 35-amino-BHFs methylated at the C-3 position (see appendix) including 3β-methyl-35-amino/bacteriohopane-30,31,32,33,34-pentol (3-methylaminotetrol; IIIc) and the pentafunctionalised homologue 3-methylenaminotetrol (Va). Production of any C-3 methylated BHP appears to be limited to those organisms which contain the recently identified hpnR gene (Welander and Summons, 2012). The hpnR gene was identified in only 3 of 9 methanotroph genomes including the Type I Methylococcus capsulatus, as expected based on prior culture studies (e.g. Neunlist and Rohmer, 1985a; Summons et al., 1994; Jahnke et al., 1999). Although not currently present in the genomic database, the presence of hpnR must also be inferred for the related Type I genera Methylocaldum, for which there is supporting data from culture studies demonstrating production of C-3 methylated aminotetrol and aminopentol (Cvejic et al., 2000). The hpnR gene was also identified in two species of another Type I genus Methyloptrobiurn (Welander and Summons, 2012); however, to the best of our knowledge, actual synthesis of methylated BHFs has not been demonstrated for this genera and the one literature report we know of identified production of aminotriol and aminotetrol but not aminopentol (Talbot et al., 2001), so it may produce C-3 methylated homologues but possibly only the tetra- and pentafunctionalised aminoBHFs.

Although the identity of the other methanotroph genomes interrogated in the Welander and Summons (2012) study are not indicated, culture based investigations of the BHP composition of other Type I species have revealed that production of aminopentol without the co-occurrence of the methylated homologue is a common feature, particularly in members of the Type I genera Methylovorans (e.g. Neunlist and Rohmer, 1985a; Summons et al., 1994; Jahnke et al., 1999; Talbot et al., 2001; van Winden et al., 2012a) and more recently in Methylophilum (van Winden et al., 2012a). Furthermore, no Type II methanotrophs have ever been found to make methylated BHFs (e.g. Neunlist and Rohmer, 1985b; Cvejic et al., 2000; Talbot et al., 2001; Van Winden et al., 2012a) and were not reported as containing the hpnR gene (Welander and Summons, 2012).

Whilst a range of non-methanotroph sources are known for aminotriol (e.g. Talbot and Farrimond, 2007 and references therein), reports of additional sources of aminotetrol (IV) are limited to low levels (aminotriol: aminotetrol in the range 20–100:1) in a few members of the genus Desulfovibrio (deltaproteobacteria, sulphate reducing bacteria [SRB]; Blumenberg et al., 2006, 2009a, 2012). Trace levels of aminopentol were also reported in one of these organisms (D. salexigens, ratio aminotriol: aminopentol of 1352:1; Blumenberg et al., 2012). Given the extremely low level of aminopentol in D. salexigens the same authors stated in a subsequent paper that aminopentol “remains an excellent biomarker for Type I aerobic methanotrophs” (Berndmeyer et al., 2013).

2.2. Aminopentol – a diagnostic marker for AMO in the sedimentary record

The development of liquid chromatography-mass spectrometry techniques has allowed the direct identification of intact biohopanoid structures including aminopentol in bacterial cultures and various environmental matrices (soils, peat, sediments, water column particulates (e.g. Talbot et al., 2001; Talbot and Farrimond, 2007; Talbot et al., 2008; Sáenz et al., 2011a; van Winden et al., 2012a). Subsequently, the observation of aminopentol (III) has been proposed as a diagnostic marker for Type I methanotrophs and its occurrence in a range of settings was used to infer AMO activity of Type I methanotrophs. For example, it has been identified (without the co-occurrence of the C-3 methylated homologue) in a wide range of environmental samples including Antarctic lake sediment where Methylomonas sp. DNA was also recovered (Ace Lake; Coolen et al., 2008). Other examples include Yangtze River Estuary sediments where peak aminopentol concentrations were found precisely at the point in the river where water column methane concentrations showed a rapid decline (Zhu et al., 2010). Non-methylated aminopentol was also reported in a range of other locations including: a range of lake sediments but excluding sites with high sulfate levels (Talbot et al., 2003; Talbot and Farrimond, 2007; Coolen et al., 2008), Amazonian wetland, shelf and deep sea fan sediments (Wagner et al., 2014), soils (e.g. Cooke, 2010), peat (Kim et al., 2011; van Winden et al., 2012a,b), a hot spring microbial mat (Zhang et al., 2007), one sample of River water from the Pacific coast of Panama (Sáenz et al., 2011a) and the water column of the Baltic Sea (Berndmeyer et al., 2013).

In combination with earlier work using less specific gas chromatography techniques which allowed the BHFs to be identified only at the level of degree of functionalisation (tetra-, penta- or hexafunctionalised [e.g. IV, V, III respectively]; e.g. Innes et al., 1997; Farrimond et al., 2000; Talbot et al., 2003), it has repeatedly been demonstrated that hexafunctionalised BHFs are only common and abundant in terrestrial systems. Conversely, tetrafunctionalised BHFs always dominate in marine settings (e.g. Farrimond et al., 2000; Watson, 2002; Blumenberg et al., 2009b, 2010; Sáenz et al., 2011a,b).

In comparison to observations of aminopentol, reports of 3-methylaminopentol (IIIc) (with or without 3-methylaminotetrol, Va) in modern samples are extremely limited, including one lake sediment (Talbot and Farrimond, 2007), a geothermal silica sinter deposit (Gibson et al., 2008) and the water column of the Black Sea (Blumenberg et al., 2007); although another study of Black Sea water column samples found only the non-methylated aminopentol
homologue (Wakeham et al., 2007) and aminopentol has not been reported from Black Sea sediments (Blumenberg et al., 2009b). This as yet unexplained miss-match between observations of the C-3 methylated biohopanoids in modern studies and the more widespread reports of 3-methyl degradation products (see Section 2.3) was first noted by Farrimond et al. (2004).

2.3. C30 hopanoids and geohopanoid markers of AMO

The hopanoid biomarkers diploptene and diplopterol (I and II respectively; see Appendix for structures) reported from recent sediments, such as the Santa Barbara Basin (northwestern Pacific; 44°ka; Hinrichs, 2001; Hinrichs et al., 2003), the western North Pacific (Uchida et al., 2004) and the Sea of Marmara (northeastern Mediterranean; during the last deglaciation; Menot and Bard, 2010), have been used to infer methane cycling. However, these biomarkers are not specific to aerobic methane oxidisers as they are also produced by a variety of other organisms (e.g. cyanobacteria, nitrogen-fixing bacteria; e.g. Rohmer et al., 1984), and therefore complimentary, more diagnostic evidence is required to confirm such a source, such as a highly depleted carbon isotope composition of the biomarkers.

Methanotrophs use different carbon assimilation pathways for methane with Type I methanotrophs using the ribulose monophosphate (RuMP) pathway and Type II methanotrophs using the serine pathway (e.g. Hanson and Hanson, 1996). An additional group of Type I organisms (formerly known as Type X), including Methylococcales sp., use the RuMP pathway as well as the reductive pentose phosphate and serine pathways (Jahnke et al., 1999). Differences in these assimilation pathways result in significant variation in carbon isotope fractionation between the carbon source, which are also linked to the particular type of methane monooxygenase enzyme (MMO) which is expressed by the organism. The membrane bound particulate methane monooxygenase (pMMO) enzyme typically results in greater fractionation leading to more depleted δ13C lipid values in Type I and X methanotrophs. Conversely, studies have shown that lipids produced by Type II methanotrophs, capable of expressing both pMMO and a soluble form of the enzyme sMMO, can range from 10‰ enriched to 12‰ depleted (Summons et al., 1994; Jahnke et al., 1999). However, this differentiation between Type I and Type II organisms can be further complicated by the fact that some Type I Methylococcales-like species isolated from a freshwater environment contained both pMMO and sMMO (Auman et al., 2000; Auman and Lidstrom, 2002).

Hopanes, including C-3 methylated hopanones are the defunctionalised diagenetic products of the BHPs (e.g. Farrimond et al., 2004). They have been reported from rocks dating back to the late Archean (e.g. Eigenbrode et al., 2008) and, as for the C30 biohopanoids, in combination with 13C depleted carbon isotope values, have been used as molecular evidence for AMO (e.g. Collister et al., 1992). A particular focus has been marine cold seeps where geohopanoids (hopanoic acid and hopanes) with a wide range of depleted C isotope compositions have been reported (−60 to −110‰, e.g. Burhan et al., 2002; Thiel et al., 2003; Peckmann and Thiel, 2004; Birgel et al., 2006a,b).

2.4. Summary for this study

Biological sources of the 3-methylhopanoids appear relatively restricted (Welander and Summons, 2012) and when supported by significantly depleted C isotope compositions have been demonstrated to be useful indicators of AMO (e.g. Collister et al., 1992; Burhan et al., 2002; Peckmann and Thiel, 2004; Birgel et al., 2006a,b). However, these compounds are in fact only produced by a small subset of methanotrophs (Neunlist and Rohmer, 1985a; Cvejic et al., 2000) and their precursor BHPs in modern systems are proving to be highly elusive. Therefore in this study we take the relatively novel approach of using aminopentol (III) as a biomarker for Type I methanotrophs and AMO.

3. MATERIALS AND METHODS

3.1. Site location and sample description

ODP Site 1075 was drilled as part of Ocean Drilling Program (ODP) Leg 175 and is situated on the Northern part of the Congo deep-sea fan (4°47.1198’S, 10°4.4989’E; Fig. 1) at 2996 m water depth (e.g. Wefer et al., 1998). The collected sediments are bioturbated monotonous greenish-grey clays. The age model for ODP 1075 was adopted from Jahn et al. (2005) with the age assignment of marine isotope stages and sub-stages according to Lisiecki and Raymo (2005).

The sediment sample from the estuary of the Congo River (‘Anker 24’) was taken as grab sample (Eisma et al., 1978) and stored as dried sediment before analyses. Additional lipid data were published earlier (Schefuß et al., 2004).

Soil samples were collected from 22 sites spanning a wide range of land cover types, ranging from scrub savannah and grasslands, secondary forest and pristine tropical mixed forest, to tropically seasonally flooded and swamp forest environments within the Congo Basin (Fig 1; Table S2). Surface soils (0–5 cm) were collected during the months of November 2010 and August 2011. Sites were located approximately 5–30 m from nearby streams and rivers. Samples were wrapped in clean foil and shipped to Newcastle University (UK) within three weeks of collection. Samples were stored frozen on arrival and were freeze-dried and ground prior to lipid extraction.

Malebo Pool floodplain wetland sediments were collected along a transect at three sites encompassing sediment that is permanently flooded, sediment inundated during high discharge months only and sediment from above the seasonal high water point (Fig. 1). At each of the three sites sediment was collected at two distinct depths (0–5 cm and 5–15 cm), i.e. a surface and sub-surface sample. Samples were immediately frozen and shipped to the University of Newcastle-upon-Tyne (UK). Previous work has shown that OM exported from this site at the Malebo Pool is geochemically similar to OM at the head of the estuary (~350 km
downstream) and no major tributaries join the Congo River between this site and the Atlantic Ocean (Spencer et al., 2012).

3.2. Total organic carbon analysis

TOC of the ODP 1075 samples was measured as detailed in Holtvoeth et al. (2001). TOC of the soils and Malebo pool samples was measured at Newcastle University using a LECO CS244 Carbon/Sulfur Analyser after removal of inorganic carbon by treatment with hydrochloric acid.

3.3. Lipid extraction

Freeze-dried sediments and soils (ca. 3 g) were extracted using a modified Bligh and Dyer extraction method as described by Cooke et al. (2008). Material was extracted in a Teflon centrifuge tube by adding a monophasic solution of water/methanol (MeOH)/chloroform (4 ml:10 ml:5 ml). The mixture was sonicated at 40 °C for 1 h followed by shaking at room temperature for a further 2–4 h. The mixture was then centrifuged at 12,000 rpm for 15 min and the supernatant transferred to a second centrifuge tube. The same monophasic solution was again added to the initial tube and the extraction procedure repeated twice with the supernatant decanted each time. Chloroform and water (5 ml each) were added to the tubes containing the supernatants and these were centrifuged for 5 min to complete the separation of the organic (chloroform) and MeOH/water phases. The organic fractions were then transferred to a round-bottomed flask and rotary evaporated to near dryness, transferred to a glass vial using a solution of warm (ca. 50 °C) chloroform/MeOH (2:1, v/v) and then evaporated to dryness under a stream of N₂ to yield the total lipid extract (TLE). A 5α-pregnane-3β,20β-diol internal standard was added to the TLE, which was then split into three equal aliquots following dilution with 5 ml chloroform/MeOH (2:1, v/v; heated at 50 °C for 10 min).

3.4. Bacteriohopanepolyol analysis

An aliquot (one third) of the TLE was used for bacteriohopanepolyol (BHP) analysis: the aliquot was evaporated to dryness under N₂ and acetylated by adding acetic anhydride and pyridine (1 ml each) and heating at 50 °C for 1 h then left at room temperature overnight to yield acetylated BHPs. The acetic anhydride and pyridine were removed under a stream of N₂ and the resulting acetylated extract was dissolved in 1 ml MeOH/propan-2-ol (3:2, v/v).

BHP analysis was performed by reversed-phase high performance liquid chromatography-atmospheric pressure chemical ionisation-mass spectrometry (HPLC-APCI-MS) using a ThermoFinnigan surveyor HPLC system fitted with a Phenomenex Gemini C₁₈ column (150 mm; 3.0 mm i.d.; 5 µm particle size) and a security guard column cartridge of the same material coupled to a Finnigan LCQ ion-trap.
mass spectrometer equipped with an APCI source operated in positive ion mode. Chromatographic separation was accomplished at 30 °C with a flow rate of 0.5 ml min⁻¹ and the following mobile phase solvent gradient: 90% A, 10% B (0 min); 59% A, 1% B, 40% C (at 25 min); isocratic to 45 min returning to the starting conditions in 5 min and stabilising for 10 min (where A = MeOH, B = water and C = propan-2-ol). APCI was achieved at 155 °C capillary temperature and 490 °C APCI vapouriser temperature with a corona discharge current of 8 μA, sheath and auxiliary gas flow of 40 and 10, respectively (arbitrary units). MS² analysis was carried out in data-dependent mode with three scan events: SCAN 1: full mass spectrum, m/z 300–1300; SCAN 2: data-dependent MS² spectrum of most intense ion from SCAN 1; SCAN 3: data-dependent MS³ spectrum of most intense ion from SCAN 2. Detection was achieved at an isolation width of m/z 5.0 and fragmentation with normalised collisional dissociation energy of 35% and an activation Q value (parameter determining the m/z range of the observed fragment ions) of 0.15. The semi-quantitative estimate of BHP concentrations was achieved employing the characteristic base peak ion peak areas of individual BHPs in mass chromatograms (from SCAN 1) relative to the m/z 345 mass chromatogram base peak area of the acetylated 5α-pregnane-3β,20β-diol internal standard. Averaged relative response factors relative to the internal standard, determined from a suite of acetylated BHP standards, were used to adjust the BHP peak areas. Typical error in absolute quantification was ±20%, based on selected replicate analyses and BHP standards of known concentration (Cooke, 2010; van Winden et al., 2012a).

### 3.5. Conversion of BHPs to primary hopanols

Seven samples (Table 1) were selected for compound-specific carbon isotope analysis. Highly functionalised BHPs cannot be analysed directly by gas chromatography-isotope ratio mass spectrometry (GC-IRMS) due to their low volatility and high polarity. However, it is possible to analyse the primary alcohols that are produced from BHP precursors via periodic acid reduction followed by sodium borohydride reduction (e.g. Crossman et al., 2001; Coolen et al., 2008) which converts hexa-/penta-/tetrafunctionalised BHPs to terminal C₃₀/C₃₁/C₃₂ hopanols, respectively as described in detail below.

**Table 1**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age (ka)</th>
<th>Carbon isotope values (% VPBD)</th>
<th>% Hopanol derived from aminoBHPa</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C₃₀ avg.</td>
<td>S.D.</td>
<td>N/A</td>
</tr>
<tr>
<td>3H1W 105</td>
<td>65.88</td>
<td>-38.9</td>
<td>0.6</td>
<td>N/A</td>
</tr>
<tr>
<td>6H3W 105</td>
<td>372.7</td>
<td>-41.3</td>
<td>0.7</td>
<td>-39.1</td>
</tr>
<tr>
<td>6H7W 74</td>
<td>425.23</td>
<td>-40.3</td>
<td>0.7</td>
<td>-34.0</td>
</tr>
<tr>
<td>7H4W 5</td>
<td>481.38</td>
<td>-40.7</td>
<td>0.6</td>
<td>-34.3</td>
</tr>
<tr>
<td>7H6W 15</td>
<td>521.12</td>
<td>-40.2</td>
<td>0.7</td>
<td>-34.6</td>
</tr>
<tr>
<td>8H3W 65</td>
<td>580.41</td>
<td>-34.7</td>
<td>0.1</td>
<td>-36.6</td>
</tr>
<tr>
<td>8H7W 15</td>
<td>629.87</td>
<td>-37.9</td>
<td>0.2</td>
<td>-34.5</td>
</tr>
</tbody>
</table>

a Based on relative contribution of aminoBHP to total BHP (of each degree of functionalisation: Hexa-, penta- and tetrafunctionalised respectively) based on LCMS analysis.

6 N/A not analysed (due to low intensity and/or absence of peak).

### 3.6. Compound-specific carbon isotope analysis

Compound-specific stable carbon isotope ratios were determined for the acetylated terminal hopanols (produced by the reactions described in Section 3.5) by gas chromatography–combustion–isotope ratio mass spectrometry (GC–C-IRMS) using a Thermo Trace Ultra GC with splitless injection (280 °C) via a Combustion III interface linked to a Thermo Delta V + IR-MS. Chromatographic separation was achieved on a fused silica capillary column (30 m × 0.25 mm i.d.) coated with a 0.25 μm dimethyl polysiloxane stationary phase (HP-5) with the following temperature program: 50–200 °C at 15 °C/min (held for 1 min),
200–250 °C at 10 °C/min (held for 1 min), 250–350 °C at 5 °C/min, followed by an isothermal for 8 min. Values, measured in duplicate, are determined from a pulsed reference gas calibrated from a reference Alkane A4 mixture with peak specific known isotopic values (Arndt Schimmelmann, Indiana University, USA) and reported in standard per mil notation (δ13C) relative to Vienna PeeDee Belemnite (VPDB); analytical precision on the basis of replicate analysis of standards, was typically ±0.3% and accuracy is represented by 1 sigma standard deviation (Table 1). Measured δ13C values were corrected to take into account the added acetate moiety, the δ13C value of which was determined from an acetylated androstanol standard of known isotopic composition. This approach assumes that the fractionation of the acetate group during acetylation is the same for the standard and the hopanols.

4. RESULTS

4.1. Bacteriohopanepolyol concentrations

A variety of fully functionalised BHPs have previously been reported from ODP Site 1075 sediments, with abundant BHPs in all samples (Cooke et al., 2008; Handley et al., 2010). Here, we confirm that aminopentol (III) is present in 120 of the 122 sediments analysed, as are the other aminoBHPs aminotriol (IV) and aminotetrol (V; Fig. 2) together with 2 other minor BHPs related to aminopentol (IIIa and IIIb; Table S1), up to 115 metres below sea floor (mbsf) and an estimated age of ca. 1.2 Ma based on the age model of Jahn et al. (2005). We did not find any C-3 methylated hopenoids in this study (see also Handley et al., 2010). No statistically relevant co-variation is observed between aminopentol concentration and TOC (R² = 0.006, p = 0.392). Aminopentol concentrations vary widely throughout the core, reaching several maxima of ca. 170 µg g⁻¹ Corg (Fig. 2). Such values are an order of magnitude greater than previously reported sedimentary aminopentol concentrations in river and estuarine sediments (Cooke et al., 2009; Zhu et al., 2010) and the highest reported to date from any marine sediment. Aminotriol concentrations are, generally, higher than aminopentol, with maximum values reaching ca. 360 µg g⁻¹ Corg. Conversely, aminotetrol is present in subordinate quantities, with concentrations consistently a factor of ca. 5 lower than aminotriol. The concentrations of these two 35-aminobacteriohopanepolyols also vary significantly over the last 1.2 Ma and their changes generally mirror those in aminopentol concentration (Fig. 2). Linear regression of concentrations of aminopentol vs. aminotriol and aminotetrol show highly significant correlations with R² values of 0.69 (p = <0.001) and 0.81 (p = <0.001), respectively.

Within the uncertainties of accuracy of the age model (Jahn et al., 2005) there appears to be a relationship between aminopentol concentrations and marine isotope stages (MIS), which are defined by shifts in global ocean oxygen isotopic composition (Lisiecki and Raymo, 2005): concentrations are generally higher during interglacial stages (Fig. 2). Some slight mismatches in the positioning of the aminopentol maxima at the glacial/interglacial transitions (e.g. around 680 and 245 kyr) may arise due to the limited resolution of the aminopentol record and limitations inherent in the age model. It should also be noted that the pronounced shifts in aminoBHP concentrations observed in the Congo core are unlikely to be the result of a preservational bias as the same relationship is also observed if the aminoBHPs are plotted as a proportion of total BHPs.

![Fig. 2. TOC and 35-AminoBHP concentrations from ODP Site 1075 on the Congo deep sea fan from ca. 10 ka to 1.2 Ma. (a) TOC (%). (b) 35-Aminobacteriohopane-30,31,32,33,34-pentol (aminopentol). (c) 35-amino-bacteriohopane-32,33,34-triol (aminotriol). (d) 35-Amino-bacteriohopane-31,32,33,34-tetrol (aminotetrol). BHP concentrations are expressed as weight per gram of sedimentary organic carbon (µg g⁻¹ Corg). Note that a reported concentration of 0 µg g⁻¹ Corg does not necessarily represent the complete absence of aminoBHPs but indicates that concentrations were below confidence levels for integration. Error bars represent analytical error of ±20% associated with the quantification of BHPs using LC-MS. The grey shaded areas highlight glacial marine oxygen isotope stages (MIS) as defined by Lisiecki and Raymo (2005).](image-url)
(not shown). We argue that if diagenetic processes exerted a significant influence on downcore changes in concentrations they would likely affect structurally related BHPs similarly. However, other BHP records (Handley et al., 2010) do not mirror the trends observed in the aminoBHP records and there is no indication of significant diagenetic overprinting of other sedimentary BHP records at depth, except for the more labile soil marker adenosylhopane as reported previously (Cooke et al., 2008; Handley et al., 2010).

4.2. Compound-specific carbon isotope analysis

Hexa-, penta- and tetrafunctionalised biohopanoids were converted to terminal C$_{30}$, C$_{31}$ and C$_{32}$ hopanols, respectively, for compound specific isotope analysis. The measured value is therefore an integrated one for all converted biohopanoids as well as free-hopanols possessing the same degree of functionality, which were already present in the sediment. Assuming a minimum contribution from pre-existing free hopanols, the relative contribution of 3S-aminobacteriohopanepolyol to the total hopanol concentration can be estimated via quantification using LC-MS. The measured hopanol $\delta^{13}$C values are plotted against the % contribution of amino-BHP to total BHPs of each specific degree of functionality (Fig. 3). Hopanol $\delta^{13}$C values decrease as the % contribution of amino-BHP to total BHPs of each specific degree of functionality increases (Fig. 3; Table 1) e.g. for the C$_{30}$ hopanol, the most depleted values of around $-41\%_o$ are observed when the aminopentol precursor contributes ca. 90% of the total hexafunctionalised BHPs.

4.3. BHP composition of Congo surface estuarine, wetland and soil samples

The BHP composition of one surface sediment sample from the Congo River estuary, 3 surface and 3 subsurface sediments from the Malebo Pool and 22 soils from the continental hinterland (see Fig. 1 for locations) was investigated. All samples contain a wide variety of BHPs, with similar compounds to those found in the ODP 1075 sediments (Cooke et al., 2008; Handley et al., 2010). Here we focus on the aminoBHPs only, which are present in all samples (Table S2; Fig. 4). The BHP composition of the estuarine and wetland sediment samples closely resemble that of the Congo core sediments with strong contributions from aminotriol and aminopentol relative to aminotriol, whereas that of the soils shows marked differences with aminotriol (Fig. 4) being by far the most dominant aminoBHP and with aminotriol observed in only 7 of the 22 samples. Aminopentol was a significant component (>50 µg g$^{-1}$org) in only one soil (closed evergreen lowland forest sample C18B; Table S2; Fig. 4) collected from a swampy area, approximately 2 m from a small 7 m wide river channel.

5. DISCUSSION

5.1. A 1.2 Myr record of aerobic methane oxidation intensity

As described above (Section 2.1), Type I methane oxidising bacteria are the only known significant source of aminopentol (III). The methanotrophic origin of the 35-aminoBHPs is supported, although not finally conclusive, by the compound-specific carbon isotope analyses. $\delta^{13}$C values of the hopanol derivatives with a primary hydroxyl group decrease markedly when the % derived from aminoBHP increases (Fig. 3), confirming that the aminoBHPs are substantially more $^{13}$C-depleted than most other BHPs and bulk organic matter (average $\delta^{13}$C$_{org}$ = $-21\%_o$; Holtvoeth et al., 2001).

The most negative carbon isotope values of ca. $-41\%_o$ are not as negative as some previously reported values for biohopanoids produced by aerobic methanotrophs (e.g. Hinrichs, 2001; Coolen et al., 2008; Birgel et al., 2011), but is equivalent to the most depleted values reported for diploptene by Uchida et al. (2004) where they used variations between $-41.0\%_o$ and $-27.9\%_o$ to infer enhanced incorporation of $^{13}$C depleted methane via methanotrophic processes. However, we also postulate that a likely source organism for the aminoBHPs in the Congo fan could be members of the Type I genera Methyleneomonas given the significant amount of aminopentol without the corresponding C-3 methylated homologue (see Section 2.1). Although we can only speculate, this could potentially include members of this genera which can express both pMMO and sMMO
(see Section 2.3), depending on prevailing conditions as reported in freshwater isolates from Lake Washington (Auman et al., 2000; Auman and Lidstrom, 2002). Previous studies of Type II methanotrophs using sMMO have shown that their lipid carbon isotope compositions can be much more variable potentially resulting in $^{13}$C enrichment as well as depletion (Jahnke et al., 1999). The studies of Type II organisms also showed that the type of MMO expressed had no effect on the BHP distribution (Jahnke et al., 1999). It may therefore be possible that the limited $^{13}$C depletions observed in our study are at least partially the result of BHP production by sMMO-expressing aminopentol producers as no difference in BHP composition was observed in studies of Type II organisms when expressing sMMO vs. pMMO (Summons et al., 1994; Jahnke et al., 1999).

To the best of our knowledge there is no data on methane carbon isotope composition from the Congo system (river and/or wetlands). Tyler et al. (2007), report typical values of $-56^{\circ}/_{oo}$ for swamps and marshes and $-59^{\circ}/_{oo}$ for “bogs, fens and lakes” at latitudes below $60^\circ$N, although biogenic methane produced locally under substrate limiting conditions or by methylo trophic methanogenesis may be somewhat enriched (in the region of those reported for thermogenic methane ($-50^{\circ}/_{oo}$ to $-30^{\circ}/_{oo}$; Jahnke et al., 1999 and references therein). At this time we can only speculate if any of these values might be representative for our source areas but if valid, then we would not expect the highly depleted values observed previously in other settings including marine cold seeps (typically in the range $-65^{\circ}/_{oo}$ to $-110^{\circ}/_{oo}$; e.g. Collister et al., 1992; Burhan et al., 2002; Thiel et al., 2003; Birgel et al., 2006a,b). However, less depleted $^{13}$C values between $-31^{\circ}/_{oo}$ and $-40^{\circ}/_{oo}$, for example, have recently been reported for hopanoids with an inferred methanotrophic origin from peat bogs (van Winden et al., 2010), demonstrating the wide range of negative carbon isotope signatures derived from methanotrophs occurring in natural samples.

The strong correlation between the different aminoBHP records (Fig. 2) combined with the carbon isotopic values suggest that, although other sources cannot be discounted entirely, aminotriol and aminotetrol are likely primarily derived from the same source organism as aminopentol, or organisms that respond in similar ways to changes in environmental conditions. As aerobic methanotrophic bacteria are the only significant source of aminopentol (Cvejic et al., 2000; van Winden et al., 2012a) and, at least partially, aminotetrol and aminotriol, we conclude that changes in concentration of these biomarkers in the Congo sediments are mainly proportional to changes in methanotrophic biomass and thus the intensity of aerobic methane oxidation. Consequently, the intervals of increased sedimentary aminopentol concentration are interpreted as periods of increase in aerobic methanotroph activity, although no quantitative estimates are currently possible.

Fluctuations in the intensity of aerobic methane oxidation appear to follow the pattern of late Quaternary glacial-interglacial climate cycles and marine isotope stages (Fig. 2; based on the age model of Lisiecki and Raymo, 2005). Within the limitations of resolution of our aminopentol record, the highest activity is observed during interglacials, possibly focussed to the beginning of interglacials (near or at Terminations) when temperature and sea level rose at the highest rates. This relationship is somewhat less clear in the deeper sections of the core, which may also be due to larger uncertainties in the age model in this part of the core and/or the more sparse data coverage.

![Fig. 4. Ternary plot of the relative abundance of aminoBHPs including the 3 major (aminopentol - III, aminotriol - IV and aminotetrol V) and 2 minor (IIIa and IIIb combined with aminopentol) compounds present in ODP Site 1075 sediments (122 samples), the Congo estuarine sediment, 6 wetland sediments from the vicinity of Malebo Pool and 22 soils from the Republic of Congo. See Fig. 1 for locations.](image)
We recognise that aminopentol concentration is not generally elevated during MIS 3, compared to other interglacial periods. It has been proposed that MIS 3 was generally less pronounced than other interglacials (e.g. Shackleton et al., 1990), suggesting that the methane cycling mechanism responsible for the aminopentol response in the sediments may also have been attenuated, in comparison to other interglacials. If correct, this suggests a causal link between shifts in Quaternary climate and aerobic methane oxidation intensity.

5.2. Possible sources of the methane oxidation signatures on the Congo fan

Considering the location of ODP Site 1075, aminopentol, and other BHPs, may have an allochthonous terrigenous or an autochthonous marine origin, or a combination of both. We therefore discuss possible sources of the aminopentol and, by association, the bacteria from which it was derived.

5.2.1. Aerobic methane oxidation on land

Aerobic methane oxidation is a widespread process in the terrestrial realm (e.g. Hanson and Hanson, 1996) and aminopentol has been reported from a range of continental environments, including soils (Cooke, 2010) peat bogs (van Winden et al., 2012a,b), river sediments (Cooke et al., 2009; Zhu et al., 2010) and lakes (Talbot and Farrimond, 2007; Coolen et al., 2008). The elevated BHP concentrations observed in the Congo core are uncommon in marine sediments and BHP concentrations are typically greater in estuarine and coastal sediments and waters than in the open ocean (Zhu et al., 2011; Sáenz et al., 2011a). In such settings, surface sediment concentrations usually decrease markedly with distance from the coast (Zhu et al., 2010, 2011; Dogru Selver et al., 2012). A terrestrial source for aminopentol must therefore be considered very likely.

The full sedimentary aminoBHP composition provides insight into this possible terrestrial source. We compare the aminoBHP composition for all samples from the Congo core with that of the surface sediment sample from the Congo estuary, the 6 floodplain wetland sediments and 22 soil samples from throughout the Congo River Basin (Fig. 4). The BHP distribution of the soils from the hinterland is markedly different to that from the Congo fan sediments, with the exception of one soil from swampy ground (sample C18B, Table S2), implying that sedimentary BHPs in the estuary and at Site 1075 are not primarily derived from soil settings. The aminoBHP composition of the 6 floodplain wetland sediments are, however, more closely related to the composition of ODP 1075 (Fig. 4), although there are some variations, particularly in the proportion of aminotetrol (V) which is always a minor component (and frequently absent) throughout the core when compared to aminotriol and aminopentol (See Fig. 2). A terrestrial source is, however, indeed supported by compound-specific carbon isotope data as their signatures are too heavy for a purely marine microbiological source (about $-40^{\%}$, as opposed to $< -60^{\%}$, e.g. Jahnke et al., 1995; Burhan et al., 2002; Peckmann and Thiel, 2004; Birgel et al., 2006a,b).

Consequently, terrestrial BHP sources other than soils must be considered. The Congo River Basin is associated with a complex system of inland waters, seasonally inundated wetlands and includes the World’s largest swamp forest found within its central depression (Cuvette Congolaise or Cuvette Centrale) and the Malebo Pool (Fig. 1), close to the outflow of the Congo River where organic matter can be produced, stored, degraded and ultimately exported to the Atlantic Ocean (Spencer et al., 2013). These processes in the Congo River Basin and their integrated export signal are poorly constrained at the moment. However, floodplain lakes and seasonally inundated wetland systems are widespread across all terrestrial ecosystems including the tropical Congo (Fig. 1; Spencer et al., 2013) and Amazon (Melack and Hess, 2010) Basins. For the latter, first BHP signatures have recently been reported and linked with marine sedimentation on the Holocene to deglacial shallow and deep Amazon fan (Wagner et al., 2014). Comparable and highly invariant BHP compositions were observed in two marine sediment records, with high relative abundance of C-35 aminoBHPs, including aminopentol, that ranged between 40% and 50% of all BHPs. The strong similarity in BHP composition in marine and floodplain sediments was used to suggest that BHPs measured in the marine sediments have initially been produced within continental wetland regions and therefore document export from terrestrial wetland regions to the marine environment, both during recent and past glacial climate conditions.

Previous studies have proposed that variations in the hydrological cycle at low latitudes are the dominant control on atmospheric methane levels due to expansion/contraction of tropical and boreal wetlands (e.g. Blunier et al., 1995; Fischer et al., 2008; Loulergue et al., 2008). We consequently propose that wetland areas, one apparent source of the observed aminoBHP signatures, would have been expanded during more humid (interglacial) conditions concurrent with enhanced precipitation and river outflow to the shelf and deep fan. At this point it is not yet possible to deconvolute both controls, but we suggest that expansion of wetland environments during humid climate periods may have been more important than enhanced runoff, considering that large tropical rivers constantly export large volumes of freshwater and organic matter to the coastal ocean, irrespective of glacial or interglacial climate conditions.

5.2.2. Aerobic methane oxidation from marine sources

Alternatively, or in addition, the aminopentol record from the Congo fan may represent an autochthonous marine signal. However, to the best of our knowledge there are no reports of aminopentol from other marine sites such as cold seeps (including cold seep mussel gill symbionts) where only tetra- and pentafunctionslised biohopanoids have been reported, including but limited to aminotriol and aminotetrol (e.g. Jahnke et al., 1995; Burhan et al., 2002; Pancost et al., 2005; Birgel et al., 2011). If there is a marine source of aminopentol, it could be interpreted as an indirect record of methane emission intensity into the oxic bottom and/or pore water during or slightly after sediment deposition. The inferred increase in shallow pore water or bottom water column methane concentrations could be the result of an
increase in sedimentary methanogenesis, linked to high surface water productivity and elevated OM flux to the sediments. The Congo records, however, do not show a clear correlation between sedimentary total organic carbon (TOC) concentration and glacial-interglacial climate cycles (Fig. 2; Holtvoeth et al., 2001), driven by fluctuations in productivity (Schneider et al., 1997) in response to variations in upwelling intensity and nutrient availability (Schneider et al., 1994; Holmes et al., 1997; West et al., 2004). We therefore conclude that changes in productivity and associated increases in organic carbon flux and shallow sedimentary methanogenesis are unlikely to be the primary cause for the observed fluctuations in methane oxidation intensity.

A second potential marine methane source is deep subsurface gas reservoirs. There is ample evidence for seafloor methane seepage in the Congo fan region based on pockmark structures (Gay et al., 2006a; Sahling et al., 2008). Giant structures of over 800 m in diameter have been reported that correlate with elevated methane concentrations in overlying waters (up to 20 μmol/l; Gay et al., 2006b) and authigenic carbonate precipitates (Feng et al., 2010). Gay et al. (2007) demonstrated that most of the pockmarks on the deeper Congo Fan are currently dormant, but that they were periodically active in the past (see Noethen and Kasten, 2011 for discussion), possibly connecting to gas sources in the deep subsurface. The episodic nature of such outgassing events, however, makes a direct link to the observed glacial-interglacial climate variability unreasonable. However, there is currently no biohopanoid data from any pockmarks available to support or refute this idea.

A third and final potential marine source for methane outgassing is the presence of gas hydrates, which have been inferred on the Northern Congo Fan from mud volcanoes and along the Congo continental slope near the presence of pockmark structures (Charlou et al., 2004; Sultan et al., 2004). Gas hydrates have also been recovered from the shallow subsurface at water depths of 3100 m in the vicinity of ODP Site 1075 (Sahling et al., 2008). Considering the observed stratigraphic relationship between aminopen- tol concentrations and late Quaternary climate, a shallow gas hydrate source would be consistent with enhanced methane emission into the aerobic water column especially during early interglacial periods, assuming that the AOM filter (see Jorgensen and Kasten, 2006 for further information) was not quantitative in the subsurface at temporarily very high methane fluxes from an unstable gas hydrate layer. Still, even if a gas hydrate source of the methane seems feasible within the Congo fan area, it must be considered that ODP Site 1075 is situated at 3000 m water depth, which under modern conditions is within the lower part of the hydrate stability zone and further discussion is beyond the scope of this study.

6. CONCLUSIONS

This study confirms molecular evidence for AMO, in the form of specific aminobHPs produced by aerobic methanotrophs, in marine sediments from the Congo deep sea fan (ODP Site 1075) dating back to 1.2 Ma. Our record from the Congo fan is the first to document aerobic methane oxidation over such an extended time interval in this way and highlights natural variability in methane emission and cycling in the past. At this point we cannot categorically identify the source of the molecular signature with absolute confidence, but available data favour a continental source of the BHP signals in the Congo deep sea fan sediments. The exact origin of the BHP signature in the Congo River Basin is difficult to constrain, but close similarities between average BHP signatures in one estuarine sample from the Congo mouth and the fan sediments with those from tropical floodplain wetland sediments from the interior of the Basin strongly point towards wetland areas as the most likely source environment. At this point we cannot exclude additional in situ production of aminoBHPs in aerobic marine surface sediments and/or the overlying water column linked to short periods of enhanced destabilisation of shallow subsurface marine gas hydrates, however, there is no direct evidence to support such a source and mechanism. With the primary scope of this study being to introduce a new molecular proxy for methane cycling from the sedimentary record, more systematic studies are required to further constrain the variability of BHP composition and isotopic signatures within different source areas, both marine and terrestrial, and associated rates of methane flux.

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APPENDIX A.

All BHP side structures shown (III, IV, V) have previously been unambiguously identified by NMR and side chain stereogenic centres determined by ORD measurements for all three mentioned aminopolylols (Zhou et al., 1991). When identified in this study using LC-MS only, where stereochemistry cannot be confirmed, we have assumed the structure to be the same as that previously characterised but the occurrence of additional/alternative isomers cannot be excluded. Structure IIIb includes double bond at either position C-6 or C-11 (cf. Cvejic et al., 2000). Structure, IIIb is a novel, early eluting (on reversed phased HPLC) isomer of aminopentol (III) proposed to comprise 2 hydroxyl groups located on one C atom which hinders full acetylation (see van Winden et al., 2012a).
APPENDIX B. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.gca.2014.02.035.

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