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Carbon isotopic composition of branched tetraether membrane lipids in soils suggest a rapid turnover and a heterotrophic life style of their source organism(s)

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Abstract

Branched Glycerol Dialkyl Glycerol Tetraethers (GDGTs) are membrane spanning lipids synthesised by as yet unknown bacteria that thrive in soils and peat. In order to obtain more information on their ecological niche, the stable carbon isotopic composition of branched GDGT-derived alkanes, obtained upon ether bond cleavage, has been determined in various soils, i.e. peat, forest, grassland and cropland, covered by various vegetation types, i.e., C_3- vs. C_4-plant type. These $\delta^{13}C$ values are compared with those of bulk organic matter and higher plant derived *n*-alkanes from the same soils. With average $\delta^{13}C$ values of −28‰, branched GDGTs in C_3 soils are only slightly depleted (ca. 1‰) relative to bulk organic carbon and on average 8.5‰ enriched relative to plant wax-derived long-chain *n*-alkanes (nC_{29}–nC_{33}). In an Australian soil covered with C_4 type vegetation, the branched GDGTs have a $\delta^{13}C$ value of −18‰, clearly higher than observed in soils with C_3 type vegetation. As with C_3 vegetated soils, branched GDGT $\delta^{13}C$ values are slightly depleted (1‰) relative to bulk organic carbon and enriched (ca. 5‰) relative to *n*-alkanes in this soil. The $\delta^{13}C$ values of branched GDGT lipids being similar to bulk organic carbon and their co-variation with those of bulk organic carbon and plant waxes, suggest a heterotrophic life style and assimilation of relatively heavy and likely labile substrates for the as yet unknown soil bacteria that synthesise the branched GDGT lipids. However, a chemoautotrophic lifestyle, i.e. consuming respired CO_2, could not be fully excluded based on these data alone. Based on a natural labelling experiment of a C_3/C_4 crop change introduced on one of the soils 23 years before sampling and based on a free air CO_2 enrichment experiment with labelled CO_2 on another soil, a turnover time of ca. 17 years has been estimated for branched GDGTs in these arable soils.

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1 Introduction

Branched glycerol dialkyl glycerol tetraethers (GDGTs; I–IX in Fig. 1) are microbially derived membrane lipids that were first inferred to exist based on the presence of unexpected branched alkanes in BBr_3-treated (ether-cleaved) Messel Shale sediments (Chappe et al., 1979). The core tetraether form was subsequently isolated from a peat bog from The Netherlands and characterised by NMR (Sinninghe Damsté et al., 2000). Such a membrane spanning tetraether lipid structure was believed to be a hallmark of the Domain of Archaea. Branched GDGTs, however, are not composed of isoprene units, as is typical for such archaeal tetraethers, but comprise C_{28} alkyl moieties with methylation at the C-13 and C-16 positions. Structural variation in the branched GDGTs occurs by additional methylation at the C-5 position and/or the occurrence of a cyclopentyl moiety through internal cyclisation of a methyl branch with a δ-carbon (Weijers et al., 2006a). Although the cyclopentyl moieties are a seemingly archaeal trait, a bacterial origin of branched GDGTs was eventually postulated, based on the stereochemical configuration of the glycerol backbone, which is the 1,2-di-*O*-alkyl-*sn*-glycerol configuration as found in bacterial membrane lipids and not the 2,3-di-*O*-alkyl-*sn*-glycerol configuration found in archaeal lipids (Weijers et al., 2006a). Branched GDGTs are abundant in peat bogs and have been reported in virtually every soil for which they have been specifically analysed, although concentrations can vary to a large extent (Kim et al., 2006; Peterse et al., 2009a; Weijers et al., 2006a, b). The total concentration of branched GDGTs seems to be determined primarily by organic carbon content and soil pH, whereas differences in the distributions of specific GDGTs are most strongly related to soil pH and temperature (Peterse et al., 2009b; Sinninghe Damsté et al., 2008; Weijers et al., 2006b, 2007b).

So far, the branched GDGTs still are the molecular coelacanths waiting for a living "avatar" (Chappe et al., 1979) and as such the ecological niche of the source bacteria remains enigmatic. As branched GDGT concentrations are much higher in the water-saturated, and thus, anoxic part of peat bogs (catotelm) than in the partly oxygenated

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upper horizon (i.e., acrotelm, Weijers et al., 2006a), their source organisms are likely anaerobic bacteria. A combined organic geochemical and microbiological analysis of a 50 cm peat core from a Swedish ombrotrophic bog hints to the Acidobacteria as the phylum potentially containing GDGT-synthesising bacteria (Weijers et al., 2009). Unfortunately, only very few cultured bacteria are available from this large phylum hampering a proper screening of the Acidobacteria for these compounds.

The carbon isotopic composition of an organism is primarily dictated by the isotopic composition of the source carbon and the mechanism by which this carbon is assimilated. These are, in turn, related to the ecology of an organism (e.g. Pancost and Sinninghe Damsté, 2003). Lipids of chemoautotrophic microorganisms oxidizing methane, for example, generally have low δ^{13}C values (e.g. Brassell et al., 1981; Schouten et al., 1997; Summons et al., 1998). In contrast, lipids of heterotrophic organisms involved in fermentation of organic matter, are generally assumed to resemble the carbon isotopic composition of this organic matter (e.g. Hayes, 1993), although variability exists (e.g. Zhang et al., 2003). These δ^{13}C values could, in turn, be different again from the lipid carbon isotopic composition of autotrophic microbes that utilise CO_2 as their primary carbon source, e.g. Cyanobacteria (e.g. Sakata et al., 1997). In the absence of cultured species of branched GDGT-synthesising bacteria, the carbon isotopic composition of branched GDGT lipids in the environment might, thus, provide additional information on the ecological role of the source organisms. This is important for two reasons. First, based on the abundance of branched GDGT lipids in soils and peats, these microorganisms seem to be an important constituent of soil ecosystems, however, we do not know what processes they are involved in. Second, information on the ecology of branched GDGT-synthesising bacteria might aid in a better interpretation of proxy records based on these compounds, for example, whether or not preferential seasonal activity might cause a bias in temperature records reconstructed using the relative abundance of these compounds (Weijers et al., 2007b).

Thus far, only three studies reported the carbon isotopic composition of branched GDGTs (Oppermann et al., 2010; Pancost and Sinninghe Damsté, 2003; Smittenberg

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et al., 2005). The foremost reason for such few studies is that GDGT lipids themselves are not GC-amenable, and carbon isotopic determinations typically require either offline isolation (using, for example, preparatory liquid chromatography) of GDGTs or the chemical cleavage of the ether bonds, thereby releasing the GC-amenable octacosane components. It should be noted that the second approach does not incorporate the carbon isotopic composition of the glycerol component of the GDGTs. Using the first approach, Smittenberg et al. (2005) reported δ^{13}C values of −28.4‰ and −28.6‰ in sediment samples from Drammensfjord, Norway. These branched GDGTs are most likely derived from forested soils in the catchment of the Drammen river and soils directly surrounding the fjord (Huguet et al., 2007). Using the chemical cleavage of ether bonds, Pancost and Sinninghe Damsté (2003) reported δ^{13}C values of 13,16-dimethyloctacosane to be ca. −29‰, based on 8 samples from the Bargerveen peat bog, The Netherlands. Although not diagnostic, comparison to other organic matter pools led the authors to speculate that the source organism is either a fermentative bacterium acting as intermediate between aerobic heterotrophs and methanogens or an as yet uncharacterised methanogenic archaeon. The latter, however, seems implausible as a bacterial origin of the branched GDGTs has now been more firmly established. Following a similar protocol, recently, Oppermann et al. (2010) reported a carbon isotopic value of ca. −31‰ for branched GDGT derived alkanes in a control soil near a CO_2 vent.

To expand the range of environments for which GDGT δ^{13}C values are known, we investigated a set of various soils and an additional peat. Following ether-bond cleavage, the isotopic composition of the branched alkane components of GDGTs were determined and compared to the δ^{13}C values of total organic carbon (TOC) and the δ^{13}C values of plant wax-derived long chain *n*-alkanes, which are relatively recalcitrant compounds of the TOC. These values are used to test proposed metabolisms of the source organisms. Due to the presence of two natural labelling experiments in the dataset, i.e. a C_3–C_4 crop change and a free air CO_2 enrichment (FACE) experiment using ^{13}C depleted CO_2, the stable carbon isotopic composition of branched GDGTs

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in these soils could also be used to estimate the turnover time of branched GDGTs. In addition, where possible, carbon isotopic compositions of the common isoprenoid GDGT-0 (X in Fig. 1), derived from a range of archaea, and of crenarchaeol (XV), derived from soil Crenarchaeota, have been determined for comparison.

2 Site descriptions

Four soils were obtained from the long-term experiment of the "Höhere Landbauschule" at Rotthalmünster, Germany (48°21′47″ N, 13°11′46″ E). The soil type at this site is a loess-derived stagnic Luvisol with a silty loam texture. Soil samples were taken from: (i) a continuous wheat plot (*Triticum aestivum* L.) established on a former grassland in 1969; (ii) a continuous maize plot (*Zea mays* L.) where only grains were harvested; prior to establishment of the maize culture in 1979, this plot was grassland until 1970 followed by wheat cultivation; (iii) a grassland established in 1961; and (iv) a nearby forest soil from a ca. 80 year old spruce stand (*Picea abies* L.). More details on the site and soils are provided by John et al. (2005). All soils were sampled in September 2002; the wheat and maize cropped soils from a depth interval of 0–30 cm, the grassland soil from a depth of 0–10 cm and the forest soil was sampled from a 0–7 cm depth interval.

Two additional C_3-type soils were obtained from the UK. The first is a grassland soil from the long term experimental research platform site at Rowden Moor (Devon, SW England). The clayey pelo-stagnogley soil from the Hallsworth series (classified as a Stagni-vertic Cambisol in the FAO system) has a silty clay texture and remains very wet from autumn until early spring due to the virtually impermeable clay layer at 30 cm depth (Harrod and Hogan, 2008). The vegetation consists of *Lolium perenne* with patches of *Juncus effuses*. Five small 30-cm long cores were taken in February 2008 in an X-shape and sliced in 10-cm increments. Similar depth increments were pooled and homogenized, resulting in three composite samples spanning the top 30 cm. A second soil was sampled from a beech and oak dominated deciduous forest in Leigh Woods, along the Avon Gorge near the city of Bristol, UK. These forests represent

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some of the oldest (150–250 yrs) woodlands in the South West of England (Rackham, 1980) and have been designated a National Nature Reserve. The soil, a brown ranker falling within the Crwbin association, is a well drained loamy brown earth with a clayish silty texture mixed with coarse limestone gravel and has developed on carboniferous limestone. The soil was sampled in November 2007 at a 3–15 cm depth interval.

A C_4-grassland soil was obtained from an open, mixed woodland-grassland site in the "Blue Range" study area located 130 km north-west of Charters Towers, Queensland, Australia (19°09′ S, 145°24′ E), within the catchment of the Burdekin River. The site is an uncleared, remnant woodland consisting of a mosaic of open grassy areas and more densely wooded areas. The main vegetation type is eucalyptus woodland (*Eucalyptus crebra*; C_3-type vegetation), but the groundcover was dominated by *Urochloa* (*Urochloa mosambicencis*) and *Chrysopogon* (*Chrysopogon fallax*) grasses (C_4-type). More details on the site and soil are given in Krull and Bray (2005). The soil analysed was sampled from an open site dominated by C_4-type grass vegetation in June 2002 at a depth interval of 0–20 cm.

A peat soil was sampled at 34–38 cm depth, i.e. the catotelm, from a mesotrophic mire in the Stordalen mire in northern Sweden (68°21′ N, 18°49′ E) near the village of Abisko. The site is situated at 385 m above sea level, ca. 200 km north of the Arctic Circle and is characterised by discontinuous permafrost. The vegetation on this mire is dominated by Sphagnum species.

A soil from a Free Air CO_2 Enrichment (FACE) study plus a control soil from the same plot were obtained from the Swiss Federal Institute of Technology (ETH) experimental trial near Eschikon, 20 km north-east of Zürich, Switzerland. The soil, classified as Eutric Cambisol (FAO) was covered by clover (*Trifolium repens* L.) and on neighbouring plots experienced either ambient (ca. 360 ppm on average) or elevated (ca. 600 ppm) atmospheric CO_2 concentrations for 10 years. The $\delta^{13}C$ value of the added CO_2 was ca. −48‰. Mixed with atmospheric CO_2 this resulted in a $\delta^{13}C$ value of the CO_2 above the plot of −18‰, significantly lower than that of atmospheric CO_2 (−8‰). For experimental details see Zanetti et al. (1997) and Hebeisen et al. (1997). Samples were

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taken in summer 2002 after the termination of the FACE experiment and combined with corresponding samples from replicate plots and homogenized. For this study, samples from the top soil layer (0–10 cm) have been used.

3 Methods

3.1 Bulk analyses

Total organic carbon content (TOC%) of dried soil samples was determined after decarbonisation of the inorganic carbon with 1 M HCl, using a Fisons Instruments NA1500-NCS elemental analyzer. The carbon isotopic composition of TOC ($\delta^{13}C_{toc}$, in per mill notation relative to the Vienna Pee Dee Belemnite standard) has been determined using a ThermoFinnigan Delta^{Plus} isotope ratio mass spectrometer, with an internal precision of 0.07‰, which is coupled to the elemental analyzer via a Finnigan MAT ConFlo II continuous flow system interface.

3.2 Extraction

Depending on availability of sample material and TOC content, up to 45 g of freeze dried and ground soil material has been extracted ultrasonically using Methanol (MeOH), Dichloromethane (DCM):MeOH 1:1 (v/v) and DCM solvents, sequentially, 3 times each for 15 min, or with a Soxhlet system for 24 h using a solvent mixture of DCM:MeOH 2:1 (v/v). The obtained total extracts were concentrated by rotary evaporation and remaining clay particles were removed by eluting over small columns plugged with pre-extracted cotton wool. The extracts were then separated over a column packed with activated Al_2O_3 into a nominally apolar fraction by eluting with DCM and a polar fraction by eluting with a DCM:MeOH 95:5 (v/v) azeotrope. The polar fraction, containing the GDGT lipids, was dried under a gentle N_2 stream, ultrasonically redissolved in *n*-hexane:propanol 99:1 (v/v) to a concentration of ca. 2 mg/ml and filtered over a 0.45 µm mesh PTFE filter (Alltech).

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3.3 HPLC/MS

Small aliquots of the polar fractions were analysed for their GDGT content using high performance liquid chromatography – mass spectrometry (HPLC/MS) on an Agilent 1100 series/Hewlett-Packard 1100 MSD series machine equipped with HP Chemstation software according to Hopmans et al. (2000, 2004). Separation of GDGTs was achieved on an analytical Alltech Prevail Cyano column (150 mm×2.1 mm, 3 µm) with *n*-hexane:propanol 99:1 (v/v) as eluent (flow rate 0.2 ml min^{-1}), isocratically for the first 5 min, thereafter with a linear gradient to 1.8% propanol in 45 min. Ion scanning was performed in selective ion monitoring (SIM) mode scanning for the masses of interest. Quantification was achieved by integrating the $[M+H]^+$ (protonated mass) peak areas and comparison with an external standard curve based on injections of known amounts of crenarchaeol.

3.4 Purification

GDGT fractions were obtained using semi-preparative HPLC (see Smittenberg et al., 2002). In short, by means of multiple injections, a sample was separated over a semi-preparative Alltech Prevail Cyano column (250 mm×10 mm; 5 µm) with a flow rate of 3 ml min^{-1}, using a *n*-hexane:propanol 99:1 (v/v) mixture as eluent, isocraticaly for the first 5 min, thereafter increasing to 1.8% propanol in 45 min. The eluent with sample was collected in 1 min fractions into collection vials using a Foxy Junior fraction collector (Isco, Lincoln, NE, USA). Flow injection analysis into the mass spectrometer (Agilent 1100 MSD series) was used to determine which collection vials contain the GDGTs that were subsequently pooled and concentrated using rotary evaporation and dried under a gentle N_2 flow.

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3.5 Ether bond cleavage

The alkyl moieties of the GDGTs were released by cleaving the ether bonds that connect them to the glycerol backbone (e.g. Schouten et al., 1998). To this end, 4–5 ml HI (57 wt%) were added to the purified GDGT fraction and refluxed for 1.5 h at 150 °C under continuous stirring. After cooling, the sample was transferred, with *n*-hexane and bi-distilled and extracted water, into a separatory funnel. The organic layer was washed twice with bi-distilled, extracted water, once with a 5 wt% $Na_2S_2O_3$ solution and twice again with bi-distilled, extracted water. The organic phase was concentrated using rotary evaporation and dried over a small column packed with Na_2SO_4. The HI treated sample was then separated over a column packed with activated Al_2O_3 into an apolar fraction (released alkyl iodides) and a polar fraction (remaining polar compounds) using *n*-hexane:DCM 9:1 (v/v) and DCM:MeOH 1:1 (v/v) solvent mixtures, respectively. Subsequently, the alkyl iodides were converted to hydrocarbons using $LiAlH_4$. To this end, the fraction was refluxed for 1.5 h at 150 °C in ca. 4 ml 1,4-Dioxane (stabilized) with a spatula tip of added $LiAlH_4$, with continuous stirring. After cooling, the excess $LiAlH_4$ was neutralized by adding a few drops ethyl acetate, and the sample was centrifuged for 5 min at 2500 rpm. The supernatant was removed and transferred into a separatory funnel filled with 30 ml bidistilled and extracted water and 0.5 ml 5N HCl. The test tube was rinsed 3 times with DCM which was, after centrifugation, also added to the separatory funnel. The 1,4-Dioxane/water mixture was washed three times with DCM. The DCM, containing the alkyl compounds, was taken out of the funnel, concentrated using rotary evaporation and dried over a small column packed with Na_2SO_4.

3.6 Analysis

Released alkyl moieties were identified using a ThermoQuest TraceMS gas chromatograph mass spectrometer (GC-MS). Chromatographic separation was achieved using a Chrompack fused silica capillary column (50 m × 0.32 mm i.d.) coated with a CP Sil-5CB stationary phase (dimethylpolysiloxane equivalent, 0.12 µm film thickness) and using

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He as the carrier gas; the following temperature program was used: 70 °C (1 min) to 130 °C at 20 °C min^{-1} and 130 °C to 300 °C (20 min) at 4 °C min^{-1}. The mass spectrometer operated with electron spray ionization at 70 eV and a scan range of *m/z* 50–650 with a cycle time of 0.6 s. The interface was set at 300 °C and the ion source at 200 °C.

Carbon isotopic composition of *n*-alkanes in the apolar fraction and of the GDGT-derived branched alkanes were determined using a ThermoFinnigan DELTA^{plus}XP Isotope Ratio Mass Spectrometer (IRMS; electron ionization, 100 eV electron voltage and 1 mA electron energy; three faraday cup collectors for *m/z* 44,45 and 46) instrument linked to a Thermo Trace GC via a ThermoFinnigan mark III combustion interface with an oxidation reactor (Cu, Ni, Pt) maintained at 940 °C and a reduction reactor (Cu) at 600 °C. The column and temperature program details were the same as described for the GC-MS with He as carrier gas. Precision was checked twice a day by running a standard mix of fatty acid methyl esters of known isotopic composition. Based on this, the internal precision of the measurements has been determined to be ±0.3‰. All samples were run in duplicate and reported values are means of duplicate measurements. Carbon isotopic values are reported in standard per mill notation ($\delta^{13}C$) relative to the Vienna Pee Dee Belemnite (VPDB) international standard.

4 Results and discussion

4.1 GDGT concentration and distributions

The complete set of branched (I–IX; Fig. 1) and isoprenoid (X–XV) GDGTs is present in all soil samples as analyzed by HPLC/MS in selective ion monitoring (SIM) mode. Branched GDGTs are clearly most abundant in the peat sample with a concentration of ca. 290 µg g^{-1} TOC (Table 1). In the soils, concentrations are lower and range from 3 to 84 µg g^{-1} TOC, with the highest concentrations occurring in Rowden grassland. Branched GDGT concentrations in the soils seem to vary irrespective of the type of soil. No systematic difference seems apparent between woodland and grassland, for

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example; concentrations are not notably higher in the FACE experiment than in other soils, nor are they notably lower in the C_4 soils compared to the C_3 soils (Table 1).

For the peat as well as the soils, concentrations of the archaeal derived isoprenoid GDGT lipids are generally lower than those of the bacterial derived branched GDGT lipids, consistent with earlier observations (Weijers et al., 2006a, b). Concentrations of the isoprenoid GDGT-0 (X), a common archaeal tetraether membrane lipid, range from less than 0.1 $\mu g\,g^{-1}$ TOC in the forest soils to 7.2 $\mu g\,g^{-1}$ TOC in the Rowden grassland soil. In contrast to the branched GDGTs, the concentration of GDGT-0 in the peat, 3.6 $\mu g\,g^{-1}$ TOC, is not notably higher than in the soils for this data set. Concentrations of crenarchaeol (XV), most likely derived from soil crenarchaeota (Weijers et al., 2004, 2006b), range from <0.1 $\mu g\,g^{-1}$ TOC, i.e. at or below detection limit, in the forest soils, the peat and Rowden grassland soil to 6.9 $\mu g\,g^{-1}$ TOC in the FACE soil under elevated CO_2. Relatively high concentrations also occur in the FACE control soil and in the cropped soils (Rotthalmünster wheat and maize). Also the Rotthalmünster grassland soil shows slightly higher crenarchaeol concentrations, certainly relative to the branched GDGT concentration (Table 1). In general, concentrations of crenarchaeol seem to be higher in soils where branched GDGT concentrations are lower, though some exceptions exist. This observation was made earlier in a larger set of soils and was suggested to be largely governed by soil pH (Weijers et al., 2006b). In addition, saturation of the soil with water, and thus oxygen availability, might play a role. Branched GDGT and GDGT-0 concentrations are highest in the water saturated peat sample and in the Rowden grassland soil, which is water saturated large parts of the year, whereas crenarchaeol concentrations in these samples are very low.

4.2 Alkyl moieties released by ether bond cleavage of GDGTs

Initially, we performed ether bond cleavage on polar fractions; unfortunately, with the exception of the peat, none of the GDGT-derived alkanes were of sufficient abundance for compound-specific isotopic determinations. One cause of this is that HI/$LiAlH_4$ treatments of complex mixtures suffer from low yields resulting in such low recovered

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amounts of released hydrocarbons that IRMS analyses of these render impossible. In addition, GDGT concentrations in soils are low compared to those in peat, i.e. generally one to three orders of magnitude lower (Weijers et al., 2006b, see also Table 1). Released alkanes are therefore easily swamped by other hydrocarbons that might be released by ether bond cleavage of other compounds but most likely are formed via acid-catalysed dehydration reactions of alcohols. Therefore, an additional preparative HPLC purification step was used whereby the GDGT containing solvent extract was cleaned by removing the more apolar and more polar parts of the fraction (see Smittenberg et al., 2002). Although preparative HPLC can also be associated with some loss of material, this step resulted in far simpler chromatograms dominated by putatively GDGT-derived hydrocarbons, most notably 13,16-dimethyloctacosane (Fig. 2). However, even after this additional step, carbon isotopic determinations were not possible for all GDGT-derived hydrocarbons.

13,16-Dimethyloctacosane (structure a in Fig. 1) is generally the most abundant GDGT-derived hydrocarbon released by ether bond cleavage, consistent with GDGT I and II typically being the most abundant core tetraether lipids present. Its $\delta^{13}C$ value could be determined for all soils (Table 2). 5,13,16-Trimethyloctacosane (structure b) was abundant in almost all samples, but notably not in the Blue Range soil. Presumably because this Australian soil is from a dry and hot environment resulting in low branched GDGT concentrations and a predominance of GDGTs composed of 13,16-dimethyloctacosane (Weijers et al., 2007b). GDGTs containing one or two cyclopentyl moieties (structures c, d) generally are an order of magnitude lower in abundance than GDGTs without cyclopentane moieties. As a consequence, the cyclopentane containing hydrocarbon (structure c) could only be detected in a few samples, and no carbon isotopic compositions could be determined except for the FACE soil under ambient CO_2 conditions. Concentrations of the isoprenoid GDGTs are markedly lower than those of the branched GDGTs, except for the FACE soils, and consequently biphytanes were not detected in all soils. The acyclic biphytane (structure e in Fig. 1) was obtained by ether bond cleavage of GDGTs in most of the soils, although often in trace amounts

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preventing determination of its carbon isotopic composition in some of the soils. The tricyclic biphytane containing a cyclo-hexane moiety (structure h), released from crenarchaeol (structure XV) and its regioisomer, could only be detected in some samples of which 5 had sufficient abundances for $\delta^{13}C$ analysis. The mono- and dicyclic biphytanes (structures f and g) were generally low in abundance and only for two samples amounts of the dicyclic biphytane (structure g) were sufficient for $\delta^{13}C$ analysis.

In the $HI/LiAlH_4$ treated fraction of the Stordalen mire peat, an unknown compound was observed eluting at 34.8 min (structure z in Fig. 2d), right in sequence behind 13,16-dimethyloctacosane (structure a) and 5,13,16-trimethyloctacosane (structure b), respectively and thus possibly a tetramethyloctacosane. The molecular ion is *m/z* 450, based on the $[M^+-15]$ ion being *m/z* 435, consistent with this being a $C_{32}H_{66}$ hydrocarbon. The mass spectrum of compound z (Fig. 3) shows elevated peaks at *m/z* 211 and 267, similar to the fragmentation pattern of the 5,13,16-trimethyloctacosane, but also shows elevated peaks at *m/z* 365 and 393 which is consistent with the presence of a 4th methyl group at the C-24 position. In addition, the calculated Kovats Retention Index (RI) of 2961 for the peak representing compound z is similar to the theoretical RI of 2960 for 5,13,16,24-tetramethyloctacosane calculated based on the additivity principle (Kissin et al., 1986). Therefore, compound z is tentatively identified as 5,13,16,24-tetramethyloctacosane (Fig. 3). This suggests that GDGT *m/z* 1050 (III) could not only be composed of two 5,13,16-trimethyloctacosanyl moieties (Fig. 1), but also of a combination of a 13,16-dimethyloctacosanyl and a 5,13,16,24-tetramethyloctacosanyl moiety. If these isomers exist, this might explain the fact that in HPLC-MS chromatograms, the peak representing GDGT III in the *m/z* 1050 mass chromatogram is often broader and more irregularly shaped than observed for the peaks representing GDGTs I and II. This should, however, be confirmed by additional analyses. The preliminary identification of compound z also suggests that GDGTs with *m/z* 1064 and *m/z* 1078 could exist, i.e. with a combination of tri- and tetra-methylated, or two tetra-methylated carbon moieties, respectively. However, these compounds have so far not been reported, although the SIM approaches often used in GDGT analyses, especially of ancient sediments,

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would not have detected such components. A screening with HPLC-MS of several peat samples for these compounds did not reveal clear signals that reached above background levels. Together with compound z being rather small relative to structures a and b in the Stordal mire peat (Fig. 2d), this suggests that they are far less common.

4.3 Stable carbon isotopic compositions

The isotopic composition of bulk organic carbon ranges from −25.7 to −29.3‰ for soils under C_3 type vegetation (Table 2), consistent with a C_3 vegetation source (Boutton et al., 1998 and references therein). The $\delta^{13}C_{TOC}$ of the FACE soil kept under elevated CO_2 conditions is depleted (−29.8‰), relative to the control soil under ambient CO_2 conditions, due to the use of ^{13}C-depleted CO_2 for fumigation. Bulk organic carbon $\delta^{13}C$ values are −17 to −21.3‰ for the two soils under C_4 type vegetation, consistent with a significant C_4 vegetation input (Boutton et al., 1998, and references therein). However, the $\delta^{13}C_{TOC}$ of the Rotthalmünster maize soil is somewhat lower than that of the Australian C_4 soil, likely reflecting the presence of a pool of recalcitrant carbon that is older than 23 years and derived from the C_3 vegetation that was present before the change to maize cultivation.

Lipids, like plant leaf wax derived long chain *n*-alkanes, constitute a major component of fresh plant material and subsequently soil organic carbon (Gregorich et al., 1996; Kögel-Knabner, 2002). A suite of *n*-C_{27} to *n*-C_{33} plant leaf wax *n*-alkanes were present in the apolar fraction of all soils, with *n*-C_{31} typically being the most abundant. For the FACE soils and Rotthalmünster soils these results are consistent with earlier measurements (Wiesenberg et al., 2004, 2008a). For most soils, the carbon isotopic composition of odd *n*-C_{27} to *n*-C_{33} alkanes could be determined. The $\delta^{13}C$ values typically vary between −34 and −39‰ for the C_3 soils, with generally the lower values for the longer *n*-alkanes (Table 2). The carbon isotopic composition of *n*-C_{31}, the most abundant *n*-alkane, ranges from −35.6‰ to −38.1‰ for the C_3 top soils with the forest soils showing the higher values (Table 2). In the Rowden grassland, where a

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shallow depth profile has been sampled, $\delta^{13}C$ values of both TOC and the *n*-C_{31} alkanes increase slightly with depth, an observation that has been previously made and for which potential explanations include increasing biodegradation and incorporation of microbial biomass with depth (and time) and, though small, the Suess effect, i.e. the decrease in $\delta^{13}C$ values of atmospheric CO_2 over the last 200 years (Bostrom et al., 2007; Ehleringer et al., 2000, and references therein; Huang et al., 1996). Also, carbon input via root biomass and root exudates into the deeper soil layers might have an important, though not yet well investigated, effect on carbon isotopic compositions along a soil profile (e.g. Wiesenberg et al., 2010). The $\delta^{13}C$ value of *n*-C_{31} in the Stordalen mire is higher than those of the soils (−32.7‰) but comparable to values reported for the Bargerveen bog (Pancost et al., 2003; −31.5‰, Pancost and Sinninghe Damsté, 2003) and other peats (e.g. Xie et al., 2004). The carbon isotopic composition of the *n*-C_{31} alkane in the FACE soil kept 10 years under elevated CO_2 conditions is, like the TOC, depleted relative to the control soil kept under ambient CO_2 conditions, resulting from the fact that CO_2 above the enriched plot was 10‰ depleted in ^{13}C relative to atmospheric CO_2. This is in line with earlier results for the same soils obtained by Wiesenberg et al. (2008b), although they acknowledge that these differences vary strongly among individual *n*-alkanes. The carbon isotopic compositions of the *n*-alkanes in C_4 soils, like those of the TOC, are clearly higher than in C_3 soils. This is especially true for the Blue Range soil, whereas values for the maize soil are somewhere intermediate between typical C_3 and C_4 *n*-alkane $\delta^{13}C$ values (Chikaraishi and Naraoka, 2003; −36 and −21‰, repectively, Collister et al., 1994). This is related to the presence of a large proportion (60%) of old (>23 yr) and recalcitrant C_3-plant derived *n*-alkanes in the Rotthalmünster maize cropped soil (Wiesenberg et al., 2004).

The $\delta^{13}C$ values of the branched GDGT-derived alkanes (structures a and b) from the C_3 vegetated soils vary between −26.7 and −30.1‰ (Table 2); values for trimethyloctacosane (structure b) are enriched by ca. 0.3‰ relative to dimethyloctacosane (structure a), however, this is within the analytical error. In general, these values are clearly higher than the plant wax lipids but slightly lower (0.6‰ on average) than

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the carbon isotopic composition of TOC. Moreover, within a given soil type, branched GDGT-derived alkane $\delta^{13}C$ values exhibit less variability than those of *n*-alkanes. For example, GDGT-derived alkane $\delta^{13}C$ values exhibit no depth-dependant variation in the Rowden grassland soil, with values constant around −30‰. For the FACE soil kept under elevated (and ^{13}C-depleted) CO_2, a depletion is observed for the GDGT-derived hydrocarbons relative to the control soil under ambient CO_2 conditions (ca. −4‰), which is slightly larger than that observed for TOC (−3‰), and certainly larger than that observed for the long chain *n*-alkanes (ca. −2‰; Table 2). The $\delta^{13}C$ values for dimethyloctacosane in the C_4 soils are between −18 and −19‰. These values are clearly higher than values found in C_3 soils. Strikingly, and in contrast to the TOC and *n*-alkanes, the carbon isotopic composition of GDGT-derived alkanes in the maize soil are similar to those in the Blue Range soil (cf., ca. −1‰ for dimethyloctacosane, ca. −4‰ for TOC and ca. −8‰ for the *n*-alkanes; Table 2).

The FACE soil under ambient CO_2 conditions was the only soil for which a carbon isotopic composition could be determined for 1-(3′-methylpentadecyl)-4-nonylcyclopentane (structure c), i.e. the dimehtyloctacosane in which one methyl group has formed a pentacyclic moiety by internal cyclisation to a δ-carbon (Schouten et al., 2000; Weijers et al., 2006a). This value is slightly lower than those of the acyclic component (structure a), −29.1‰ as opposed to −27.1‰. We have, however, insufficient data to examine whether or not this is a typical characteristic.

Carbon isotopic values for the acyclic biphytane (structure e), derived mainly from GDGT X, could be determined for half of the soils (Table 2). The $\delta^{13}C$ values range from −28.6‰ to −33.4‰ and are generally depleted by 2 to 3‰ relative to the branched GDGT-derived alkanes, with the exceptions being the peat and FACE soil kept under elevated and ^{13}C-depleted CO_2. Carbon isotopic compositions of the tricyclic biphytane (structure h), derived from crenarchaeol (GDGT XV), could be determined for five soils and are −29.5 to −28.8‰ in the C_3 soils and −23‰ in the C_4 maize soil (Table 2). Notably, in the FACE control soil, the dicyclic biphytane (structure g) is also largely derived from crenarchaeol. This is evident from the fact that the monocyclic biphytane

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(structure f), derived from GDGTs XI–XIII and not sourced by crenarchaeol, is only just above detection limit (Fig. 2b). The $\delta^{13}C$ value of the dicyclic biphytane (structure g) in this soil is −28.0‰ and thus closely resembles that of the tricyclic biphytane (structure h). It has to be noted, though, that crenarchaeol derived biphytane peaks were generally small and that their $\delta^{13}C$ values are associated with slightly larger errors, on average ±1.1‰.

4.4 The metabolism of branched GDGT producers

The carbon isotopic compositions of branched GDGT-derived alkanes (structures a and b) generally track those of the $\delta^{13}C_{TOC}$. For the C_3 top soils, structures a and b are, on average, depleted by 0.6‰ relative to TOC. Only the Rotthalmünster maize cropped soil shows a different pattern, i.e. an enrichment of the dimethyl octacosane relative to TOC by 2.5‰. The close relationship between TOC and bacterial lipid $\delta^{13}C$ values could arise from two mechanisms (Fig. 4). First, the GDGT-producing bacteria could be chemoautotrophs, and the relationship between TOC and GDGT-derived alkane $\delta^{13}C$ values arises because the isotopic composition of organic matter is an important control on soil CO_2 $\delta^{13}C$ values (e.g. Kuzyakov, 2006, and references therein). The relationship between the $\delta^{13}C$ value of respired CO_2 emitted from soils and that of the substrate is complex (e.g. Crow et al., 2006), but $\delta^{13}C$ of respired CO_2 roughly equals that of the (fresh) soil organic matter. It is important to note here that soil CO_2 present in the soil and respired CO_2 emitted from a soil (and often being the one that is measured) are not isotopically the same. Generally, soil CO_2 is enriched in ^{13}C relative to CO_2 respired from the soil due to the difference in diffusion coefficients for $^{12}CO_2$ and $^{13}CO_2$ (Cerling et al., 1991). Based on theory and observation this difference is about 4.4‰ (Cerling et al., 1991). Thus, the biomass of chemoautotrophic microbes living on soil CO_2 is expected to have higher $\delta^{13}C$ values than that of soil TOC. The branched GDGT derived alkanes in our soils are, in contrast, slightly depleted in ^{13}C relative to TOC (Table 2). This difference might be accounted for by isotope fractionation during

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lipid biosynthesis (DeNiro and Epstein, 1977). This fractionation depends on substrate, synthetic pathway and growth conditions and generally ranges anywhere between −2 to −8‰ (e.g. DeNiro and Epstein, 1977; Hayes, 1993, 2001; van Breugel et al., 2005), but could be higher (Schouten et al., 2004; Teece et al., 1999; van der Meer et al., 2001), or reversed, i.e. enriched relative to biomass (Jahnke et al., 1999; e.g. van der Meer et al., 1998). In general, fatty acids and other acetogenic (straight chain) lipids in most organisms are expected to be ca. 4‰ depleted relative to biomass (Hayes, 1993), except for organisms using the reversed tricarboxylic acid cycle (van der Meer et al., 1998). Unfortunately, both the CO_2 fixation pathway and the exact biosynthetic pathway of branched GDGT lipids (Weijers et al., 2006a) are unknown, making it difficult to make inferences on this fractionation. The source bacteria are supposedly anaerobic organisms and lipid fractionation associated with anaerobic bacteria is often observed to be larger than 4‰ (e.g. Teece et al., 1999; Zhang et al., 2003), which might be consistent with the estimated difference between soil CO_2 and GDGT-derived alkane $\delta^{13}C$ values (Fig. 4). We might compare the $\delta^{13}C$ values of dimethyloctacosane with a true chemoautotroph in these soils, i.e. Crenarchaeota (see also Sect. 4.5). Crenarchaeotal derived biphytanes (structure h), are slightly depleted relative to branched GDGT-derived alkanes (structure a) for all soils in which both could be measured, except the FACE soil under elevated CO_2 conditions (Table 2). Although the differences, given the associated errors, are not large and could support a chemoautotrophic metabolism for the branched GDGT-synthesising organisms, differences in fractionation during biosynthesis of straight chain or isoprenoid lipids could be large, depending on the carbon assimilation pathway (e.g. van der Meer et al., 1998).

Secondly, the similarity of TOC and bacterial lipid $\delta^{13}C$ values could reflect a heterotrophic metabolism for branched GDGT synthesising bacteria (Fig. 4). Little fractionation occurs during bacterial heterotrophy, i.e. during conversion from plant to bacterial biomass (Hayes, 1993), and as discussed above, lipids of anaerobic bacteria are typically >4‰ (range of 2 to 8‰) depleted relative to biomass; thus, if heterotrophs are consuming bulk organic matter, we would expect the bacterial lipids to not only track

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TOC δ^{13}C values but also to be depleted by 2 to 8‰ relative to TOC δ^{13}C values. The fact that this is not observed suggests that the heterotrophic bacteria are preferentially consuming a ^{13}C-enriched component of the TOC pool. These could be carbohydrates and/or proteins, as these are generally quickly assimilated by microbes (e.g. Berggren et al., 2010) and about 0–9‰ enriched in ^{13}C relative to bulk tissue (Abelson and Hoering, 1961; Copley et al., 2003; Gleixner et al., 1993; van der Meer et al., 2001; van Dongen et al., 2002). A similar theory for heterotrophy has been proposed by Huang et al. (1996) for the source of bacterial hopenes in soil and peat. They found that hop-22(29)-enes and neohop-13(18)-enes in soil and peat were only slightly depleted in ^{13}C (ca. 2‰) relative to TOC, but 4–5‰ enriched relative to plant derived *n*-alkanes. Support for the use of more labile substrates, such as carbohydrates, might come from the Rowden grassland depth profile, where branched GDGT-derived alkane δ^{13}C values do not increase with depth, as is the case for TOC, *n*-alkane and GDGT-0 derived biphytane δ^{13}C. This suggests that the branched GDGT-synthesising bacteria feed on a readily available and continuously replenished food source, rather than the stock of OM that tends to get enriched in ^{13}C with depth. If labile components are present at depth, it is most likely that they are either derived from the surface, possibly transported as dissolved organic carbon, or derived from root exudates, which contain large amounts of low molecular weight compounds like carbohydrates, carboxylic acids and amino acids (Gransee and Wittenmayer, 2000; Kuzyakov et al., 2003; Nguyen, 2003).

Pancost and Sinninghe Damsté (2003) suggested a similar heterotrophic life style for the branched GDGT synthesising organism in the Bargerveen peat bog based on similar small differences between the isotopic compositions of 13,16-dimethyloctacosane and TOC and the fact that stratigraphic shifts in δ^{13}C values of 13,16-dimethyloctacosane corresponded to similar δ^{13}C shifts in hopanes derived from heterotrophic bacteria. Also Oppermann et al. (2010) recently suggested a heterotrophic metabolism based on a small difference between TOC and branched GDGT-derived alkane δ^{13}C values in two soils on and near a CO_2 vent. By expanding this relationship to a range of C_3 and C_4 vegetated soils, our work reinforces this hypothesis,

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suggesting that bacterial GDGT-producers have a heterotrophic life style and are likely involved in the fermentation of labile organic matter, potentially generating substrates for other anaerobic organisms such as methanogens.

Distinguishing between the two hypotheses for carbon sequestration of branched GDGT-synthesising bacteria, i.e. uptake of respired CO_2 or fermentation, is, however, difficult. The substrate material, i.e. decaying organic matter is ultimately the same and isotopic fractionations are small. This, combined with the unknown nature of the source bacteria, prevents strong conclusions on their carbon assimilation mechanism. However, in addition to the combined stratigraphic shift in $\delta^{13}C$ values of 13,16-dimethyloctacosane and heterotrophic bacterial derived hopanes shown by Pancost and Sinninghe Damsté (2003), also the study by Oppermann et al. (2010) provides some direction in this. They compared the microbial lipid composition in a soil on a CO_2 vent, receiving naturally labelled CO_2, to a nearby reference soil away from this vent. The change in $\delta^{13}C$ of the branched GDGT-derived alkanes between their CO_2 vent site and the reference site (ca. 5‰) is not as large as the change for biphytanes from presumably autotrophic methanogens (ca. 18‰), which would suggest that branched GDGT synthesising bacteria do not feed directly on the CO_2, and thus are no chemoautotrophs. The authors do acknowledge, though, that where the CO_2 vent site is dominated by autotrophic methanogens, it was not possible to determine to what extent heterotrophic methanogens contribute to the C_{40} biphytanes at the reference site, which might account for part of the observed difference. As long as the branched GDGT-synthesising bacteria remain unknown and could not be cultured, stable isotope pulse labelling experiments, for example using glucose or CO_2, could provide additional insight into the two potential mechanisms of carbon sequestration.

4.5 The metabolism of isoprenoid GDGT producers

16S rRNA gene sequences analyses have proven the presence of group I.1b Crenarchaeota in soils (e.g. Bintrim et al., 1997; Jurgens et al., 1997; Leininger et al., 2006; Schleper et al., 2005) and also their specific biomarker lipid crenarchaeol is often found

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(e.g. Leininger et al., 2006; Weijers et al., 2006b, XV in Fig. 2a). Therefore, soil Crenarchaeota are almost certainly the source of the tricyclic biphytane (structure h, Fig. 1). The metabolic strategy of soil crenarchaeota is similar to the marine pelagic Crenarchaeota, i.e., nitrifiers oxidising ammonium to nitrate (Leininger et al., 2006; Offre et al., 2009; Schleper et al., 2005). This similar strategy might also explain the relatively high concentrations of crenarchaeol (XV) in the FACE soils under *T. repens* vegetation (2–7 µg/gTOC, Table 1). Legumes, like clover, are known to symbiotically fix atmospheric nitrogen into the soil (e.g. Abberton et al., 1999), which might intensify the N-cycle and thus the presence of Crenarchaeota in the soil. The measurements of crenarchaeol-derived biphytane (structure h) $\delta^{13}C$ values, despite their relatively larger errors, are among the first measurements of any kind of soil crenarchaeotal biomass. The values are lower than those of TOC $\delta^{13}C$ by 1.5 to 2.5‰ and slightly lower than those of the branched GDGT-derived alkanes. The $\delta^{13}C$ values for the tricyclic biphytane (structure h) in the C_3 soils (−28.8 to −29.5‰, see Table 2) are considerably lower than values reported for marine crenarchaeol, which vary between −20 and −22‰ (Hoefs et al., 1997; Pancost et al., 2001, 2008; Schouten et al., 1998, 2001; Wakeham et al., 2003). These marine crenarchaeol values are relatively high because marine crenarchaeota are chemoautotrophic organisms assimilating CO_2 and HCO_3^- via a modified 3-hydroxypropionate pathway (Berg et al., 2007; Hallam et al., 2006; Kuypers et al., 2001; Wuchter et al., 2003). Given the similar metabolic strategy for soil crenarchaeota, it will be soil CO_2 that is used by these Crenarchaeota as their primary carbon source. Proof for this comes from a study by Urich et al. (2008), who showed the presence of soil-Crenarchaeotal gene products diagnostic for a CO_2 fixation pathway based on a RNA centered meta-transcriptomic analysis of a nutrient poor sandy lawn soil from Germany. Since soil CO_2 is ultimately derived from soil organic matter via respiration, this explains the depleted values relative to marine crenarchaeol.

The acyclic C_{40} isoprenoid biphytanes (structure e) could derive from either methanogenic archaea, which dominantly synthesise the acyclic dibiphytane tetraether GDGT-0 (X) and smaller amounts of GDGT-1 (XI) (reviewed in Kates et al., 1993), or

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soil crenarchaeota who synthesise similar GDGTs in addition to crenarchaeol (XV) (Sinninghe Damsté et al., 2002; Weijers et al., 2006b). Other uncultivated or unknown Euryarchaeota, however, cannot be completely excluded here as a potential source. The δ^{13}C values of the acyclic biphytanes vary between −28.6‰ and −33.4‰ (Table 2).

In group I Crenarchaeota, the ratio of GDGT X to XV is temperature dependent and generally varies between 0.2 and 2 (Schouten et al., 2002). Given the relative distributions of GDGT X and XV in the soils (Table 1), it is mainly in the Rowden soil and the Stordalen mire that a near exclusive methanogenic source is to be expected without crenarchaeotal admixture (i.e. very high ratios of GDGT X relative to XV). Carbon isotopic compositions of the acyclic biphytanes (structure e) in the Rowden soil and the Stordalen mire are indeed lower and more depleted in ^{13}C relative to TOC than the same compounds in soils where a larger contribution from Crenarchaeota is expected, i.e. the Rotthalmünster wheat soil and the FACE soils (GDGT X/XV ratio of 0.28, 0.77 and 0.71, respectively). In fact, in these latter soils the δ^{13}C values of biphytanes e and h are rather similar, pointing to a potential similar source, i.e. soil Crenarchaeota.

The carbon isotopic composition of −28‰ for the acyclic biphytanes reported in Bargerveen bog were suggested to reflect methanogenic archaea using acetate as a substrate, as opposed to autotrophic methanogens utilising CO_2/H_2 (Pancost et al., 2000; Pancost and Sinninghe Damsté, 2003). This was because the latter are expected to be considerably depleted in ^{13}C (Summons et al., 1998). Here, we see similar δ^{13}C values for biphytane structure e in the Stordalen mire sample and only slightly lower values in the Rowden grassland soil. This suggests that also in these soils a considerable amount of the methanogens present seems to be heterotrophs using acetate to produce CH_4, which seems consistent with a dominantly acetotrophic metabolism across a range of environments (Summons et al., 1998).

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4.6 GDGT turnover time

Amongst the soils analysed, two experienced a dramatic change in the isotopic composition of their vegetation cover over the past decades, and this can be used to evaluate the turnover time of GDGTs in soil. The first is the switch from a C_3 crop to maize (C_4) cultivation for the Rotthalmünster maize soil 23 years ago; the second is the free air CO_2 enrichment study, in which the plants were fumigated with elevated levels of ^{13}C-depleted CO_2 for 10 years. Twenty three years after the change to maize cultivation on the Rotthalmünster soil, *n*-C_{31} still has a carbon isotopic composition of −31.6‰, which is somewhere between the characteristic values for C_4 and C_3 vegetation but slightly closer to the latter. Although most organic molecules in soils, including *n*-alkanes and related compounds, have rather fast turnover times (e.g. Rethemeyer et al., 2004), *n*-alkanes are generally slightly more recalcitrant, showing turnover times in the order of decades (ca. 8 to 60 years in arable soils, Amelung et al., 2008 and references therein). Indeed, Wiesenberg et al. (2004) determined a turnover time of ca. 35 years for *n*-alkanes in this soil. Such a pool of slightly older C_3 plant-derived carbon also seems to persist in the bulk organic carbon, which, as a consequence, is also depleted in ^{13}C relative to a "typical" C_4 type soil. This is consistent with work indicating that the bulk organic carbon pool in soils partially comprises an older, recalcitrant pool (Marschner et al., 2008, and references therein). Strikingly, the branched GDGT-derived alkanes (structures a and b) in the Rotthalmünster maize soil appear to have an almost entirely C_4-derived carbon isotopic composition with no evidence for a significant remnant C_3 signature. Specifically, their $\delta^{13}C$ values are distinct from those found in C_3-vegetated mineral top-soils but similar to those obtained in the Blue Range soil (Table 2); they are also enriched relative to TOC, a unique characteristic of this soil. Thus, the pool of branched GDGTs in this soil has been completely refreshed since the change to maize cultivation 23 years ago. Using the $\delta^{13}C$ value of the 13,16-dimethyloctacosanes (structure a) from the Rotthalmünster maize soil, an estimate could be made on the turnover time of branched GDGTs. For this we

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need the end-member $\delta^{13}C$ values of branched GDGTs in soils covered with C_3- and C_4-type vegetation. Since the carbon isotopic composition of GDGT-derived alkanes seems to be similar to or only slightly depleted relative to that of TOC, we might assume these end-member values to be similar to that of the vegetation. Using a $\delta^{13}C$ value of −28‰ and −15‰ for C_3 and C_4 vegetation respectively, this means that ca. 75% of the GDGTs in the maize soil is carrying a C_4 carbon isotope signature and ca. 25% a C_3 isotope signature. Using the formulas given in Wiesenberg et al. (2008b) to calculate decomposition rate and turnover time based on these fractions, we arrive at a turnover time of ca. 17 years for branched GDGT core lipids in this arable soil in a temperate climate region.

Some further support for this estimate comes from the free air CO_2 enrichment experiment. Due to fumigation with ^{13}C depleted CO_2, the CO_2 above the plot was depleted by 10‰ relative to atmospheric CO_2 and this should have an effect on the carbon isotopic composition of the vegetation growing on the plot. Indeed, Wiesenberg et al. (2008b) have shown that bulk organic carbon of the vegetation on this plot was depleted by ca. 9‰ and that the vegetation derived *n*-alkanes were depleted by ca. 7‰ relative to the control plots. In the soil itself, however, where a pool of old carbon is present, a depletion of only 3‰ for the bulk carbon and 2‰ for the *n*-alkanes was observed. We find a similar minor depletion for the *n*-alkanes (C_{27}–C_{33}) and TOC in this study (Table 2). In contrast, the branched GDGT-derived alkane structure a is depleted by 4.2‰ in the soil under elevated CO_2 conditions relative to the soil under ambient CO_2 conditions. This larger depletion suggests that the branched GDGT turnover has been more rapid over the 10 years of fumigation than that of the *n*-alkanes and potentially even the bulk organic carbon of the soil. It also suggests that the branched GDGT-producing bacteria consume, at least partly, more labile organic matter, or CO_2 derived thereof, rather than the more recalcitrant part of the TOC pool. The fact that the depletion for the GDGT-derived alkane structure a does not reach the 7 to 9‰ observed for the vegetation indicates that turnover has not yet completed or that not all of the organic matter consumed is fresh material. Most likely it will be a combination

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of both. Assuming this is only related to an incomplete turnover, we might make an educated guess on the turnover time of branched GDGTs in this soil. A difference of ca. 4‰ for the 13,16-dimethyloctacosanes (structure a) and ca. 9‰ for the bulk plant organic carbon between the FACE and control soils gives a proportion of 44% of the GDGTs that has been labelled since the start of the fumigation 10 years before sampling. Using the formulas given by Wiesenberg et al. (2008b), this translates into an estimate of the turnover time for branched GDGT core lipids of ca. 17 years, which is similar to the estimate based on the C_3/C_4 vegetation change on the Rotthalmünster maize soil. Although it should be acknowledged that in both cases calculation of the turnover time depends on a single soil sample, the fact that the two, each representing a different form of labelling, arrive at a similar estimate, gives confidence to assume a turnover time of branched GDGT lipids in soils of near to two decades. This is faster than the turnover time of TOC and *n*-alkanes, but equal to that of carboxylic acids in the same soils (Table 3). It also supports a recent report by Peterse et al. (2010) who investigated distributions of both core GDGT lipids and intact polar lipid-derived GDGTs in a series of soil plots of which the pH has been manipulated for the last 45 years. The distribution of the IPL derived GDGTs, assumed to be dominantly derived from living biomass, matched with the current pH of the soil and therefore a turnover time for GDGTs of less than 45 years has been assumed.

Thus, our data suggest that in biologically active materials like soils, fossil microbial lipid biomass, at least the branched GDGTs, is not accumulating; rather, it appears to be continually recycled. This seems in paradox with occurrences of soil GDGT membrane lipids in sediments as old as the early Eocene (Weijers et al., 2007a). However, these deep time occurrences of GDGTs often represent sedimentary settings, like the marine environment for example, to which soil organic matter is transported. In addition, it is not only the recalcitrance of molecules that determine their preservation, it is also, or even mainly, other factors like surface interactions with minerals (e.g. Kögel-Knabner et al., 2008), hydrophobicity (e.g. Bachmann et al., 2008) or incorporation into macromolecular matrices (de Leeuw et al., 2006; Pancost et al., 2008) that determine

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the long-term preservation of lipids. Considering the latter, it is perhaps important to our interpretation that we considered the isotopic composition solely of core GDGT lipids in the extractable (i.e. "free") lipid fraction; if there are GDGTs incorporated into macromolecular matrices, their turnover time would likely be longer. Also, intact polar GDGT lipids (IPLs), i.e. possessing a functional head group at the OH position on the glycerol backbone and precursor to the core GDGT lipids, are not considered in this study. Being the precursor molecule, their turnover time is likely to be shorter, although IPLs might also get preserved as such, either or not within the macromolecular matrix.

5 Conclusions

Compound specific $\delta^{13}C$ analysis of branched GDGT-derived alkanes shows that the carbon isotopic composition of branched GDGTs is similar to that of TOC. This suggests that the bacteria synthesising branched GDGTs, which might be looked for in the large phylum of Acidobacteria (Weijers et al., 2009), likely are heterotrophic organisms assimilating isotopically lighter and probably more labile substrates, although an autotrophic lifestyle, i.e. assimilating CO_2, could not be fully excluded. Given the dominance of branched GDGT lipids, and likely also of their source organism, over methanogen-derived GDGT lipids in the anaerobic horizons of peat bogs (Weijers et al., 2006a, 2009), it might be speculated that the branched GDGT-synthesising organisms are associated with the terminal processes in organic matter degradation, i.e. obtaining labile substrates from other microorganisms and potentially providing substrates to methanogenic Archaea. Additionally, our data suggest that branched GDGT-synthesising microorganisms in a particular sediment or soil will not cause branched GDGTs to accumulate over long periods of time; if so, it would be expected that branched GDGTs in, for example, the Rotthalmünster maize soil have a time-integrated $\delta^{13}C$ value rather than a more contemporary one. Instead, the (natural) labelling experiments studied here suggest a turnover time of ca. 17 years for branched GDGTs in arable soils in temperate climates, which fits well in the emerging view that

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most microbial biomass in soils, including membrane lipids, are relatively short lived (Amelung et al., 2008, and references therein; e.g. Rethemeyer et al., 2004; Wiesenberg et al., 2008b).

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Table 1. Soils used in this study, with concentrations of the different GDGT membrane lipids. I–IX are the branched GDGTs, X is the acyclic isoprenoid "GDGT-0" and XV is the cyclohexane-bearing isoprenoid GDGT "crenarchaeol". Roman numerals refer to Fig. 1.

	depth (cm)	TOC (%)	sediment extracted (g)	extraction method	Yield (ng/g dry weight sed.) I–IX	X	XV	Yield (µg/g TOC) I–IX	X	XV	% of total I–IX	X	XV
Forest soil													
Leigh Woods	3–12	14.9	2.2	soxhlet	2500	3	6	17	<0.1	<0.1	100	0	0
Rotthalmunster	0–7	4.0	20.1	soxhlet	420	3	0	11	0.1	<0.1	99	1	0
Grassland soil													
Rotthalmunster	0–8	2.5[a]	17.3	ultrasonic	82	4	26	3	0.2	1.0	73	4	23
Rowden I	0–10	5.7	10.2	ultrasonic	4800	410	1	84	7.2	<0.1	92	8	0
Rowden II	10–20	3.7	10.3	ultrasonic	710	28	1	19	0.8	<0.1	96	4	0
Rowden III	20–30	1.7	12.1	ultrasonic	270	5	1	16	0.3	0.1	98	2	0
Cropland soil													
Rotthalmunster wheat	0–30	1.7	25.1	soxhlet	82	8	29	5	0.5	1.7	69	7	24
Peat soil													
Stordalen mire	34–38	44.8	0.3	soxhlet	130 000	1600	<0.5	290	3.6	<0.1	99	1	0
FACE soil													
Trifolium repens ambient CO_2	0–10	3.1	47	soxhlet	120	43	56	4	1.4	1.8	55	20	26
Trifolium repens elevated CO_2	0–10	3.5	46	soxhlet	360	170	240	10	4.9	6.9	47	22	31
C_4 vegetated soil													
Blue Range	0–20	0.9	19.5	ultrasonic	38	2	1	4	0.2	0.1	93	5	2
Rotthalmunster maize	0–30	1.8	25.5	soxhlet	210	44	62	12	2.4	3.4	66	14	20

[a] data from John et al. (2005)

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Table 2. Overview of the carbon isotopic composition (with standard deviation) of TOC, plant wax-derived odd-numbered *n*-alkanes and the different alkyl moieties released from the GDGTs by ether bond cleavage. The bold letters refer to the structures of the carbon chains drawn in Fig. 1.

	δ^{13}C (‰ vs. VPDB)													
	TOC	*n*-C_{27}	*n*-C_{29}	*n*-C_{31}	*n*-C_{33}	**a**	**b**	**c**	**e**	**f**	**g**	**h**		
Forest soil														
Leigh Woods	−27.9	−33.9 (0.1)	−34.7 (0.1)	−35.6 (0.7)	−35.6 (0.1)	−28.5 (0.1)	−28.1 (0.8)	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	
Rotthalmünster	−25.7[c]	−34.0 (0.7)	−36.6 (0.4)	−36.2 (0.0)	−38.2 (0.6)	−27.5 (0.1)	−27.4 (0.5)	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	
Grassland soil														
Rotthalmünster	−28.0[c]	−36.7 (0.2)	−36.8 (0.3)	−37.4 (0.0)	−38.0 (0.4)	−29.2 (1.6)	−28.8 (1.8)	b.d.	b.d.	b.d.	b.d.	−29.3 (0.4)	−29.5 (0.7)	
Rowden I	−29.3	−37.0 (0.4)	−37.6 (1.9)	−37.0 (1.6)	−37.3 (2.1)	−30.1 (1.0)	−29.0 (0.1)	b.d.	b.d.	−33.4 (0.8)	b.d.	b.d.	b.d.	
Rowden II	−27.9	−35.0 (0.3)	−34.4 (0.4)	−34.6 (0.4)	−34.4 (0.1)	−30.5 (0.4)	−30.1 (0.7)	b.d.	b.d.	−32.0 (n.d.)	b.d.	b.d.	b.d.	
Rowden III	−27.0	b.d.	−33.3 (0.5)	−34.4 (0.4)	−34.8 (0.8)	−30.0 (0.1)	−29.7 (0.6)	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	
Cropland soil														
Rotthalmünster wheat	−26.6[c]	−36.8 (1.8)	−38.8 (0.2)	−38.1 (0.1)	−39.5 (0.1)	−26.9 (0.1)	−26.7 (1.0)	b.d.	b.d.	−29.4 (0.6)	b.d.	b.d.	−29.1 (0.6)	
Peat soil														
Stordalen mire	−23.8	−36.6 (1.7)	−33.7 (1.2)	−32.7 (0.1)	−33.5 (0.1)	−29.1 (0.0)	−28.7 (0.0)	b.d.	b.d.	−29.7 (0.2)	b.d.	b.d.	b.d.	
Bargerveen[a]	−27.5	−31.0 (0.4)	−31.0 (0.4)	−31.5 (0.4)	−31.5 (0.4)	−26/−30.5 (n.d.)	n.d.	b.d.	b.d.	−25/−29 (1.0)	−34.5/−36.5 (1.0)	b.d.	b.d.	
FACE soil														
T. repens ambient CO_2	−26.8[d]	−36.3 (0.3)	−36.5 (0.0)	−36.9 (0.3)	−36.7 (0.0)	−27.1 (0.1)	b.d.	−29.1 (0.8)	b.d.	−28.6 (0.4)	b.d.	−28.0 (1.4)	−28.8 (1.8)	
T. repens elevated CO_2 [b]	−29.8[d]	−39.0 (0.1)	−38.7 (0.4)	−38.3 (0.4)	−38.7 (0.8)	−31.3 (0.8)	b.d.	b.d.	b.d.	−29.2 (n.d.)	b.d.	b.d.	−30.4 (0.9)	
C_4 vegetated soil														
Blue Range	−17.0[e]	b.d.	b.d.	−23.9 (2.0)	−22.7 (1.8)	−18.0 (1.5)	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	
Rotthalmünster maize	−21.3[c]	−29.2 (0.2)	−31.9 (0.6)	−31.6 (0.5)	−31.8 (0.4)	−18.8 (0.8)	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	−23.0 (1.4)	

[a] all data from Pancost and Sinninghe Damsté (2003) and Pancost et al. (2003)
[b] note that the CO_2 used for the FACE experiment (during day time only) was depleted in δ^{13}C by 10‰ relative to atmospheric CO_2
[c] data from John et al. (2005)
[d] data from Wiesenberg et al. (2008b)
[e] data from Krull and Bray (2005)
b.d. = below detection limit or co-elution that prevents carbon isotope determination
n.d. = not determined

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Table 3. Turnover time estimates (in years) for TOC, long chain odd-numbered *n*-alkanes, carboxylic acids and branched GDGT-derived 13,16-dimethyloctacosanes based on (natural) labelling experiments in the Rotthalmünster maize soil and the FACE soils (structure a in Fig. 1).

	TOC	long chain *n*-alkanes	carboxylic acids	13,16-dimethyl-octacosane (a)
Rotthalmnster maize soil	40[a]	35[a]	21[a]	17[c]
FACE soil	25[b]	55[b]	20[b]	17[c]

[a] data from Wiesenberg et al. (2004)
[b] data from Wiesenberg et al. (2008b)
[c] this study

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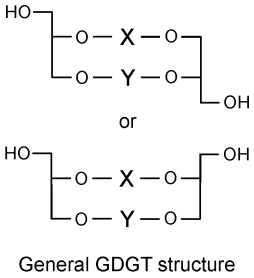

GDGT no.	GDGT m/z	X	Y	GDGT no.	GDGT m/z	X	Y
I	1022	a	a	X	1302	e	e
II	1036	a	b	XI	1300	e	f
III	1050	b	b	XII	1298	f	f
IV	1020	a	c	XIII	1296	f	g
V	1018	c	c	XIV	1294	g	g
VI	1034	b	c	XV	1292	g	h
VII	1032	c	d				
VIII	1048	b	d				
IX	1046	d	d				

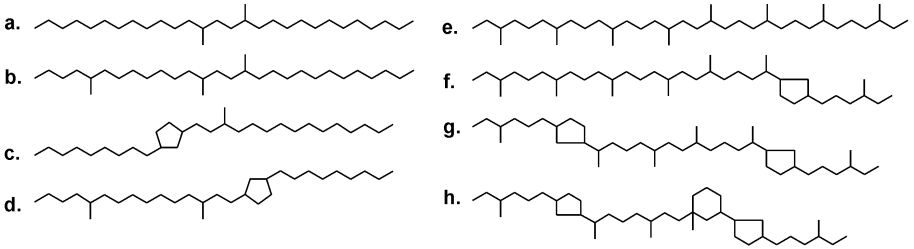

Fig. 1. Structures of the branched glycerol dialkyl glycerol tetraether (GDGT) membrane lipids (**I**–**IX**) and the isoprenoid GDGTs (**X**–**XV**). GDGT **X** is also referred to as GDGT-0 and GDGT **XV** is known as crenarchaeol. The alkyl moieties (**a**–**h**) are located at positions X and Y in the general GDGT structure (these positions are interchangeable) and are released upon chemical cleavage of the ether bond. GDGT structures can also occur as a stereoisomer of the general structure, i.e. parallel and anti-parallel configuration of the alcohol groups.

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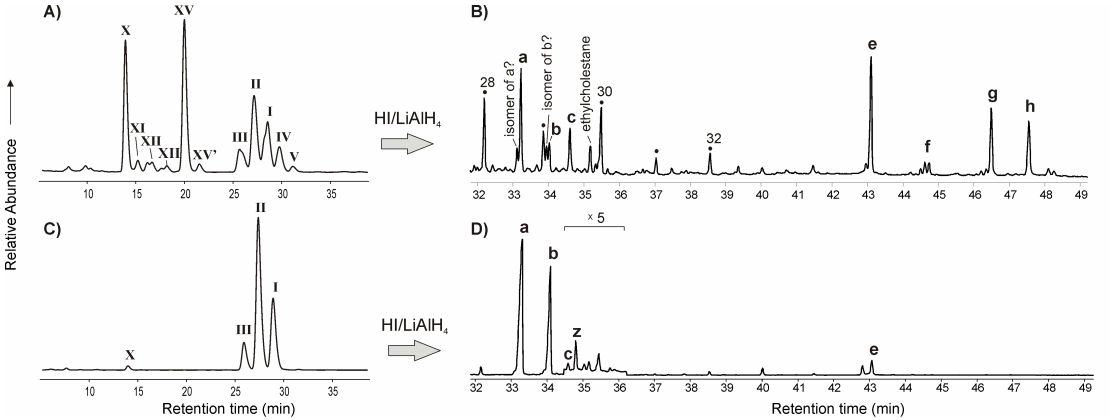

Fig. 2. Partial LC and GC chromatograms of the FACE soil under ambient CO_2 conditions (**A** and **B**, respectively) and of the Stordalen mire peat (**C** and **D**, respectively). The polar fraction of the total extracts, containing the GDGT compounds (roman numerals in (A) and (C), numbers refer to Fig. 1), has been enriched in GDGTs by means of preparative HPLC. Subsequently, the alkyl moieties have been released by cleaving the ether bonds with an $HI/LiAlH_4$ treatment. The GC chromatograms shown in (B) and (D) represent the hydrocarbon fraction obtained from the respective samples after this treatment. Letters a–h refer to the structures in Fig. 1. Bullets represent *n*-alkane series with corresponding chain length that most likely derive from dehydration of higher plant derived *n*-alkanols and subsequent reduction of the double bond by $LiAlH_4$. The peaks eluting just before peak b and c have the exact same mass spectrum and are therefore suggested to be stereoisomers of b and c, respectively. The peak indicated wit z in panel (D) is tentatively identified as 5,13,16,24-tetramethyloctacosane, see Fig. 3. Note that the vertical scale in panel (D) is magnified 5 times between 34.5 and 36.5 min.

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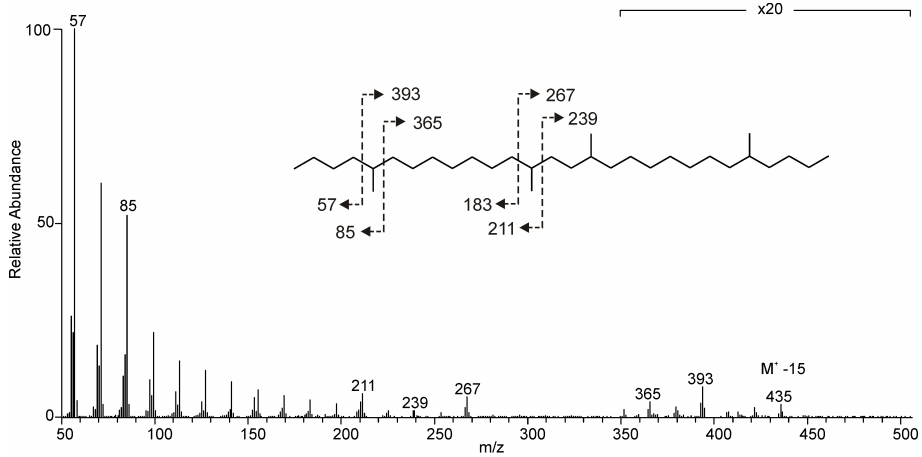

Fig. 3. Mass spectrum (electron spray ionisation) of compound "z" from Stordalen mire (Fig. 2d) with the structure tentatively identified as 5,13,16,24-tetramethyloctacosane. The mass spectrum has been corrected for background. Note that the vertical scale is magnified by a factor 20 from *m/z* 350 onward.

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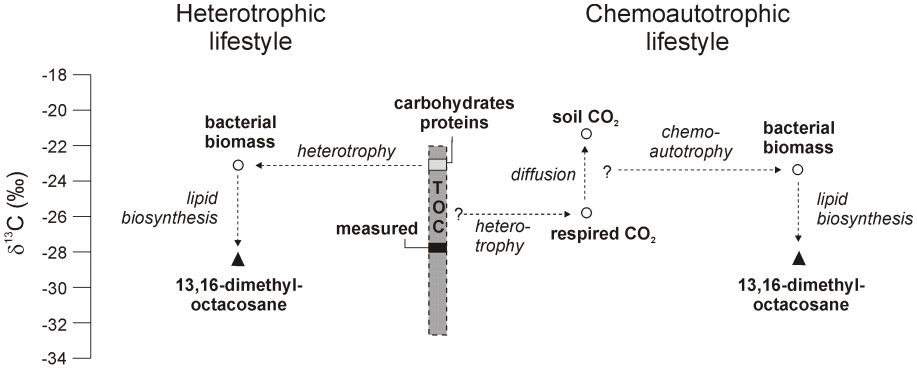

Fig. 4. Schematic representation of the potential heterotrophic metabolism (left hand side) and chemoautotrophic metabolism (right hand side) of the branched GDGT-synthesising bacteria. Filled symbols represent averages of measured values (for the C_3 top-soils), open symbols represent estimated values based on fractionation factors from the literature.

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