

Bacterial Tetraether Lipids in Lacustrine Environments

Implications for their use as Paleoclimate Proxies

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Summary

Anthropogenic global warming poses a long-term threat to human society. The reliable prediction of future climate evolution, however, not only builds upon our understanding of present-day processes, but also requires profound knowledge of Earth system responses on timescales exceeding the range of instrumental observations. Current numerical simulations steadily grow in complexity, increasing the need for high-quality paleoclimate data as a means of model evaluation, especially from the spatially heterogeneous terrestrial realm. One promising approach for the reconstruction of past continental temperatures is based on organic-geochemical analyses of bacterial tetraether lipids (i.e., branched GDGTs), which are prevalent in the environment, and show systematic compositional changes with ambient temperature. Several studies established quantitative relationships between atmospheric T and brGDGT distribution in modern lake sediments, yet, application of these transfer functions to different limnological settings does not always yield robust paleotemperature estimates. Attempts for further improvement of the brGDGT proxy are currently hindered by the fact that the vast majority of brGDGT-producing microbes are still unidentified, and the ecophysiological mechanisms behind the apparent T response are unknown. In order to shed light on the ecology and lifestyle of these microbes in lacustrine systems, we investigated a variety of recent lake sediments from the Swiss and Italian Alps, as well as suspended- and sinking biomass from the permanently stratified north basin of Lake Lugano (Southern Switzerland), by a combination of stable isotope-, geochemical-, and molecular biological methods.

Almost 50 % of the investigated lake deposits contained an as yet unknown brGDGT isomer that was not detectable in soils collected from the catchments, which provided unprecedented molecular evidence for brGDGT biosynthesis within lacustrine systems. This compound was also abundantly present in hypoxic and anoxic deep water of Lake Lugano. Strikingly, however, it was completely absent from the overlying oxic waters, implying an exclusive origin from microbes adapted to low dissolved oxygen concentrations. In contrast, concentration profiles and stable carbon isotope data show that another structural brGDGT isomer is dominantly synthesized by bacteria inhabiting oxygenated waters, attesting to a vertical differentiation of the brGDGT-producing microbial community. In order to constrain the identity of the source organisms, we investigated prokaryotic diversity by sequencing of ribosomal DNA, with special emphasis on members of the phylum *Acidobacteria* — the only group known thus far to produce brGDGTs. Indeed, members of different acidobacteria subdivisions showed a depth differentiation that is reminiscent of the concentration gradients found for individual brGDGT lipids, with a number of uncultured representatives exclusively occurring in the anoxic water column.

In Lake Lugano, we further studied the vertical transport of brGDGTs by deploying three sediment traps, (i) at the base of the thermocline, (ii) at the redox transition zone, and (iii) 90 m within the anoxic water body. The brGDGT content of settling organic particles increased successively from the upper towards the lower trap, attesting to pronounced hypolimnetic in-situ production under both, oxic and hypoxic/anoxic conditions. Subsequent laboratory experiments showed quick uptake of isotopically labeled organic C into brGDGTs from surface water, and a ~100-fold abundance increase within six weeks, demonstrating a high potential for aerobic brGDGT production by heterotrophic bacteria. Surprisingly, however, a corresponding anoxic incubation with biomass from the bottom waters of Lake Lugano did not result in measurable label uptake, suggesting that anaerobic brGDGT production may be limited to the redox transition zone (RTZ) where alternative electron acceptors such as nitrate are still present.

Our work in Lake Lugano also revealed a steep decline in the natural ^{13}C content of brGDGTs ($\delta^{13}\text{C}_{\text{brGDGT}}$) at the RTZ, most likely related to the cycling of ^{13}C -depleted organic compounds derived from the oxidation of methane. This is consistent with the fact that in the majority of the investigated lakes, brGDGTs were substantially depleted in ^{13}C (up to 10 ‰) compared to the bulk of sedimentary organic C ($\delta^{13}\text{C}_{\text{TOC}}$). Indeed, we found significantly lower $\delta^{13}\text{C}_{\text{brGDGT}}$ values in meso-eutrophic lakes that exhibit bottom water anoxia, than in oligotrophic and fully-oxygenated settings, which is best explained by the co-occurrence of methane-oxidizing- and brGDGT-producing bacteria in the vicinity of the RTZ.

The 36 lakes investigated in this study span an altitudinal air temperature difference of ~10 °C. However, the correlation between mean annual air temperature (MAT) at the lake sites and commonly applied brGDGT proxy indices in the surface sediments was poor ($R^2=0.1$), contradicting previously reported data from lakes of other regions. In light of the important role of deep water-derived brGDGTs in lake sediments we show here, we reason that the brGDGT–MAT relationship in our data set is likely compromised by the variable contribution of brGDGTs from terrestrial, shallow-, and deep water pools, each of which carry distinct temperature imprints.

Specialized lipid extraction techniques are required for the recovery of GDGTs from living biomass. We tested three commonly used protocols on suspended particulate matter from Lake Lugano, and found substantial extraction biases affecting the intact polar lipid (IPL) fraction of brGDGTs. Up to 90 % of the IPL pool were not accessible by means of normal ultrasonic solvent extraction, nor were they recovered with the widely applied, phosphate-buffered ‘modified Bligh-Dyer’ protocol. Near complete IPL recovery was, however, only achieved after substitution of the P-buffer with a solution of 5% trichloroacetic acid. Our results therefore suggest that previous studies may have substantially underestimated the ‘living fraction’ of brGDGTs and its contribution to the total lipid pool.

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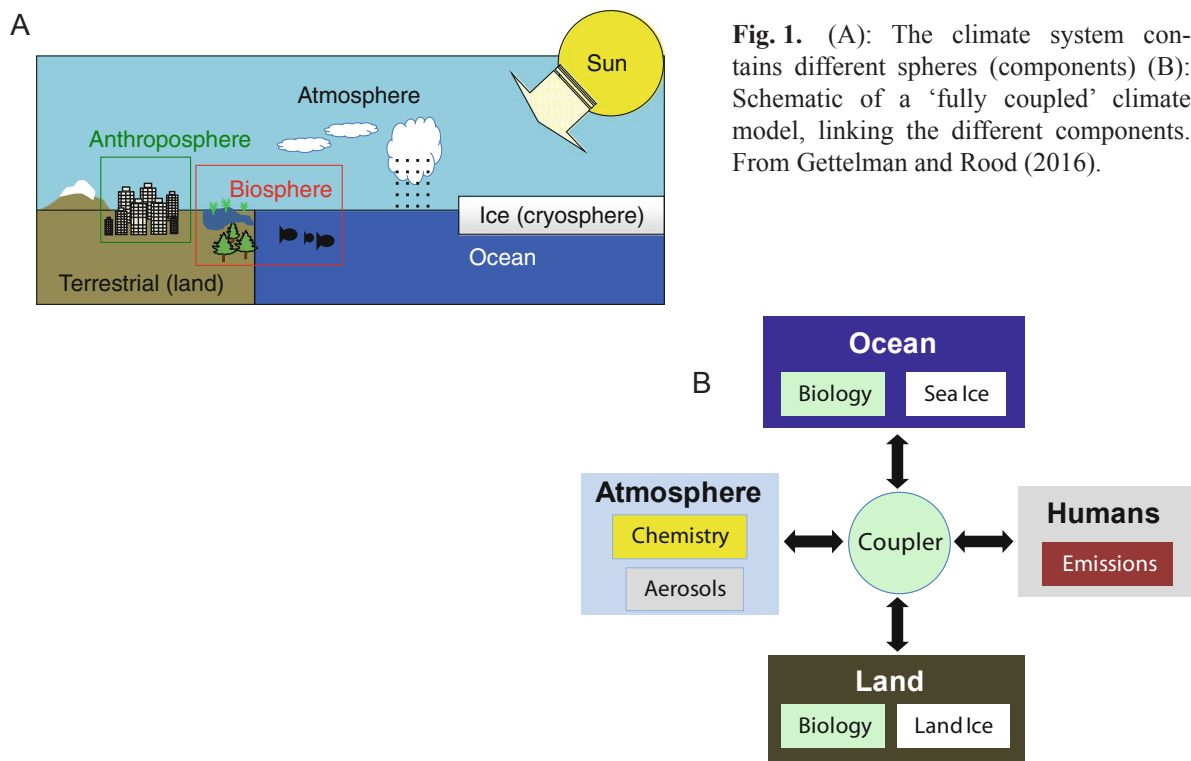
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Chapter 1

1.1. Rationale

Why paleoclimate research?

Global mean air T has increased by ~ 1 °C since 1900 (Hartmann et al., 2013) and caused observable physical changes on Earth's surface, including the retreat of continental ice sheets and mountain glaciers (Zemp et al., 2006; Mougintot et al., 2015), sea level rise (Holgate and Woodworth, 2004; Church and White, 2006), decline of arctic sea ice (Parkinson and Comiso, 2013; Parkinson and DiGirolamo, 2016), thawing of permafrost soils (Hinzman et al., 2005; Eriksson et al., 2006), and altitudinal shifts of vegetation zones (Kelly and Goulden, 2008). The effects of these changes on human settlements and infrastructure are already visible (Field et al., 2014), and the expected increase of climatic extreme events such as droughts and tropical cyclones (AghaKouchak et al., 2013; Walsh et al., 2016) pose societal challenges for many generations to come. While a causal link between anthropogenic green house gas emissions and modern global warming is considered a scientific fact, there is still great uncertainty in projecting the future evolution of climate change (Stocker et al., 2007; Flato et al., 2013). Despite the recent advances made in numerical modeling (Gettelman and Rood, 2016), a considerable amount of prediction error stems from the intrinsic difficulty to account for processes and feedbacks that act across different components of the climate system (Flato et al., 2013) (Fig. 1). As a means of model evaluation, therefore, records of past climate variations that reach back beyond instrumental records provide independent control on model accuracy and



-resolution, assuming that our concepts of present-day processes are similarly applicable throughout Earth's history (Hargreaves and Annan, 2009; Harrison et al., 2014).

Particularly, the adequate numerical representation of the terrestrial system component still poses challenges to climate modelers, owing to the steep gradients in topography, relative humidity, and land cover that can cause highly differential fluxes of heat-, water-, and carbon on small spatial scales (Gettelman and Rood, 2016). At the same time, in large parts of the landlocked world, the reliable prediction of future *continental* climate change is essential for policy makers to implement appropriate measures against climate-related threats. Because the complexity, as well as the temporal and spatial resolution of these models steadily increases (e.g. Gobiet et al., 2014; Williams et al., 2015), so does the need for high-resolution paleoclimate records from terrestrial environments for their evaluation. In contrast to the oceans, however, most of the land surface is exposed to erosion, and thus preservation of paleoclimatic information is restricted to specific settings. Although, a variety of terrestrial deposits are commonly used to assess past environmental change, including speleothems (McDermott, 2004), peat (Zheng et al., 2015; Inglis et al., 2017), and paleosol successions (Peterse et al., 2011; Zech et al., 2012; Schreuder et al., 2016), lake sediments play an exceptional role as continental climate archives (e.g. Swain, 1978; O'Sullivan, 1983; Kiage and Liu, 2006; Castañeda and Schouten, 2011; Berke et al., 2012; Sinninghe Damsté et al., 2012; Morrissey et al., 2017; Randlett et al., 2017; Thienemann et al., 2017). Firstly, the preservation of organic remains from plants and aquatic organisms is often good, as the absence of oxygen in many cases hinders bioturbation by burrowing organisms and chemical oxidation of the sediments. Moreover, the water shed from which allochthonous materials such as mineral- and plant detritus are washed into the lake, is mostly well-constrained, allowing for a high degree of geographic specificity. Finally, lakes cover ~2 % of the Earth's continents (Messenger et al., 2016) and are, with the exception of extremely dry regions, widely distributed over the globe (Fig. 2). Lacustrine paleoclimate records are therefore of great value, especially in evaluation of regional climate models that implement much higher spatial resolutions than the global ones (e.g. Polanski et al., 2014; Peyron et al., 2016), and will continue to be an integral component of future climate research.

Lake sediments as paleoclimate archives

Paleoclimatic information is extracted from sedimentary archives via measurements of proxy indicators (proxies), which are thereafter translated to quantitative or qualitative environmental variables through analogy with modern processes and observations. For instance, (i) bulk sediment parameters such as C/N ratios, total organic carbon- (TOC) and biogenic silica content (Colman et al., 1995) indicate changes in organic matters sources and primary productivity, (ii) clay mineral composition can be used to infer the intensity of chemical weathering and soil-forming processes within the water shed (Fagel and Boës, 2008), and (iii) assemblages of pollen give insight into the diversity of seed-producing plants (spermatophytes) from the lake's catchment (Kiage and Liu, 2006). Also a

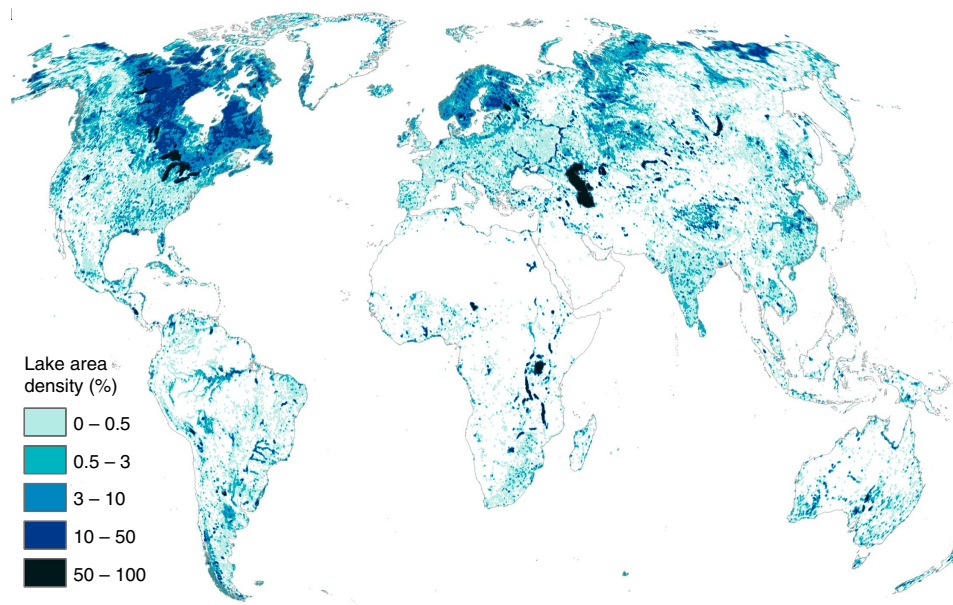


Fig. 2. Lake area density (limnicity) calculated as percent area covered by lakes within a 25 km radius. From Messenger et al. (2016).

number of aquatic organisms including unicellular algae (diatoms, chrysophytes), small crustaceans (cladocerans) and other zooplankton (e.g. testate amoebae) are routinely investigated to track changes in hydrochemical characteristics of the water column over time (Lotter et al., 1998; Buczkó et al., 2009). Such qualitative measures of past ecosystems and climate regimes are valuable, yet, they do not provide concrete atmospheric temperature estimates that are needed for paleoclimate–model comparisons. Various studies have therefore investigated quantitative relationships between the diversity of lacustrine aquatic biota and mean air temperatures along climatic gradients (e.g. Lotter et al., 1998). To date, however, only two groups of micro-fossils, namely the siliceous resting stages of chrysophytes (golden algae) (Kamenik and Schmidt, 2005), and larval head capsules of chironomids (non-biting midges) (Walker et al., 1991; Luoto and Ojala, 2016) show systematic responses to cold season- and summer air T, respectively, which are likely caused by differential T optima of the individual species. Particularly, midge assemblages have yielded meaningful paleoclimatic information throughout the Holocene of N-America (Levesque et al., 1997) and Europe (Brooks and Langdon, 2014), and inferred temperatures seem to agree with both, instrumental T records (Luoto and Ojala, 2016) and paleoclimate models (Heiri et al., 2014). There are, however, several drawbacks to these micropaleontological methods, that is (i) time-intensive visual classification and counting that is susceptible to inter-observer biases, (ii) the need for extensive modern calibration data sets that capture the whole diversity within a given study area, often requiring distinction of >100 species (Brooks and Birks, 2001; de Jong et al., 2013), (iii) the influence of variables other than T on species distributions (e.g., pH, lake depth, alkalinity; Brooks and Birks, 2001; Hernández-Almeida et al., 2014), which constrains the selection of appropriate study sites.

The use of molecular T proxies such as microbial membrane lipids has the potential to circumvent some of these issues. Ideally, the variations in lipid composition are solely controlled by ambient T through physiological adaptations on the cellular level (i.e., membrane lipid homeostasis; Sinensky, 1974; Zhang and Rock, 2008), and thus reconstructed T should be largely independent from other environmental variables. Further, given that the lipids derive from single species, transfer functions can be derived from studies of pure cultures under controlled laboratory conditions, providing a more robust basis for the interpretation of proxy signals than empirical environmental calibrations. Finally, both sample preparation and analysis are less laborious and probably more reproducible, as automated analytical chemical methods based on chromatography and mass spectrometry are employed, allowing for higher sampling resolution and sample throughput. However, while lipid-based T proxies are well-established in marine settings (e.g. long chain alkenones; Brassell et al., 1986) their potential in lacustrine systems is just being explored. This work focuses on a quite recently discovered class of tetraether lipids known as ‘branched GDGTs’ that occur ubiquitously in all terrestrial and freshwater environments, and thus bear great promise as a universal continental paleo-T proxy.

1.2. Introduction

Prokaryotic cell envelopes

The cell envelope of prokaryotes (bacteria and archaea) are complex and multi-layered structures of up to ~100 nm thickness, consisting of (i) the plasma membrane at the interior that defines the physiological boundary of unicellular organisms, and (ii) the cell wall to the exterior that provides structural support and protection from the environment (Silhavy et al., 2010). The composition of the cell envelope is heterogeneous among bacteria and archaea and includes polysaccharides (peptidoglycan or pseudomurein), lipopolysaccharides (LPS), teichoic acids, and different types of proteins (i.e., surface proteins [S-layer], proteinaceous sheaths, lipoproteins) (Silhavy et al., 2010; Albers and Meyer, 2011). The organization of bacterial cell walls follows either of two basic architectures, which can be determined by the Gram staining test (Gram and Friedlaender, 1884). Gram-negative bacteria possess a much thinner peptidoglycan layer than the gram-positive ones, but are instead surrounded by a second asymmetric membrane consisting of phospholipids on the interior, and LPS on the exterior (Fig. 3A). Moreover, certain bacteria cover the cell wall with S-layer proteins or a polysaccharide capsule, both of which likely serve as additional protective barriers (Kasper, 1986; Sara and Sleytr, 2000). Archaeal cell walls, in contrast, often comprise a simple S-layer that is directly anchored to the plasma membrane, and polysaccharides are generally less common (Albers and Meyer, 2011). Nevertheless, various more complex arrangements of protein- and polysaccharide layers have also been reported from the archaeal domain (Fig. 3B).

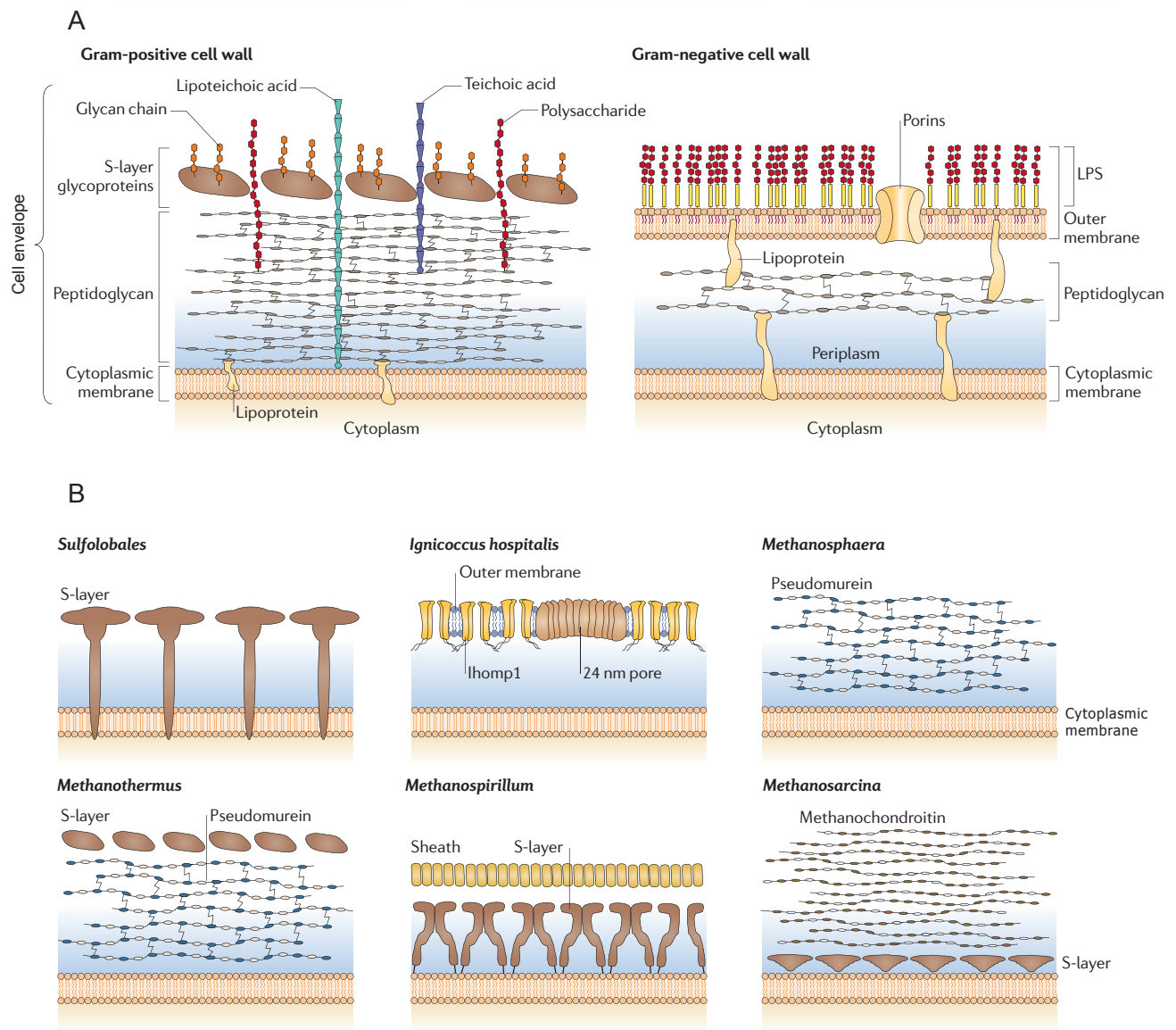


Fig. 3. (A): The cell envelope of gram-positive and gram-negative bacteria (A), and different types of cell wall architectures in archaea (B). Methanochondroitin is also a polysaccharide. From Albers and Meyer, (2011).

Glycerol-based membrane lipids

The cytoplasmic membranes of all eukaryotic cells and most bacteria are lipid bilayers, dominantly composed of glycerol-esters in which hydrophobic fatty acid chains are ester-linked to a hydrophilic glycerol backbone (Fig. 4A) (Kates, 1964). Different to the ester-bound lipids, some organisms and tissues synthesize lipids in which the glycerol- and alkyl moieties are linked via chemically more inert ether bonds (Fig. 4B). These can occur as mono-, di-, and tetraethers, the latter of which form mono-layered instead of bi-layered membranes (Fig. 4B), and are referred to as glycerol-dialkyl-glycerol-tetraethers (GDGTs). Different types of ether bond-containing (membrane) lipids are found throughout all domains of life (Pearson, 2014) (i.e., *Archaea*, *Bacteria*, and *Eukaryotes*; Woese et

al., 1990), yet, they are most common and abundant within the *Archaea* (De Rosa and Gambacorta, 1988). The glycerol- and the alkyl moieties together form the GDGTs' 'core lipids' (CLs), which are persistent in the environment and are preserved in sediments over geological timescales (Kuypers et al., 2001; Lengger et al., 2013). In living cells, however, the majority of lipids additionally possess polar 'head groups' that are attached to the glycerol moieties via ester- (phospholipids) or glycosidic bonds (glycolipids), which are then referred to as intact polar lipids (IPLs) (Fig. 5).

Although the plasma membranes of all microorganisms are dominantly composed of glycerol-based lipids, there are diagnostic differences in the lipid structures. Most importantly, the stereo configuration of the glycerol moiety in archaeal lipids is opposite of that in bacteria and eukaryotes (Kates, 1977). This difference arises from distinct stereo-specific enzymes that provide the precursor of the glycerol backbone. In case of the archaea, this is *sn*-glycerol-1-phosphate (G1P) that is synthesized from dihydroxyacetone-phosphate via G1P-dehydrogenase, finally resulting in *sn*-2,3-glycerol lipids (Nishihara and Koga, 1995) (*sn*: stereospecific numbering; Cevc et al., 1993) (Fig. 4B). In bacteria and eukaryotes, however, the glycerol backbone is built from *sn*-glycerol-3-phosphate (G3P) (Kito

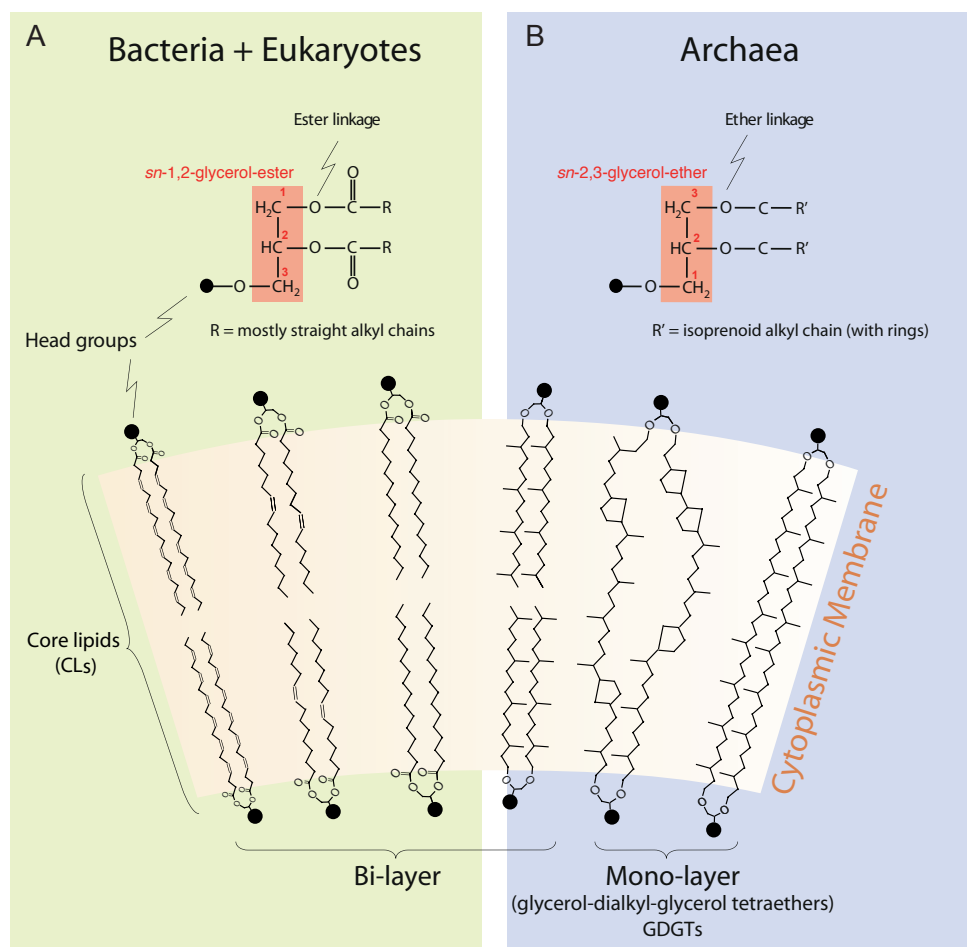


Fig. 4. The 'lipid divide'. Note that the alkyl chains (i.e., fatty acids) in bacteria are much more diverse than shown, and comprise for instance methyl branches and cyclopropane moieties. Modified from Valentine (2007).

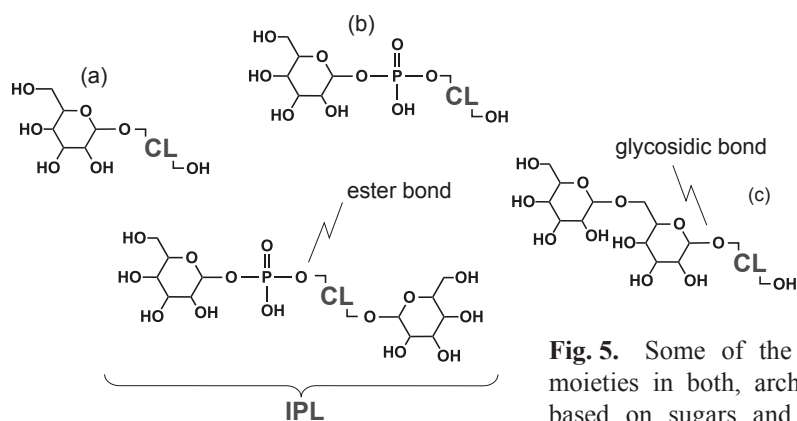


Fig. 5. Some of the most common head group moieties in both, archaeal- and bacterial GDGTs, based on sugars and phosphate. (a) hexose, (b) phospho-hexose, and (c) di-hexose. Together with the core lipid (CL), they form the intact polar lipid (IPL).

and Pizer, 1969), leading to a *sn*-1,2-glycerol stereo-configuration (Weijers et al., 2006a; Pearson, 2014) (Fig. 4A).

Another important structural characteristic of membrane lipids are the hydrophobic moieties. Alkyl chains of archaea have an isoprenoid branching pattern (Fig. 4B; De Rosa and Gambacorta, 1988) because they are formed by polymerization of C₅ monomers (isopentyl diphosphate and dimethylalyl diphosphate), which in turn are synthesized from acetyl-CoA through the mevalonic acid (MVA) pathway (Koga and Morii, 2007). In contrast, the fatty acid tails of eukaryotic and bacterial lipids are formed by step-wise addition of C₂ units (malonyl-CoA) to an acetate ‘primer’, resulting in linear hydrocarbon chains (acetogenic lipids) (Cohen, 2014). Methyl-branched fatty acids are produced when the primer molecule is derived from a branched amino acid (Valine, Leucine, or Isoleucine) instead of acetate (Kaneda, 1977).

These fundamental differences in membrane lipid structures, particularly the unique glycerol stereochemistry of archaea to which no exception is known thus far from microbial cultures, have been termed the ‘lipid divide’, and gave rise to the hypothesis that phospholipid membranes evolved independently in bacteria and archaea (Koga et al., 1998). However, the demarcation between bacterial and archaeal lipids is not as clear as often stated. Straight-chain alkyl moieties have been detected in a number of archaeal species (Kates et al., 1968; Nishihara et al., 2000; Gattinger et al., 2002), and homologue genes encoding fatty acid synthesis and -oxidation are found in many archaeal genomes (Peretó et al., 2004; Dibrova et al., 2014; Villanueva et al., 2017). Vice versa, certain bacteria are known to produce ether-linked- and membrane-spanning lipids (Langworthy et al., 1983; Sinnighe Damsté et al., 2007; see next section). Also the seemingly irrefutable opposition of the glycerol stereoisomers has been put into perspective, as metagenomic studies revealed the presence of G1P- and G3P dehydrogenase-coding genes in members from the respectively opposite clade (Peretó et al., 2004; Villanueva et al., 2017), pointing towards widespread inter-domain gene transfers between prokaryotes (Frigaard et al., 2006; Rinke et al., 2013; Youssef et al., 2014). Moreover, despite the contrasting structural features of core lipids, the most common types of polar head groups are

shared between bacteria and archaea (Kates, 1964, 1977). As also the enzymes catalyzing polar head linkage are homologous (Daiyasu et al., 2005), this implies that the last common prokaryotic ancestor possessed a phospholipid membrane (Koga and Morii, 2007; Lombard et al., 2012). In summary, most recent phylogenomic analyses of lipid-biosynthetic genes favor an ancestral origin of the glycerol-based membrane architecture (Lombard and Moreira, 2011; Lombard et al., 2012). However, it still remains a matter of controversy through which evolutionary force the ‘lipid divide’ occurred, ultimately leading to the present-day segregation of the two glycerol enantiomers between bacteria and archaea (e.g. Wächtershäuser, 2003; Koga, 2014).

Structural diversity of glycerol-dialkyl-glycerol-tetraethers (GDGTs)

GDGTs are subdivided into two major groups according to the type of their hydrophobic moieties, which comprise either (i) branched alkanes (branched GDGTs; Fig. 6A), or (ii) isoprenoid hydrocarbons (isoprenoid GDGTs; Fig. 6B) (Schouten et al., 2013). The core lipid structures of the environmentally most abundant GDGTs contain alkyl chains that comprise homologue series with differing numbers of cyclic moieties (i.e., cyclopentyl- or cyclohexyl rings; De Rosa and Gambacorta, 1988; Sinninghe Damsté, 2002; Weijers et al., 2006a). The isoprenoid (iso-) GDGTs can contain up to two OH groups (Liu et al., 2012a), and the branched (br-) GDGTs up to four additional methyl side chains (Sinninghe Damsté et al., 2000; De Jonge et al., 2013). The enzymes involved in the formation of the cyclic moieties are unknown at present, and it has been proposed that cyclization takes place either after (Weijers et al., 2006a) or concomitantly with alkyl chain polymerization (Villanueva et al., 2014). Moreover, the peripheral methyl branches within the brGDGTs’ alkyl moieties can be located either at the C5/C5’- (Sinninghe Damsté et al., 2000) or C6/C6’ position (De Jonge et al., 2013), entailing two separate isomer series of ‘C5-methylated’ and ‘C6-methylated’ brGDGTs, which can only be analytically distinguished by high-resolution liquid chromatography (De Jonge et al., 2014a; Hopmans et al., 2016; see section 1.4).

In addition, recent analyses of aquatic sediments by tandem mass spectrometry revealed complementary suites of tetraethers, including isoGDGTs with up to three additional methyl branches (Knappy et al., 2015), brGDGTs with a total of seven to twelve methyl branches (overly branched GDGTs), brGDGTs with zero to three methyl branches (sparsely branched GDGTs), and ‘hybrid’ GDGTs with alkyl chains that only partly show an isoprenoid methylation pattern (isoprenoid-branched [IB]-GDGTs; Liu et al., 2012b; Fig. 7). Their potential as biomarkers and/or environmental proxy indicators, however, have not yet been explored.

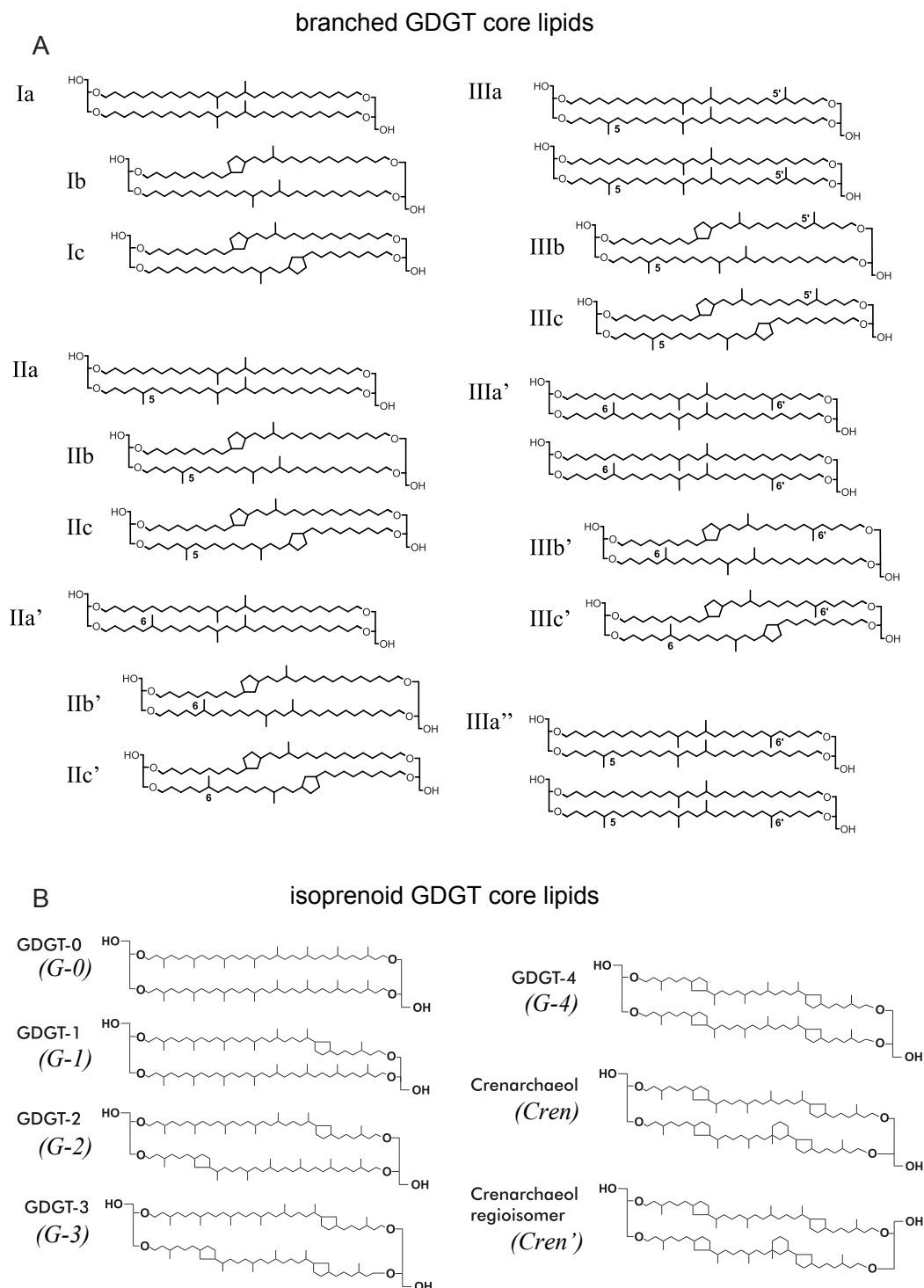


Fig. 6. Structures of the most important branched- (A) and isoprenoid GDGTs (B) in mesophilic environments. Arabic numerals (1–3) and letters (a–c) indicate the numbers of cyclopentyl rings. C6-methylated isomers are indicated with a prime and positions of the peripheral methyl branches are given. Branched GDGTs I_[i] are referred to as ‘tetramethylated’, II_[i] as ‘pentamethylated’, and III_[i] ‘hexamethylated’.

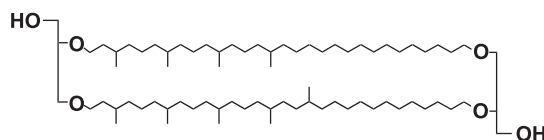


Fig. 7. One of the recently discovered hybrid ‘isoprenoid-branched’ GDGTs (Liu et al, 2012b). Note that the chain length is 30 C atoms, identical with branched GDGTs and shorter than for isoprenoid GDGTs (32 C atoms).

Biological sources of GDGTs

Both ether-linked lipids and mono-layered membrane architectures were for a long time assumed to be restricted to extremophilic microbes, because these traits were first reported from halophilic archaea (i.e., *Euryarchaeota*; Kates, 1964) and thermophilic bacteria (Langworthy et al., 1983; Jeanthon et al., 1995). Moreover, the chemical and thermal stability of ether-bonds (Koga, 2012) and the lower H⁺-permeability of mono-layered membranes (van de Vossenberg et al., 1998; Gabriel and Lee Gau Chong, 2000) were interpreted as exceptional adaptations to hot or chemically harsh environments. This simplified view on GDGT-producing microbes has changed dramatically.

isoprenoid GDGTs — Although isoGDGTs have been reported previously from ‘normal’ marine sediments (Chappe et al., 1982; DeLong et al., 1998), it was not until the development of sensitive analytical methods (Hopmans et al., 2000) that the environmental distribution of tetraethers lipids could be fully explored. Subsequent analyses revealed the ubiquitous occurrence of archaeal isoGDGTs in marine and lacustrine sediments (Schouten et al., 2000), and high-throughput nucleic acid sequencing (DNA/RNA) showed that mesophilic and psychrophilic archaeal lineages (i.e., growth optima ~10–40 °C) inhabit virtually all surface environments on Earth, including oceans, lakes, rivers, and soils (Fuhrman et al., 1992; Bintrim et al., 1997; Schleper et al., 1997; Galand et al., 2006). One of the most abundant and wide-spread group of archaea are the *Thaumarchaeota* (formerly marine Group I *Crenarchaeota*; Brochier-Armanet et al., 2008), which play an important role in the aerobic oxidation of ammonia (Könneke et al., 2005). *Thaumarchaeota* alone are estimated to represent >20 % of all microbial cells present in the Earth’s oceans (Karner et al., 2001; Church et al., 2003), and a growing amount of data suggest similar ecological importance in lacustrine water columns (Pouliot et al., 2009; Lliros et al., 2010; Buckles et al., 2013; Callieri et al., 2016). Other important sources of isoGDGTs are methanogenic *Euryarchaeota* (Koga et al., 1993), and uncultured heterotrophic archaea of the ‘Miscellaneous Crenarchaeotic Group’ (MCG) (Takai et al., 2001; Biddle et al., 2006; Seyler et al., 2014), both of which are widespread in anoxic aquatic environments and probably contribute substantially to sedimentary GDGTs in lakes (e.g. Jiang et al., 2008; Blaga et al., 2009; Borrel et al., 2012). The lipid composition of *Thaumarchaeota* is, however, unique within the archaeal domain, as they are the only group known to produce crenarchaeol (Cren) and its regio-isomer (Cren’), which are characterized by the presence of a cyclohexyl ring (Sinninghe

Damsté, 2002) (Fig. 6B). The remainder of isoGDGT CL types are not specific to any archaeal clade (e.g., Koga and Nakano, 2008).

branched GDGTs — The branched GDGTs were first identified in the Bargerveen peat (Netherlands; Sinninghe Damsté et al., 2000) and subsequently detected in soils (Hopmans et al., 2004; Weijers et al., 2006b), hot springs (Schouten et al., 2007b), coastal marine sediments (Weijers et al., 2007a), as well as in deposits of freshwater lakes (Blaga et al., 2009), indicating that they derive from a microbial group that is prevalent in the environment. In contrast to the isoGDGTs, however, the biological sources of brGDGTs are much less understood, although nuclear magnetic resonance (NMR) spectroscopy of purified brGDGT IIa from the Bargerveen peat unambiguously showed a bacterial *sn*-1,2 glycerol stereochemistry (Weijers et al., 2006a). Nonetheless, reports of brGDGTs in microbial isolates are limited to two species from the phylum *Acidobacteria* that contained relatively minor amounts of brGDGT Ia (compound 1 in Fig. 8) (Sinninghe Damsté et al., 2011). However, more common within the *Acidobacteria* are derivatives of 13,16-dimethyl octacosanedioic acid (iso-Diabolic acid [IDA]) (compounds 2–4 in Fig. 8), which possess an identical alkyl chain as the major, non-cyclic brGDGTs Ia, IIa, and IIIa. Briefly, di-carboxylic IDA (compound 2 in Fig. 8) comprised 20–43 % of membrane lipids in all of the 17 investigated strains from subdivision (SD) 1 and 3 of the *Acidobacteria* (Sinninghe Damsté et al., 2011), and IDA-mono glycerol ethers (MGEs) (compounds 3 and 4 in Fig. 8) were abundant components (10–30 %) in six isolates of SD4 (Sinninghe Damsté et al., 2014). It is important to note that IDA-containing lipids could not be extracted from the cultures by means of the commonly applied ‘modified Bligh-Dyer protocol’ (Bligh and Dyer, 1959), and acid hydrolysis of the cells was required to recover IDA and IDA-derivatives from *Acidobacteria*. Because, glycosidic bonds and ester linkages were cleaved during hydrolysis, information on the functional form IDA-based IPLs were lost. Nonetheless, the non-IDA-containing IPLs that were successfully extracted prior to hydrolysis, dominantly comprised phosphatidyl-ethanolamine- (SD1, SD2; Sinninghe Damsté et al., 2011) and phospho-choline head groups (SD4; Sinninghe Damsté et al., 2014). Additional support for an acidobacterial origin of brGDGTs comes from environmental observations. In peat, for instance, where brGDGTs are found to be exceptionally abundant, acidobacterial gene sequences affiliated with SDs 1,3, and 4 represented ~90 % of the bacterial community (Weijers et al., 2009). Furthermore, absolute brGDGT concentrations were found to correlate inversely with pH in soils (Peterse et al., 2010), similar to the relative abundance of *Acidobacteria*, which are an important component of soil microbial assemblages (Jones et al., 2009). However, comparably little is known about *Acidobacteria* in lacustrine environments. Although a few studies reported their presence in lake sediments (3–21 % of the community; Kalyuzhnaya et al., 2008; Shao et al., 2013; Sheng et al., 2016; Wang et al., 2016), only one investigated acidobacterial diversity in detail, showing the dominance of either SD1 or SD6 in three freshwater ponds from Doñana National Park (Spain) (Zimmermann et al., 2012). Despite the great diversity of acidobacterial 16S-rDNA sequences found in the environment (26 SDs; Ludwig et al., 1997; Hugenholtz et al., 1998; Zimmermann et al., 2005; Barns et al., 2007), only 37

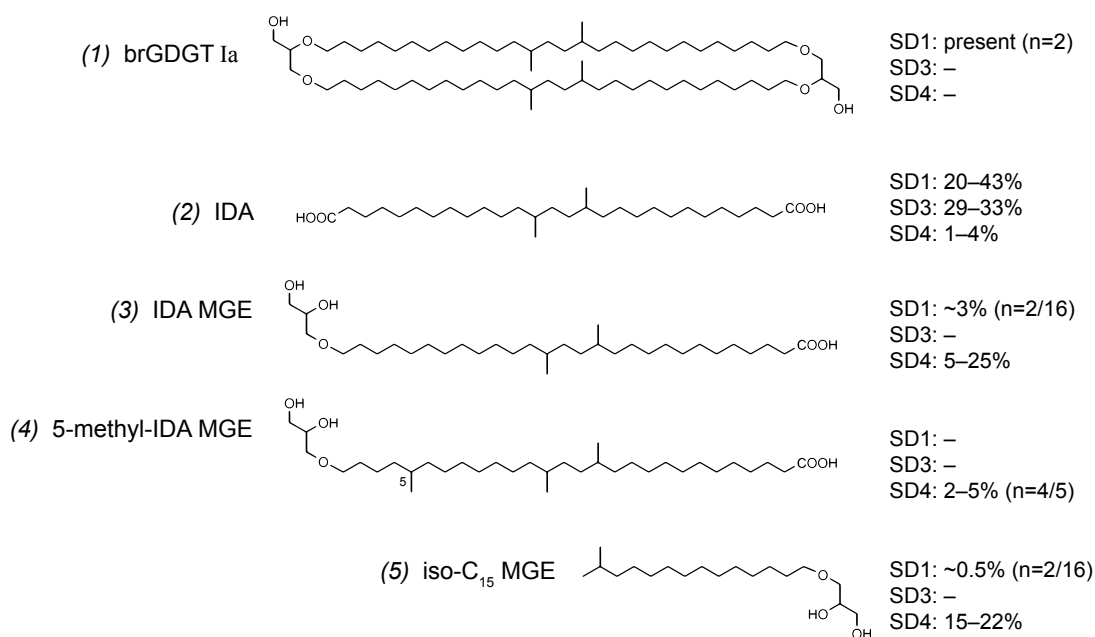


Fig. 8. Reported occurrences of brGDGT Ia, iso-Diabolic-acids (IDAs), and mono-glycerol-esters (MGE) in mesophilic isolates from the *Acidobacteria* subdivisions (SD) 1 (n=16), 3 (n=2), and 4 (n=5) (Sinninghe Damsté et al., 2011, 2014). Percentages are relative to all lipids recovered by acid hydrolysis of the cultures. If not indicated differently, all analyzed strains contained the respective compound in quantifiable amounts. Note that brGDGT Ia was detected but not quantified.

representatives have to date been obtained in pure cultures (NCBI, Feb. 2017). The vast majority of isolates are members of SD1, followed by SDs 3, 4, 8, 10, and most recently 6 (Huber et al., 2016), while the metabolic capabilities of all other subgroups remain uncharacterized. All of the thus far cultured representatives are heterotrophs, which can utilize a broad spectrum of carbohydrates and other complex organic compound as a source of cellular carbon (Ward et al., 2009; Kielak et al., 2016). Most require oxygen for growth (aerobes), although facultative or strict anaerobes have been isolated, some of which are capable of Fe^{III}- and/or nitrate reduction (Liesack et al., 1994; Coates et al., 1999; Izumi et al., 2012; Pankratov et al., 2012; Kulichevskaya et al., 2014).

Taken together, the global prevalence of bacterial and archaeal lipids with ‘extremophilic’ structural features (i.e., ether bonds, cyclopentyl rings, and membrane-spanning character) in mesophilic environments calls for a reinterpretation of these traits. The key for understanding the physiological function of GDGTs may lie in the lower H⁺ permeability of tetraether-based membranes (van de Vossenberg et al., 1998; Gabriel and Lee Gau Chong, 2000), which reduces the metabolic energy needed to maintain a trans-membrane proton gradient for ATP synthesis (Hoehler, 2004). Mesophilic microorganisms possessing such energy-efficient membranes may thus be considered ‘energetic extremophiles’ (Valentine, 2007) that are adapted to low substrate concentrations and operate close to the thermodynamic equilibrium, as for example the pelagic marine thaumarchaeon *Nitrosopumilus*

maritimus (Martens-Habbena et al., 2009; Könneke et al., 2014) and anaerobic methanotrophic archaea (Hinrichs et al., 1999; Dale et al., 2006).

brGDGTs in lacustrine environments

The first comprehensive survey of GDGTs in lake environments (n=47; Blaga et al., 2009) reported a dominance of branched over isoprenoid GDGTs in the majority of the investigated lake sediments and suspended particulate matter- (SPM) samples from the water column, yet, it remained unclear whether brGDGTs were derived from allochthonous soil organic matter, or were additionally produced by aquatic microbes. Subsequently, it was realized that the brGDGT composition in Lake Challa (Kenya; Sinninghe Damsté et al., 2009) and Lake Towuti (Indonesia; Tierney and Russell, 2009) was markedly distinct from that in soils surrounding the lakes, suggesting that sedimentary brGDGTs are substantially contributed by autochthonous in-situ production, either within the water column or the sediments. Although following studies arrived at a similar conclusion, at the time this PhD project started, no unambiguous evidence for aquatic brGDGT biosynthesis existed in the literature. Also, the preferred habitat and the ecological role of the source organisms within the lacustrine water column were elusive. For instance, high brGDGT concentrations (up to 43 ng L⁻¹) and high relative IPL contents (30–50 %) in SPM suggested in-situ production within surface waters (2 m) of some North American lakes (Schoon et al., 2013). In Lake Challa, on the other hand, a steep increase in brGDGT concentrations just below the anoxic interface and elevated IPL percentages (30–40 %) pointed towards a deep water source of brGDGTs (Buckles et al., 2014). However, based on distribution patterns in settling particles from a sediment trap, these authors concluded that brGDGTs are mainly exported to the sediment from the base of the thermocline. Other studies that installed sediment traps at different depth levels in Lake Lucerne (Blaga et al., 2011), Lake Superior (Woltering et al., 2012), and Huguangyan Maar Lake (SE China; Hu et al., 2016), all reported an increase of brGDGT fluxes with depth (5 to 10-fold), in support of deep water in-situ production. Remarkably, the fluxes in dimictic Lake Superior showed stark seasonality in the hypolimnion (peak amplitudes of 500–1000 ng m⁻² d⁻¹), which occurred regularly in January during winter overturning, and in June shortly after spring mixing, the latter coinciding with the diatom bloom period. Notably, the seasonal flux maxima observed in Lower King Pond (VT, USA; Loomis et al., 2014b) and Huguangyan Maar Lake (Hu et al., 2016) likewise corresponded to water column overturning and the spring primary productivity season, providing further circumstantial evidence for pronounced aquatic production of brGDGTs. In contrast, there are comparably little constraints on the possibility of post-depositional brGDGT production within sediments. These include (i) compositional differences between water column samples and surface sediments (Buckles et al., 2014), and (ii) high relative amounts of IPL-brGDGTs in sediments (~30 %; Tierney et al., 2012).

Microbial adaptation to environmental conditions

In order to sustain the functionality of the cytoplasmic membrane under varying environmental conditions, microbes have evolved mechanisms to adjust the physicochemical properties of their membrane lipids according to environmental conditions, which is known as ‘membrane lipid homeostasis’ (Zhang and Rock, 2008). Critical requirements to the plasma membrane are for instance the maintenance of (i) the liquid-crystalline state and ‘fluidity’ at different temperatures (Sinensky, 1974), and (ii) a certain degree of (im)permeability with respect to H^+ as well as other ionic species and solutes under varying pH or salinity conditions (Cronan and Gelmann, 1975). The most commonly observed response of bacteria exposed to T lower than the growth optimum, is an increase in the ratio of unsaturated vs. saturated fatty acids (FAs) in glycerol-phospholipids (Marr and Ingraham, 1962; Cronan, 1975), which is assumed to result in a less dense membrane ‘packing’ and a lower phase transition point (Zhang and Rock, 2008). Other species respond to low T stress by reducing the average chain length of their FAs (McGibbon and Russell, 1983; Russell et al., 1995), or produce more of the lower-boiling branched FAs, particularly those from the ‘anteiso’ series (McElhaney et al., 1976; Kaneda, 1977). Furthermore, exposing bacterial cultures to acid stress can lead to the conversion of mono-unsaturated FAs to cyclopropane FAs by activation of a specific enzyme (Lepage et al., 1987; Zhang and Rock, 2008), supposedly reducing the H^+ permeability of the plasma membrane (e.g. Brown et al., 1997). Archaea, in turn, are found to respond to high-T and low-pH stress by increasing the number of cyclopentyl rings (Uda et al., 2001; Boyd et al., 2011), which has been found to enhance the thermal stability of archaeal membranes (Gliozzi et al., 1983), while at the same time possibly decreasing H^+ leakage (Yamauchi et al., 1993; van de Vossenberg et al., 1999).

First molecular organic temperature proxy

Analogue responses to growth temperature have been observed in unicellular haptophyte algae from the class *Prymnesiophyceae* (e.g. *Emiliania huxleyi*) that produce di-, tri-, and tetra-unsaturated C_{37} -methyl- and -ethyl ketones, known as long-chain alkenones (LCAs) (Marlowe et al., 1984). As shown by culture studies, the relative amount of double bonds within LCAs correlates inversely with growth T (Prahl and Wakeham, 1987; Sun et al., 2007; Toney et al., 2012), suggesting that they play a role in controlling membrane properties, although their physiological functions in the cells are not fully understood and may be diverse (Epstein et al., 2001). LCAs represent ~20–30 % of the plasma membrane in *E. huxleyi* (Sawada and Shiraiwa, 2004), one of the most widely distributed oceanic species (Read et al., 2013), and are abundant components in recent and ancient marine sediments as old as the Cretaceous (Farrimond et al., 1986). Because *Prymnesiophyceae* thrive within the euphotic zone of the oceans’ mixed layer, their LCA composition (expresses as the alkenone unsaturation index $U^{K^*}_{37}$; Brassell et al., 1986; Prahl and Wakeham, 1987) is believed to reflect sea surface water temperatures (SST) (Müller et al., 1998). Thus, the LCAs that are exported to the sediments (e.g. by fecal pellets) and preserved in the geological record, can be used to reconstruct SST at the time of deposition. The *Prymnesiophyceae* are also important members of lacustrine planktonic communities

(Cranwell, 1985; D'Andrea et al., 2006; Randlett et al., 2014), and LCAs were detected in a lake core reaching back to the early Miocene (10.5 Ma; Sun et al., 2012). Indeed, the $U^{K'}_{37}$ index showed a strong correlation with average summer LST in modern lake sediments from Germany and Austria ($r^2=0.90$, $n=9$; Zink et al., 2001). However, recent studies point towards a strong genetic heterogeneity among haptophytes from different study sites (Toney et al., 2010, 2012), which seems to be related to chemistry and salinity of the lake water (Toney et al., 2011; Longo et al., 2016). These confounding effects currently hamper the broad application of LCAs for paleolimnological reconstructions, and addressing these issues is a matter of ongoing research.

isoGDGT-based paleothermometry

Also, the isoprenoid GDGTs produced by *Thaumarchaeota* show T-dependent compositional variations that are applied in paleoclimatology. These, however, refer to the relative amount of cyclopentyl rings, which is expressed as the 'Tetraether index' of tetraethers consisting of 86 carbon atoms'

$$TEX_{86} = \frac{G-2 + G-3 + Cren'}{G-1 + G-2 + G-3 + Cren'} \quad (\text{Eq. 1; Schouten et al., 2002;})$$

compound names refer to Fig. 6B, and Arabic numerals indicate the amount of cyclopentyl ring in the respective molecule. Note that Cren' contains four cyclopentyl rings and one cyclohexyl ring).

Several calibration studies were carried out using surface sediment samples covering the Earth's major ocean basins (Schouten et al., 2002; Kim et al., 2008, 2010; Liu et al., 2009; $n=426$), yielding linear transfer function that relate TEX_{86} values to SST. However, while a dominant T control on isoGDGT composition was supported by a mesocosm incubation experiment with North Sea water (~800 L; Wuchter et al., 2004), recent experimental work with pure thaumarchaeal cultures showed that (i) the T response differs markedly between species (Elling et al., 2015; Qin et al., 2015), (ii) that TEX_{86} values of *N. maritimus* vary substantially depending on growth phase (Elling et al., 2014) and ammonia oxidation rates (Hurley et al., 2016), and (iii) that low O_2 concentrations may potentially bias TEX_{86} -reconstructed SST (Qin et al., 2015). Hence, taxonomic differences between thaumarchaeal population in different ocean basins (e.g. Ionescu et al., 2009) may contribute to the residual error in the TEX_{86} calibrations (typically 2–5 °C), particularly in the Red Sea for which a separate transfer function has been proposed (Trommer et al., 2009). It is also unclear at present, by which mechanism *Thaumarchaeota* respond to changes in SST, as they are commonly found to reside in subsurface water, at the base of the thermocline (Varela et al., 2007). Despite these complications, the TEX_{86} proxy has provided paleo-SST estimates that are in general agreement with independent proxy data, and thus represents an indispensable geochemical tool in recent paleoceanographic research (e.g.

Schouten et al., 2003; Jenkyns et al., 2004; Schefuß et al., 2011; O'Brien et al., 2014; Zhang et al., 2014), particularly in settings where LCAs are absent (e.g. Castañeda et al., 2010).

As *Thaumarchaeota* are also abundant in freshwater lakes (Schouten et al., 2000; Callieri et al., 2016), the TEX₈₆ proxy bears great potential for paleoclimate reconstructions in lacustrine deposits, and has been applied particularly in the tropics (e.g. Powers et al., 2005, 2011; Tierney et al., 2008; Morrissey et al., 2017). However, sedimentary isoGDGT concentrations are often too low for TEX₈₆ measurements, especially in small lakes (Powers et al., 2010). More importantly, lake deposits may additionally contain isoGDGTs derived from (i) allochthonous soil organic matter, (ii) methanogenic *Euryarchaeota*, and/or (iii) other uncultured archaeal clades (e.g. MCG) thriving in anoxic bottom water and/or the sediments (Blaga et al., 2009; Borrel et al., 2012). The latter issue is certainly of particular concern, as methanogenesis and other anaerobic microbial processes are prevalent in lacustrine environments (Garcia et al., 2000). This can be (at least partly) accounted for by excluding samples that show high relative amounts of G-0 (Fig. 6B) (e.g., Sinninghe Damsté et al., 2012), as this GDGT is the dominant membrane lipid in methanogens (Koga et al., 1993), and possibly also in archaea of the MCG clade (Biddle et al., 2006). It further seems that there are marked taxonomic differences in lacustrine thaumarchaeal communities between lakes, possibly depending on water chemistry and nutrient concentrations (Auguet and Casamayor, 2013). Nonetheless, a global lacustrine TEX₈₆ calibration was proposed that allows for the reconstruction of mean annual lake surface temperatures (LST) with uncertainties of ~4.5 °C (root mean square error; Powers et al., 2010).

The brGDGT paleo-T proxy

Soon after the recognition of branched GDGTs as abundant environmental lipids, a survey of 134 soil samples from 90 globally distributed locations showed a systematic relationship between mean annual air temperature (MAT) and brGDGT composition. This led to the definition of (i) the methylation index of branched tetraethers

$$MBT = \left(\frac{Ia + Ib + Ic}{\sum(\text{all brGDGTs})} \right) \quad (\text{Eq. 2; Weijers et al., 2007b}),$$

describing the relative amount of methyl branches, and (ii) the cyclization index of branched tetraethers

$$CBT = -\log \left(\frac{Ib + IIb + IIb'}{Ia + IIa + IIa'} \right) \quad (\text{Eq. 3; Weijers et al., 2007b}),$$

reflecting the relative amount of cyclopentyl moieties (compound names refer to Fig. 6A). The MBT was correlated with MAT ($r^2=0.62$) and to a lesser extent with soil pH ($r^2=0.37$), whereas the

CBT was strongly related to pH ($r^2=0.70$). Thus, by determining both MBT and CBT, it was possible to predict instrumental MAT values at a given location with an uncertainty of ca. ± 5 °C ($r^2=0.77$; Weijers et al., 2007b) (Fig. 9).

This empirical correlation gave rise to the hypothesis that the brGDGT-producing bacteria adapt to ambient T by introducing additional methyl branches into their lipids, thereby increasing membrane fluidity at lower T (Weijers et al., 2007b). In turn, the pH-dependency of the CBT index made the authors speculate that *less* cyclopentyl rings may result in a denser membrane packing and a *lower* H⁺ permeability, which would be the opposite response as previously reported from archaeal cultures (e.g. Uda et al., 2001). Later, field and laboratory experiments with peat showed shifts in brGDGT distributions in response to artificially induced T changes on time scales of 1–2 years (Huguet et al., 2013, 2014), corroborating that T is a major determinant of brGDGT composition in terrestrial environments. Yet, a direct T effect on the membrane lipid chemistry of the source organism remains to be unequivocally established, because microbial isolates that cover the whole diversity of naturally occurring brGDGTs are not available at present.

The initial soil calibration was subsequently expanded (Peterse et al., 2012) and later revised (De Jonge et al., 2014a), as it became clear that previous analyses may have been biased due to the incomplete analytical separation of C5- and C6-methylated brGDGT isomers. The new transfer function reduced the root mean squared error (RMSE) of the regression from previously 6.2 °C (Peterse et al., 2012) to 4.8 °C. More importantly, however, the novel methylation index MBT'_{5me} that only considers the C5-methylated brGDGTs was independent from soil pH and CBT values (Fig. 10), and enabled T reconstruction without quantification of cyclic brGDGTs that often have low abundances. Instead, pH correlated strongly with the relative amount of the 6_{me} brGDGTs, demonstrating that co-elution of the isomers caused the co-variation of MBT and CBT values in the previous calibrations.

The MBT/CBT–temperature function was first applied to a sediment core from the submarine Congo River fan (Weijers et al., 2007a). The reconstructed paleo-MAT record indicated a ~ 4.5 °C warming of the Congo Basin since the early Holocene (17.2 ka bp), which agreed well with climate model estimates and available proxy data from E-Africa (Powers et al., 2005). In assumption of a dominantly terrigenous brGDGT source, the soil-based calibration has also been applied to lake sediments. For example, brGDGT-inferred MAT from a Pleistocene lake (Valle Grande, NM, USA) showed glacial–interglacial cycles, which were in agreement with vegetation shifts inferred from pollen records (Fawcett et al., 2011). Moreover, Holocene paleo-MAT estimates derived from Lake Cadagno (central Alps, Switzerland) showed subtle variations (± 1 °C) that correspond to fluctuations of Norwegian glaciers and north-west European winter precipitation, indicating an influence of the North Atlantic Oscillation on central European climate (Niemann et al., 2012). Further, the brGDGTs in Lake Cadagno sediments compositionally resembled those of nearby soils, which made the authors speculate that the exceptionally high H₂S concentrations in the lake's stratified water column (~ 1 mM) may inhibit the growth of aquatic brGDGT-producers.

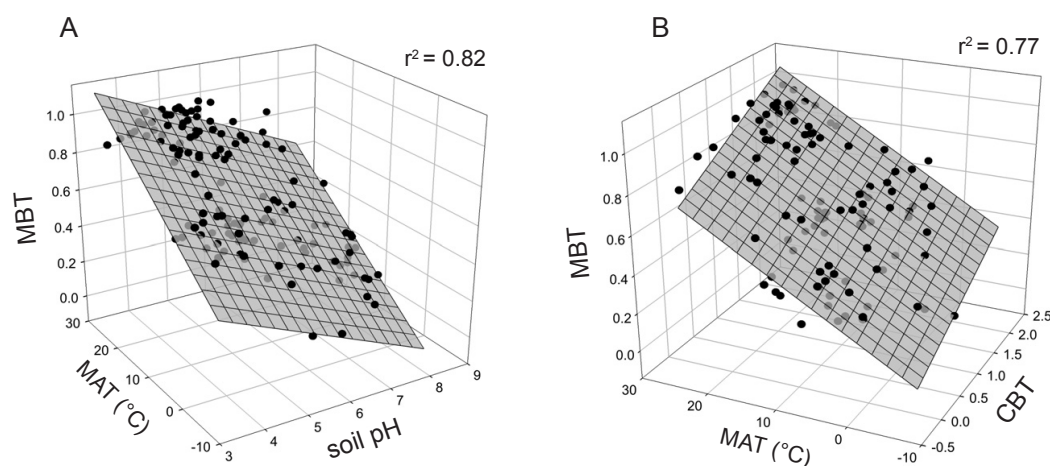


Fig. 9. Initial calibration of the brGDGT paleothermometer using a set of 134 globally distributed soil samples. The methylation index of branched tetraethers (MBT) is correlated to both, mean annual mean air temperature (MAT) and soil pH (A), the latter of which can be replaced by the cyclization index of branched tetraethers (CBT) (B). From Weijers et al. (2007a).

Application of the brGDGT proxy to lake sediments

In contrast to the above examples, application of the soil-based transfer function(s) to lake sediments, in most cases, leads to a substantial ‘cold-bias’ in MBT/CBT-based T estimates (often >10 °C; Fig. 11A; Tierney and Russell, 2009; Blaga et al., 2010; Tierney et al., 2010; Pearson et al., 2011). This is caused by higher relative amounts of penta- and/or hexamethylated brGDGTs (i.e., IIa/IIa’ and IIIa/IIIa’) in sediments compared to coexisting soils of the same climate zone, suggesting that the aquatic brGDGT-producing bacteria generally express a higher degree of membrane lipid methylation. Remarkably, despite these offsets, sedimentary brGDGT compositions still show T-dependent variations. All thus far reported data from modern lake sediments indicate that altitudinal/latitudinal air T gradients (among all environmental variables considered) exert the dominant control on brGDGT composition (e.g. Tierney et al., 2010; Loomis et al., 2014a), emphasizing that the lacustrine source organisms are systematically affected by climatic variables. The underlying physiological and/or ecological response mechanisms, however, are as yet unclear.

Building on these empirical data, lake sediment-based transfer functions have been devised for different regions, that is East Africa (Tierney et al., 2010; Loomis et al., 2012), New Zealand (Zink et al., 2010, 2016), Tibet (Wang et al., 2015), and Antarctica (Foster et al., 2016). The most comprehensive study thus far integrated brGDGT data from 139 globally distributed lakes (Sun et al., 2011), and found that exclusion of 39 alkaline lakes with $\text{pH} > 8.5$ substantially improved r^2 (0.73 vs. 0.62) and RMSE of the regression (4.27 °C vs. 5.24 °C). A much better correlation was achieved with a set of 90 sediment samples spanning a transect from the Scandinavian Arctic to Antarctica ($r^2=0.88$; RMSE=2.0 °C; Pearson et al., 2011; Fig. 11B). A variety of regression techniques have been used in the above studies. Some authors rely on the MBT and CBT indices known from the soil

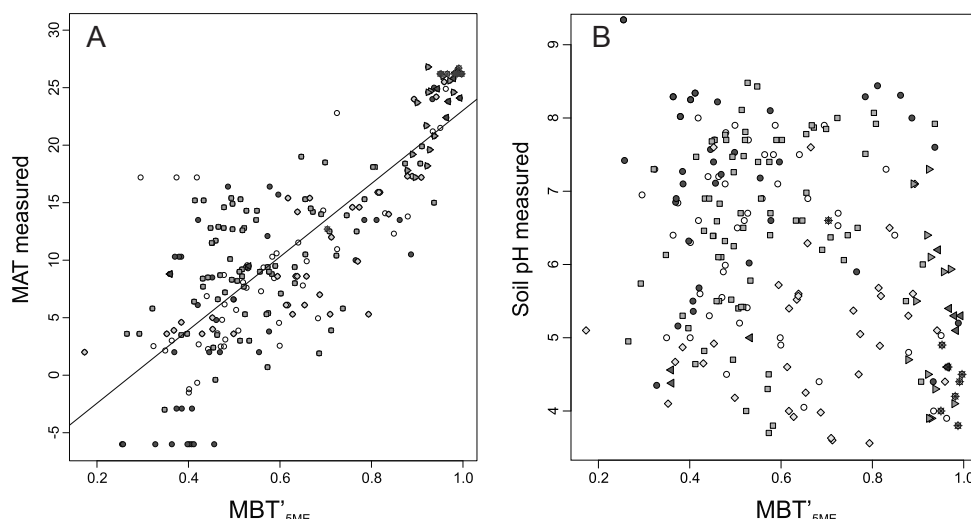


Fig. 10. The new MBT'_{SME} index correlates with mean annual air T (MAT) (**A**), but is independent from soil pH (**B**), due to the separation of C5- and C6-methylated brGDGT isomers. From De Jonge et al. (2014a).

calibration, and fit intercept and slope coefficients to account for the bias induced aquatic production (e.g. Sun et al., 2011). Other studies performed least squares multiple linear regression (MLR) on the fractional abundances of individual brGDGTs (i.e., normalized to the sum of all brGDGTs; Pearson et al., 2011; Loomis et al., 2012). The major difference between these calibration types is that MLR procedures only consider a specific subset of the brGDGTs as variables. These are picked either (i) stepwise in an iterative process in which the effect of each variable is evaluated separately (stepwise forward selection [SFS]; Blanchet et al., 2008), or (ii) from among all possible combinations of variables, according to adjusted r^2 values and the Mallows' C_p -statistic (best subsets regression [BSR]; e.g. Graumlich and Brubaker, 1986). Notably, in all published MLR-based transfer functions, the correlation with MAT is driven by the higher-methylated brGDGTs IIIa and IIa, which is in general agreement with the supposed homeostatic increase in membrane lipid branching at lower T. Some calibrations, therefore, only consider the three major brGDGTs Ia, IIa, and IIIa as predictive variables (e.g. Tierney et al., 2010). Moreover, other authors chose to use mean summer air T (MST) rather than MAT in their regression analyses, arguing that biological and microbial activity is greater in summer, and that ice cover may inhibit brGDGT synthesis in winter (Pearson et al., 2011; Foster et al., 2016). Indeed, biases in brGDGT-derived T towards the warm season have been reported from soil transects in Tibet (Deng et al., 2016), however, there is little evidence for an explicit warm season preference of lacustrine brGDGT production, as indicated by sediment trap data (e.g. Woltering et al., 2012).

Application of the lacustrine brGDGT proxy calibration(s) to lake sediment records is, nevertheless, not straight forward. It has been frequently noted that brGDGT-reconstructed MAT values differ substantially, depending on the choice of calibration function. For instance, markedly distinct paleo-T trends were reconstructed from the 50,000 year sediment record of Sacred Lake (Kenya) by means

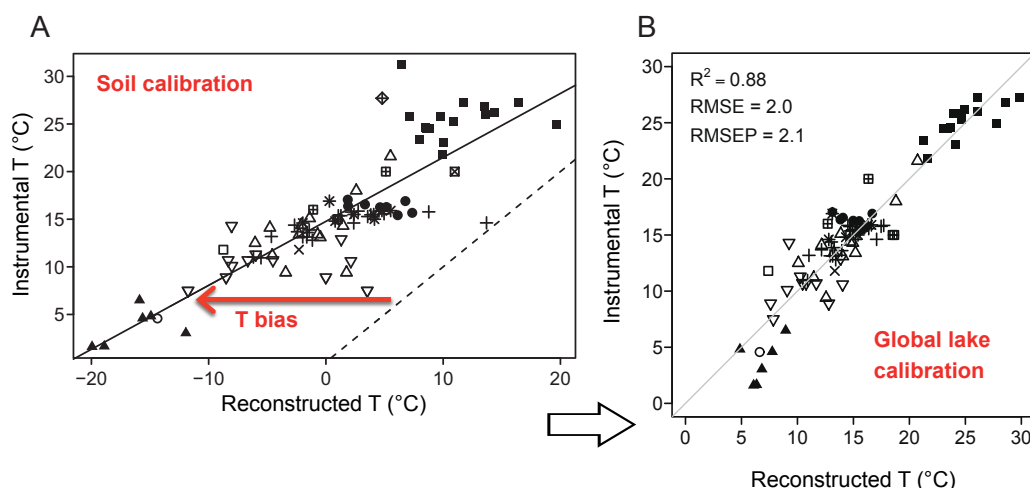


Fig. 11. Application of the soil calibration to 90 globally distributed lake surface sediments leads to ‘cold-biased’ reconstructed MAT (A), which has been accounted for by a ‘de-novo’ multiple linear regression (B). Modified from Pearson et al. (2011).

of previous transfer functions (gray panels in Fig. 12; Loomis et al., 2012). Subsequent recalibration with an extended set of modern E-African lake sediments substantially improved the correlation, with best results achieved by the SFS-MLR technique (red panels in Fig. 12). Even greater discrepancies have been reported from Lake McKenzie (Australia) (Woltering et al., 2014), with offsets of almost 30 °C between MAT estimates derived by available transfer function from different regions (e.g. New Zealand, E-Africa). Biases were also substantial in a Holocene sediment record of Fan Lake (South Georgia) where global calibrations were surprisingly T-insensitive ($\Delta T \approx 2$ °C), whereas application of the Antarctic transfer function suggested a strong cooling ($\Delta T \approx 8$ °C) after the Holocene Climate Optimum (~ 3.8 ka bp; Foster et al., 2016).

The specific reason for these discrepancies are unknown, but it has been speculated that differences in (i) oxygen availability (Tierney et al., 2010; Colcord et al., 2015), (ii) water depth (Tierney et al., 2010; Pearson et al., 2011), and (iii) salinity (Günther et al., 2014) may influence lacustrine brGDGT compositions. It is further important to consider that all lacustrine calibration work to date used the analytical method of Schouten et al. (2007a) for brGDGT analysis, which does not allow for the separate quantification of C5- and C6-methylated brGDGTs. Therefore, some of the previously noted pH-dependent effects on brGDGT calibrations (e.g., Loomis et al., 2011) may have been induced by the co-elution of these structural isomers, as the C6brGDGTs are found to be more abundant in alkaline vs. acidic environments (De Jonge et al., 2014a; Dang et al., 2016). Additionally, C6brGDGTs seem to play a particular role in freshwater environments, as they are exceptionally abundant in riverine and lacustrine SPM from Siberia (De Jonge et al., 2014b, 2015a, 2015b), as well as in the Tagus river (Portugal) and its flood plain deposits (Warden et al., 2016). As such, the MBT'_{6me} index has been proposed that only considers the C6-methylated brGDGTs (Dang et al., 2016). Remarkably, this novel ratio correlated with MAT in lake sediments from E-China ($r^2=0.66$, $n=18$; $p<0.01$), whereas

the MBT'_{5me} did not show any T response ($r^2=0.14$), pointing towards different microbial sources of C5- vs. C6-methylated brGDGTs (Dang et al., 2016). Incomplete separation of these isomer classes may thus have contributed to the errors in previous lake calibrations, and differential environmental effects on the distribution of C5- vs. C6 brGDGT may have remained hidden.

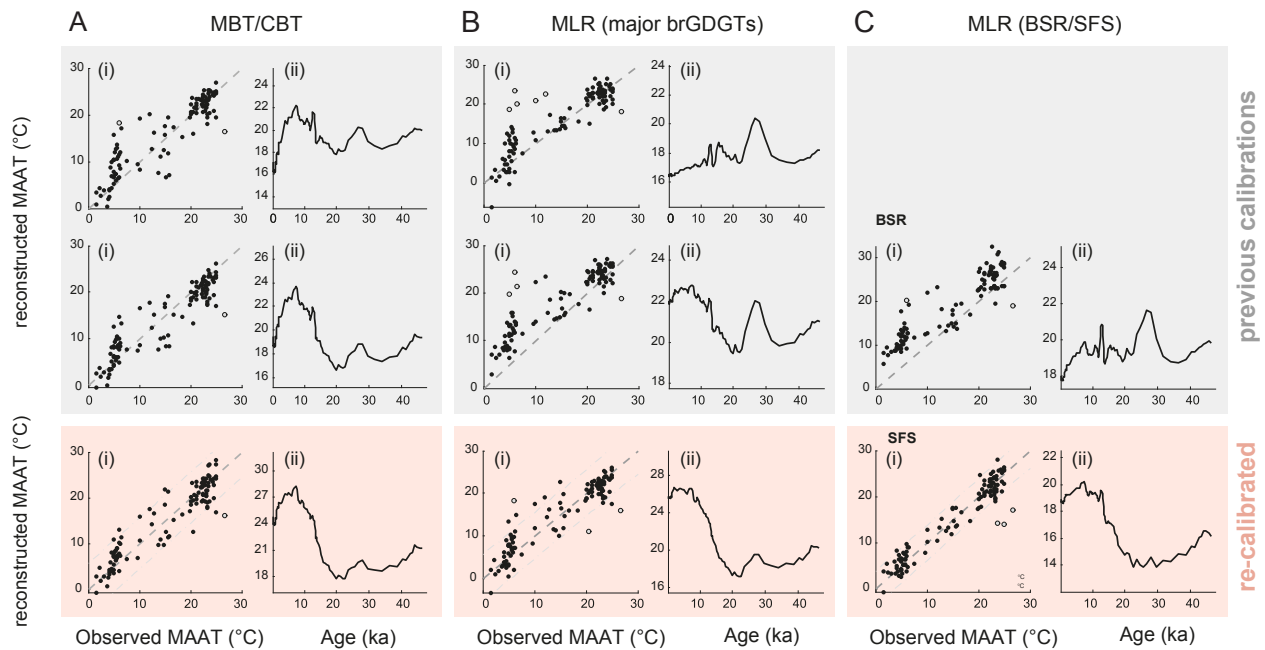


Fig. 12. Reconstructed MAT vs. (i) observed MAT in a calibration set of 111 modern lake surface sediments from E-Africa, and (ii) sediment age (10^3 years before present [ka]) of Sacred Lake (Kilimanjaro, Kenya), calculated with previously published transfer function (gray) and after re-calibration (red). Three different types of calibrations were used, based on (A) methylation- and cyclization index (MBT/CBT), (B) the fractional abundances of the major brGDGTs Ia, IIa, and IIIa, and (C) multiple linear regression (MLR) on the fractional abundances of all brGDGTs, using either best subsets regression (BSR) or step-wise forward selection (SFS). Grey circles denote outliers excluded from the calibrations. Modified from Loomis et al. (2012).

1.3. Research objectives

Current research on brGDGTs clearly attests to their potential as paleoclimate indicators in lake systems. However, the broad application of the brGDGT proxy to lacustrine sediment records is hampered by

- (i) the regional variability of proxy calibrations that leads to uncertainties regarding the choice of transfer function,
- (ii) the differential contribution of terrigenous and aquatic brGDGT sources to the sediments, each of which carries a distinct lipid signature,
- (iii) potential in-situ production in deep hypolimnetic water that may introduce T-independent bias to sedimentary brGDGT records, and
- (iv) other environmental factors such as redox conditions and salinity, which possibly affect brGDGT-producing microbes.

In order to facilitate further improvements of the lacustrine brGDGT proxy, and thus our ability to assess past continental climates, the main goals of this PhD project were

- (i) **to locate the ‘hotspots’ of brGDGT synthesis within the lacustrine water column,**
- (ii) **to constrain the identity, metabolism, and ecological niche of the as yet unidentified source organisms within lake systems, and**
- (iii) **to obtain a lacustrine brGDGT–MAT calibration for the Alpine region.**

1.4. Methods

GDGT preparation and -analysis

Lipid Extraction — Lipids are generally hydrophobic, and thus are soluble in organic, apolar solvents rather than in aqueous (polar) solutions. Therefore, dried samples are typically extracted with solvent mixtures of methanol (MeOH) and dichloromethane (DCM). There are, however, differences in polarity between lipid classes, particularly between core lipids (CLs) and intact polar lipids (IPLs). The overall polarity of the lipid molecules is controlled by the balance between hydrophobic- and hydrophilic building blocks, and is further influenced by the presence of ionic moieties such as phosphate and choline ($[\text{CH}_3]_3\text{N}^+ - [\text{CH}_2]_2$). As such, the protocols used for the extraction of living tissues dominated by more hydrophilic IPLs are different from those primarily targeting refractory CLs in sediments and soils, and use solvent mixtures containing both, organic solvents and aqueous solutions (Bligh and Dyer, 1959). This is believed to equally solubilize CLs and IPLs contained within environmental samples. Nevertheless, some studies successfully employed water-free solvent protocols (i.e., MeOH/DCM mixtures) to extract IPLs from aquatic suspended biomass (e.g. Ingalls et al., 2012; Basse et al., 2014). In contrast to IPLs, CLs are dominantly apolar, because the hydrocarbon chains mainly govern the physicochemical properties of the molecule. Nevertheless, differences do exist also between different CL types. For example, isoGDGTs are considerably more apolar than brGDGTs, because their hydrophobic alkyl moieties are larger (40 vs. ~30 C atoms), in relation to the hydrophilic glycerol backbone. Environmental samples commonly contain a great variety of different lipid types ranging from apolar leaf wax n-alkanes, over moderately polar ketones, to the more polar alcohol fraction. These polarity classes are typically separated before analysis by manual column chromatography, using either silica gel or aluminum oxide as stationary phases. To this end, the stationary phase is loaded with the total lipid extract and sequentially flushed (i.e., eluted) with solvent mixtures of increasing polarity, each of which yields a compound mix with roughly similar polarity.

Chromatographic separation — The analysis of complex mixtures of organic compounds first requires separation of the individual molecular species. This is achieved by analytical chromatography (Ettre and Sakodinskii, 1993) that separates molecules according to their differential interaction with a stationary phase, resulting in a sequential elution of the compounds at the end of the column, where they are detected and quantified. Two different chromatographic methods were applied in this study, (i) gas chromatography (GC) in which the sample is vaporized and carried through a capillary column to the detector (Hesse and Tschachotin, 1942), and (ii) liquid chromatography (LC; Tswett, 1911) where the dissolved sample is pumped through a steel column packed with a granular stationary phase. LC was applied for GDGT analyses as they are not easily vaporized at reasonable temperatures (~320 °C for normal GC). The alkyl chains of GDGTs obtained by ether cleavage, however, were analyzed by GC. Because the molecular structure of the analyte determines its affinity

to the stationary phase, the retention times (i.e. the time a specific compound needs for the passage of the column) lie close together for structurally similar compounds. As a consequence, the presence of 6_{me} brGDGT isomers has remained hidden until recently, because they co-eluted (i.e., overlapped) with the 6_{mc} brGDGT on the commonly used LC stationary phase (Fig. 13).

Detection — After analytical chromatographic separation, the compounds were identified and quantified by mass spectrometry (Aston, 1920). In this technique, the molecules are first ionized, and then accelerated in a static electric field. Subsequently, the mass-over-charge ratio (m/z) of each ion is determined by mass-selective magnetic- or electric fields, and the abundance of each individual ion is recorded in real time.

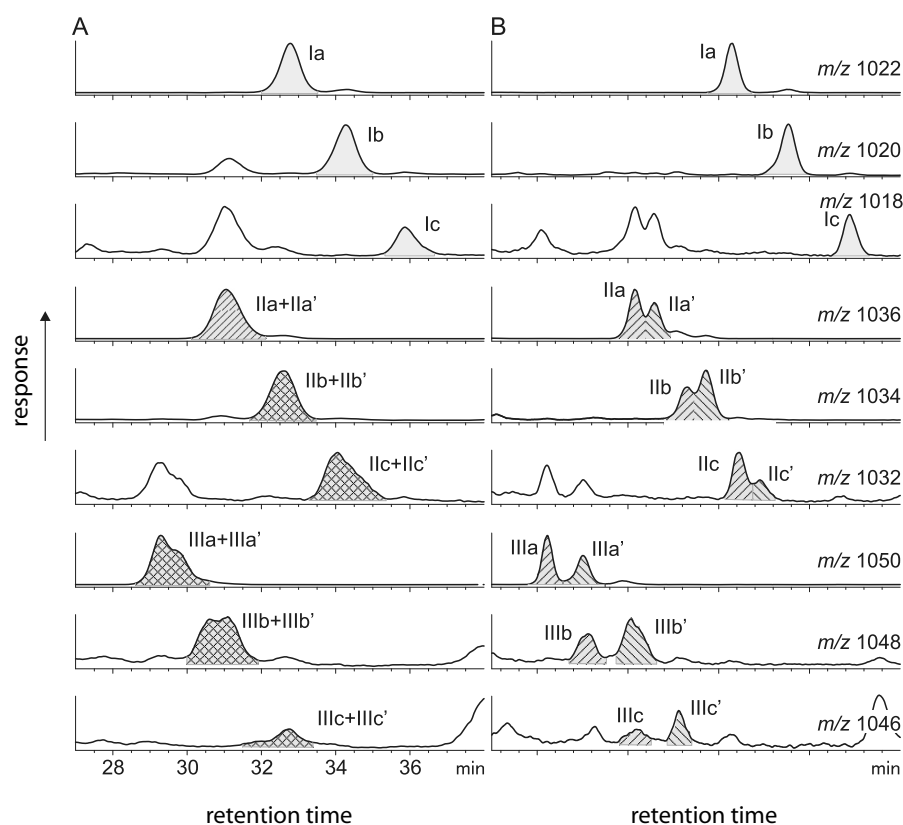


Fig. 13. Improved separation of brGDGT isomers using high-resolution liquid chromatography (B), compared to the previously used analytical setup. From De Jonge et al. (2014a).

Stable C isotope ratios as indicators of microbial metabolism

Stable isotope ratios of the elements C, N, O, S, and H are widely used in biochemical, ecological, and (paleo)environmental studies, because they tend to fractionate during biologically-catalyzed reactions (Hayes, 2001; Fry, 2006; Michener, 2007; Sachse et al., 2012). Particularly the ratio of $^{13}\text{C}/^{12}\text{C}$ in biomolecules or bulk organic matter (typically expressed in ‰ values relative to the ‘Vienna Pee Dee Belemnite’ standard; VPDB; Eq. 4) is an important tool in ecology, biogeochemistry, geology and paleoclimatology for distinguishing C sources and metabolic pathways (e.g. Pancost and Sinninghe Damsté, 2003). Applications of $\delta^{13}\text{C}$ values build upon (i) the natural fractionation of C isotopes in the environment by biosynthetic- and metabolic processes on the organism level, and (ii) the isotopic differentiations of C pools across biogeochemical compartments on larger spatial scales. Autotrophic organisms such as plants, algae, and chemo- or photoautotrophic bacteria, for example, strongly discriminate against ^{13}C during assimilation of inorganic C (i.e., CO_2 or HCO_3^-), which is caused by the enzymes involved in the various pathways of C fixation (e.g. RuBisCO). The biomass produced by these organisms is therefore characterized by lower $\delta^{13}\text{C}$ values (typically ~ 20 ‰) relative to the C substrate (House et al., 2003). Heterotrophic assimilation of organic substrates, on the other hand, largely conserves $\delta^{13}\text{C}$ values and may lead to ^{13}C enrichment of only a few ‰ (DeNiro and Epstein, 1976). There is, however, considerable biosynthetic ^{13}C fractionation *within* all uni- and multicellular organisms during biosynthesis of carbohydrates, proteins, and lipids (DeNiro and Epstein, 1977). Lipids, in general, exhibit a more or less pronounced ^{13}C depletion relative to the bulk isotopic composition of the whole organism, which can be as large as -10 ‰ in some bacteria (Tece et al., 1999), but on mostly ranges between 2 ‰ and 5 ‰.

$$\delta^{13}\text{C} = \left(\frac{^{13/12}\text{C}_{\text{sample}}}{^{13/12}\text{C}_{\text{VPDB}}} - 1 \right) \times 1000 \text{ [‰]} \quad (\text{Eq. 4})$$

Reservoir-scale isotopic differentiation in the environment, for instance, occurs within lacustrine and marine water columns, when algae convert large amounts of $^{12}\text{CO}_2$ from surface water into biomass, leading to a local ^{13}C enrichment in the dissolved inorganic carbon (DIC) pool (e.g. Lehmann et al., 2004). On the other hand, many methanogenic archaea use the reductive acetyl-CoA pathway (Wood–Ljungdahl pathway; Ljungdahl, 1986) that strongly discriminates against ^{13}C (<30 ‰; (Fuchs, 1986; Preuß et al., 1989), and thus the methane produced in anoxic lake water and/or -sediments typically carries a ‘light’ C isotope signature. Organisms that directly or indirectly feed on CH_4 or CH_4 -derived biomass, respectively, are hence expected to have a low- ^{13}C isotopic imprint (e.g. Brees et al., 2014). As such, heterotrophic organisms thriving at different depths within a stratified water column with an oxic (upper) and an anoxic (bottom) layer, may exhibit distinct $\delta^{13}\text{C}$ values, although their metabolic lifestyle is identical.

Another application of stable C isotopes in biogeochemistry is the deliberate introduction of substrates artificially enriched in ^{13}C , in order to trace the fate of ‘ ^{13}C -labeled’ compounds within bio(geo)chemical systems, on scales ranging from whole lakes (e.g. Cole et al., 2002) to individual C positions within molecules (Rohmer et al., 1993; Lin et al., 2012). The type of organic- or inorganic ^{13}C substrate provided may further give insight into the C metabolism of the target organisms (e.g. hetero- vs. autotrophic). It has to be considered, however, that labeled C may be cycled through different intermediates, i.e., organic substrates may first be mineralized by decomposing microbes, and ^{13}C -enriched CO_2 (or HCO_3^-) may then be taken up by autotrophic organisms (e.g. algae).

Stable C isotope analysis — For determination of natural $^{13}\text{C}/^{12}\text{C}$ ratios, the C-containing compounds are converted to CO_2 , which is then introduced to an Isotope Ratio Mass Spectrometer (IRMS), typically providing an accuracy of ca. ± 0.1 ‰ to ± 0.5 ‰ under normal conditions. The peripheral instrumentation depends on the type of sample. Organic C is combusted with O_2 , either as bulk sample or after gas-chromatographic separation of individual compounds (compound-specific stable C isotope analysis; Gilmour et al., 1984). Inorganic carbonates or DIC are converted to CO_2 by acidification, and dissolved organic compounds are oxidized to CO_2 by sodium persulfate (Kaplan, 1992).

1.5. Publication outline

The results of this thesis are summarized in three separate manuscripts that are presented in the following chapters, of which one (Chapter 2) has been published as a research paper. If not stated differently, sample preparation and analysis were performed by myself at the laboratories of the Aquatic and Stable Isotope Biogeochemistry group of Prof. M. Lehmann (*Institute of Environmental Sciences* – Univ. Basel), or in the facilities of our collaborators at the *Department of Marine Microbiology and Biogeochemistry* (Royal Netherlands Institute for Sea Research – NIOZ). All manuscripts were written by myself with contribution from the listed co-authors. Third party contributions are detailed below, or in the individual manuscripts.

Chapter 2: Identification and carbon isotope composition of a novel branched GDGT isomer in lake sediments: Evidence for lacustrine branched GDGT production

Yuki Weber, Cindy De Jonge, W. Irene C. Rijpstra, Ellen C. Hopmans, Alina Stadnitskaia, Carsten J. Schubert, Moritz F. Lehmann, Jaap S. Sinninghe Damsté, Helge Niemann

Published as: Weber et al. (2015), Geochimica et Cosmochimica Acta 154: 118–129
doi: 10.1016/j.gca.2015.01.032

In this first study, we report on the discovery of a novel brGDGT compound that was previously unknown from terrestrial environments, as well as its ¹³C-depleted C isotope signature that was markedly distinct from surrounding catchment soils. The *Swiss Federal Institute of Aquatic Science and Technology* (EAWAG) organized fieldwork at Lake Hinterburg, and I received strong support from Ellen Hopmans and Cindy De Jonge during analytical and preparatory work at the NIOZ. Irene Rijpstra (NIOZ) performed the ether cleavage and part of the GC analyses.

Chapter 3: Incomplete recovery of intact polar glycerol dialkyl glycerol tetraethers (GDGTs) from lacustrine suspended biomass

Yuki Weber, Jaap S. Sinninghe Damsté, Ellen C. Hopmans, Moritz F. Lehmann, Helge Niemann

Published as: Weber et al. (2017), Limnology and Oceanography: Methods 15(9):782–793
doi: 10.1002/lom3.10198

Here I investigated the recovery of brGDGTs from water column samples of Lake Lugano by means of two commonly applied extraction methods, revealing that brGDGT-producing bacteria are resistant towards methanol extraction. Marco Simona (*Institute of Earth Sciences, University of Applied Sciences of Southern Switzerland – SUPSI*) and I collected the samples with support from others. Ellen Hopmans (NIOZ) performed HPLC-ESI-Orbitrap-MS analyses (results not included).

Chapter 4: Redox-dependent niche differentiation of tetraether producing bacteria: Evidence for multiple branched GDGT sources in lakes

Yuki Weber, Jaap S. Sinninghe Damsté, Jakob Zopfi, Cindy De Jonge, Adrian Gili, Carsten J. Schubert, Marco Simona, Fabio Lepori, Moritz F. Lehmann, Helge Niemann

In review: Proceedings of the National Academy of Sciences of the United States of America

This manuscript represents a synthesis of the most significant results from this thesis, which comprise water column- and sediment trap data from Lake Lugano, as well as a collection of 36 lake surface sediments from Switzerland and northern Italy. Besides brGDGT quantification, we performed compound-specific $\delta^{13}\text{C}$ measurements on the brGDGTs' alkyl moieties, as well as sequencing of ribosomal DNA from water column samples of Lake Lugano. The results provide clues on the putative metabolism and ecological niche of brGDGT-producing microbes, and have important implications for future paleoclimate work. Marco Simona and I collected suspended biomass and a sediment core from Lake Lugano. Sediment traps were deployed, sampled, and evaluated (mass flux and organic C) by Marco Simona and M. Lehmann. Adrian Gili coordinated the collection of the other lake sediment samples. Jakob Zopfi extracted and quantified DNA, and Cindy De Jonge performed HPLC-MS analyses of lake sediments. Processing and phylogenetic analysis of sequencing data were performed by myself.

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Chapter 2

Identification and carbon isotope composition of a novel branched GDGT isomer in lake sediments: Evidence for lacustrine branched GDGT production

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Abstract

Branched glycerol dialkyl glycerol tetraethers (brGDGTs) are bacterial membrane lipids that occur ubiquitously in soils and lacustrine sediments and have great potential as proxy indicators for paleotemperature and pH reconstructions. Initially, brGDGTs in lakes were thought to originate from soils of the watershed. The composition of the lacustrine brGDGT pool, however, often differs substantially from that in catchment soils, complicating the application of the brGDGT paleothermometer to lake sediments. This suggests that terrigenous brGDGT signals in lacustrine sedimentary archives may be affected by aquatic in situ production. In sediments of a Swiss mountain lake, we detected a novel hexamethylated brGDGT, which elutes between the known 5- and 6-methyl brGDGT isomers during HPLC-MS analysis. This novel isomer accounted for 8.5 % of the total brGDGTs. Most remarkably, this brGDGT was not detected in soils collected from the catchment of the lake, providing circumstantial evidence for an in situ brGDGT source in the lake's water column or sediments. Isolation of the compound by preparative HPLC and subsequent GC-MS analysis of the alkyl chains revealed that the novel brGDGT comprises two structural isomers. One possesses a 5,13,16- and a 6,13,16-trimethyloctacosanyl moiety and constitutes 84 % of the new brGDGT; the second contains a 13,16-dimethyloctacosanyl and a 5,13,16,23-tetramethyloctacosanyl moiety. The $\delta^{13}\text{C}$ values of both the alkyl chains derived from the novel brGDGT (-46 ‰) and all other major brGDGTs (-43 to -44 ‰) were significantly lower than those of brGDGT-derived alkanes in catchment soils (-27 to -28 ‰) further attesting in situ production of brGDGTs in the studied lake.

1. Introduction

Non-isoprenoidal, branched glycerol dialkyl glycerol tetraethers (brGDGTs) are bacterial membrane lipids (Weijers et al., 2006) that are ubiquitously detected in soils, and peat, as well as in sediments and suspended particulate matter (SPM) of lakes, rivers and coastal marine environments (see Schouten et al., 2013 for a review). BrGDGTs contain two branched alkyl chains, which may contain up to four methyl branches and one cyclopentyl moiety (Sinninghe Damsté et al., 2000; Weijers et al., 2006; De Jonge et al., 2013) (for structural information see Fig. 1). The degree of methyl branching and cyclisation of brGDGTs can be expressed using the methylation- and cyclisation indices (MBT and CBT, Weijers et al., 2007a), which are quantitatively related to mean annual air temperature (MAAT) and pH in soils. Consequently, brGDGT distributions can be used as potential paleoclimate proxy in deposits (e.g. lake sediments) that receive substantial amounts of soil organic matter. Indeed, previous soil-based MBT/CBT calibrations (Weijers et al., 2007a; Peterse et al., 2012) allowed for the reconstruction of temporal MAAT and soil pH variations in marine and lacustrine sediments and paleosol successions (Weijers et al., 2007b; Peterse et al., 2011a; Fawcett et al., 2011; Niemann et al., 2012). However, with a few exceptions (Niemann et al., 2012; Günther et al., 2014; Woltering et al., 2014), soil calibrations of the MBT/CBT-paleothermometer yielded unrealistically low MAAT values (up to 10 °C) when applied to lake sediments (e.g. Zink et al.,

2010; Blaga et al., 2010; Pearson et al., 2011), complicating quantitative MAAT reconstructions in lacustrine environments. At least in part, this bias is caused by elevated abundances of the brGDGT IIIa in lake sediments (Tierney et al., 2010; Sun et al., 2011; Naeher et al., 2014).

It has been suggested that substantial amounts of brGDGTs can be produced in situ, i.e. within the water column or the sediments of a lake, thereby altering or overprinting the terrigenous proxy signal in the lacustrine sedimentary archives (e.g. Tierney and Russell, 2009; Loomis et al., 2011; Tierney et al., 2012; Wang et al., 2012; Buckles et al., 2014a, b). However, investigations of a potential aquatic source of brGDGTs are complicated because the identity and ecological niche of brGDGT-producing organisms is still not fully understood, even in well-studied terrestrial environments such as soils and peat (e.g. Dirghangi et al., 2013; Huguet et al., 2013). So far, only one out of the 15 known brGDGTs (structure Ia, Fig. 1) has been identified in two strains of the *Acidobacteria* (subdivision 1, Sinninghe Damsté et al., 2011). Recently, ether- and ester-bound lipids containing identical alkyl moieties as brGDGTs were identified in subdivision 4 *Acidobacteria*, indicating that they are probably more widely distributed within this group of bacteria (Sinninghe Damsté et al., 2014). In peat, where

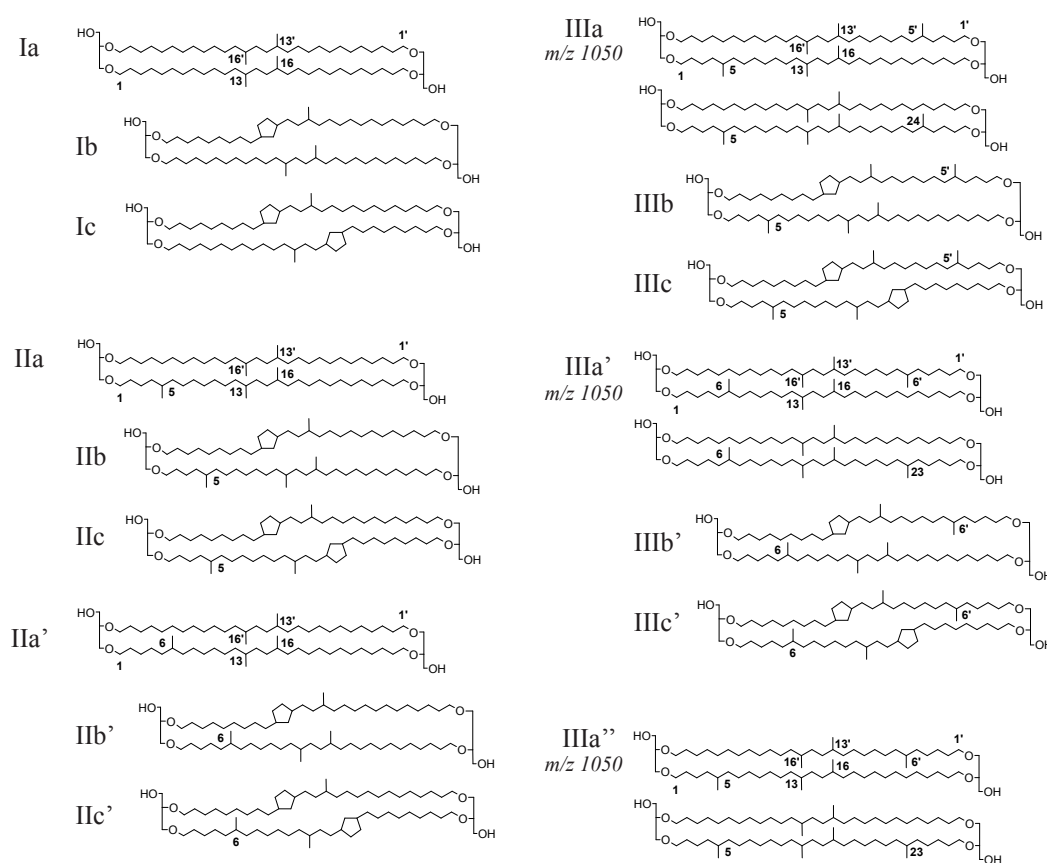


Fig. 1: Structures of all known brGDGTs (including 5- and 6-methyl isomers) and the novel 5/6-methyl isomer (IIIa''). BrGDGTs Ia, Ib and Ic are referred to as 'tetra-methylated'; IIa, IIa', IIb, IIb', IIc and IIc' as 'penta-methylated'; IIIa, IIIa', IIIb, IIIb', IIIc and IIIc' as 'hexa-methylated'. The asymmetric isomers exist for brGDGTs IIIa, IIIa' and IIIa'' but are not named separately since they cannot be resolved by HPLC-based chromatography. Structures of the hexa- and penta-methylated brGDGTs with cyclopentyl moiety(ies) IIb', IIc', IIIb' and IIIc' are tentative.

brGDGTs are found to be most abundant among all environments studied thus far, 16S rRNA gene sequencing showed that the bacterial community was dominated by *Acidobacteria* (Weijers et al., 2009). Furthermore, total brGDGT abundances were found to correlate inversely with pH in soils, further supporting the hypothesis that *Acidobacteria* are the most likely biological source of brGDGTs (Jones et al., 2009; Peterse et al., 2010). Moreover, the source organisms seem to have a high affinity to anoxic environments since concentrations of brGDGTs tend to increase below the oxic/anoxic transition zones in peat sections (Weijers et al., 2009; Peterse et al., 2011b) and the water column of stratified lakes, respectively (Sinninghe Damsté et al., 2009; Buckles et al., 2014b).

Recently, isomers of the penta- (structure IIa', Fig. 1) and hexamethylated brGDGTs (IIIa') were identified in a Siberian peat (De Jonge et al., 2013) and in a set of globally distributed soil samples (De Jonge et al., 2014a) by using preparative HPLC and improved HPLC/MS analysis, respectively. The 6-methyl isomers differ from the previously known brGDGTs by having methyl groups at positions 6 and 6' instead of positions 5 and 5' (see Fig. 1). The hexa-methylated 6-methyl brGDGTs (IIIa', IIIb', IIIc') were observed to be dominant over their 5-methyl counterparts (IIIa, IIIb, IIIc) in suspended particulate matter (SPM) of a Siberian river system (De Jonge et al., 2014b), suggesting that these 6-methyl isomers can also be produced within aquatic environments.

Here we demonstrate, by adopting the HPLC method of De Jonge et al. (2014a), the existence of an unknown hexa-methylated brGDGT isomer with a strongly ^{13}C -depleted isotopic signature in sediments of a small mountain lake in Switzerland. This novel brGDGT was not detectable in soils from the lake's catchment, which for the first time provides direct evidence for in situ production of brGDGTs in a lacustrine system.

2. Materials and methods

2.1 Sampling site

Lake Hinterburg (46.7177°N; 8.0675°E) is a small (0.045 km²) and shallow (~11 m) mountain lake with a catchment area of 1.6 km², located 1516 m above mean sea level (asl) (Fig. 2). The bedrock in the catchment consists exclusively of marine limestone, forming steep slopes (30-45°) and rock cliffs reaching up to ca. ~2320 m asl at the S and SE sides of the lake. Vegetation in the area comprises subalpine forest (spruce and green alder) and mountain pastures up to an elevation of ca. 1800 m asl. The lake is fed by surface runoff and a small creek at its western shore. Lake Hinterburg is not drained by a surface outflow. The lake level, however, stays fairly constant throughout the year, indicating a balanced hydrology that is maintained by subterranean in- and outflow. Hydrochemical analyses yielded meso- to eutrophic conditions with 33 $\mu\text{g l}^{-1}$ total phosphorus and 1.16 mg l⁻¹ total nitrogen (water column average; Heiri et al., 2003). At least during the summer productivity season from May through September the lake is stratified with anoxic conditions in the bottom 3 m of the water column (data from Federal Environment Agency, BAFU).

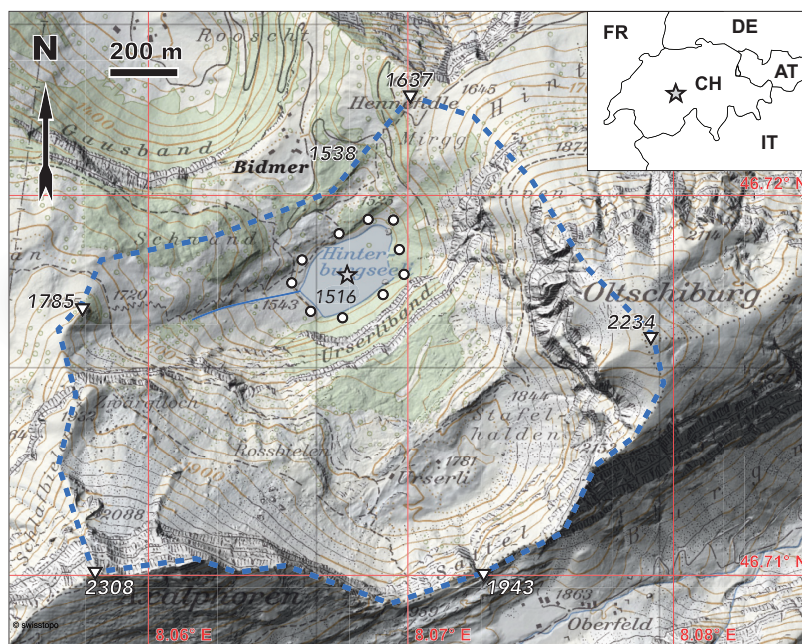


Fig. 2: Topographic map of the study area, showing the catchment of Lake Hinterburg (dashed line, 1.6 km²) as well as sampling locations for soils (filled circles) and sediment (star). Reproduced by permission of Swisstopo (BA14064).

Sampling — Using a hand-held grab sampler operated from a rubber boat, ~2000 cm³ of sediment (top ~15 cm) were collected from one position at the center of the lake (Fig. 2). The lake's surface sediment consists of a black-colored and fine-grained, sapropel mud with a distinct H₂S smell and a high organic carbon content of 10 %. Along the circumference of the lake, we additionally collected topsoil samples (0-10 cm) from 10 locations about 20-30 m away from the shore (Fig. 2). All samples were immediately frozen after sampling and stored at -20 °C until further processing. Sediment and soil samples were lyophilized, sieved (2 mm mesh size) and homogenized with a mortar. Finally, equal volumes of the 10 discrete soil samples were pooled before lipid extraction.

2.2 Lipid extraction, separation and brGDGT analysis

Due to the large volume of sediment required for extraction prior to isotope- and structural analyses, automated extraction methods (e.g. Accelerated Solvent Extraction) were not feasible. Instead, total lipid extracts (TLEs) were obtained by successive ultrasonic extraction (200 g sediment and 100 g of the combined soil sample) with two solvent mixtures consisting of dichloromethane (DCM) and methanol (MeOH) (DCM/MeOH 1:2, v/v; DCM/MeOH 2:1, v/v) and finally twice with DCM. The extracts were combined, concentrated by rotary evaporation, passed through a column packed with sodium sulfate, and completely dried under a gentle stream of N₂. The TLEs were separated into neutral polar fractions over activated Al₂O₃ columns (2.5 x 27 cm, 130 g packing) using 300 ml DCM and 500 ml DCM/MeOH (95:5, v/v), respectively. The brGDGT-containing polar fractions were dried

under N₂ and re-dissolved in hexane (Hex) / isopropanol (IPA) (99:1, v/v) at a concentration of 10 mg ml⁻¹. Small aliquots of these fractions (100 µl) were spiked with a synthetic C₄₆-GDGT as an internal standard (Huguet et al., 2006), passed through a 0.45 µm PTFE filter and analyzed with high performance liquid chromatography - atmospheric pressure positive ion chemical ionization - mass spectrometry (HPLC-APCI-MS). Chromatographic separation and identification of brGDGT isomers were achieved with the analytical setup reported in De Jonge et al. (2014a), while brGDGT concentrations were determined as described in Schouten et al. (2007).

2.3 Isolation of brGDGT isomers by preparative HPLC

Prior to the isolation of specific brGDGT isomers by preparative HPLC, we applied an additional column chromatography step for pre-enrichment. The polar fractions were dried under N₂, re-dissolved in hexane/IPA (99:1 v/v) and transferred onto a column (2.5 x 20 cm) of activated silica gel (58 g). The column was eluted with (a) hexane/IPA (96:4 v/v) followed by (b) hexane/IPA (9:1 v/v) and finally (c) MeOH (250 ml each). Small aliquots of these three fractions were spiked with an internal standard (Huguet et al., 2006) and GDGTs were quantified by HPLC-APCI-MS (Schouten et al., 2007). The first fraction (a) comprised crenarchaeol and all isoprenoidal GDGTs, whereas the second fraction (b) contained >99 % of all brGDGTs. In summary, this additional purification step resulted in a ~15-fold enrichment of brGDGTs in fraction (b) compared to the polar fraction eluted from the Al₂O₃ column. The brGDGT-containing fraction (b) was dried, re-dissolved in hexane/IPA (99:1 v/v) at a concentration of 10 mg ml⁻¹ and separated on a semi-preparative NH₂ column (Econosphere, 10 × 250 mm, particle size 10 µm, pore size 80 Å; Alltech Associates, USA) by multiple injections of 300 µl aliquots. The column was eluted isocratically with 1 % IPA in hexane for 5 min followed by a linear gradient to 2 % IPA in 55 min. The system was cleaned with 10 % IPA and re-equilibrated to initial conditions (10 min each). Individual 1 min fractions were collected, and the GDGT content was assessed by HPLC-MS using conditions described by De Jonge et al. (2014a). The fractions that were most enriched in the new hexamethylated brGDGT isomer (still containing ~30 % of the closely eluting isomers IIIa and IIIa') were pooled subsequently, split into aliquots (10-20 µg in 10 µl solvent) and separated on four analytical Alltima Silica HPLC columns in series (150 mm x 2.1 mm; Grace Alltech; USA) by isocratic elution with 2 % IPA in hexane (0.25 ml min⁻¹). Fractions were collected in 15-s intervals, analyzed following De Jonge et al. (2014a), and pooled into three groups according to the dominance of either compound IIIa, the new isomer IIIa'' or compound IIIa'. The pooled fractions still contained substantial amounts of the "flanking" isomers and were thus re-injected onto the same setup of four consecutive HPLC columns to improve separation of the individual brGDGT isomers. This process was repeated until a minimum of 40 µg of each of the three isomers (IIIa, IIIa'' and IIIa') was obtained with a purity >95 % (as assessed by HPLC-MS).

2.4 Structural assessment and compound-specific stable carbon isotope analysis

The structure and stable carbon isotope composition of the hexamethylated brGDGT isomers isolated from the sediments were determined using the approach of De Jonge et al. (2013). Briefly, aliquots of the three isolates were subjected to 56 % HI in H₂O as described by Schouten et al. (1998a) to cleave the ether bonds linking the alkyl chains with the glycerol backbone. The resulting alkyl iodides were reduced to hydrocarbons with H₂/PtO₂ (Kaneko et al., 2011) and analyzed by gas chromatography (GC) and gas chromatography mass spectrometry (GC-MS).

The carbon isotope composition of the alkyl chains was determined by gas chromatography/combustion- isotope ratio mass spectrometry (GC/C-IRMS) using a Delta V™ Advantage IRMS (Thermo Scientific, USA). Samples containing the hydrocarbons released by ether cleavage were injected at 70°C on a 50 m low-bleed Rxi®-5ms GC column (0.2 mm iD, 0.33 µm df, Restek, USA). After 2 min hold time, the temperature was increased to 130 °C at 20 °C min⁻¹ and then further increased at 4 °C min⁻¹ to 300 °C and held at 300 °C for 35 min. The carbon isotopic composition of the analytes (reported in the δ-notation with respect to the V-PDB carbonate standard) were calibrated externally using an alkane mixture (C₁₆ - C₃₀) with known isotopic compositions (n-alkane mixture B3 provided by A. Schimmelmann, Indiana University, USA). GC/C-IRMS performance was monitored by regular analysis of this standard mixture. The analytical error was ±0.5 ‰ for the HPLC-purified fractions and ±1.5 ‰ for non-purified polar fractions, which were also analyzed. Multiple injections per sample were performed to determine the error in the reported δ¹³C values.

2.4 Bulk analyses

The total organic carbon (TOC) contents of soil and sediment samples were determined by combustion-IR spectrometry using a RC612 multiphase carbon analyzer (LECO, USA). The δ¹³C of bulk samples was assessed by elemental analysis - isotope ratio mass spectrometry (EA-IRMS) on an Integra2 instrument (Sercon, UK). USGS 40 (-26.39 ‰) and EDTA#2 (-40.38 ‰, A. Schimmelmann, Indiana University, USA) were used as reference materials for calibration. For inorganic carbon removal, samples were moistened with Milli-Q® water, exposed to fumes of concentrated HCl for 12 h in a desiccator, and subsequently dried at 50°C prior to analysis by EA-IRMS.

3. Results

3.1 brGDGT distributions in Lake Hinterburg sediments and catchment soils

We applied the recently developed HPLC setup for the analyses of brGDGT isomers (De Jonge et al., 2014a) to lake sediments and catchment soils of lake Hinterburg, allowing us to individually quantify brGDGTs with methyl groups at the 5 (and 5') and 6 (and 6') position. Our analysis revealed that all 15 known brGDGTs (Fig. 1; Weijers et al., 2006; De Jonge et al., 2014a) were present in both the combined catchment soils and in the surface sediment of Lake Hinterburg. The fractional

abundance pattern of brGDGT in the sediment was markedly different to that of the combined catchment soils (Fig. 3). In the sediment, the hexamethylated brGDGTs (III) accounted for 54 % of the total brGDGTs, whereas in the catchment soils, they contributed only 23 %. The total fraction of the penta-methylated brGDGTs (II), on the other hand, was smaller in the lake sediment (34 %) than in the soil sample (47 %). In particular compound IIa was more abundant in the soil (the abundance of compound IIa' was similar in both samples). Also the tetra-methylated brGDGTs (I) were substantially more abundant in soils (33 %) compared to the sediment (11 %).

3.2 Isolation and identification of a novel brGDGT isomer

The HPLC-MS mass chromatogram of the hexamethylated brGDGTs (m/z 1050, Fig. 4C) of the lake sediment indicated the presence of a third, yet unknown isomer (compound IIIa'') eluting between the 5-methyl isomer (IIIa; Sinninghe Damsté et al., 2000) and the recently identified 6-methyl isomer (IIIa', De Jonge et al., 2013). This novel compound accounted for 8.5 % of all analyzed brGDGTs in the lake sediment sample. We isolated the three hexamethylated brGDGT isomers (IIIa, IIIa'' and IIIa') by preparative HPLC (Fig. 4D-F) and investigated the structure of these brGDGTs by analyzing their alkyl moieties. The brGDGT-derived alkanes were analyzed by GC and GC-MS

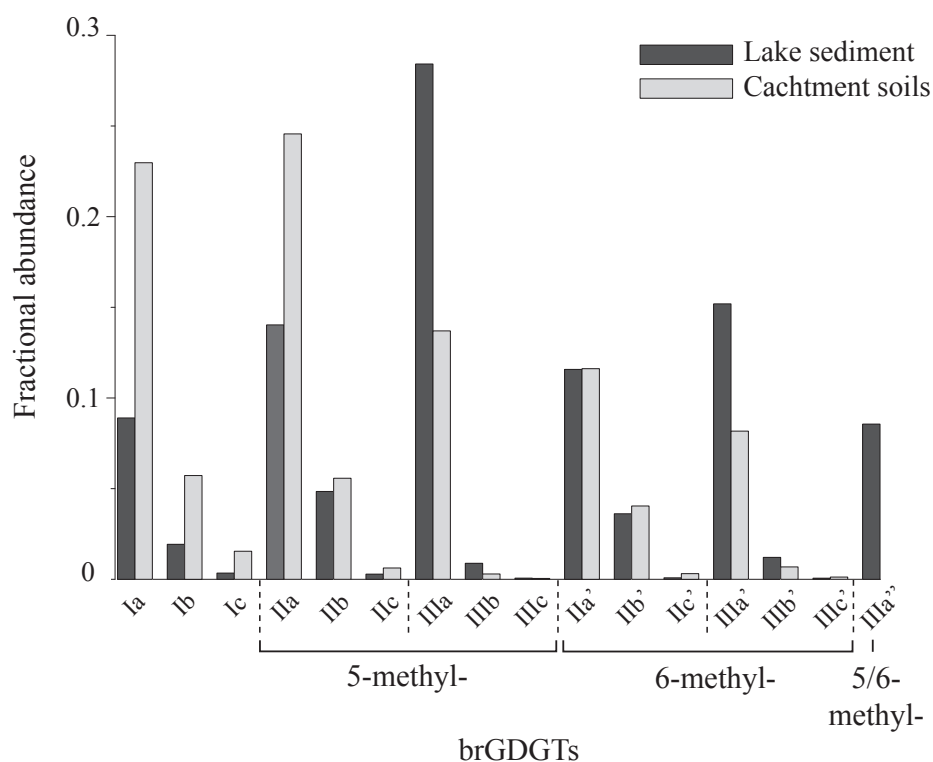


Fig. 3: Fractional abundances of all previously known brGDGTs (including 5- and 6-methyl isomers) and the novel 5/6-methyl brGDGT isomer in the mixed soil and the lake sediment. Compounds are grouped according to the degree of methylation (I: tetra-, II: penta-, III: hexa-methylated) and cyclization (b: one, c: two cyclopentyl moieties; see Fig. 1 for structural information). The novel isomer represents 8.5 % of all brGDGTs. Total brGDGT concentrations were $4.7 \mu\text{g g}^{-1}$ ($36 \mu\text{g g}^{-1}$ TOC) in the sediment and $4.1 \mu\text{g g}^{-1}$ ($30 \mu\text{g g}^{-1}$ TOC) in the mixed soil sample.

(Fig. 5A-C, Table 1) and identified by comparing their Kovats retention indices and electron ionization (EI) mass spectra with previously published data (Sinninghe Damsté et al., 2000; Weijers et al., 2010; De Jonge et al., 2013). The products of brGDGT IIIa were octacosanes with 2, 3 and 4 methyl substituents (hydrocarbons a, b, d; Fig. 5A), confirming its identity as a 5-methyl brGDGT. Minor amounts of the alkyl chains a and d in the brGDGT IIIa-derived hydrocarbon fraction indicate the existence of an asymmetrical isomer of brGDGT IIIa (Fig. 4D, cf. Fig. 1), which cannot be resolved from the symmetrical one with current HPLC setups (De Jonge et al., 2013; Becker et al., 2013). Analysis of the alkyl moieties of brGDGT IIIa' (compounds a, c, e, Fig. 5C) attests to its identity as a 6-methyl brGDGT (De Jonge et al., 2013). The hydrocarbons a and e again reflect the presence of an asymmetric isomer (Fig. 4F). The percent contribution of asymmetric brGDGTs was calculated from the relative abundances of the released alkanes (Table 1), yielding 18 and 29 % for brGDGT IIIa and IIIa', respectively, which is similar to data reported for a Siberian peat (20 and 33 %, respectively; De Jonge et al., 2013).

Ether cleavage of the novel brGDGT IIIa'' (Fig. 5B) yielded the known di- and trimethyloctacosanes (compounds a, b and c) and a novel tetramethyloctacosane (compound f). The relative abundance of the trimethyloctacosanes b and c and their approximate 1:1 ratio indicates that the symmetric form of the novel compound IIIa'' possesses two different trimethyloctacosanyl chains (compounds b and c) with terminal methyl branches at position C5 and C6, respectively, which we refer to as the "5/6-methyl isomer". This assignment is consistent with its HPLC behavior because brGDGT IIIa'' elutes exactly in between the 5-methyl brGDGT IIIa and the 6-methyl brGDGT IIIa' (Fig. 4C). As for the isolated brGDGTs IIIa and IIIa', the minor amounts of hydrocarbons a and f are assumed to originate from an asymmetric isomer, which accounts for 16 % of the novel brGDGT (Fig. 4E). The EI mass spectrum of the unknown branched hydrocarbon f (Fig. 5E) is similar to those of hydrocarbons d and e (data not shown). It contains an ion at m/z 435, representing the molecular ion after loss of one methyl group $[M-15]^+$, and suggesting that compound f is indeed a tetramethyloctacosane. The fragment ions at m/z 365 and 393 indicate a methyl group at position C5 (Weijers et al., 2010). However, compound f also yielded abundant ions at m/z 379 and 351 (both also present in the mass spectrum of compound e), which are characteristic for a methyl group at position C23 (De Jonge et al., 2013). We, therefore, tentatively assign this novel hydrocarbon as 5,13,16,23-tetramethyloctacosane. The C5/C23 positioning of the external methyl groups is also reflected by the fact that it elutes between the 5/24 and the 6/23-methyl isomers (compounds e and d). The experimental Kovats index of compound f (2954) is precisely intermediate between those observed for alkanes e (2950) and d (2959), consistent with the more internal position of one of the methyl groups (Kissin and Feulmer, 1986). In combination with the presence of dimethyloctacosane (compound a), this identification is fully consistent with the existence of the asymmetrical form of brGDGT IIIa''.

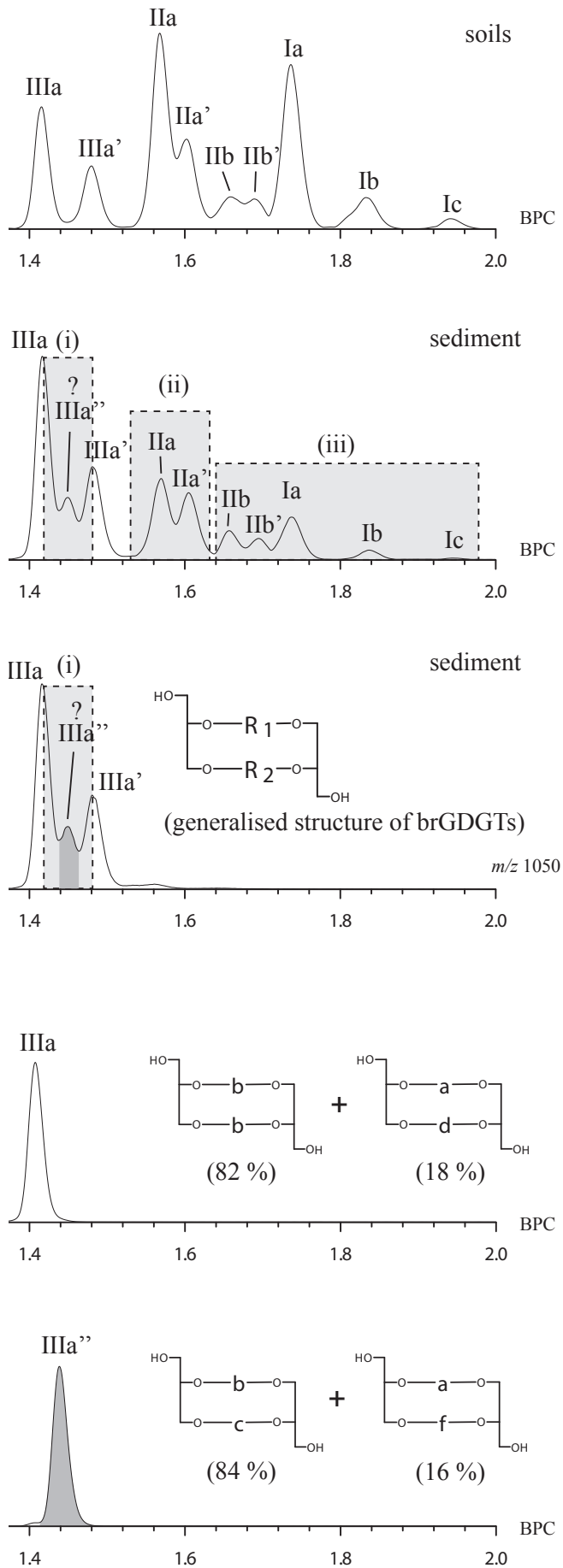


Fig. 4: Base peak chromatograms (BPC) of polar fractions showing the brGDGT content of the combined catchment soil sample (A) and the lake surface sediment (B). Peaks are indexed according to the predominant brGDGTs (upper case roman numerals, cf. Fig. 1). Note the sole presence of a novel brGDGT (IIIa'') in the sediment sample. The m/z 1050 mass trace of the sediment sample (C) further reveals the isomeric nature of the three closely eluting peaks. The first- (IIIa) and the last-eluting peak (IIIa') are the 5-methyl and the 6-methyl isomers of the hexa-methylated brGDGT, respectively. Fractions obtained from the first preparative HPLC step are indicated with lower case roman numerals (i)-(iii) in (B). (D-F) BPCs of fractions obtained from fraction (i) in the second preparative HPLC step. Each isolated peak contains a major symmetric and a minor asymmetric brGDGT isomer. Lower-case letters (a-f) refer to structures in Fig. 5. Retention times were normalized to an internal standard.

3.3 Stable carbon isotope composition of brGDGT-derived alkanes

Alkanes derived from the isolated, hexa-methylated brGDGTs (IIIa, IIIa' and IIIa'') of the sediment sample were all characterized by low $\delta^{13}\text{C}$ values ranging from -42.4 to -46.6 ‰ (Table 1). The averaged $\delta^{13}\text{C}$ value of alkyl chains was slightly lower for the novel brGDGT IIIa'' (-46.5 ‰) than for IIIa and IIIa' (-44.2 and -43.5 ‰, respectively). Since these fractions were purified using preparative HPLC, and brGDGTs were not quantitatively recovered from the parent material, our C-isotope data may to some degree be biased as a result of ^{13}C -isotope fractionation during HPLC purification (Caimi and Brenna, 1997; Smittenberg et al., 2002). In order to estimate the potential biasing effect of the preparation process, we additionally determined the $\delta^{13}\text{C}$ of brGDGT-derived alkanes in the polar fraction, which have not been subjected to HPLC-based purification. There, the trimethyloctacosanes (compounds b and c), which dominantly originate from brGDGTs IIIa

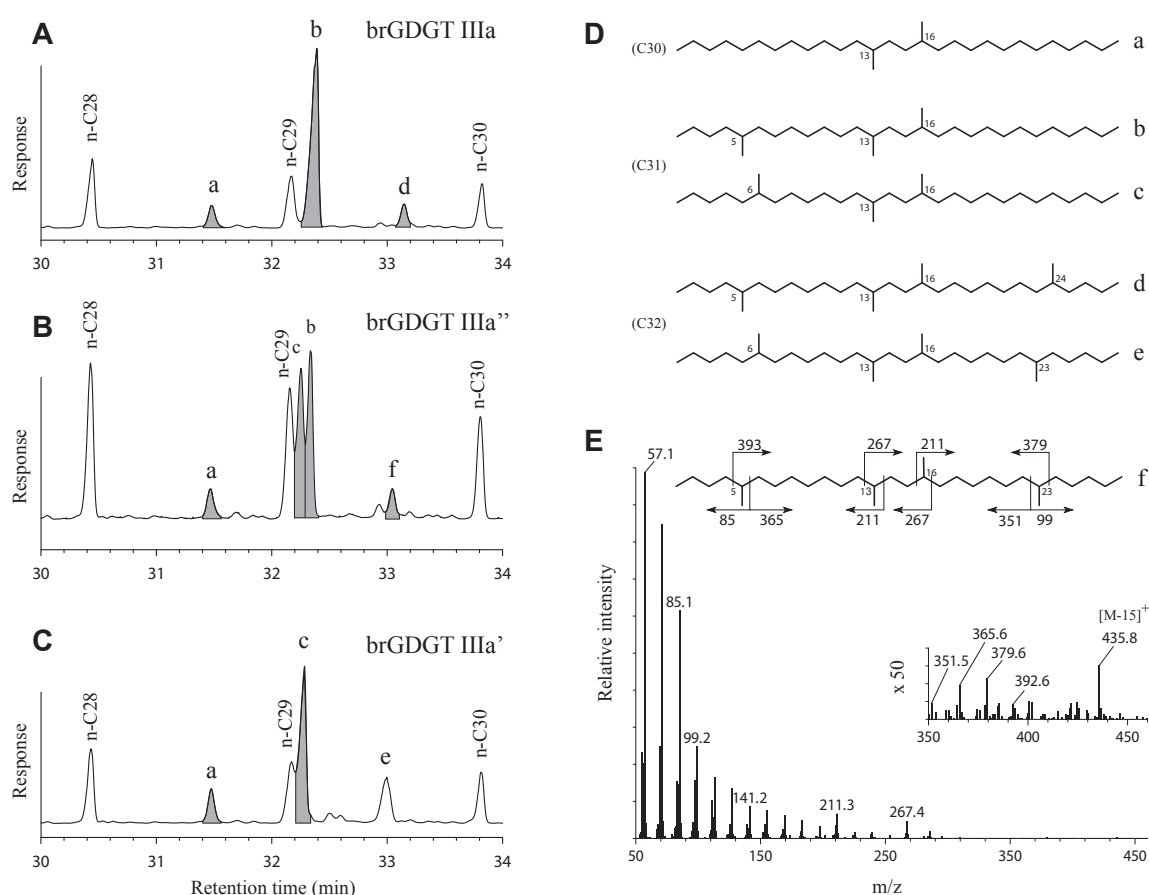


Fig. 5: Partial gas chromatograms of hydrocarbons formed from the three isolated brGDGT isomers IIIa (A), IIIa'' (B) and IIIa' (C) by ether cleavage and subsequent reduction. An n-alkane standard mixture was co-injected along with the samples. The isolates released alkanes with 30, 31 and 32 C-atoms and varying positions of methyl branches (D) upon ether cleavage. A previously unknown C32 branched alkane (f) was released by the novel brGDGT isomer (IIIa''), which was identified as 5,13,16,23-tetramethyloctacosane according to its mass spectrum (E). Note that 6,13,16,23 tetramethyloctacosane (e) was not quantified because it co-eluted with an unknown compound. Instead, we assume equal quantities of compounds (a) and (e), since they both originate from the asymmetrical isomer of IIIa' (De Jonge et al., 2013; cf. Fig. 1).

(45 %), IIIa' (34 %) and IIIa'' (11 %) also had a low $\delta^{13}\text{C}$ signature (-42.4 ‰), similar to the fractions obtained by preparative HPLC (Table 1). This, in combination with the fact that also hydrocarbons generated from other sedimentary brGDGTs displayed similar $\delta^{13}\text{C}$ values (see below), suggests that the isotopic bias caused by preparative HPLC is, if present at all, small in relation to the isotopic offsets we observed in this study (see below).

To assess possible isotopic differences between hexa-, penta- and tetramethylated brGDGTs (cf. structures in Fig. 1), we also investigated the two fractions collected in the first preparative HPLC step, dominantly containing brGDGTs IIa and IIa' (fraction ii in Fig. 4B) and brGDGTs Ia, Ib, IIb, and IIb' (fraction iii in Fig. 4B). Upon ether cleavage, fraction (ii) yielded di- and trimethyloctacosanes at a ratio of about 1:1 (Table 1), reflecting the structure of the pentamethylated brGDGTs IIa and IIa'. GDGT-derived alkanes in fraction (iii), on the other hand, were dominated by dimethyloctacosane (80 %) originating from the tetra-methylated brGDGTs Ia and Ib. The relatively small amounts of trimethyloctacosanes in the latter fraction (20 %) originate from brGDGT IIb and IIb', which co-eluted on the semi-preparative NH_2 column. $\delta^{13}\text{C}$ values of these alkyl chains range between -42.9 and -43.8 ‰, which is, within the analytical error, equivalent to what we observed for the hexamethylated brGDGTs IIIa and IIIa'. Thus, all major brGDGTs in Lake Hinterburg sediments (i.e. without cyclopentyl moieties) are equally depleted in ^{13}C (-43 ‰), with ~3.5 ‰ lower $\delta^{13}\text{C}$ values in the novel brGDGT IIIa'' (-46.5 ‰).

We also investigated the stable carbon isotopic composition of brGDGTs in soils from the Lake Hinterburg watershed. For this purpose, the entire brGDGT fraction was subjected to ether cleavage without pre-separation. The resulting di- and trimethyloctacosanes were characterized by significantly higher $\delta^{13}\text{C}$ values, on average -27.3 ‰ (Table 1), than the hydrocarbons generated from sedimentary brGDGTs.

4. Discussion

4.1 Evidence for *in situ* production of brGDGTs in lakes

The novel, hexamethylated brGDGT IIIa'' is a major component (8.5 %) of the sedimentary brGDGT pool in Lake Hinterburg. Remarkably, it was not detectable in the soils collected from the lake's catchment (Fig. 4A), strongly suggesting an origin from bacteria thriving in the lake water column and/or sediments. Indeed, brGDGT IIIa'' was also not present in quantifiable amounts in a set of 239 globally distributed soil samples (De Jonge et al. 2014a), showing that this 5/6-methyl brGDGT is typically not produced in soil environments. The substantial difference in brGDGT distribution of sediments vs. soils (beyond the presence vs. absence of the novel brGDGT IIIa'') provides additional evidence for a pronounced *in situ* production of brGDGTs in Lake Hinterburg.

Table 1: Carbon isotope composition and relative abundances (RA) of brGDGT-derived alkanes and $\delta^{13}\text{C}$ values of total organic carbon (TOC). Roman numerals (fractions) refer to Fig. 4 and lower case characters (ID) refer to Fig. 5.

sample	fraction	brGDGT content	brGDGT-derived alkanes			$\delta^{13}\text{C}$ (‰ VPDB)	
			<i>preparation</i>	-octacosane	ID	RA	average ^b
sediment	IIIa	IIIa (>95 %) <i>2-step prep. HPLC</i>	13,16-dimethyl-	a	0.09	-44.5 (± 0.5)	-44.2
			5,13,16-trimethyl-	b	0.82	-44.2 (± 0.5)	
			5,13,16,24-tetramethyl-	d	0.09	-44.1 (± 0.5)	
	IIIa''	IIIa'' (>95 %) <i>2-step prep. HPLC</i>	13,16-dimethyl-	a	0.08	-45.9 (± 0.5)	-46.5
			5,13,16-trimethyl-	b	0.40	-46.6 (± 0.5) ^a	
			6,13,16-trimethyl-	c	0.44	-46.6 (± 0.5) ^a	
			5,13,16,23-tetramethyl-	f	0.08	-46.3 (± 0.5)	
	IIIa'	IIIa' (>95 %) <i>2-step prep. HPLC</i>	13,16-dimethyl-	a	0.13	-43.1 (± 0.5)	-43.5
			6,13,16-trimethyl-	c	0.64	-43.9 (± 0.5)	
			6,13,16,23-tetramethyl-	e	0.13 ^d	-42.7 (± 0.5)	
	(ii)	IIa and IIa' <i>1-step prep. HPLC</i>	13,16-dimethyl-	a	0.51	-43.3 (± 0.5)	-43.6
			5,13,16-trimethyl-	b	0.25	-43.8 (± 0.5) ^a	
6,13,16-trimethyl-			c	0.24	-43.8 (± 0.5) ^a		
(iii)	Ia, Ib, IIb, IIb' <i>1-step prep. HPLC</i>	13,16-dimethyl-	a	0.80	-42.9 (± 0.5)	-43.0	
		5,13,16-trimethyl-	b	0.10	-43.1 (± 0.5) ^a		
		6,13,16-trimethyl-	c	0.10	-43.1 (± 0.5) ^a		
polar fraction	all brGDGTs <i>no pre-separation</i>	13,16-dimethyl-	a	0.37	-43.3 (± 1.5) ^c	-42.8	
		5,13,16-trimethyl-	b	0.36	-42.4 (± 1.5) ^{a,c}		
		6,13,16-trimethyl-	c	0.27	-42.4 (± 1.5) ^{a,c}		
		TOC	-	-	-	-34.1 (± 0.2)	-34.1
soil	polar fraction	all brGDGTs <i>no pre-separation</i>	13,16-dimethyl-	a	0.55	-26.9 (± 1.5) ^c	-27.3
			5,13,16-trimethyl-	b	0.22	-27.9 (± 1.5) ^{a,c}	
			6,13,16-trimethyl-	c	0.23	-27.9 (± 1.5) ^{a,c}	
		TOC	-	-	-	-26.3 (± 0.1)	-26.3

^a combined $\delta^{13}\text{C}$ value, determined for the b/c double peak (cf. Fig. 5B)

^b averaged $\delta^{13}\text{C}$ of alkanes in each fraction, weighted by their relative abundances

^c larger errors caused by interferences from background compounds

^d relative abundance inferred from compound a (cf. Fig. 5C)

This offset in brGDGT distribution has been observed in a number of earlier studies. For instance, surface sediments of Lake Challa (Kenya) also displayed substantially higher fractional abundances of brGDGTs IIa and IIIa than soils from the watershed (Buckles et al., 2014b). Buckles et al. (2014b) further found that brGDGT distributions in Lake Challa's water column (SPM and settling particles) were similar to those of the surface sediments, pointing to a dominantly aquatic origin of sedimentary brGDGTs. An increase of hexamethylated brGDGTs (III) in sediments vs. catchment soils has also been reported from high-elevation lakes in Uganda (Loomis et al., 2011) and Tibet (Günther et al., 2014), whereas various lakes from E-Africa (Tierney et al., 2010), Nepal and China (Sun et al., 2011), as well as an eutrophic Swiss lake (Naeyer et al., 2014) showed enrichment in both hexamethylated (III) and pentamethylated (II) brGDGTs. In contrast to this, Wang et al. (2012) found a thus far unique increase of tetramethylated brGDGTs (I) in sediments of a saline lake from Tibet relative to the soil of its watershed. The relative increase observed for certain brGDGTs (sediments vs. soils) is probably related to selective in situ production of these compounds within the lakes. The great

diversity in lacustrine brGDGTs observed thus far, therefore, suggests that in situ production is not restricted to any of the known tetra-, penta- and hexamethylated brGDGTs.

In order to isotopically distinguish between terrestrial and aquatic brGDGT sources, we compared the $\delta^{13}\text{C}$ of brGDGT-derived alkanes from the lake sediment with those from the catchment soils. It needs to be noted that we did not investigate the glycerol moieties of the brGDGTs. These carbohydrates might be enriched in ^{13}C relative to the lipid chains (van der Meer et al., 2001; van Dongen et al., 2002). However, since they only represent ca. 9 % of the brGDGT's C-atoms, we assume the C-isotopic composition of the alkyl chains to be sufficiently representative for the $\delta^{13}\text{C}$ of any given brGDGT (Table 1). In stark contrast to the sedimentary brGDGTs, the di- and trimethyloctacosanes derived from soil brGDGTs were characterized by significantly higher $\delta^{13}\text{C}$ values, on average -27.3 ‰ (Table 1), comparing well with previously published data (-26.7 to -30.1 ‰ in soils covered by C3 vegetation, Weijers et al., 2010). The 16 ‰ offset in the $\delta^{13}\text{C}_{\text{brGDGTs}}$ between sediments and catchment soils allows for a clear distinction between soil and aquatic sources and provides further evidence for in situ brGDGT production within the lake system (see also section 4.2). Moreover, considering the very different abundance pattern of brGDGTs in soils compared to sediments (Fig.3), any admixture of soil-derived brGDGTs to the aquatically produced fraction would affect the $\delta^{13}\text{C}$ values of sedimentary brGDGTs differentially. Therefore, the uniformly low $\delta^{13}\text{C}$ values for all major brGDGTs (not only for the lake-specific brGDGT IIIa'') attest to a predominantly aquatic origin of the total sedimentary brGDGT pool in the studied lake, with no or only a minimal terrigenous contribution.

4.2 Ecology and putative lifestyle of the aquatic source organisms

The identity and ecology of brGDGT-producing bacteria are still not fully understood. Nevertheless, the highly diverse *Acidobacteria* (26 subdivisions, Jones et al., 2009 and references therein) are considered to be the most likely biological source of brGDGTs in terrestrial environments (Weijers et al., 2009; Peterse et al., 2010; Sinninghe Damsté et al., 2011, 2014). Whole-genome sequencing of three members from *Acidobacteria* subdivisions 1 and 3 (Ward et al., 2009) did not yield any evidence for enzymes involved in autotrophic carbon fixation pathways, and all thus far available isolates (including subdivisions 1, 3, 4, 8, 10 and 23) are heterotrophic (see Sinninghe Damsté et al., 2014 for an overview). Heterotrophy of the brGDGT-producing bacteria is also indicated by earlier isotope work. $\delta^{13}\text{C}$ values of brGDGT-derived dimethyloctacosane in a peat core closely followed stratigraphic $\delta^{13}\text{C}$ variations of 17 α ,21 β (H)-homohopane, a compound that has previously been found in heterotrophic bacteria (Pancost and Sinninghe Damsté, 2003). Moreover, the $\delta^{13}\text{C}$ of brGDGTs in arable soils changed from values that are common for C3 vegetation (-27.5 ‰) to more positive values (-18 ‰) after a C3-to-C4 crop change (Weijers et al., 2010), highlighting that plant-derived carbon was incorporated into brGDGTs. On the other hand, there was no indication for the incorporation of ^{13}C -enriched CO_2 emitted from a volcanic vent in Central Italy into the brGDGT

pool of the adjacent soils (Oppermann et al., 2010) showing that, in this setting, brGDGTs do not originate from autotrophic organisms fixing CO₂. Whereas all these studies imply a heterotrophic origin of brGDGTs in terrestrial environments, we can only speculate about the potential modes of nutrition and carbon sources of brGDGT-synthesizing bacteria in lakes.

The reported range for C isotope fractionation associated with autotrophic CO₂ fixation through the most common Calvin-Benson-Bassham (CBB) pathway is large (~5 to 30 ‰, on average 18 ‰) (House et al., 2003). Furthermore, the δ¹³C of the dissolved inorganic carbon substrate (DIC) in lake water may vary seasonally (e.g., Lehmann et al., 2004) and between different sites (Bade et al., 2004). However, assuming a δ¹³C_{DIC} of -10 ‰ (as reported for three eutrophic Swiss lakes; Lee et al., 1987; Lehmann et al., 2004; Teranes and Bernasconi, 2005), and further assuming C-isotopic equilibrium between the dissolved inorganic carbon species (translating into a δ¹³C_{CO₂} of -20 ‰ at 5 °C and near neutral pH; Zhang et al., 1995), autotrophic CO₂ fixation (e.g. via CBB) and additional ¹³C discrimination associated with lipid biosynthesis (~2 to 10 ‰, Schouten et al., 1998b; Hayes, 2001; Sakata et al., 2008) is not inconsistent with the negative δ¹³C values of sedimentary brGDGTs (-43 to -46.5 ‰) observed in Lake Hinterburg.

Nevertheless, previous research suggests that heterotrophic *Acidobacteria* are the most probable source of brGDGTs, at least in terrestrial environments. The bulk biomass of heterotrophic microorganisms generally reflects the C isotopic composition of the carbon source (DeNiro and Epstein, 1976, 1978), with lower δ¹³C signatures in the lipid fraction due to the additional ¹³C discrimination associated with lipid biosynthesis. In soils, our (Table 1) and previously published isotope data (Weijers et al., 2010), however, show that the Δδ¹³C between brGDGTs and TOC (the latter comprising the potential C source of brGDGT-producing organisms) is often smaller than expected (~0.5-3 ‰). This may point to a selective microbial uptake of ¹³C-enriched components of the bulk organic matter pool, e.g. proteins and/or carbohydrates (Weijers et al., 2010 and references therein), or exceptionally small ¹³C discrimination associated with brGDGT biosynthesis. Assuming that the aquatic brGDGT-producing microbes in Lake Hinterburg are equivalent to those in the nearby catchment soils (in terms of heterotrophic lifestyle and enzymatic machinery), the much lower δ¹³C values found for the sedimentary brGDGTs can be only explained with a ¹³C-depleted carbon source of about -42 ‰ within the lake environment. This carbon source could be (1) sedimentary organic matter or (2) suspended/dissolved organic carbon in the water column. In light of the substantially larger Δδ¹³C between brGDGTs and TOC in the sediments of Lake Hinterburg (-9 ‰ at a given δ¹³C_{TOC} of -34‰, Table 1) compared to what has been found in soil environments (max. -3 ‰; Weijers et al. 2010), sedimentary organic matter as a prime carbon source seems rather unlikely. However it is unclear to what extent the bulk δ¹³C_{TOC} of the sediment is representative of the C substrate ultimately incorporated into the bacterial biomass. The δ¹³C_{TOC} may for example be affected by the admixture of refractory terrestrial organic matter that is more inaccessible for microbes, so that we cannot rule out the possibility of (additional) brGDGT production within the lake sediments.

Nevertheless, in eutrophic, seasonally stratified water bodies (such as Lake Hinterburg), recycling of organic carbon, chemoautotrophy and methanotrophy often lead to the buildup of ^{13}C -depleted algal and bacterial biomass, with $\delta^{13}\text{C}$ values in SPM as low as -40 to -45 ‰ (e.g. France, 1995; Hollander and Smith, 2001; Blees et al., 2014a, b). The $\delta^{13}\text{C}$ values of brGDGT reported here are consistent with heterotrophic uptake of such ^{13}C -depleted organic carbon within the water column, and lipid biosynthesis with relatively small C isotope fractionation. Water column production of brGDGTs has also been suggested for other lakes, i.e. Lake Challa, Kenya (Buckles et al. 2014) and Lower King Pond, Vermont, USA (Loomis et al. 2014) where the relative abundances of brGDGTs in catchment soils and the water column were strongly different.

5. Conclusions

We identified a novel hexamethylated brGDGT (5/6-methyl isomer, IIIa'') in sediments of Lake Hinterburg (Switzerland). This brGDGT comprises a symmetric isomer with a 5,13,16- and a 6,13,16 trimethyloctacosanyl chain and, to a lesser extent (16%), an asymmetric isomer containing a 13,16-dimethyloctacosanyl and a 5,13,16,23-tetramethyloctacosanyl moiety. Its absence in soils from the lake's catchment as well as in a set of 239 globally distributed soil samples (De Jonge et al., 2014a) provides circumstantial evidence that this novel brGDGT is produced in situ within the lake environment. Additional evidence for in situ production of brGDGTs in Lake Hinterburg is provided by relatively low $\delta^{13}\text{C}$ values of sedimentary brGDGTs, which are isotopically distinct from soil brGDGTs by ~16 ‰. In analogy to terrestrial systems, a heterotrophic life style of the aquatic, brGDGT-producing microbes is suggested; however autotrophy cannot be fully excluded. Our results have important implication for brGDGT analysis in lacustrine systems. The recognition of the novel, lake-specific isomer clearly highlights the necessity of improved HPLC techniques to further explore the links between brGDGT isomer abundances and environmental parameters such as lake water temperature and pH. Moreover, we demonstrated that the stable carbon isotope composition of brGDGTs can be used to discriminate aquatic from terrigenous sources and thereby help to disentangle the contributions of either brGDGT pool in depositional archives.

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Chapter 3

Incomplete recovery of intact polar glycerol dialkyl glycerol tetraethers (GDGTs) from lacustrine suspended biomass

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Abstract

Branched and isoprenoid glycerol dialkyl glycerol tetraethers (GDGTs) are membrane lipids of bacteria and archaea, respectively, which are often used as proxy indicators in paleolimnological studies. Previous studies, however, have shown that GDGTs are not quantitatively recovered from intact microbial cells by means of commonly applied extraction methods. In order to examine possible biases induced by incomplete extraction, we analyzed suspended particulate matter ($\geq 0.7 \mu\text{m}$, Lake Lugano, Switzerland) using (i) ultrasonic extraction with methanol (MeOH) and dichloromethane (DCM) as solvents, and (ii) modifications of the Bligh-Dyer protocol. Lipid recovery was subsequently assessed by acid hydrolysis of the post-extraction residues, revealing that particularly *branched* GDGTs were poorly recovered from SPM by the MeOH/DCM mixtures (25% – 62%), whereas much better extraction ($>85\%$) was achieved with the Bligh-Dyer method, which includes water as an extraction solvent. However, up to 75% of the Bligh-Dyer-extracted *intact polar* branched GDGTs partitioned into the aqueous phosphate buffer during subsequent phase separation and were not recovered in the total lipid extract (TLE). In contrast, when the P-buffer was substituted with 5% aqueous trichloroacetic acid (TCA), only 13% were lost into the aqueous phase. Depending on the used protocol, the distribution of the intact polar lipid (IPL) -derived GDGTs varied substantially, which may complicate the IPL-based calibration of GDGT proxies (e.g. MBT/CBT; TEX_{86}). Our results suggest that IPL-GDGTs of both bacteria and -archaea thriving in lakes can be difficult to extract, and caution is advised when targeting the ‘viable’ GDGT pool in aquatic environments.

1. Introduction

Glycerol dialkyl glycerol tetraethers (GDGTs) are organic compounds synthesized by microorganisms that thrive ubiquitously in both aquatic and terrestrial environments. They are cellular membrane lipids of many *Archaea* and certain *Bacteria*, and contain isoprenoid (isoGDGTs) or branched (brGDGTs) hydrocarbon chains, respectively, as their central structural building blocks (Fig. S1; S2). It has been shown that the relative abundances of specific GDGTs correlate with environmental parameters, such as sea or lake surface temperature (Schouten et al., 2002; Powers et al., 2010), mean annual air temperature, and soil pH (Weijers et al., 2007; Peterse et al., 2012; De Jonge et al., 2014a; Ding et al., 2015), attesting to their potential as paleoclimatic proxy indicators in aquatic sediments (see Schouten et al., 2013 for a review).

Despite the widespread application of GDGTs in paleolimnological studies (e.g. Fawcett et al., 2011; Niemann et al., 2012; Woltering et al., 2014; Buckles et al., 2015; Loomis et al., 2015; Keisling et al., 2016), the identity and ecology of the source organisms (in particular those of bacterial, i.e., branched, GDGTs) is not well constrained. One way to gain insight into GDGT biosynthesis in the environment is to specifically target and quantify the intact polar lipid (IPL) -derived GDGT fraction (Schoon et al., 2013; Buckles et al., 2014; De Jonge et al., 2014b). IPL-GDGTs represent the functional form of GDGTs within the membranes of living cells, and contain polar head groups

(e.g. phosphohexose and [poly] hexose) that are linked to the core lipid (CL) via ester and glycosidic bonds (Biddle et al., 2006; Schouten et al., 2008; Schubotz et al., 2009; Liu et al., 2010; Peterse et al., 2011). Because these head group moieties are comparably labile after cell death (Harvey et al., 1986; Logemann et al., 2011), higher relative amounts of IPL-GDGTs in the environment are commonly attributed to the presence of living GDGT-producing organisms (Biddle et al., 2006; Schouten et al., 2012; Buckles et al., 2013). Nevertheless, recent studies suggest that IPLs, at least to some extent, may be preserved in sediments over geological timescales (Lengger et al., 2013; Xie et al., 2013). GDGTs without polar head groups (i.e., CL-GDGTs), on the other hand, are only minor components in the lipid pools of living microbes ($\leq 10\%$, Huguet et al., 2010a; Elling et al., 2014). Most of the CL-GDGTs abundantly present in environmental samples are therefore believed to derive from the corresponding IPL precursors after loss of their polar head group(s). IPL-GDGTs in lipid extracts are commonly analyzed in their intact form using electro spray ionization mass spectrometry (ESI-MS) when information on the type of head groups is primarily desired (e.g. Peterse et al., 2011; Zhu et al., 2013), whereas for quantification and for the determination of paleoenvironmental proxy indices, head groups are typically removed by acid hydrolysis, and the ‘IPL-derived CL-GDGTs’ are then analyzed via atmospheric-pressure chemical-ionization (APCI) -MS (e.g. De Jonge et al., 2014b).

Extraction of IPL-GDGTs from environmental samples commonly follows a modified Bligh-Dyer protocol (Bligh and Dyer, 1959), which was specifically designed for the extraction of intact eukaryotic and bacterial phospholipids. Currently, two basic variants of the original protocol are widely applied, in which the solvent mixture contains either phosphate buffer (White et al., 1979), an aqueous solution of trichloroacetic acid (Nishihara and Koga, 1987), or a combination of both (Sturt et al., 2004). It has been reported, however, that during extraction of archaeal cultures, only a minor fraction of the membrane IPLs is recovered in the total lipid extract (TLE) (Huguet et al., 2010a; Cario et al., 2015). Similarly, branched GDGTs and their putative precursor molecules produced by acidobacterial isolates were almost completely inaccessible by common extraction techniques, including the Bligh-Dyer method (Sinninghe Damsté et al., 2011, 2014). In these studies, the lipids of both living archaea and bacteria could only be fully recovered after acid hydrolysis of the cells, suggesting that also the GDGT-synthesizing microbes in the environment may be, to some degree, resistant towards solvent extraction. Since hydrolysis of samples and/or the extracted residues is not commonly applied in environmental studies, the ‘living fraction’ of GDGTs and its contribution to the total lipid pool could be substantially underestimated. Moreover, selective GDGT recovery from living microbes may lead to biases in the measured GDGT distributions, potentially affecting GDGT-based proxy indices derived from the IPL pool.

In order to further examine the effect of, and the possible reasons for, incomplete lipid recovery, we determined and compared yields of IPL- and CL-GDGTs in lacustrine suspended particles (i) after ultrasonic extraction using methanol (MeOH) and dichloromethane (DCM) as solvents (Huguet et al., 2010b; Ingalls et al., 2012), and (ii) after extraction following modifications of the Bligh-Dyer

protocol. In addition, we quantified the fraction of GDGTs not recovered in the TLE by acid hydrolysis of (i) the extraction residues, and (ii) the aqueous phase of the Bligh-Dyer solvent mixture, both of which are normally discarded.

2. Materials and Procedures

2.1 Materials

Suspended particulate matter (SPM) was collected from Lake Lugano (Switzerland; 46°00'12"N, 9°00'14"E) at two depths by *in situ* filtration of lake water in September 2014 (Experiment#1; 70 m and 275 m) and June 2016 (Experiment#2; 275 m). In order to prevent rapid clogging, and to increase loading capacity, particles were collected on a double layer of glass fiber filters, consisting of a 0.7 μm base- and a 2.7 μm pre-filter (nominal pore size; Whatman GF/F and GF/D, respectively), which were subsequently processed together. The filters were frozen immediately on dry ice after sampling, stored at -80 °C, and lyophilized prior to lipid extraction.

2.2 Procedures

2.2.1 Lipid Extraction

Filters were split into equal parts and cut into small pieces. For the MeOH/DCM extraction (modified from Ingalls et al., 2012) the lyophilized filter material was placed in a PTFE tube and repeatedly extracted in an ultrasonic bath (six times; 5 min each), twice using methanol (MeOH), twice with dichloromethane (DCM)/MeOH (1:1, v:v), and twice with DCM (30 ml each). The tube was centrifuged after each extraction, and the supernatants were combined. The resulting total lipid extract (TLE) was concentrated by rotary evaporation (27 °C), filtered through a pasteur pipet plugged with pre-cleaned quartz wool, and completely dried under a gentle stream of N_2 . In order to estimate the reproducibility of this extraction procedure, the filter material from 70 m was extracted in triplicate.

Another part of lyophilized filter material was placed in glass centrifuge tubes and extracted using a modified Bligh-Dyer protocol (e.g. Pitcher et al., 2009). Briefly, the filter pieces were sonicated in an ultrasonic bath (three times, 10 min each) in 20 ml of a solvent mixture consisting of MeOH:DCM:phosphate buffer (2:1:0.8, v:v:v), centrifuged, and all supernatants were combined. Phase separation was achieved by altering the solvent composition to MeOH:DCM:phosphate buffer (1:1:0.9, v:v:v) and subsequent centrifugation. The lipid-containing DCM phase was transferred to a round-bottom flask, and the aqueous phase was extracted twice more with DCM. The DCM phases were combined, and the resulting TLE was concentrated by rotary evaporation (27 °C), filtered through pre-cleaned quartz wool and dried under N_2 . For SPM from 275 m (in Experiment#2), a second part of the filter material was subjected to a modified Bligh-Dyer extraction as described above, however, the phosphate buffer was substituted with 5% trichloroacetic acid (TCA) (Nishihara and Koga, 1987).

All glass ware involved in subsequent transfers of the extracts obtained by either extraction protocol was rinsed at least 3 times with MeOH and subjected to sonication in order to minimize the loss of IPL-GDGTs with sugar head groups (i.e., glycolipids), as the latter have been shown to adsorb on glass surfaces (Pitcher et al., 2009).

2.2.2 Sample processing and GDGT analysis

The concentration and composition of IPL-GDGTs were determined indirectly following the ‘subtraction method’ described by Huguet et al., 2010a). Briefly, CL-GDGTs were quantified in the TLE before and after acid hydrolysis (Fig.1) and the excess of GDGTs detected in the hydrolyzed aliquot is assumed to derive from the IPL-GDGT pool. To this end, the TLEs obtained by either extraction method were re-dissolved in DCM:MeOH (1:1, v:v) using an ultrasonic bath, and 0.1 μg of an internal standard (a C_{46} tetraether; Huguet et al., 2006) was added. The TLE was then split in two halves, one of which was subsequently processed for CL-GDGT analysis (see below). The other half was transferred to a glass reaction vial, and hydrolyzed in 2 ml 1.5 N methanolic HCl (4 h, 70 °C). After cooling, 2 ml MilliQ water were added to the vial. Subsequently, the hydrolyzed

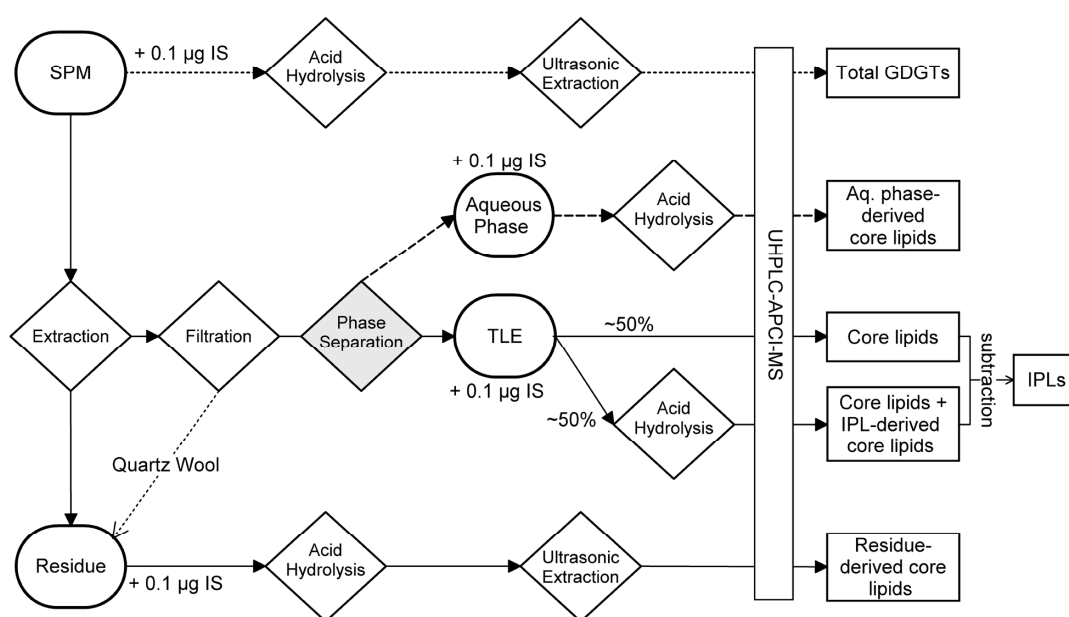


Fig. 1: Flow chart illustrating the major sample processing steps (rhombuses) for the quantification of GDGTs. An internal standard (IS) was added to the total lipid extracts (TLEs), sample residues, and aqueous phases, respectively. Note that the ‘phase separation’ step (gray) only applies to the Bligh-Dyer extractions. For Experiment#2, the aqueous Bligh-Dyer solvent phases were acid-hydrolyzed and analyzed separately (dashed arrows). Direct acid hydrolysis of the sample (dotted arrows) was used to independently determine the total amount of recoverable GDGTs (Experiment#2 only).

lipids (i.e., CL- plus IPL-derived CL-GDGTs) were extracted twice with 2 ml DCM and dried under a stream of N₂.

To quantify the GDGTs still remaining in the SPM samples, the residual filter material from both extraction methods, together with the quartz wool used for filtration of the respective TLE was placed into a capped glass flask containing 0.1 µg internal standard (IS). The residue was then hydrolyzed with 25 ml 1.5 N methanolic HCl (5 h, 70 °C). After cooling, the reaction mixture was transferred into a PTFE tube, placed in an ultrasonic bath for 5 min, and centrifuged. The supernatant was decanted into a separatory funnel, and the residue was subsequently extracted by ultrasonication, twice with 25 ml DCM:MeOH (1:1, v:v), and once with 25 ml DCM (5 min each). After centrifugation, all supernatants were combined in the separatory funnel. MilliQ water (60 ml) was added to the mixture to achieve phase separation, and the DCM phase (containing the core lipid GDGTs released from the residue) was concentrated by rotary evaporation, and dried completely under N₂. To independently quantify the total pool of recoverable GDGTs, another aliquot of the lyophilized filter material from 275 m (Experiment#2), together with 0.1 µg IS, was directly subjected to acid hydrolysis and subsequently extracted, as described above for the residual material.

We also probed for the presence of GDGTs in the aqueous phases of the Bligh-Dyer solvent (Experiment#2 only) by subjecting the P-buffer- and the TCA phase (~50 ml each), respectively, to acid hydrolysis (5 h, 70 °C), by adding 10 ml concentrated HCl (32%) and 0.1 µg IS. After cooling, 50 ml MeOH and 25 ml DCM were added and the mixture was sonicated (20 min). Phase separation was achieved in a separatory funnel by addition of DCM and MilliQ water (50 ml each), and the organic phase was concentrated and dried under a stream of N₂.

Prior to analysis, all TLE aliquots and hydrolysates were separated over activated Al₂O₃ using DCM and DCM:MeOH (1:1, v:v) as eluents. The second, GDGT-containing fraction was dried under N₂, re-dissolved in hexane:isopropanol (99:1, v:v), and passed through a 0.45 µm PTFE filter, prior to analysis by ultra-high-performance liquid chromatography positive-ion atmospheric-pressure chemical-ionization mass spectrometry (UHPLC-APCI-MS). Analytical separation of GDGTs was achieved on an Agilent 1260 UHPLC device equipped with two UHPLC silica columns (BEH HILIC columns, 2.1 × 150 mm, 1.7 µm; Waters) in series, and quantified on an Agilent 6130 quadrupole MSD in selected ion monitoring mode under conditions described in Hopmans et al. (2016). A 1:1 mixture of the IS (m/z 744) and Crenarchaeol (m/z 1292) was analyzed before each sequence of samples.

2.2.3 Data treatment and statistical analysis

The GDGT concentrations in the individual fractions were calculated using the added internal standard (Fig.1) and its MSD response factor relative to Crenarchaeol. The CL-GDGT content measured in the non-hydrolyzed aliquot of the TLE was then subtracted from the content determined in the hydrolyzed one, yielding the amount of IPL-GDGTs that were present in the TLE prior to hydrolysis (i.e., IPL-derived core lipids; Huguet et al., 2010a).

Statistical analyses of GDGT distributions were performed using the software package R ('vegan' package, Oksanen et al., 2015). To this end, GDGTs that were below the methodological detection limit in any of the analyzed fractions were removed, and the concentrations of the remaining branched and isoprenoid GDGTs, respectively, were normalized separately. The fractional abundances of each compound were then scaled through division by the standard deviation and subjected to non-metric multi dimensional scaling (NMDS), using the Canberra distance metric (Lance and Williams, 1966).

3. Assessment

3.1 GDGT yields

Core-lipid GDGTs (CL-GDGT) accounted for 20% – 70% of the all recoverable GDGTs (i.e., IPL + CL + residue + aqueous phase), and showed remarkably consistent extraction yields across all three tested protocols (Tab. 1; Fig.2). In the MeOH/DCM-extracted residues, we found substantial amounts of branched GDGTs after acid hydrolysis (38% to 75%), whereas the residue yields of isoprenoid GDGTs were much lower (14% to 36%). All Bligh-Dyer-extracted residues (P-buffer and TCA), in contrast, contained comparably minor amounts of either GDGT class (4% to 13%). In turn, substantially more IPL-GDGTs were observed in the TLEs obtained by the Bligh-Dyer method as compared to MeOH/DCM extractions. Yet, while absolute isoprenoid IPL-GDGT yields were similar for either modification of the BD protocol ($\pm 4\%$), ~ 5 times more branched IPL-GDGTs were detected in the TLE when the phosphate buffer was substituted by 5% trichloroacetic acid (TCA), as revealed by Experiment#2 (SPM from 275 m). In other words, when the BD solvent contained phosphate-buffer (P-BD), more than 50% of the brGDGTs were not recovered in the TLE (neither as CLs nor as IPLs) (Table 1; Fig.2).

In order to examine this apparent loss of GDGTs during sample processing, we investigated the aqueous phases of the BD-extracts obtained from Experiment#2 (i.e., P-buffer and 5% TCA) by subjecting them to acid hydrolysis. We found that almost 60% of the total brGDGTs were present in the P-buffer after P-BD extraction. However, when 5% TCA was used instead (TCA-BD), the brGDGTs were almost entirely recovered in the TLE (78%), and only a minor fraction (9%) was lost into the aqueous TCA phase. In stark contrast to the brGDGTs, much less isoGDGTs were present in the aqueous phases, accounting for $\sim 1\%$ and 9% of total recovery in the P-BD and TCA-BD extractions, respectively (Experiment#2; Table 1).

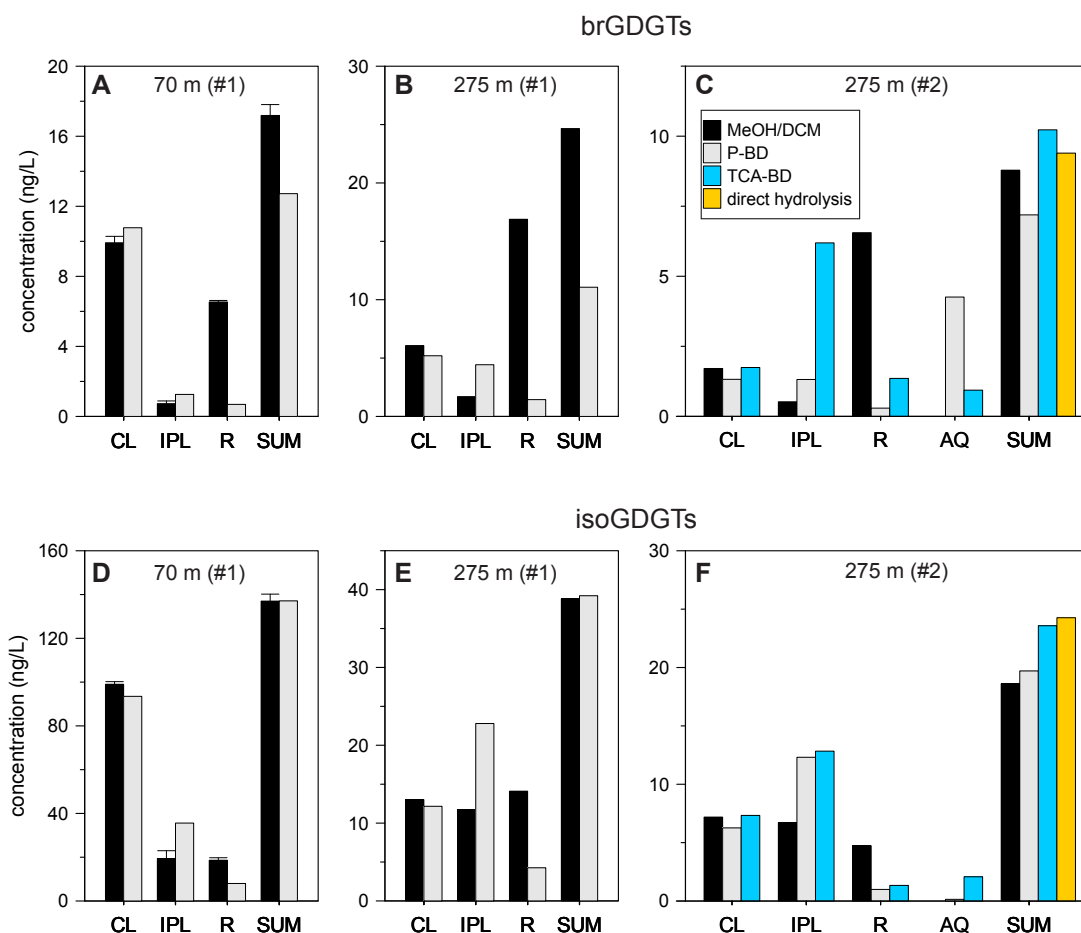


Fig. 2: Concentrations of branched (A-C) and isoprenoid (D-F) GDGTs in the core lipid (CL), intact polar lipid (IPL), and residue (R) fractions, as well as in the aqueous phase of the Bligh-Dyer solvent (AQ) for suspended particulate matter (SPM) collected from two depths in Lake Lugano. Cumulative concentrations are given for each experiment (SUM). Note that the increase in IPL-brGDGT yields in the TCA-BD- vs. MeOH/DCM extraction (Experiment#2; Panel C) is accompanied by a corresponding decrease in residual brGDGTs (cf. Table 1). For Experiment#1, the aqueous phase of the Bligh-Dyer extracts were not tested for the presence of GDGTs. Error bars indicate the standard deviation for SPM from 70 m depth (Experiment#1), where MeOH/DCM extractions were performed in triplicate.

In Experiment#2, the sum of GDGTs in all recovered fractions ranged between 77% and 109% relative to the amounts released by direct hydrolysis of the SPM (Table 1). The totaled yields of both branched- and isoprenoid GDGTs were highest for the TCA-BD method, while reasonable recovery of brGDGTs (94%) was still achieved with the MeOH/DCM protocol.

Table 1: Concentrations of GDGTs in SPM.

Sample	Method	fraction	brGDGTs (ng L ⁻¹)	isoGDGTs (ng L ⁻¹)
70 m (#1)	MeOH/DCM	CL ^a	9.9 ±0.37	99 ±1.20
		IPL ^b	0.7 ±0.15	19.4 ±3.61
		R ^c	6.5 ±0.09	18.6 ±1.14
		SUM ^e	17.2 ±0.62	137 ±3.19
70 m (#1)	Bligh-Dyer (P-buffer)	CL ^a	10.8	93
		IPL ^b	1.3	36
		R ^c	0.7	8.0
		SUM ^e	12.7	137
275 m (#1)	MeOH/DCM	CL ^a	6.1	13.0
		IPL ^b	1.7	11.7
		R ^c	16.9	14.1
		SUM ^e	24.7	38.8
275 m (#1)	Bligh-Dyer (P-buffer)	CL ^a	5.2	12.2
		IPL ^b	4.4	22.8
		R ^c	1.4	4.3
		SUM ^e	11.1	39.2
275 m (#2)	MeOH/DCM	CL ^a	1.7	7.2
		IPL ^b	0.5	6.7
		R ^c	6.6	4.7
		SUM ^e	8.8	18.6
275 m (#2)	Bligh-Dyer (P-buffer)	CL ^a	1.3	6.3
		IPL ^b	1.3	12.3
		R ^c	0.3	1.0
		AQ ^d	4.3	0.1
		SUM ^e	7.2	19.7
275 m (#2)	Bligh-Dyer (TCA)	CL ^a	1.7	7.3
		IPL ^b	6.2	12.8
		R ^c	1.4	1.3
		AQ ^d	0.9	2.1
		SUM ^e	10.2	23.6
275 m (#2)	acid hydrolysis		9.4	24.3

(a): Core lipids; (b) intact polar lipids; (c) released by hydrolysis of the extraction residue; (d) released by hydrolysis of the aqueous phase; (e) sum of all analyzed fractions; Standard deviations are given for the triplicate extraction (italic face).

3.2 GDGT distributions

In order to assess potential method-dependent compositional biases, we examined the core lipid distribution of br- and isoGDGT in the individual fractions by multivariate statistics. Ordination by non-metric multi dimensional scaling (NMDS) revealed that the GDGT composition in the CL pools were consistent between all extraction methods applied in this study (Fig. 3; S3A), in agreement with the concentration data. However, the type of extraction protocol strongly affected the core lipid composition in the IPL-, the residue-, and the aqueous phase-derived GDGT fractions (Fig. 3; S3B-D). Remarkably, brGDGT distributions in the aqueous P-buffer, the IPL fraction obtained by the

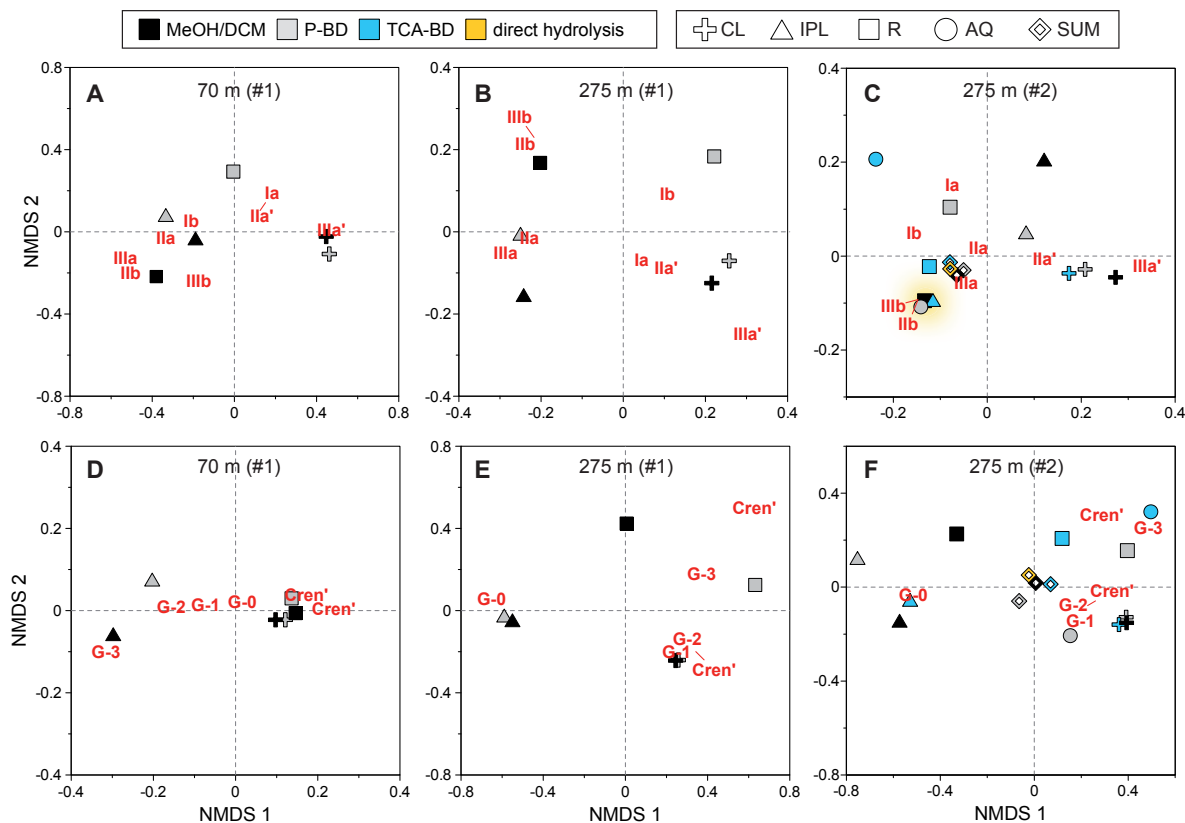


Fig. 3: Non metric multi dimensional scaling (NMDS) plots showing method-dependent differences in branched (A-C) and isoprenoid GDGTs (D-F) compositions in the core lipid- (crosses), intact polar lipid- (triangles), and residual fractions (squares), as well as in the aqueous phases of Bligh-Dyer extracts (circles). Bulk GDGT compositions (open diamonds) are based on the sum of all recovered fractions (Experiment#2 only). The distance between symbols reflects the dissimilarity in GDGT distribution. Note the almost identical compositions in the three highlighted fractions. Scores of the most abundant brGDGTs and all analyzed isoGDGTs (cf. Figs. S1; S2) are shown in red. Data of the sample analyzed in triplicate (70 m) were averaged before statistical analysis.

TCA-BD method, and the residue pool after MeOH/DCM extraction were almost identical (Fig.3C). The isoGDGT compositions in these three fractions, however, were markedly distinct (Fig.3F). In addition, these compositional offsets among the recovered fractions varied between the two sampled depths (70 m and 275 m), and to a lesser extent, between the two sampling campaigns (#1 and #2), for both GDGT classes (Fig.3). When the GDGT abundances in all recovered fractions from Experiment#2 were totaled and subsequently normalized, the resulting ‘cumulative’ branched GDGT distributions were fairly similar to the composition in the direct sample hydrolysate (obtained by extraction *after* acid hydrolysis), for both branched and isoprenoid GDGTs (Fig. 3C,F; S3E).

4. Discussion

4.1 Differential extractability of branched and isoprenoid IPL GDGTs

The consistent yields and distributions of branched and isoprenoid CL-GDGTs obtained by either of the three extraction protocols indicate that CLs represent a well-defined and readily extractable lipid pool in SPM from Lake Lugano. In contrast, the remaining fraction of the GDGT pool (i.e., the ‘non-CL’ pool) displayed a highly differential behavior depending on the type of extraction method used. Most obviously, near-quantitative GDGT extraction from SPM (in particular of IPL-*br*GDGTs) could only be achieved with the Bligh-Dyer protocol that includes an aqueous phase (P-buffer or 5% TCA) as part of the solvent mix, which corroborates the importance of water in enhancing IPL recovery (c.f. Fig. 2C,F). The majority of these ‘recalcitrant’ *br*GDGTs extracted with the P-BD method showed a strong affinity to the aqueous phase, attesting to a pronounced hydrophilicity of the IPL-*br*GDGTs present in the extract prior to phase separation. This suggests that the bacterial IPL-GDGTs possess extraordinarily large and/or polar head groups, which may still be attached to (polar) macromolecular components of the cell wall. The use of 5% TCA, however, seems to strongly reduce these hydrophilic properties, indicating either (i) pH-controlled polarity changes within the IPLs’ head groups, e.g. by protonation of ionic moieties such as carboxylic acids or phosphate groups or (ii) partial hydrolysis of the (macromolecular) polar moieties originally attached to the *br*GDGTs.

In stark contrast to the bacterial tetraethers, the archaeal *iso*GDGTs did not show any considerable affinity to the aqueous P-buffer (<1% of total recovery) but were, however, markedly more hydrophilic in the presence of TCA (9% of total recovery). Although the longer alkyl chains of archaeal GDGTs (c.f. Fig. S1; S2) make them slightly less polar than the bacterial ones, we argue that structural differences within the lipid cores cannot explain such contrasting physicochemical properties and their alteration by TCA. Instead, we reason that considerable differences must exist between the two lipid classes with regard to the type (and/or size) of the polar moieties bound to the core lipids. Thus far, IPL head group chemistry was established for *br*GDGTs extracted from peat (Liu et al., 2010; Peterse et al., 2011), and for *iso*GDGTs derived from marine SPM and sediments (e.g. Schubotz et al., 2009; Meador et al., 2015), as well as from archaeal cultures of the mesophilic *Thaumarchaeota* (Schouten et al., 2008; Pitcher et al., 2010, 2011; Sinninghe Damsté et al., 2012). Interestingly, while (poly)hexoses, phospho-hexoses and phospho-glycerol were common head groups in both GDGT classes, only the *branched* IPL-GDGTs contained glucuronic acid (GlcA). Given the acid equilibrium of GlcA ($\text{R-COOH} \rightleftharpoons \text{R-COO}^- + \text{H}^+$), we further hypothesize that GlcA-IPL-*br*GDGTs are more polar/hydrophilic under neutral- (P-buffer) vs. acidic (TCA) pH, which may contribute to the much larger amounts of branched vs. isoprenoid GDGTs in the aqueous P-buffer. Moreover, our findings suggest that previous studies analyzing the *br*GDGTs’ head groups in P-BD extracts were likely biased towards IPL classes that are dominantly hydrophobic. That is to say, because the aqueous P-buffer phase (potentially containing hydrophilic IPLs) is commonly not further analyzed,

IPL-brGDGTs comprising larger and/or highly polar moieties may have remained un-detected. At this point, we can only speculate that GDGTs in bacteria may comprise an as of yet unconstrained class of IPLs that are not, or to a lesser extent, synthesized by archaea. Future studies should attempt to obtain a more complete image of the polar lipidomes of GDGT-producing microbes, i.e., by extending the analytical window towards hydrophilic- and higher molecular weight IPLs.

4.2 Fractionation of specific IPL-derived core lipid GDGTs during extraction

To further elaborate on the method-dependent compositional shifts in the ‘non-CL’ GDGT fractions, we took a closer look at the relative partitioning of individual core lipids between the fractions recovered in Experiment#2 (i.e., IPL-, residue-, P-buffer-, and TCA fractions). Consistent with the observed compositional differences (Fig. 3C,F; S3), the fractionation of individual core lipids showed considerable variability between each of the three extraction protocols tested (Fig. 4). For instance, brGDGTs IIc and IIc’ exhibited a much weaker solvent resistance and hydrophilicity than the average of all bacterial tetraethers, whereas the affinity to the TCA phase was noticeably increased for IIIc, Ia, and Ic. As for the *iso*GDGTs, the crenarchaeol regio-isomer (Cren’) was extracted much less effectively than the remaining archaeal lipids, and GDGT-3 (G-3) was disproportionally more abundant in the TCA fraction (Fig. 4C, S3D). This different ‘behavior’ may be due to a preferential association of certain types of head groups with specific GDGT cores (Sinninghe Damsté et al., 2012; Elling et al., 2014), and could further indicate that protective cellular structures that may hamper effective extraction are unevenly distributed among the multitude of GDGT-producing microbial taxa. In fact, core lipid distributions are known to be highly variable within the clade of ammonia oxidizing archaea (AOA) (Pitcher et al., 2010, 2011; Kim et al., 2012), with the fractional abundance of Cren’, for example, spanning a factor of 50 among Group I.1a and I.1b *Thaumarchaeota* (Sinninghe Damsté et al., 2012). Based on our results, we therefore speculate that particularly the Cren’-enriched archaea in Lake Lugano may possess extraordinarily robust cell walls that are extracted quantitatively only with the aid of TCA. Moreover, we reason that the distinct compositional trends in the IPL- and residue fractions at 70 m vs. 275 m (Fig.3) are likely linked to phylogenetic differences in the GDGT-producing microbial communities residing at different water depth and redox regimes.

4.3 Recalcitrance of brGDGT-producing microbes towards organic solvent extraction

It has previously been reported that not all GDGTs present in lake sediments, soils, and/or peatlands are accessible through common extraction techniques (i.e., P-BD, Soxhlet-, and accelerated solvent extraction – ASE) (Huguet et al., 2010c, 2010d; Tierney et al., 2012; Chaves Torres and Pancost, 2016). In all of these studies, considerable amounts of GDGTs could still be released from the post-extraction residues by acid hydrolysis, giving rise to the hypothesis that parts of the GDGT pool had been ‘insolubilized’ (i.e., by encapsulation in organic macromolecules, in organo-mineral

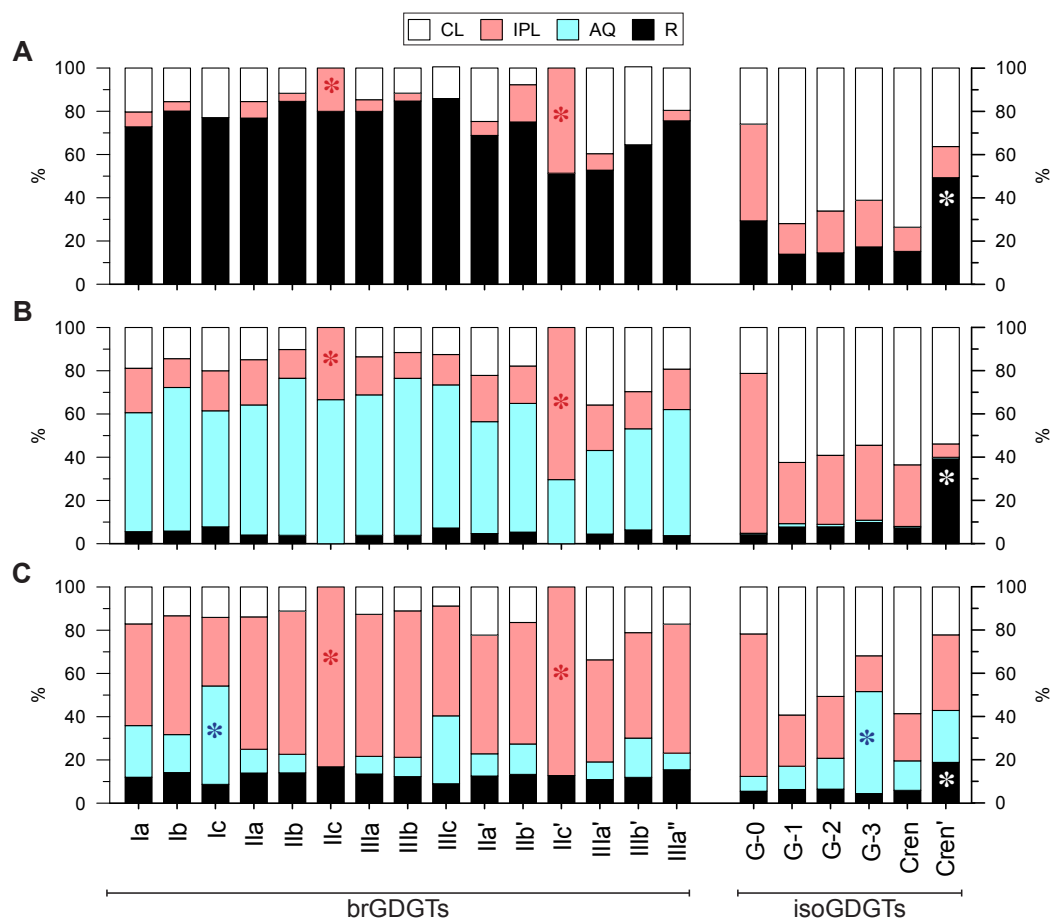


Fig. 4: Relative partitioning of individual branched- and isoprenoid GDGT core lipids in Experiment#2 (275 m SPM) between CL- (white), IPL- (pink), residue- (black), and aqueous fractions (light blue), for different extraction methods: (A) MeOH/DCM, (B) Bligh-Dyer with P-buffer, (C) Bligh-Dyer with TCA. Shown is the percentage relative to the totaled amount of the respective GDGT in all recovered fractions (i.e., CL + IPL + residue + aqueous phase). Compound names refer to structures in Figs. S1 and S2. Above average contributions mentioned in the text are indicated with an asterisk. brGDGT IIIc' was below detection in some fractions and is not shown.

complexes, or in soil- or sediment aggregates) (see Lützow et al., 2006 for review). The ‘recalcitrant’ *br*GDGT fraction in Lake Lugano SPM may originate either from (i) allochthonous organic matter from catchment soils and/or re-suspended slope sediments that may have undergone such ‘insolubilization’ through pedogenic/diagenetic reactions, or (ii) from autochthonous (i.e., ‘fresh’) microbial biomass produced within the water column. Here, we examine this issue by comparing the amount and composition of GDGTs in the various lipid pools. The striking similarity between the MeOH/DCM-insoluble fraction and the IPL-*br*GDGTs obtained by the TCA method, both in terms of composition (Fig. 3C) and absolute abundance (Table 1), strongly suggests that the *br*GDGTs resilient to ultrasonic extraction with MeOH/DCM mixtures are, in fact, dominantly IPLs and thus probably derive from (recently) living microbes that possess an ‘intrinsic recalcitrance’ towards

organic solvent extraction. Indeed, previous work on pure cultures of *Acidobacteria* subdivisions 1,3, and 4, has demonstrated that *brGDGTs* and iso-diabolic acid-derivatives (i.e., the structural building blocks of the major *brGDGTs*) almost exclusively occur in a form that is not recoverable by regular P-BD extraction, whereas other types of membrane lipids (mostly branched C15 – C17 fatty acids) were readily extracted. (Sinninghe Damsté et al., 2011, 2014). This suggests that either (i) *brGDGT* synthesis is associated with a specific solvent resistant phenotype of the investigated strains, or (ii) the intact polar *brGDGTs* and -iso-diabolic acids comprise extraordinarily hydrophilic head group moieties, which may have resulted in their selective loss during phase separation (c.f. Section 4.1).

In fact, several studies have reported bacterial strains that are capable to survive and grow in the presence of high concentrations of organic solvents (up to 90% vol.), which are usually toxic for most microorganisms (Inoue and Horikoshi, 1989; Kato et al., 1996; Junker and Ramos, 1999; Sardesai and Bhosle, 2002). Besides other possible mechanisms of solvent-tolerance (Rodríguez-Herva et al., 1996; Ramos et al., 1997; Torres et al., 2011; Segura et al., 2012), it was suggested that hydrophilic, external polysaccharide capsules (Kasper, 1986) may serve as a physical barrier, preventing solvents from penetrating and damaging underlying parts of the cell wall and the plasma membrane (Zahir et al., 2006). It therefore seems reasonable to assume that the water contained in the Blich-Dyer solvent mix may have facilitated the permeabilization of such polysaccharide layers, leading to the disintegration and solubilization of the cells through ultrasonication. Members of the phylum *Acidobacteria*, in fact, are known to form such capsules (Kishimoto et al., 1991; Kulichevskaya et al., 2010, 2012; Okamura et al., 2011). Nevertheless, since the microbial sources of most *brGDGT* core lipid types have not yet been undoubtedly identified through culturing, we cannot draw any definite conclusion with regards to the cellular mechanisms behind the solvent-resistance we observed.

Conclusions

We demonstrated that up to 75% of the *bacterial* IPL-GDGTs present in lacustrine SPM can be lost into the aqueous P-buffer phase during Bligh-Dyer extraction, suggesting that previous studies may have substantially underestimated the ‘viable’ fraction of brGDGTs in aquatic environments. Furthermore, the choice of the extraction protocol has a marked effect on the IPL-derived core lipid distributions of both branched and isoprenoid GDGTs, which has consequences for the calculation of GDGT-based environmental proxy indices, and may thus complicate their calibration using the IPL fraction (e.g. in cultures or within the water column). For example, we observed method-dependent compositional variations within the IPL-GDGT pool that translate into relative offsets of up to $\pm 4.6^{\circ}\text{C}$ for TEX_{86} -derived lake surface temperatures and ± 0.9 for brGDGT-based pH estimates (Table S1). Such procedural biases are, however, less pertinent to paleo applications, in which the CL-GDGTs are the analytical target. Moreover, our data strongly suggest that particularly *br*GDGT-producing bacteria thriving in the water column of lakes are resistant towards extraction techniques that solely rely on organic solvents. For near-quantitative recovery of both bacterial and archaeal IPL-GDGTs, we therefore recommend using a modified Bligh-Dyer extraction with 5% TCA (instead of P-buffer). If only the core lipid- (and not the head group-) composition of GDGTs is of interest, residue hydrolysis after MeOH/DCM extraction may be employed instead to access the recalcitrant GDGT pool. Finally, for quick determination of bulk GDGT composition, direct acid hydrolysis prior to extraction represents a valid alternative.

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3.1. Supplementary information

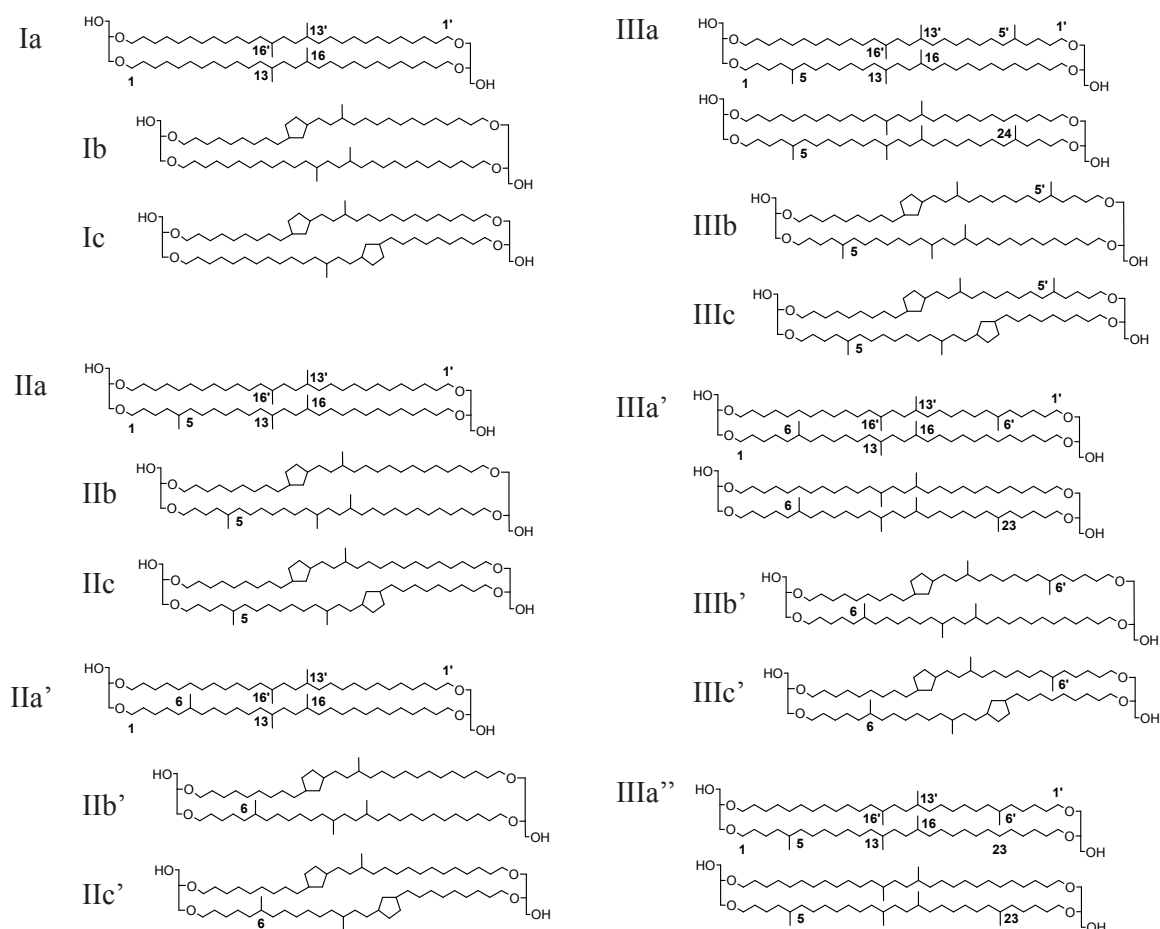


Fig. S1: Structures of the analyzed branched GDGTs including 5-methyl isomers (Sinninghe Damsté et al., 2000), 6-methyl isomers (indicated with ' '); De Jonge et al., 2013, 2014), and the recently discovered 5/6-methyl isomer (IIIa''; Weber et al., 2015).

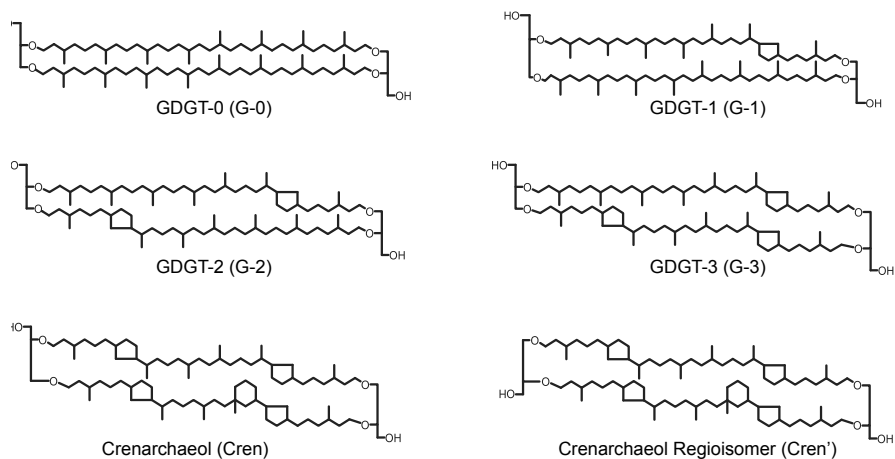


Fig. S2: Structures of analyzed isoprenoid GDGTs, modified from (Schouten et al., 2002)

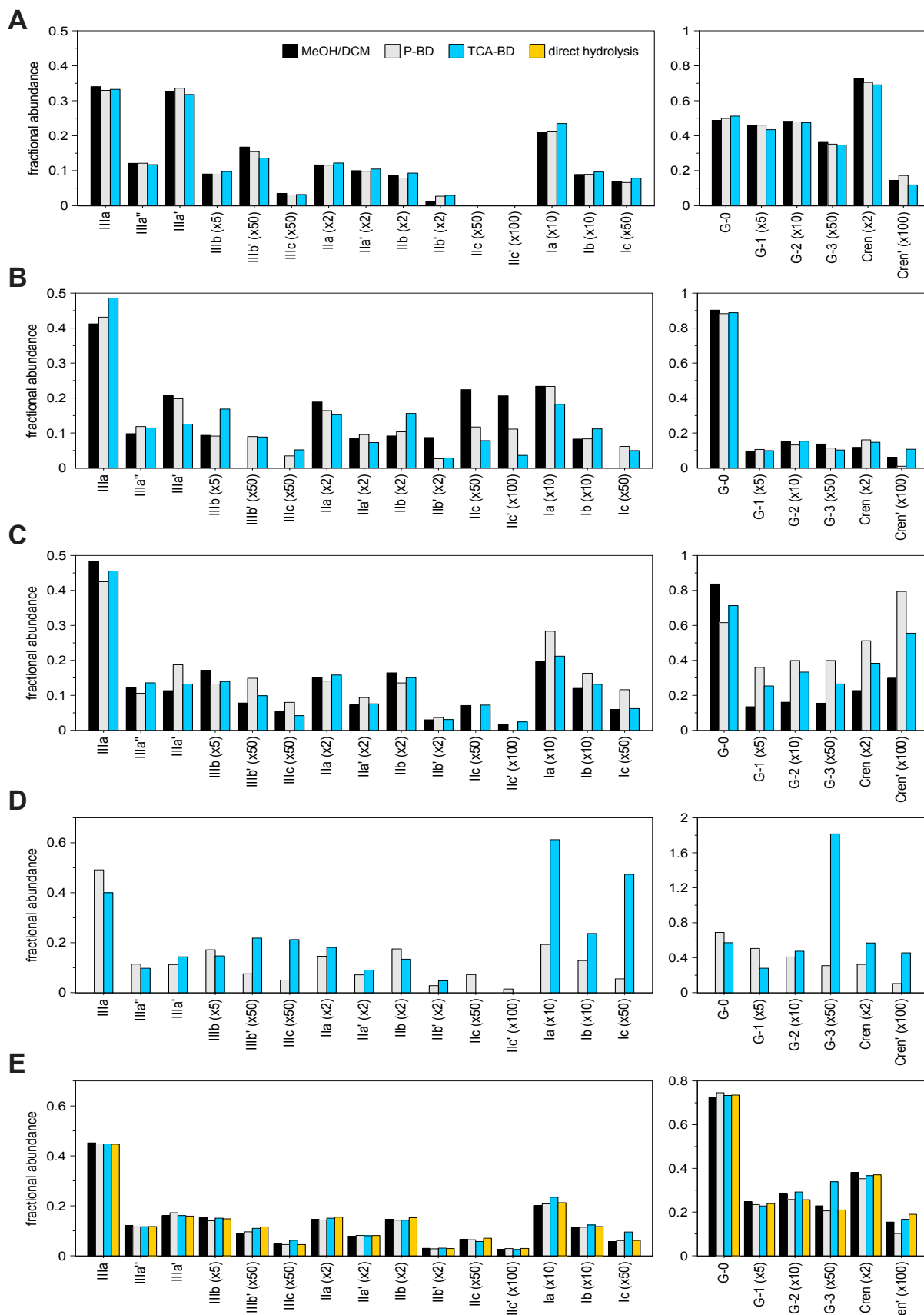


Fig. S3: Comparison of branched- and isoprenoid GDGT distributions in the CL- (A), IPL- (B), residue- (C), and aqueous fractions (D) from Experiment#2 (SPM 275 m). Cumulative compositions (E) are based on the sum of all recovered fractions. For better visibility, data of minor GDGTs were scaled by a factor (value in parentheses).

Table S1: Proxy indices and corresponding mean annual air temperature- (MAT), lake surface temperature- (LST), and pH estimates based on branched- (a-c), isoprenoid- (d, e), and both GDGT classes (f).

Sample	Method	fraction	MBT _{5me} ^a	CBT _{5me} ^a	MBT _{5me} ^b	IBT ^c	MAT _{mm} (°C) ^d	pH(CBT _{5me}) ^a	pH(IBM) ^d	TEX86 ^e	LST(°C) ^f	BIT ^g
70 m (#1)	MeOH/DCM	CL	0.174 (0.005)	0.411 (0.007)	0.012 (0.000)	2.87 (0.012)	7.2 (0.004)	7.1 (0.01)	2.7 (0.016)	0.41 (0.002)	8.8 (0.10)	0.16 (0.006)
		IPL	0.027 (0.017)	0.112 (0.093)	0.028 (0.015)	4.86 (0.177)	6.8 (0.238)	7.6 (0.16)	0.1 (0.226)	0.49 (0.015)	13.0 (0.80)	0.10 (0.009)
		R	0.027 (0.000)	-0.168 (0.009)	0.025 (0.001)	3.73 (0.003)	6.8 (0.004)	8.1 (0.02)	1.5 (0.004)	0.44 (0.002)	10.0 (0.09)	0.38 (0.013)
		SUM	0.050 (0.001)	0.010 (0.012)	0.016 (0.000)	3.08 (0.013)	7.1 (0.008)	7.8 (0.02)	2.4 (0.016)	0.43 (0.001)	9.8 (0.03)	0.20 (0.002)
70 m (#1)	Bligh-Dyer (P-buffer)	CL	0.160	0.425	0.012	2.90	7.2	7.1	2.6	0.41	8.7	0.18
		IPL	0.031	0.107	0.041	4.58	6.8	7.7	0.5	0.46	11.7	0.08
		R	0.096	0.129	0.020	4.20	7.1	7.6	1.0	0.43	9.6	0.14
		SUM	0.096	0.305	0.013	3.02	7.2	7.3	2.5	0.43	9.7	0.16
275 m (#1)	MeOH/DCM	CL	0.100	0.157	0.095	3.40	6.7	7.6	2.0	0.41	8.7	0.50
		IPL	0.046	0.244	0.149	4.42	5.7	7.4	0.7	0.53	15.1	0.68
		R	0.054	-0.082	0.178	3.35	5.8	8.0	2.0	0.57	17.7	0.84
		SUM	0.062	-0.024	0.142	3.06	6.0	7.9	2.4	0.48	12.7	0.71
275 m (#1)	Bligh-Dyer (P-buffer)	CL	0.109	0.148	0.104	3.43	6.7	7.6	1.9	0.41	8.4	0.48
		IPL	0.047	0.118	0.172	4.01	5.6	7.6	1.2	0.44	10.6	0.68
		R	0.092	0.015	0.166	4.23	6.5	7.8	0.9	0.62	20.2	0.43
		SUM	0.076	0.115	0.127	3.30	6.2	7.6	2.1	0.47	11.7	0.53
275 m (#2)	MeOH/DCM	CL	0.064	0.179	0.075	0.96	6.1	7.5	5.1	0.38	7.1	0.38
		IPL	0.052	0.338	0.097	1.53	5.1	7.3	4.4	0.49	12.9	0.54
		R	0.046	0.003	0.164	0.82	5.7	7.8	5.3	0.45	10.9	0.91
		SUM	0.049	0.047	0.130	0.55	5.8	7.8	5.6	0.41	8.6	0.68
275 m (#2)	Bligh-Dyer (P-buffer)	CL	0.066	0.214	0.073	1.05	6.2	7.5	5.0	0.38	7.0	0.35
		IPL	0.053	0.244	0.112	1.21	5.5	7.4	4.8	0.42	9.3	0.54
		R	0.074	0.070	0.155	1.94	6.1	7.7	3.8	0.44	10.1	0.50
		Aq.	0.046	-0.037	0.169	1.03	5.8	7.9	5.0	0.32	3.8	0.99
275 m (#2)	Bligh-Dyer (TCA)	SUM	0.051	0.048	0.127	0.61	5.8	7.8	5.5	0.40	7.9	0.64
		CL	0.070	0.177	0.082	0.93	6.1	7.5	5.1	0.39	7.6	0.38
		IPL	0.043	0.024	0.146	0.80	5.6	7.8	5.3	0.48	12.6	0.85
		R	0.053	0.054	0.159	1.40	5.7	7.7	4.5	0.47	11.7	0.82
275 m (#2)	direct hydrolysis	Aq.	0.138	0.224	0.303	1.40	6.6	7.5	4.5	0.61	19.8	0.57
		SUM	0.057	0.072	0.147	0.46	5.8	7.7	5.7	0.45	11.0	0.67
		CL (total)	0.051	0.050	0.136	0.49	5.7	7.8	5.7	0.40	8.0	0.64

(a): De Jonge et al., 2014a; (b): Dang et al., 2016; (c): Ding et al., 2015; (d): Xiao et al., 2015; (e): Schouten et al., 2002; (f): Powers et al., 2010; (g): Hopmans et al., 2004; standard deviation in parentheses

Supplementary text to Table S1:

We considered only those GDGT-based proxies and -calibrations that rely on the improved chromatographic separation of 5-methyl and 6-methyl brGDGTs (cf. De Jonge et al., 2014b; Hopmans et al., 2016, Fig. S1). These were from left to right:

$$\text{MBT}'_{5\text{me}} = (\text{Ia} + \text{Ib} + \text{Ic}) / (\text{Ia} + \text{b} + \text{Ic} + \text{IIa} + \text{IIb} + \text{IIc} + \text{IIIa}), \text{ (De Jonge et al., 2014a);}$$

$$\text{CBT}_{5\text{me}} = -\log((\text{Ib} + \text{IIb}) / (\text{Ia} + \text{IIa})), \text{ (De Jonge et al., 2014a);}$$

$$\text{MBT}'_{6\text{me}} = (\text{Ia} + \text{Ib} + \text{Ic}) / (\text{Ia} + \text{Ib} + \text{Ic} + \text{IIa}' + \text{IIb}' + \text{IIIa}'), \text{ (Dang et al., 2016);}$$

$$\text{IBT} = -\log((\text{IIIa}' + \text{IIa}') / (\text{IIIa} + \text{IIa})), \text{ (Ding et al., 2015);}$$

$$\text{MAT}_{\text{mr}} (\text{°C}) = 7.17 + 17.1 * \text{Ia} + 25.9 * \text{Ib} + 34.4 * \text{Ic} - 28.6 * \text{IIa}, \text{ (De Jonge et al., 2014a);}$$

$$\text{pH}(\text{CBT}_{5\text{me}}) = 7.84 - 1.73 * \text{CBT}_{5\text{me}}; \text{ (De Jonge et al., 2014a);}$$

$$\text{pH} = 6.33 - 1.28 * \text{IBT}; \text{ (Xiao et al., 2015),}$$

$$\text{TEX}_{86} = (\text{G-2} + \text{G-3} + \text{Cren}') / (\text{G-1} + \text{G-2} + \text{G-3} + \text{Cren}'), \text{ (Schouten et al., 2002);}$$

$$\text{LST} (\text{°C}) = -10 + 50.8 * \text{TEX}_{86}, \text{ (Powers et al., 2010);}$$

$$\text{BIT} = (\text{Ia} + \text{IIa} + \text{IIIa}) / (\text{Ia} + \text{IIa} + \text{IIIa} + \text{Cren}), \text{ (Hopmans et al., 2004).}$$

*TEX*₈₆-based lake surface temperatures derived from the IPL fractions are highlighted. (bold italic face).

Chapter 4

Redox-dependent niche differentiation of tetraether producing bacteria: Evidence for multiple branched GDGT sources in lakes

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Abstract

Terrestrial paleoclimate archives such as lake sediments are essential for our understanding of the continental climate system and for the modeling of future climate scenarios. Yet, quantitative proxies for the determination of paleotemperatures are sparse. The relative abundances of certain bacterial lipids, i.e., branched glycerol dialkyl glycerol tetraether (brGDGT), respond to changes in environmental temperature, and thus have great potential for climate reconstruction. Their application to lake deposits, however, is hampered by the lack of fundamental knowledge on the identity and ecology of brGDGT-producing microbes in lakes. Here, we show that brGDGT are synthesized by multiple groups of bacteria thriving under contrasting redox regimes in a deep meromictic Swiss lake (Lake Lugano). This niche partitioning is evidenced by highly distinct brGDGT inventories in oxic- vs. anoxic water masses, and corresponding vertical patterns in bacterial 16S-rRNA gene abundances, implying that sedimentary brGDGT records are affected by temperature-independent changes in the community composition of their microbial producers. Furthermore, the stable carbon isotopic composition ($\delta^{13}\text{C}$) of brGDGTs in Lake Lugano and 34 other (peri-)Alpine lakes attest to the widespread heterotrophic incorporation of ^{13}C -depleted, methane-derived biomass at the redox transition zone of meso-eutrophic lake systems. The brGDGTs produced under such hypoxic/methanotrophic conditions reflect near-bottom water temperatures, and are characterized by comparatively low $\delta^{13}\text{C}$ values. Depending on climate zone and water depth, lake sediment archives predominated by deep water/low- ^{13}C brGDGTs may provide much more reliable records of long-term climate variability than those where brGDGTs derive from multiple terrestrial and/or aquatic sources with distinct temperature imprints.

Significance

Reliable prediction of future climate conditions requires a thorough understanding of climate variability throughout Earth's history. Microbial molecular fossils, such as bacterial membrane-spanning tetraether lipids (brGDGTs), have proven to be particularly useful for the assessment of past climatic conditions, because they occur ubiquitously in the environment and show compositional changes related to temperature. However, the identity and ecology of brGDGT-producing bacteria is largely unknown, and a mechanistic basis for brGDGT-based paleoclimate reconstruction is still lacking. Here, we present novel insights into the ecological parameters that affect brGDGT synthesis in lakes, which will facilitate a more causative understanding of brGDGT lipid records preserved in lacustrine sedimentary archives.

Introduction

Accurate prediction of future climate depends on our understanding of the present and past climate system. As such, the assessment of paleoclimatic conditions on timescales exceeding instrumental records is indispensable for evaluating the performance of existing climate models. The numerical representation of the terrestrial climate system is particularly challenging due to the heterogeneous nature of continental environments (1), underlining the urgent need for paleoclimate data from inland areas. Lake sediments represent the most important type of terrestrial climate archives, because they are widely distributed, provide high temporal resolution due to fast and continuous sedimentation, and contain organic compounds as carriers of paleoclimatic information, which are often well-preserved (2).

Molecular fossils such as bacterial tetraether membrane lipids (branched glycerol dialkyl glycerol tetraethers – brGDGTs; Fig. 1) preserved in sediments provide important information on past climate conditions (e.g. 3). These insights into the past are possible due to the empirical correlation of the brGDGT distribution in contemporary soils with mean annual air temperature (MAT) and pH (4, 5), which in turn gave rise to the hypothesis that brGDGT-producing microbes adjust the composition of their membranes in response to changing environmental

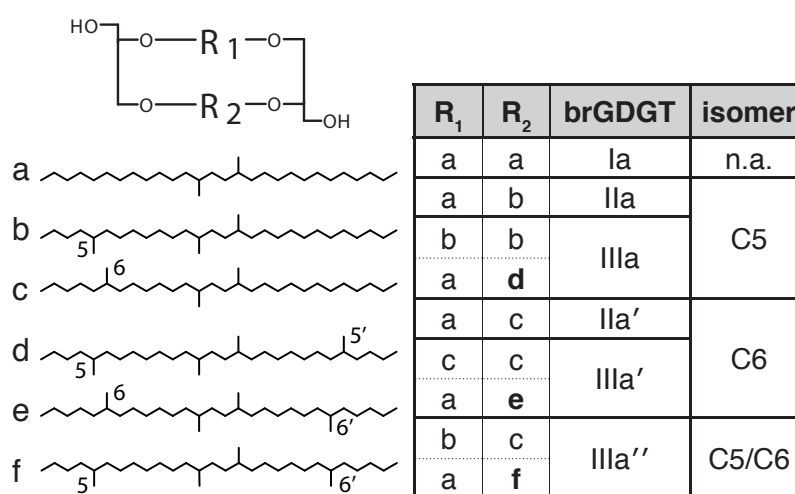


Fig.1. Structures of the major tetra- (Ia), penta- (IIa, IIa'), and hexamethylated (IIIa, IIIa', IIIa'') brGDGT core lipids, containing dimethyl- (a), trimethyl- (b, c), and tetramethyloctacosanes (d, e, f) as the central alkyl moieties (R1, R2). Positions of the peripheral methyl branches are given to denote brGDGT isomer classes, which are referred to as 'C5-', 'C6-', and 'C5/6-methylated' brGDGTs. Minor brGDGTs with cyclopentyl moieties are shown in *SI Appendix*, Fig. S1. Note that brGDGTs IIIa and IIIa' and IIIa'' each comprise a symmetric and an asymmetric isomer that are not distinguishable by liquid chromatography, with the asymmetric ones comprising distinctive alkyl moieties (d, e and f; bold face).

conditions (membrane lipid homeostasis; 6). Quantitative links between the brGDGT composition and latitudinal/altitudinal MAT gradients are evident also in surface sediments of lakes (7, 8), attesting to the potential of brGDGTs as lacustrine paleotemperature proxies.

Despite the ubiquitous presence of brGDGTs in both terrestrial and freshwater environments, knowledge on the identity and ecophysiology of their source organisms is still severely limited. The glycerol stereochemistry of brGDGTs in peat revealed that these compounds are produced by bacteria (9), which is remarkable in light of the fact that the vast majority of cultured bacterial strains exclusively contain ester- (instead of ether) bound membrane lipids. So far, only a few members of the diverse and still largely uncharacterized phylum *Acidobacteria* have been found to produce building blocks of brGDGTs, i.e. *iso* diabolic acid, and, to a much lesser degree, full brGDGT structures (10, 11). *Acidobacteria* represent an important component of microbial communities in soils and peat bogs (12, 13), and their abundance seems to be related to that of brGDGTs (9, 14). In contrast, information on their occurrence and diversity in lakes is extremely rare (e.g. 15–17), and potential links to aquatic brGDGT synthesis are uncertain.

In order to pinpoint the hotspots of brGDGT biosynthesis within the water column, and to constrain the ecology of their microbial sources in lakes, we investigated the distribution and stable carbon isotopic composition of brGDGTs in the water column of Lake Lugano (Switzerland), as well as in surface sediments of 34 other lakes in the European Alps. In Lake Lugano, we further compared the vertical distribution and abundance of bacterial 16S-rRNA genes with that of individual brGDGT isomers, and assessed the potential for aquatic brGDGT synthesis by stable isotope probing of the lipids. We provide conclusive evidence for lacustrine brGDGT production by spatially segregated microbial communities thriving under both oxic- and hypoxic/anoxic conditions, and the export of deep water-derived brGDGTs to the sediments. These novel insights into the ecology of tetraether-producing bacteria are important for unraveling the biological source(s) of these ubiquitous bacterial lipids and will allow for a more causative understanding of brGDGT paleo records in lake deposits.

Results and Discussion

Multiple microbial brGDGTs sources in Lake Lugano

The North Basin of Lake Lugano is 288 m deep, mesotrophic, and meromictic, with a permanent oxycline at ~100 m and a seasonal thermocline at 10–20 m water depth (Fig. 2A). The brGDGT pool in suspended particulate matter (SPM) was dominated by non-cyclic hexamethylated brGDGTs comprising three structural isomers (i.e., IIIa, IIIa', and IIIa''; Fig. 1), which accounted for up to 82 % of all brGDGTs in the water column (data for the less abundant brGDGTs are shown in *SI Appendix*, Fig. S2 and S3). The organic carbon-normalized total concentration of brGDGTs were low in near-surface waters (~4 ng g⁻¹ at 10 m depth), but increased ~25-fold just

below the thermocline (Fig. 2B). Similarly, the fraction of intact polar brGDGTs that still retain the labile head group moieties present in living cells, increased with depth from ~30 % at the base of the thermocline to >70 % (Fig. 2B), attesting to pronounced brGDGT synthesis in the deeper lake.

We found profound changes in the relative contribution of brGDGTs IIIa, IIIa', and IIIa'' throughout the water column (Fig. 2C). While brGDGT IIIa showed a continuous increase with depth across the redox transitions zone (RTZ), IIIa' was most abundant within oxygenated waters ~20 m above the oxic-anoxic interface, and rapidly decreased below. In stark contrast to all other brGDGTs, the IIIa'' isomer, which was recently identified in anoxic sediments of another Alpine lake (18), occurred exclusively at depths below 90 m. Moreover, it was neither

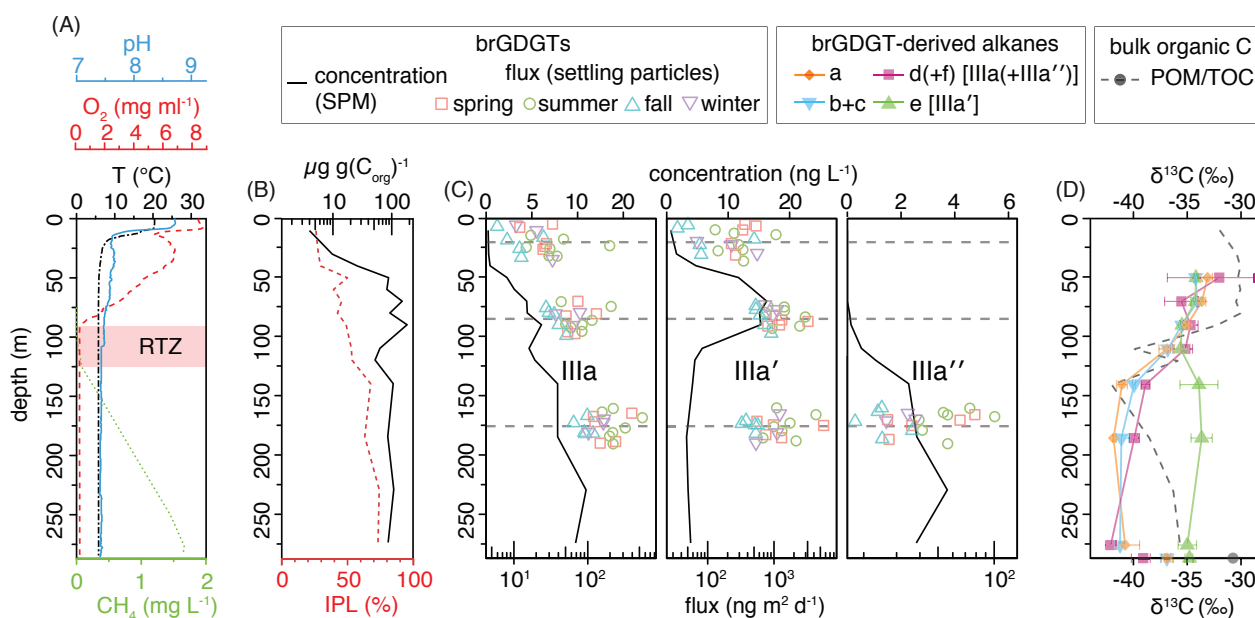


Fig. 2. Redox-dependent segregation and ¹³C depletion of brGDGTs in Lake Lugano (Switzerland). **(A)** In situ dissolved oxygen (dashed-), dissolved methane (dotted-), water temperature (dot-dashed-), and pH (solid line) in Sept. 2014. The water layer with methanotrophic activity is referred to as the redox transitions zone (RTZ) and highlighted in red. **(B)** Organic carbon- (C_{org}) normalized total concentration of brGDGTs (black solid line), as well as the relative contribution of intact polar lipids (IPLs) to the total brGDGT pool (dashed red line) in suspended particles (SPM) collected in Sept. 2014. **(C)** Concentration of the three predominant brGDGT isomers (IIIa, IIIa', and IIIa'') in the water column (black solid lines), and corresponding fluxes (open symbols) measured in sediment traps deployed at three discrete depths (dashed horizontal lines) throughout an annual cycle (Mar/1990–Mar/1991). Data for the less abundant brGDGTs are shown in *SI Appendix*, Fig. S2 and S3. Settling particles were sampled up to three times per month, and random vertical jitter was added to reduce overlap of symbols. The analytical error for absolute brGDGT quantification was $<\pm 10\%$ (2SE). **(D)** Stable C isotopic composition of the brGDGTs' alkyl moieties released by ether cleavage (a–f; solid lines), particulate organic matter (POM), and total organic carbon (TOC) (dashed line) in SPM and surface sediment (bottom symbols). Due to incomplete gas-chromatographic separation (cf. *SI Appendix*, Fig. S4), peaks of b+c and d+f (if f was present) were integrated as a whole. The characteristic association of alkanes d, e, and f with specific brGDGTs are indicated in square brackets. Isotopic analyses of brGDGTs in SPM from near-surface water (<50 m) was not possible due to low concentrations. Error bars represent the range of duplicate/triplicate GC-IRMS analyses. The range of replicate $\delta^{13}C_{POM/TOC}$ measurements was $<0.2\%$.

detected in nearby catchment soils nor in river-bed sediments collected from the lake's watershed (*SI Appendix*, Table S1), suggesting that it is exclusively produced by anaerobic bacteria within the deeper lake (cf. 18).

The spatially segregated production of these structurally similar brGDGTs in oxic and hypoxic/anoxic water masses is also reflected in their sinking fluxes, which we assessed by analyzing settling particles collected in sediment traps from three different depths throughout an annual cycle (Fig. 2C). Fluxes of the IIIa' isomer increased ~10-fold between the upper (20 m) and middle (85 m) trap, indicating that its biosynthesis is restricted to the oxygenated part of the water column. In contrast, IIIa'' was only present in settling particles below the RTZ (176 m), further attesting to its exclusive production within oxygen-deprived waters. The IIIa isomer, on the other hand, showed continuously increasing fluxes across the RTZ, suggesting that it is produced by both aerobic and anaerobic, or facultative anaerobic bacteria.

Distinct sources of these specific brGDGTs within Lake Lugano are also indicated by the stable C isotope signature of their alkyl moieties. In contrast to the bulk of brGDGT-derived alkanes that showed significantly decreasing $\delta^{13}\text{C}$ values with depth (i.e. up to 10 ‰; Fig. 2D), the C isotopic signature of alkane e that is exclusively contained in brGDGT IIIa' (cf. Fig. 1) remained unchanged across the RTZ, suggesting that it is only produced in the upper oxygenated part of the lake, from where it is exported to the sediment. To further explore the potential of brGDGT biosynthesis under oxic conditions, we amended near surface SPM from Lake Lugano (10 m depth) with ^{13}C -enriched organic substrates (see *SI Appendix*, Supplementary Materials and Methods for details). Indeed, we observed pronounced production of the brGDGT IIIa' and other C6-methylated isomers, providing evidence for an aerobic metabolism of their producers, whereas IIIa, IIIa'', and all other C5-methylated brGDGTs were not detectable at the end of the experiment (*SI Appendix*, Supplementary Information Text and Fig. S5).

The clear vertical segregation of the isomers IIIa' and IIIa'', as well as their distinct stable C isotope signatures, strongly suggest that they are produced by distinct (groups) of microorganisms with different redox requirements. In order to elaborate links between brGDGT synthesis and microbial niche differentiation, we investigated vertical trends in the bacterial population by 16S-rRNA gene sequencing. The change in brGDGT composition across the RTZ was accompanied by a marked shift in the composition of the bacterial community, with only 12 % of the operational taxonomic units (OTUs) being shared between oxic (10–80 m) and anoxic water masses (90–275 m). Furthermore, we compared OTU-specific DNA concentrations with the concentration of each individual brGDGTs in the water column (see *SI Appendix*, *Supplementary Materials and Methods* for details). A substantial fraction of the bacterial community (i.e., 2–20 % of the OTUs) showed a strong correlation ($r > 0.75$) with the abundance of one of the major brGDGTs, whereas this correlation was much weaker when all brGDGTs were considered as a whole ($r \leq 0.5$; *SI Appendix*, Fig. S6). Moreover, the bacterial clades that correlated with

individual brGDGTs were often taxonomically distinct from one another (Fig. 3). In particular, the OTUs correlating well with brGDGT IIIa' had very distinct taxonomic affiliations compared to those corresponding to brGDGTs IIIa'', in support of brGDGT synthesis by multiple (groups of) bacteria that reside in different ecological niches (e.g., oxic vs. hypoxic/anoxic).

Thus far, acidobacteria isolated from soil are the only known potential source of brGDGTs (10, 11). In Lake Lugano 4–35 % of all acidobacterial OTUs correlated well ($r > 0.75$) with the vertical concentration profiles of at least one of the brGDGTs, and their diversity varied strongly between the OTUs associated with different brGDGTs (Fig. 3). This attests to a marked niche

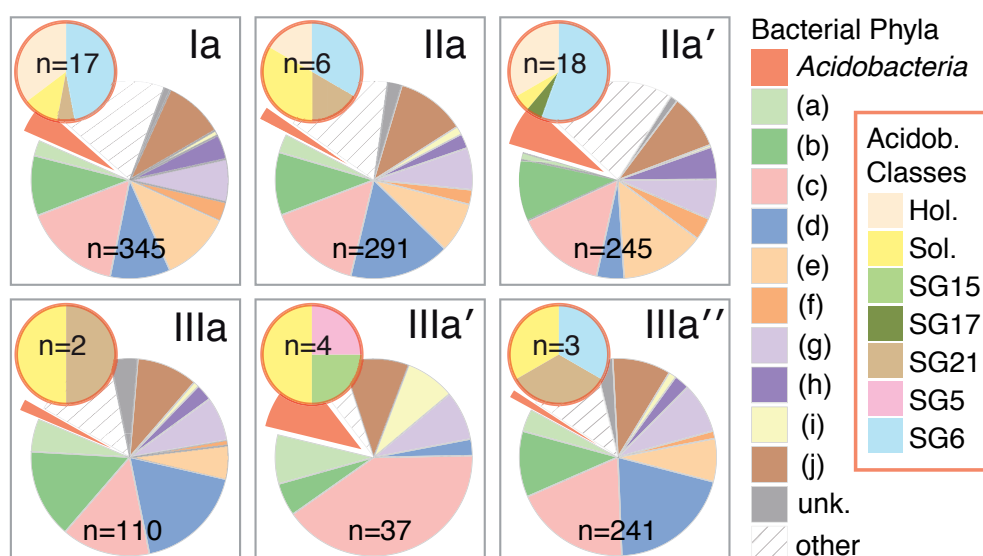


Fig. 3. Phylogenetic diversity of operational taxonomic units (OTUs) that show strong empirical correlation ($r > 0.75$) with the six major brGDGTs in Lake Lugano (2–20 % of all 1594 OTUs detected). OTU-specific DNA concentrations were estimated based on bacterial 16S-rRNA-gene abundances and total DNA concentrations (see *SI Appendix* for details), and the vertical concentration profiles of OTUs and individual brGDGTs were then compared by bivariate correlation analysis (*SI Appendix*, Fig. S6). The phylogenetic affiliation of the highly-correlated OTUs ($r > 0.75$) is shown for each brGDGT on the phylum level (large pie charts) and is further differentiated for the *Acidobacteria* (class level, smaller pie charts). The diversity of the (acido) bacteria associated with each brGDGT is markedly distinct, suggesting differential brGDGT synthesis by multiple (acido) bacterial taxa. Key: (a) Armatimonadetes; (b) Bacteroidetes; (c) Betaproteobacteria; (d) Chloroflexi; (e) Firmicutes; (f) Latescibacteria; (g) Planctomycetes; (h) Spirochaetae; (i) TM6; (j) Verrucomicrobia; unk.: unknown Phylum; SD3–21: Subdivisions of the *Acidobacteria*. Phyla contributing <3 % to the total number of OTUs in each brGDGT cluster are subsumed as 'other'. The number of OTUs (n) is indicated for each pie chart.

partitioning among members of the *Acidobacteria* (cf. *SI Appendix*, Fig. S7) that seems to correspond to the differential vertical trends of certain brGDGTs in the water column, indicating that they could indeed be one of the prime brGDGT sources in aquatic environments. Besides acidobacteria, a large number of other bacterial taxa showed similarly strong correlations with the brGDGTs (Fig. 3, *SI Appendix*, Fig. S4), suggesting that the trait of brGDGT synthesis may extend beyond the phylum of *Acidobacteria*. In this context it has to be considered that microbes with different phylogenetic affiliation may occupy similar ecological niches, due to syntrophic relationships (19) or similar metabolic lifestyles (16). The empirical co-occurrence of certain bacterial taxa and brGDGTs alone is therefore most likely insufficient to draw unequivocal conclusions on the identity of brGDGT-producing organisms in the environment.

Carbon metabolism of brGDGT-producing bacteria in Lake Lugano

The ^{13}C content of brGDGTs in Lake Lugano's water column provides insight into the potential C substrates utilized by their bacterial producers. Coinciding with the marked shift in the brGDGT distribution, we found a steep decline in the ^{13}C content of most brGDGTs (i.e., the $\delta^{13}\text{C}$ values of the alkyl moieties a, b, and c) from -33 to -42 ‰ across the RTZ (Fig. 2D). This may in parts reflect the vertical $\delta^{13}\text{C}$ gradient of the C substrate utilized by brGDGT producers, and/or may be due to a distinct biosynthetic ^{13}C fractionation expressed by different (groups of) source organisms. To elucidate this further, we analyzed the ^{13}C in the inorganic and organic C pools that may serve as substrates for brGDGT production within the water column. In fact, bulk suspended particulate organic matter (POM) showed similar vertical ^{13}C trends as the brGDGTs (i.e., a decline across the RTZ; Fig. 2D), whereas $\delta^{13}\text{C}$ values of dissolved inorganic and dissolved organic C did not change substantially with depth (*SI Appendix*, Fig. S8). We therefore conclude that brGDGT-producing bacteria in Lake Lugano are likely involved in the heterotrophic breakdown and assimilation of particulate organic C in the water column, which is in agreement with the known metabolic capabilities of acidobacterial isolates (20), and analogous to what has previously been suggested for soils (21).

The negative $\delta^{13}\text{C}_{\text{POM}}$ values encountered at the RTZ, in turn, are predominantly due to the presence of methanotrophic bacteria that oxidize and incorporate ^{13}C -depleted methane (-60 ‰) at and below the anoxic interface (22). We thus argue that at least some of the brGDGT-producers thriving within the RTZ are part of a methane-based microbial food web, in which they assimilate organic matter that is derived either directly from methanotrophic bacteria, and/or from other organisms that have previously incorporated methanotroph-derived carbon (e.g. 23–25).

Methane-fueled brGDGT production and microbial niche differentiation in other lakes

A redox-dependent differentiation of the brGDGT-producing bacterial community as we observed in meromictic Lake Lugano may be a common feature also in other stratified lake systems. Remarkably, in 20% of the lake sediments investigated in this study, the characteristic alkyl moiety derived from brGDGT IIIa' (alkane e) had substantially higher $\delta^{13}\text{C}$ values ($\geq 4\text{‰}$) than those originating from brGDGTs IIIa and IIIa'' (alkanes d and f; Fig. 4A). We therefore assume that the production of brGDGT IIIa' in these lakes is also spatially separated from that of the bulk of the other major brGDGTs, similar to what we show for Lake Lugano (cf. Fig. 2D). Moreover, in $>50\%$ of the lakes sediments, the most common alkyl moieties of brGDGTs (i.e., alkanes a, b, and c) were depleted in ^{13}C relative to total organic carbon (TOC) (i.e., $\geq 5\text{‰}$ lower $\delta^{13}\text{C}$ values) (Fig. 4B), again indicating a substantial contribution of brGDGTs produced in

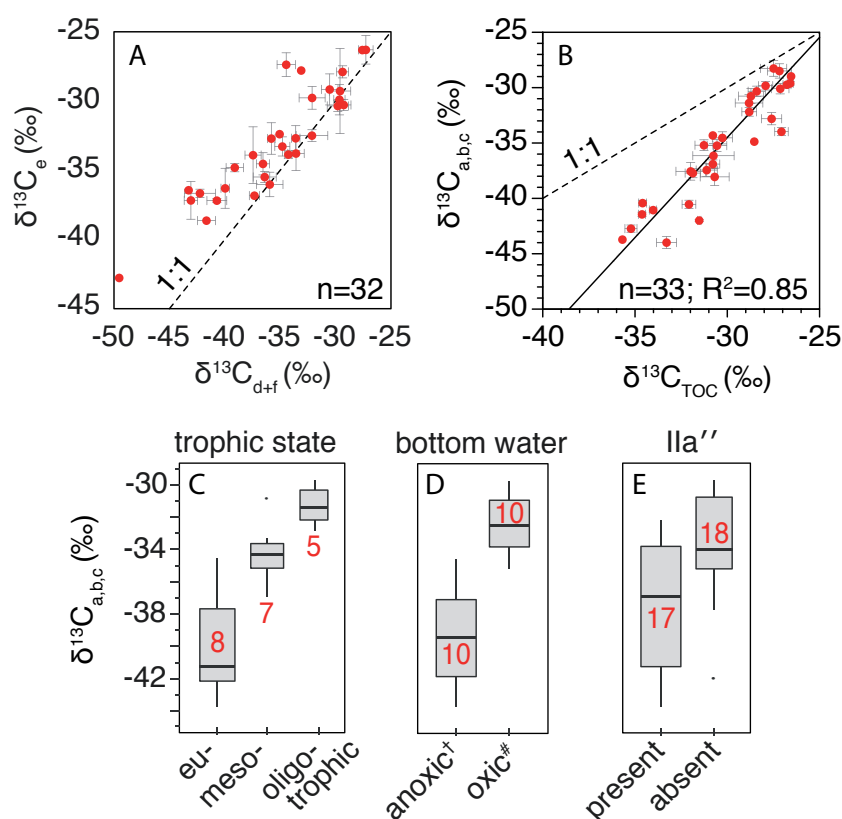


Fig. 4. Stable C isotope composition of brGDGT-derived alkanes (a–f) and total organic carbon (TOC) in lake surface sediments from the European Alps. **(A)** Alkane e that exclusively originates from brGDGT IIIa' was up to 7 ‰ ^{13}C -enriched relative to alkanes d+f derived from IIIa and IIIa''. Average values for d+f are reported because gas-chromatographic separation was incomplete (see SI Appendix, Fig. S4). **(B)** The abundance-weighted average $\delta^{13}\text{C}$ values of the most common alkanes a, b, and c ($\delta^{13}\text{C}_{a,b,c}$) shows a strong correlation with $\delta^{13}\text{C}_{\text{TOC}}$ (solid line), and were up to 10 ‰ depleted in ^{13}C relative to TOC. **(C–E)** Comparison of $\delta^{13}\text{C}_{a,b,c}$ values with trophic state, bottom water oxygenation, and occurrence of brGDGT IIIa'' in lake sediments from the European Alps. Note that water chemistry records are not available for all study sites. Trophic classification is based on epilimnetic total N and/or total P concentrations. Hydrochemical data were provided by the Swiss Federal Office of the Environment (BAFU), or taken from literature (see SI Appendix, Table S1). (†): seasonally or permanently anoxic; (#): year-round oxyc.

methanotrophic water layers, as we propose for Lake Lugano. Remarkably, these low $\delta^{13}\text{C}_{(a,b,c)}$ values are consistently associated with an elevated trophic state of the lake (Fig. 4C), which in turn gives rise to an enhanced export production, as well as anoxia (Fig. 4D) and methane accumulation in bottom waters (26). Eutrophication and redox-stratification thus likely promote the growth of brGDGT-producing microbes at the RTZ where methanotrophy may play an important role in microbial food webs, and (as indicated by the $\delta^{13}\text{C}$ data) these deep water-derived brGDGTs substantially contribute to the sedimentary brGDGT pool.

In Lake Lugano the production of brGDGT IIIa'' is restricted to hypoxic/anoxic deep water (cf. Fig. 2C). Corroboratively, we detected this compound preferentially in sediments of meso-eutrophic lakes (9 out of 11 cases; *SI Appendix*, Table S1), where biological oxygen consumption is likely to cause anoxia within the bottom water and/or the top layer of the surface sediment. The presence of brGDGT IIIa'' may thus be used as a redox indicator and, indirectly, as a proxy for the trophic state in future paleolimnological studies. Taken together, redox-dependent differentiation of brGDGT-producing bacterial communities, as exemplified in Lake Lugano, is prevalent in a significant fraction of lake systems. Compositional shifts in sedimentary brGDGT records may thus not only reflect the ability of bacteria to adapt the chemical properties of their membrane in response to environmental factors such as T (i.e., membrane lipid homeostasis), but are also strongly dependent on changes in the community composition of their microbial producers.

Implications for lacustrine paleothermometry

Observed empirical correlations between brGDGT distributions in recent lake sediments and MAT gave rise to transfer functions that allow for the quantitative reconstruction of paleoclimatic conditions from lacustrine sediment archives (e.g. 8). For instance, the Methylation Index of brGDGTs ($\text{MBT}'_{5\text{me}}$, reflecting the relative amount of C5-methyl branches; 4), has been successfully applied to East African lake sediments, where it showed a strong correlation with MAT ($R^2=0.92$; 27). Yet, this correlation was poor in the surface sediments from the European Alps investigated here ($R^2=0.10$, $n=36$). Several factors may affect the fidelity of brGDGT-based temperature proxies in lake sediments. One major concern is that terrigenous brGDGTs may contribute to the sediment through soil organic matter inputs. brGDGTs from soils have a composition that is markedly distinct from those produced within lakes of the same climate regime (7) and, as a consequence, their admixture to the aquatically-produced brGDGT pool can substantially bias T estimates derived from lake deposits.

Another factor relates to the vertical temperature distribution within the water column. Besides a possible input of soil-derived brGDGTs, the depth from where the aquatically-produced brGDGTs are predominantly transferred to the sediments will ultimately determine the 'temperature imprint' of brGDGT in the surface sediment. In Lake Lugano, the largest part of

the brGDGT sinking flux can be attributed to production in sub-thermocline (i.e., hypolimnetic) water (cf. Fig. 2C), and thus the pool of brGDGTs finally reaching the sediment reflects isothermal deep water, rather than seasonal surface water conditions (28, 29; *SI Appendix*, Fig. S9). On the other hand, in lakes with less pronounced deep water production, the sedimentary brGDGTs may more dominantly derive from surface water, shifting the proxy record towards warmer temperatures (i.e., higher MBT index values).

Thus generally, the relative contributions of soil-, (near) surface water-, and hypolimnion-derived proxy signals to the sediments may vary largely among different study sites, depending on hydrochemical parameters (e.g. trophic state/redox conditions), morphological characteristics (i.e., maximal depth), and the depositional environment (i.e., soil organic matter input), which is likely to confound the quantitative relationships between atmospheric MAT and the sedimentary brGDGT record. Knowledge of the predominant brGDGT sources, therefore, is of prime importance for the use of brGDGTs in paleoenvironmental reconstructions.

Our data from Lake Lugano show that the brGDGTs produced at the RTZ — likely by utilization of methane-derived C compounds — are much more depleted in ^{13}C than those synthesized in both the oxic part of the water column ($\geq -35\text{‰}$; cf. Fig. 2D) and in soil (-25 to -31‰ [21, 30] ; cf. *SI Appendix*, Table S1). Such ^{13}C -depleted sedimentary brGDGT pools may thus be indicative of proxy signals that almost exclusively reflect deep water T variations, and may hence be largely unaffected by above-mentioned differential brGDGT inputs from surface water and/or soils. In order to elaborate the temperature response of sediments with a deep water-dominated brGDGT source, we re-assessed the MAT-brGDGT correlation in our data set for different $\delta^{13}\text{C}_{(a,b,c)}$ domains. We found that in lakes with low $\delta^{13}\text{C}_{(a,b,c)}$ values (-45 to -36‰), the correlation between $\text{MBT}'_{5\text{me}}$ (and related proxy indices; *SI Appendix*, Fig. S10) and MAT was indeed significant ($R^2=0.45$, $n=15$, $p<0.01$), and much stronger than in lakes where the $\delta^{13}\text{C}_{(a,b,c)}$ was higher ($R^2=0.03$, $n=20$). This correlation was again substantially improved when only shallow lakes with a maximal depth of <40 m were considered ($R^2 = 0.76$; $n=10$; Fig. 5B), in which the RTZ may overlap with thermocline and the low- ^{13}C brGDGTs thus probably reflect thermocline water temperatures. In contrast, $\text{MBT}'_{5\text{me}}$ proxy values were more or less constantly low among the deeper lakes (>40 m) (Fig. 5D), which we attribute to the decoupling of atmospheric air T and hypolimnetic water T in lakes of the cool-temperate climate zone (i.e., $\text{MAT} \leq 11^\circ\text{C}$; 31). In warmer climates, on the other hand, hypolimnetic water T is in fact linearly related to air T (32, 33), and hypolimnion-derived brGDGTs from deeper lakes will therefore be more responsive to inter-annual MAT variations than in the Alpine lakes investigated here.

We thus conclude that deep water communities of brGDGT producing bacteria thriving at the RTZ 'reset' the sedimentary brGDGT 'thermometer' to (near-)bottom water T, thereby mitigating the interfering effects of surface water- and/or catchment soil-derived brGDGT signatures. Depending on climate zone and water depth, such constellations, which can be

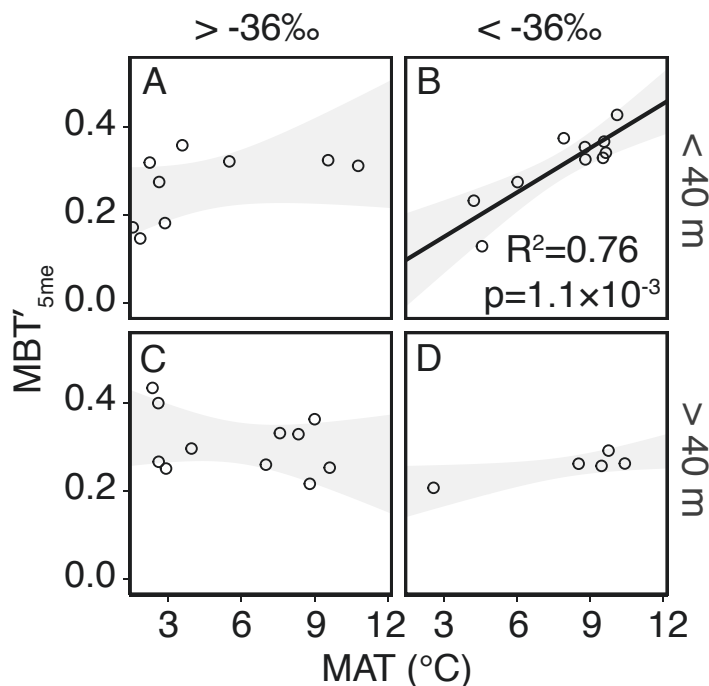


Fig. 5. Lapse rate-based mean annual air T (MAT) of the study sites plotted against the brGDGT proxy MBT'_{5me} (Methylation Index of C5-methylated brGDGTs) in lake surface sediments from the European Alps (n=35). Sites are binned by maximum water depth and the approximated average stable C isotope composition of brGDGTs ($\delta^{13}C_{a,b,c}$). Gray areas denote the 95 % confidence intervals of the linear regressions. A regression line is only shown for significant models ($p < 0.02$). The T response of related brGDGT proxy indices are shown in *SI Appendix*, Fig. S10.

identified by the C isotopic imprint inherited from biogenic methane, are expected to yield reliable records of long-term climate variability, and are preferable sites for the application of brGDGT-based paleotemperature proxies in lakes.

Materials and Methods

Water column sampling. Suspended particulate matter (SPM) was collected from Lake Lugano (northern basin) close to the deepest location (*SI Appendix*, Fig. S11) in September 2014 by large-volume in situ filtration of lake water (~50–300 L), using a double layer of glass fiber filters (2.7 and 0.7 μm ; Whatman GF/D and GF/F). The filters were frozen on dry ice immediately after sampling and stored at -80°C until further processing. Settling particles were collected at the same location, using cylindrical sediment traps (d=10 cm) deployed in 1990/1991 at three depths (20, 85, and 176 m), and were sampled up to three times per month.

Sampling of sediments and soils. Lake surface sediments (*SI Appendix*, Table S1 and Fig. S12) were obtained by gravity coring during multiple field campaigns (2008–2014). The core-tops (upper ~5 cm) were then subsampled, homogenized, and freeze-dried prior to

extraction. We further collected eight to ten top-soil samples (0-10 cm) within a perimeter of ~500 m at 19 of the 36 investigated lakes. The individual soil samples from each lake site were pooled, freeze-dried, sieved (2 mm), and homogenized with a mortar before extraction. Additionally, we collected sediment samples (0–10 cm) from two main tributaries (Cuccio and Casarate) of Lake Lugano at locations close to the river mouths (*SI Appendix*, Fig. S11), which were processed in the same way as the soil samples.

Lipid extraction. Sediments and soils were extracted three times by accelerated solvent extraction (ASE™300, Thermo Fisher) (5). For SPM samples, we employed a sequential solvent extraction and acid hydrolysis procedure to assure complete recovery of brGDGTs from living cells (intact polar brGDGTs), and to avoid losses and biases associated with modified Bligh-Dyer extraction (34). Briefly, the freeze-dried SPM was extracted by ultrasonication with methanol and dichloromethane as solvents, and the residual material was subsequently subjected to acid-hydrolysis. To maximize lipid recovery for compound-specific stable C isotope analysis of brGDGTs, SPM from selected depths (representing ~150 L of filtered water) was subjected to acid hydrolysis-prior to ultrasonic solvent extraction.

brGDGT analysis. brGDGT-containing lipid fractions were prepared as previously described (34), spiked with an internal standard (35) and analyzed by ultra-high performance liquid chromatography - positive ion atmospheric pressure chemical ionization - mass spectrometry (UHPLC-APCI-MS) in selected ion monitoring mode (SIM). For all samples from Lake Lugano, analytical separation of GDGTs was achieved using two UHPLC silica columns in series (BEH HILIC columns, 2.1 × 150 mm, 1.7 μm; Waters; 36), whereas for all remaining sites, we used an array of four Alltima Silica columns (150 mm × 2.1 mm; W. R. Grace & Co.-Conn.; USA) (4). Both 'high-resolution' UHPLC setups reliably separate C5- and C6-methylated brGDGT isomers (36). Response was monitored regularly using a known mixture of the internal standard and crenarchaeol.

Stable C isotope analyses. The $\delta^{13}\text{C}$ content of total organic C and particulate organic matter (TOC and POM, respectively) were determined after acidification of the sample (37) by elemental analysis- (EA) followed by isotope ratio mass spectrometry (IRMS) (18). For stable carbon isotope analysis of brGDGTs, we subjected bulk brGDGT fractions to ether cleavage (57 % HI), and measured $\delta^{13}\text{C}$ values of the released alkyl moieties by gas chromatography-combustion-IRMS (GC-C-IRMS) as previously described (18), with a few modifications (see *SI Appendix*).

Molecular biological methods. DNA was extracted from fractions of GF filters (38), equivalent to 2–7 L of lake water. A two-step PCR approach (Nextera, Illumina Corp., San Diego, CA, USA) was employed for library preparation, using the universal primers 515F-Y (5'-GTGYCAGCMGCCGCGGTAA) and 926R (5'-CCGYCAATYMTTTRAGTTT-3') (39) that target the V4 and V5 regions of the 16S rRNA gene. The individual samples were indexed, pooled

at equimolar concentrations, and sequenced on an Illumina MiSeq V2 platform using the 2×250 bp paired-end protocol (Microsynth Inc., Switzerland). After initial quality control and bioinformatical processing (see *SI Appendix*), taxonomy was assigned to operational taxonomic units (OTUs) by comparison with the SILVA 128 SSU reference data base. In order to compare vertical trends of brGDGTs and microbial taxa, relative OTU abundances were converted to OTU-specific DNA concentrations (ng L⁻¹) by multiplication with the total amount of extracted DNA (see *SI Appendix* for details).

Data access and availability. Abundance-filtered 16S-rDNA sequences are deposited at GenBank (NCBI) under the accession numbers MH111698 – MH113143. Raw bacterial OUT abundances, as well as brGDGT data from Lake Lugano and other Alpine lake sediments and catchment soils are available at the publisher's website.

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4.1. Supplementary information

Supplementary Information Text

In vitro production of brGDGTs under oxic vs. anoxic conditions. In order to test for the potential of brGDGT synthesis in freshwater habitats, we incubated SPM collected from the epilimnion and monimolimnion (10 m and 260 m water depths, respectively) with additions of 99 % ^{13}C -labeled organic substrates (glucose and algal biomass) under quasi in-situ conditions of the epilimnion (i.e., oxic/20 °C/light) and the deeper hypolimnion (anoxic/4 °C/dark). After six weeks of incubation, de novo brGDGT synthesis was evidenced by high levels of ^{13}C incorporation into brGDGTs (20–26 % ^{13}C) (Fig. S2B,C). Remarkably, only five out of the nine brGDGTs initially present in 10 m SPM were synthesized (i.e., Ia, Ib, and the C6-methylated brGDGTs IIa' IIb', and IIIa'; see Fig. S2A), but the concentrations of specific brGDGTs in the epilimnetic biomass increased by a factor of ~ 100 (cf. Figs. S2A,B), corresponding to a production rate of $0.62 \mu\text{g g}(\text{C}_{\text{org}})^{-1} \text{d}^{-1}$. Moreover, the production rates we observed were ~ 100 times higher than those previously reported from a peat bog ($6.3 \mu\text{g g}(\text{C}_{\text{org}})^{-1} \text{d}^{-1}$; [1]), where incubation experiments were conducted without addition of organic C substrates and under hypoxic conditions. Together this strongly suggests that the addition of organic electron donors (glucose and algal biomass) supported the growth of aerobic, heterotrophic bacteria that produce only a subset of the naturally occurring brGDGTs.

In contrast to the aerobic experiment, we did not observe considerable brGDGT production in the anoxic incubation of SPM collected from below the RTZ at 260 m depth. More precisely, both concentrations and ^{13}C contents were identical to the t_0 values within analytical error (i.e., ± 0.5 % ^{13}C for isotope analysis, and ± 10 % of t_0 concentrations). Instead, we found substantial ^{13}C incorporation into GDGT-0 (caldarchaeol), an isoprenoid GDGT derived from archaea (up to 33 % ^{13}C at t_1), providing evidence for microbial substrate turnover under the given experimental conditions (data not shown). The lack of measurable brGDGT synthesis in this anaerobic experiment therefore suggests that the brGDGT-producing bacteria at the RTZ are either (1) micro-aerobic and require at least trace amounts of O_2 , or (2) use alternative electron acceptors (e.g. nitrate) that were not present in the deeper anoxic waters from 260 m.

Supplementary Materials and Methods

^{13}C incorporation experiments with SPM from Lake Lugano. SPM was collected by in situ filtration of 140–185 L of lake water using glass fiber filters (0.7 μm ; Whatman GF/F) in August 2013. Immediately after retrieval, filters were placed in sterilized flasks together with 800 ml of in situ lake water of the respective depth, and 100 mg $^{13}\text{C}_6$ Glucose (99 %, Euriso-Top®, France) as well as 50 mg freeze-dried *Spirulina sp.* cells (99 % ^{13}C , Sigma) were added. The

flasks containing SPM from 10 m depth were closed with cellulose stoppers to facilitate gas exchange (oxic) and incubated at 20 °C under artificial daylight (12 h day⁻¹). Flasks containing SPM from 260 m were flushed with N₂, sealed airtight and left at 4 °C. Both treatments were agitated at 30 rpm on orbital shakers, and all materials were autoclaved (121 °C) prior to the experiment. After 180 days (t₁), all solids were collected by filtration (0.7 µm GF/F), freeze-dried, and processed as described for SPM samples (i.e., solvent extraction followed by acid hydrolysis of the residue). The hydrolyzed extracts and the residue hydrolysates were then analyzed on the same HPLC-MS system as the regular SPM samples (2). The remaining polar fraction was subjected to ether cleavage, and the brGDGT-derived alkanes were analyzed by GC-MS and GC-IRMS. However, due to the massive presence of sterol-derived hydrocarbons (released from the ¹³C-labelled *Spirulina* biomass), we were unable to resolve ¹³C incorporation below 0.5 % (as determined by standard co-injections). Initial brGDGT abundances (t₀) were measured in the lipid extract of unamended SPM.

Assessment of ¹³C label incorporation by UHPLC-APCI-MS. For the analysis of ¹³C-labelled brGDGTs, we monitored all single ions between *m/z* 1018 and *m/z* 1077. We then assigned brGDGTs to each peak in the base peak chromatograms (BPC; Fig. S2A), based on relative retention times (with respect to the internal standard) and comparison with unlabeled samples. For each peak, all monitored ions (*m/z* 1018 to *m/z* 1077) were integrated using the corresponding extracted mass chromatograms (EIC). We then weighted the peak areas by the number of ¹³C substitutions, e.g. 0×AREA(*m/z* 1022) + 1×AREA(*m/z* 1023) + 2×AREA(*m/z* 1024) + (...) + 23×AREA(*m/z* 1055) in case of brGDGT Ia (*m/z* 1022). The sum of the weighted peak areas was then divided by the sum of the un-weighted peak areas, yielding the average number of ¹³C substitutions in each brGDGT. The relative ¹³C content was obtained through division by the number of C atoms per brGDGT molecule. We note that co-eluting, minor brGDGTs may have contributed to the calculated peak areas in the EICs, though, we consider their contribution to be small (likely ≲5 %).

Processing of 16S-rDNA data. Bioinformatical processing of the raw sequencing data (a total of 1,741,418×2 paired raw reads from 15 water column samples) was performed with MacQIIME V.1.9.1 (3) and Cutadapt (4). Briefly, paired reads were stitched (min. overlap: 50 bp), quality-filtered (Phred≥25), cleaned from PCR primers, and de-replicated. Sequences were then subjected to both de novo- and reference-based chimera detection, and only those passing either filter were retained (UCHIME; [5]). The remaining sequences were clustered into operational taxonomic units (OTUs) at 97 % sequence identity (USAERCH 6.1 ; [6]). Taxonomy was assigned with the RDP classifier (7) (confidence threshold: 0.6), using a set of QIIME-formatted and pre-clustered (97 %) reference OTUs (SILVA 128 SSU). Downstream phylogenetic analyses were performed in 'R' (8), using the package 'Phyloseq' (9). Singletons were removed, and sequences were randomly subsampled (rarefied) to achieve an even sequencing depth (16708

reads per sample). Thereafter OTUs occurring in less than five samples, and those with less than 30 total reads across all samples, were discarded.

Comparison of 16S-rDNA data with brGDGT profiles in Lake Lugano. In order to compare vertical trends in brGDGT concentrations vs. that of OTUs, we approximated OTU-specific DNA concentrations in the water column of Lake Lugano. For this approach we accounted for the contribution of eukaryotic 16S-rDNA from chloroplast and mitochondria, because otherwise bacterial taxa would be overestimated. To this end, the raw OUT abundances were divided by the total number of acquired reads (including chloroplast- and mitochondria), and thereafter multiplied with total DNA concentrations in the water column determined by absorption spectrophotometry of the total DNA extract. Finally, the OTU-specific DNA concentrations and the concentrations of brGDGTs were subjected to bivariate correlation analysis, and the taxonomic affiliations of the highly correlated OTUs ($r > 0.75$) were summarized.

Gas chromatographic analysis of brGDGT-derived alkanes. The hydrocarbons released by ether cleavage were injected at 70 °C on a 50 m low-bleed Rxi®-5ms GC column (0.2 mm iD, 0.33 µm df, Restek, USA). After 2 min hold time, the temperature was rapidly increased to 220 °C at 20 °C min⁻¹. Temperature was held for 45 min to improve separation of the target compounds from co-eluting steranes, and subsequently increased at 4 °C min⁻¹ to 320 °C (held for 35 min). The carbon isotopic composition of the analytes was calibrated by separate analysis of an alkane mixture with known isotopic compositions (n-alkane mixture B3 provided by A. Schimmelmann, Indiana University, USA). GC-C-IRMS performance was monitored by regular analysis of this standard mixture.

Calculation of MAT and brGDGT-based proxy indices. As air temperatures were not directly measured in this study, we approximated MAT at each of the lake sites using linear lapse rate models derived from instrumental data (Federal Office of Meteorology and Climatology MeteoSwiss) by $\text{MAT}(\text{°C}) = 11.76 - 0.00494 \times h$, where h is the lake surface elevation in meters above sea level. Previously published brGDGT proxy indices were calculated from the LCMS peak areas as follows:

$$\text{MBT}'_{5\text{me}} = (\text{Ia} + \text{Ib} + \text{Ic}) / (\text{Ia} + \text{Ib} + \text{Ic} + \text{IIa} + \text{IIb} + \text{IIc} + \text{IIIa}) ; (\text{Ref. 10}),$$

$$\text{MBT}'_{6\text{me}} = (\text{Ia} + \text{Ib} + \text{Ic}) / (\text{Ia} + \text{Ib} + \text{Ic} + \text{IIa}' + \text{IIb}' + \text{IIc}' + \text{IIIa}') ; (\text{Ref. 11}),$$

$$\text{MBT}_{56} = (\text{Ia} + \text{Ib} + \text{Ic} + \text{IIa}') / (\text{Ia} + \text{Ib} + \text{Ic} + \text{IIa} + \text{IIb} + \text{IIc} + \text{IIIa} + \text{IIIa}') (\text{Ref. 12}).$$

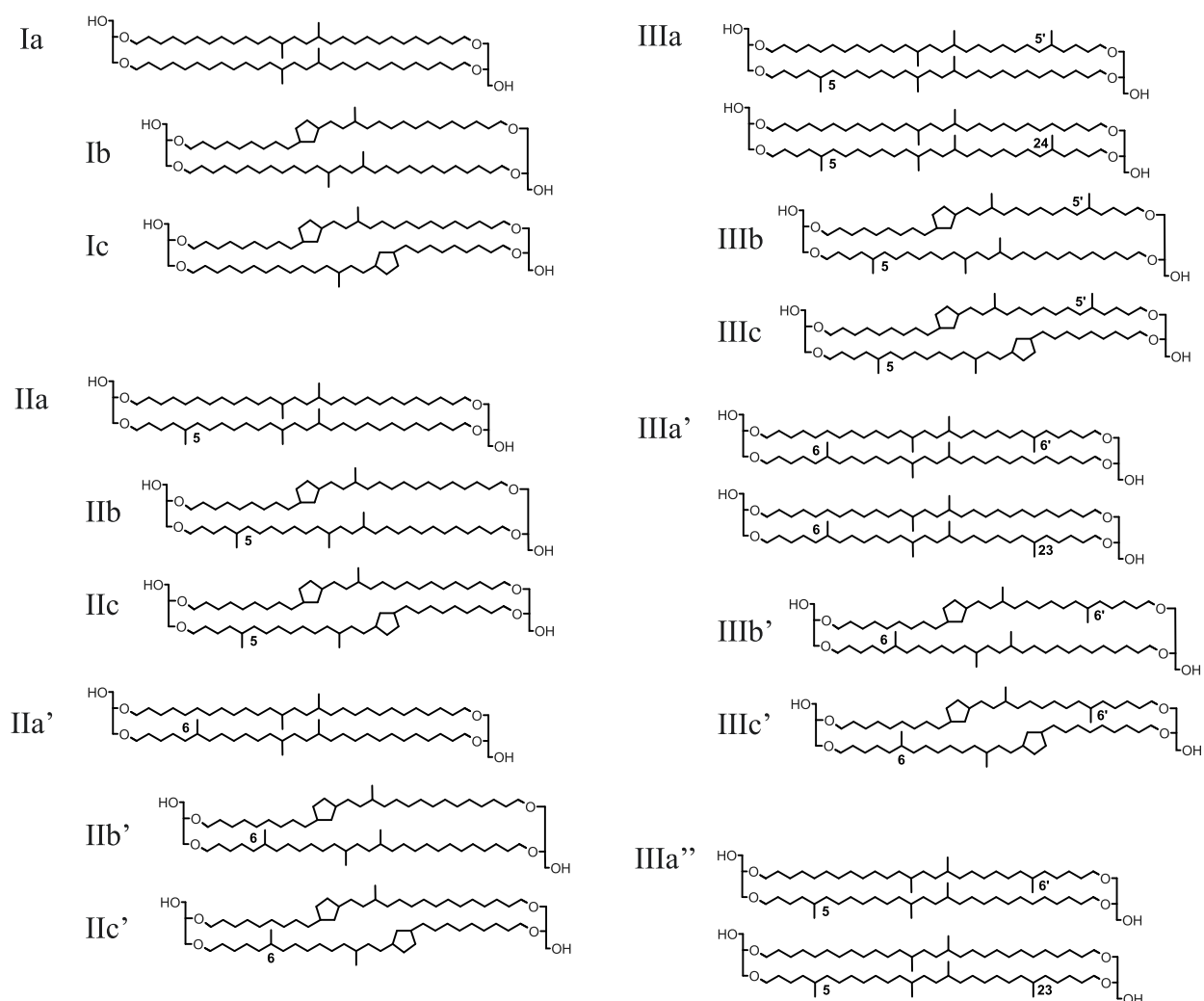


Fig. S1. Structures of all known brGDGTs, including C5 methylated isomers (13), C6 methylated isomers (indicated with a prime; 10, 14), and the recently discovered C5/6 methylated isomer (IIIa''; 15). Note that brGDGTs IIIa, IIIa' and IIIa'' each comprise a symmetric and an asymmetric isomer, which cannot be separated by HPLC-based chromatography. Structures of the hexa- and penta-methylated brGDGTs with cyclopentyl moiety(ies) IIIb', IIc', IIIb' and IIIc' are tentative.

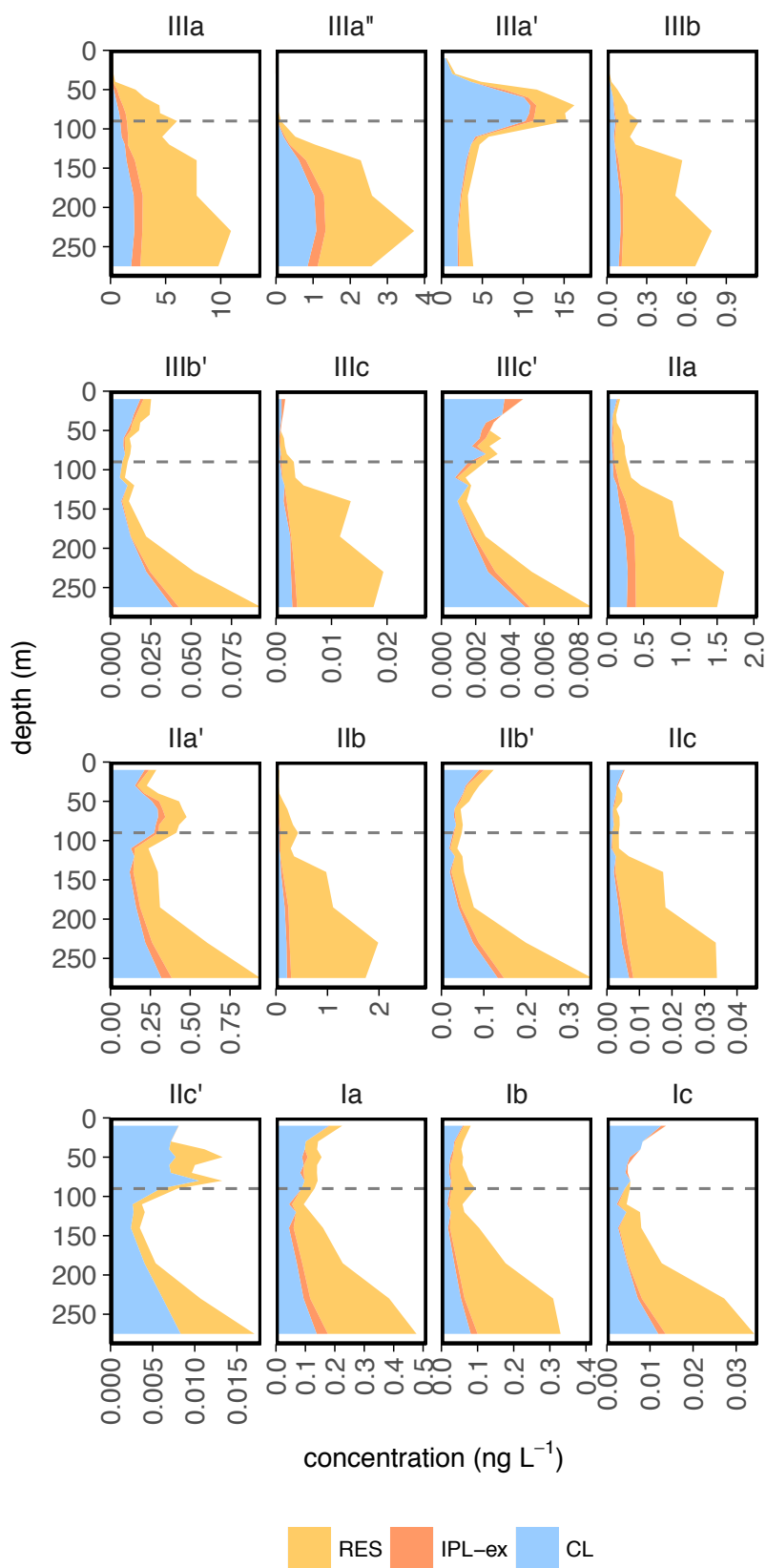


Fig. S2. Cumulative concentration of individual brGDGTs in suspended particles from Lake Lugano obtained by ultrasonic extraction with methanol and dichloromethane (CL and IPL-ex), and acid hydrolysis of the extracted residual material (RES). CL: core brGDGTs analyzed in the total lipid extract; IPL-ex: intact polar brGDGTs present in the total lipid extract, determined by acid hydrolysis of the extract (cf. 16); RES: intact polar brGDGTs released from the pre-extracted sample residues by acid hydrolysis (cf. 17).

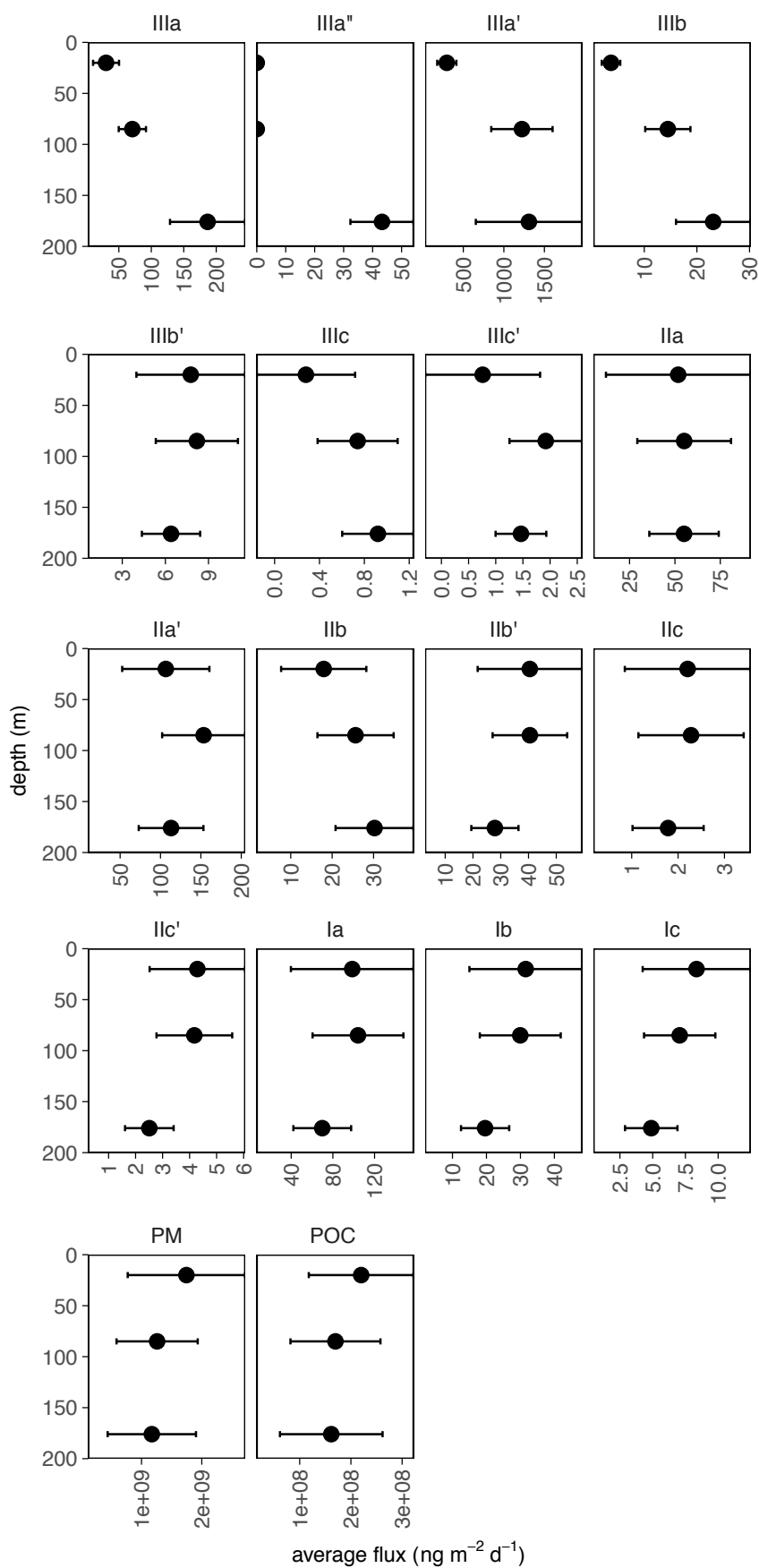


Fig. S3. Average fluxes of brGDGTs as well as total particulate matter (PM) and particulate organic carbon (POC) in sediment traps deployed at three discrete depths in Lake Lugano throughout an annual cycle (Mar/1990 – Mar/1991). Error bars show the standard deviation.

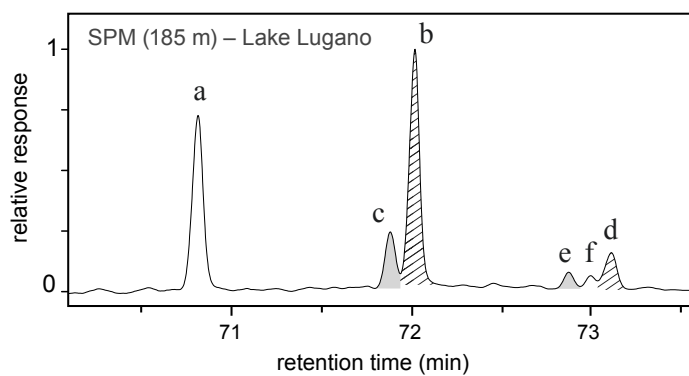


Fig. S4. GC-combustion mass chromatogram of m/z 44 (CO_2), showing partial co-elution of brGDGT-derived alkanes b and c, and d and f, respectively. Therefore, $\delta^{13}\text{C}$ values were determined by combined integration of the 'double peaks'.

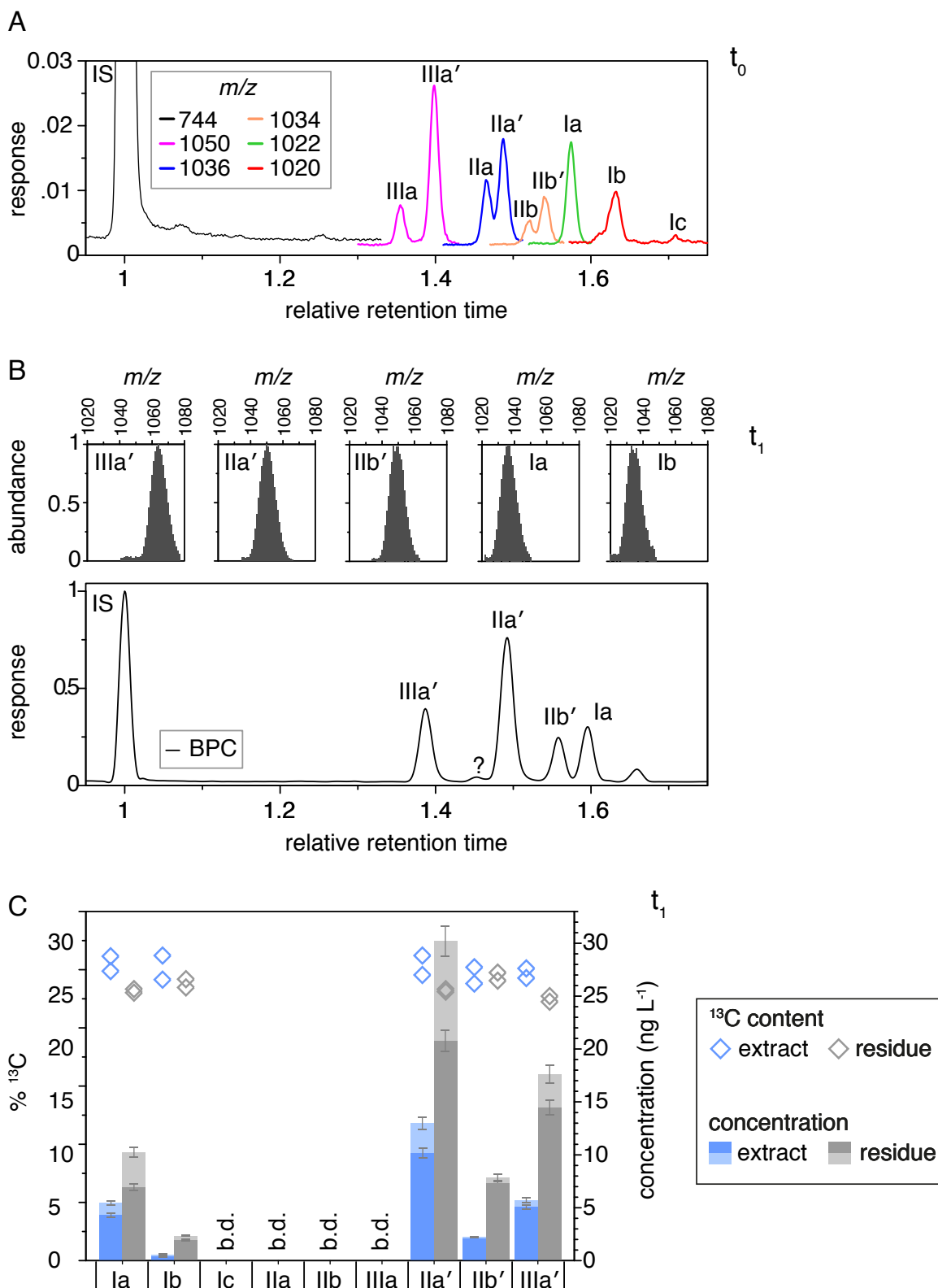


Fig. S5. Abundance and ^{13}C content of brGDGTs before (A) and after incubation with ^{13}C -labeled substrates (B+C), respectively, under oxic conditions. Cumulative mass spectra (cf. Supplementary Materials Methods) show an average mass shift of $\sim +15$ amu due to incorporation of the ^{13}C -label (B), corresponding to an average ^{13}C content of $\sim 25\%$ for all brGDGTs (C). Note the presence of C5-methylated brGDGTs (IIIa, IIa, and IIb) in the unamended SPM (t_0 ; A), which were not biosynthesized under the laboratory conditions and thus were below detection limit ('b.d.') after incubation (t_1 ; B+C). brGDGTs were analyzed in hydrolyzed total lipid extracts (TLE; blue bars and diamonds), as well as in the fraction released by acid hydrolysis of the post-extraction residues (RES; gray bars and diamonds). Experiments were performed in duplicate (light and dark colors in C). Error bars indicate the error of quantification ($\pm 10\%$; 2SE). Mass spectrometer response in A and B is shown relative to $0.1 \mu\text{g}$ of internal standard (IS, m/z 744). The concentration of brGDGTs in SPM from 10 m prior to incubation was $\sim 0.8 \text{ ng L}^{-1}$ of lake water.

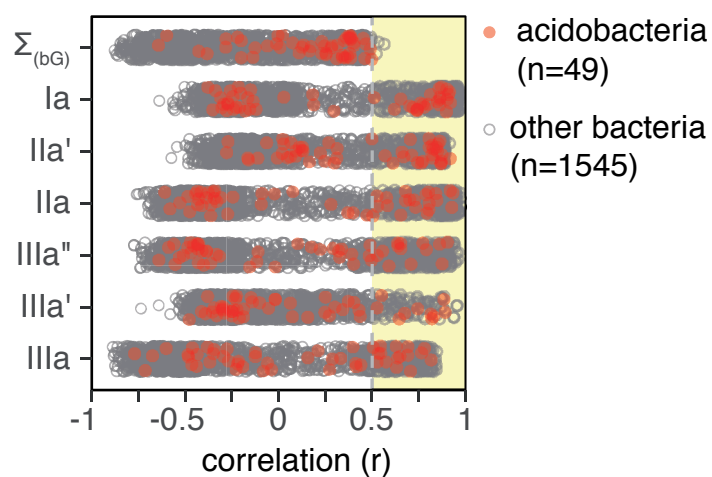


Fig. S6. Correlation coefficients (r) between OTU-specific bacterial 16S-rDNA abundances and brGDGT concentrations in the water column of Lake Lugano. Several members of the *Acidobacteria* (red filled circles) as well as other bacterial phyla (gray open circles) correlate well with each of the major brGDGTs, suggesting that the biological sources of brGDGTs are among these taxa. In contrast, none of the bacterial taxa present in Lake Lugano showed a strong correlation with the summed concentration of all brGDGTs [$\Sigma_{(bG)}$] ($r \leq 0.5$; dashed line), in support of the interpretation that individual brGDGTs have different (consortia) of source organisms. Positive correlations > 0.5 are highlighted in yellow.

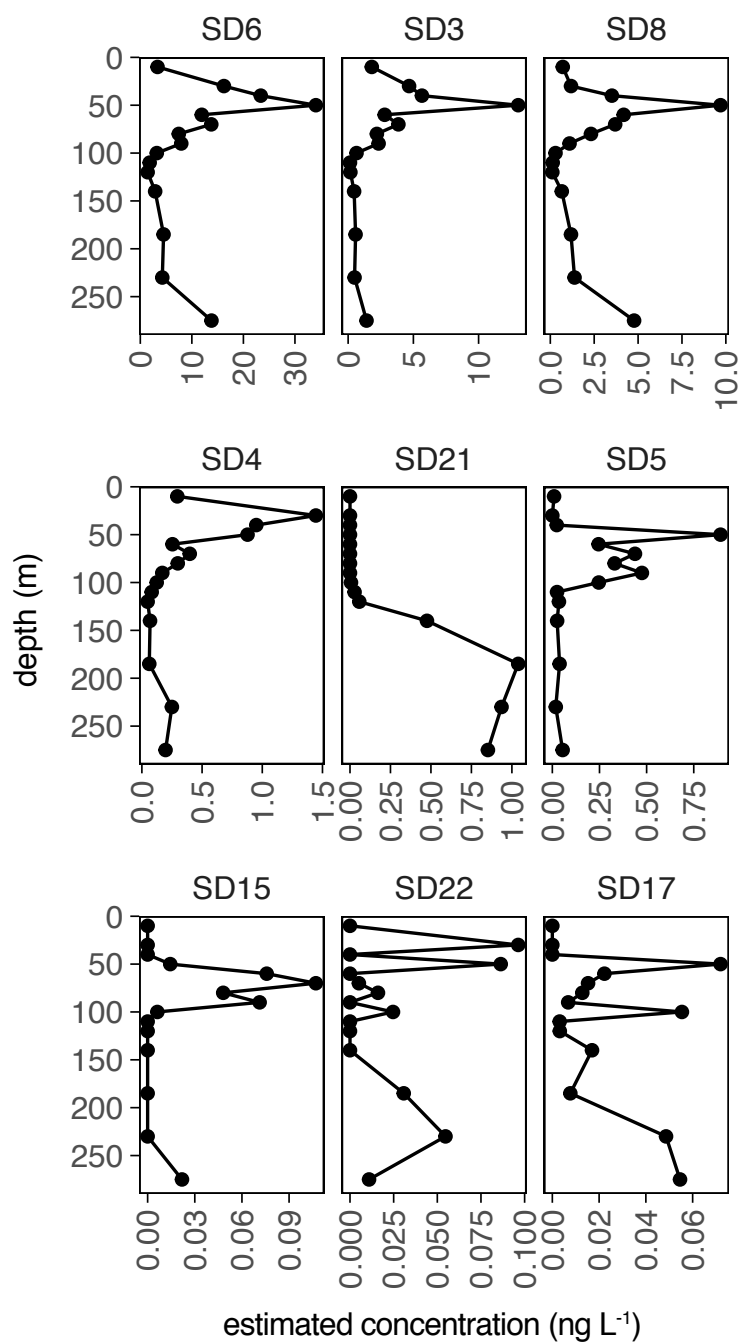


Fig. S7. Taxon-specific 16S-rDNA concentrations of OTUs affiliated with different Subdivisions (SDs) of the *Acidobacteria*. Note the differential preference of certain groups for the oxic (above 90 m) and anoxic parts (below 90 m) of the water column, resembling the differential vertical trends of brGDGTs IIIa' and IIIa'' (cf. Fig. 2C). Panels are arranged in the order of decreasing abundance (upper left to bottom right).

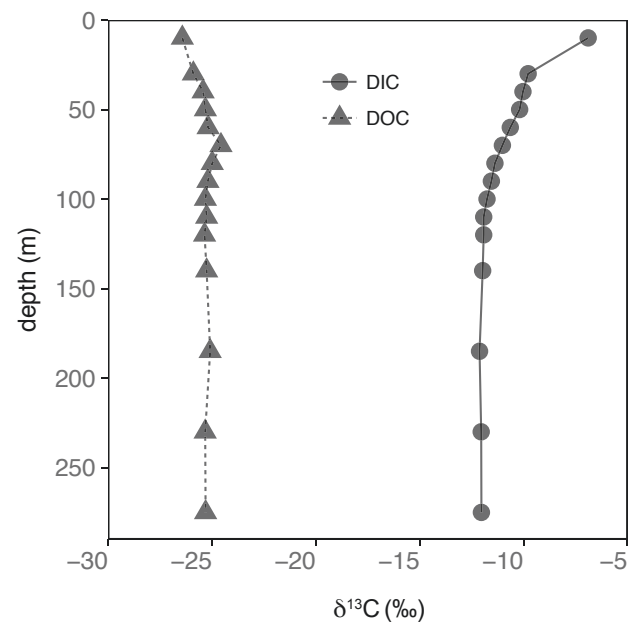


Fig. S8. Stable C isotope composition of dissolved organic (DOC) and inorganic (DIC) carbon pools in Lake Lugano (01 Nov. 2014).

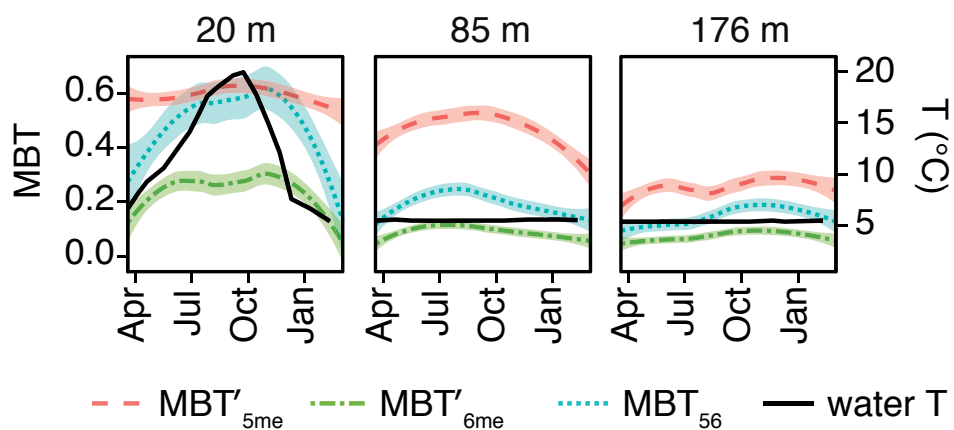


Fig. S9. Comparison of in situ water T with three different brGDGT temperature proxies (i.e., Methylation Indices of brGDGTs – MBT) in settling particles of Lake Lugano collected at three depths in 1990/1991. Despite large temporal variations in the MBT values near the surface (upper trap at 20 m), the proxy values in settling particles further below (i.e., at 85 m and 176 m) were almost invariant throughout the year, consistent with the constantly low T conditions in the hypolimnion. Locally weighted scatterplot smoothing (LOESS) was applied to the proxy data, and 95 % confident intervals are shown as shaded areas.

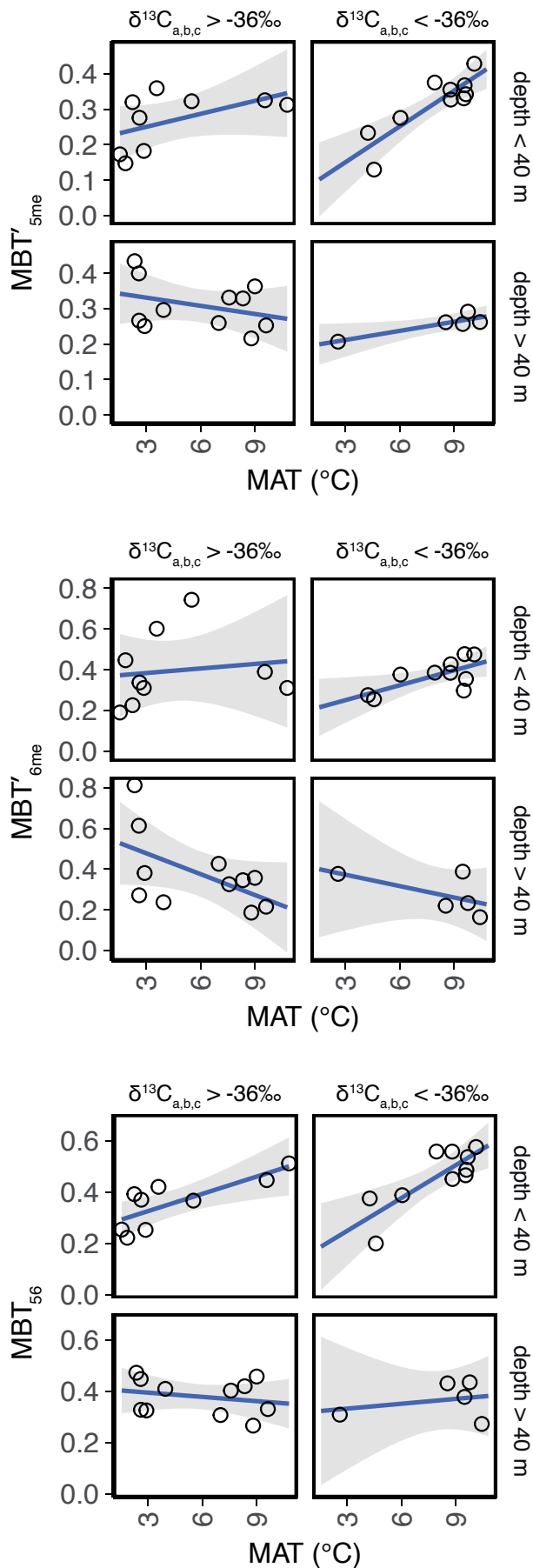


Fig. S10. Lapse rate-based mean annual air T (MAT) plotted against three recently proposed brGDGT temperature proxy indices (MBT'_{5me} [10], MBT'_{6me} [11] and MBT'_{56} [12]) in lake surface sediments from the European Alps ($n=35$). Sites are binned by maximum water depth and the average stable C isotope composition of brGDGTs ($\delta^{13}C_{a,b,c}$). Note that all three proxies show highest positive responses to MAT in shallow lakes characterized by a low ^{13}C content of brGDGTs. Gray areas denote the 95 % confidence intervals of the linear regressions.

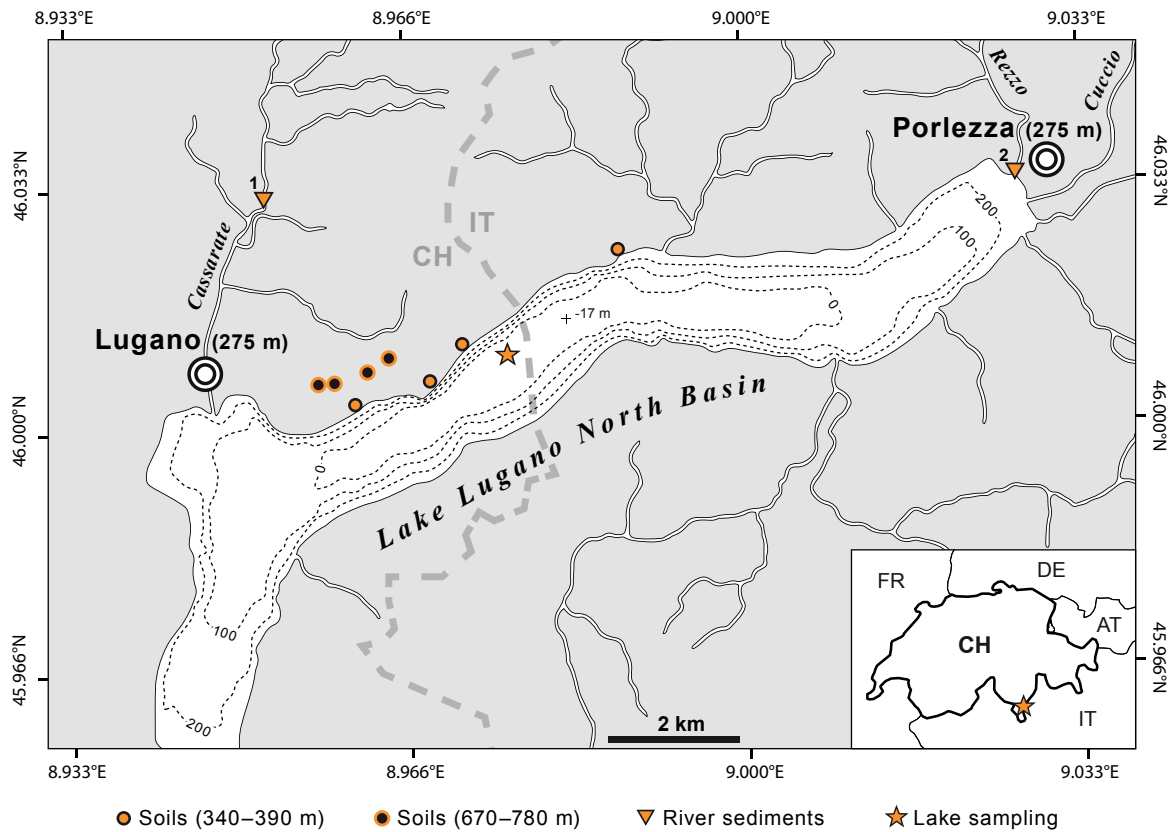


Fig. S11. Sampling sites at Lake Lugano north basin (Southern Switzerland). Lake sampling was performed at 46.01077° N; 9.02021° E, close to the deepest location. Note that catchment soil samples from Lake Lugano were collected at two distinct elevation levels.

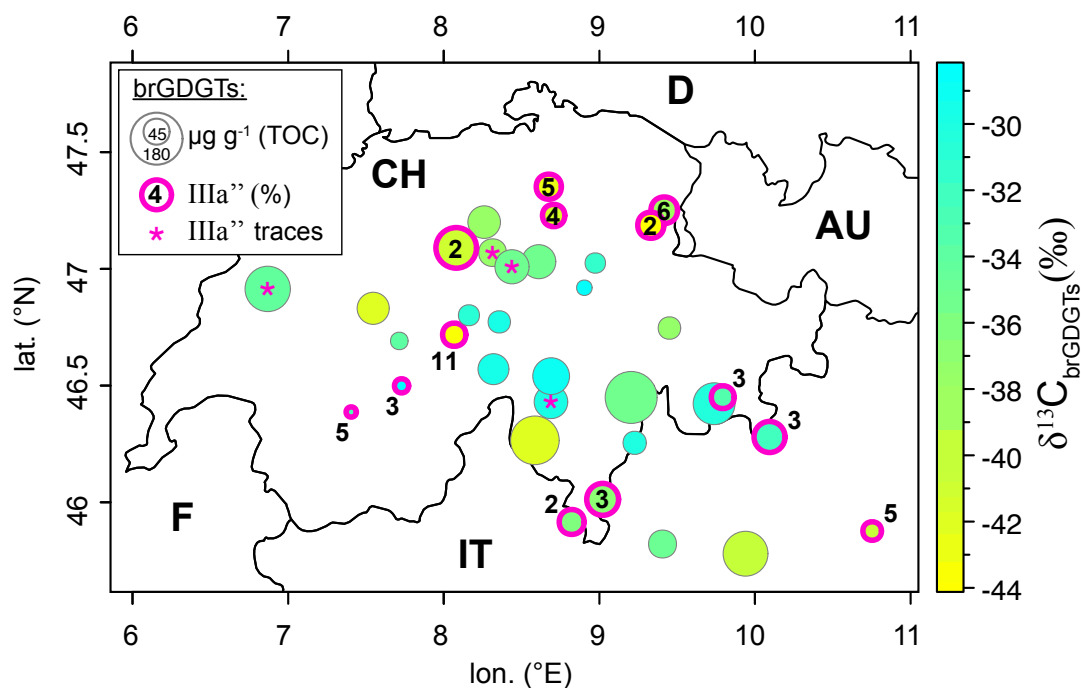


Fig. S12. Location of lake sites investigated in this study, as well as bulk sedimentary brGDGT concentration normalized to bulk organic carbon (C_{org}) (size of circles), weighted average ^{13}C content of brGDGT-derived alkyl chains (a,b,c) (color scale), and the percent contribution of the uncommon isomer IIIa'' to the sedimentary brGDGT pool (numbers).

sample type	ID	lake name	m (asl)	max. depth (m)	bottom water*	trophic state†	IIIa‡	$\delta^{13}\text{C}$ (‰)								
								TOC ¶	(a)		(b+c)		(d+f)		(e)	
									avg.	(STD)	avg.	(STD)	avg.	(STD)	avg.	(STD)
surface sediments	AZ	Alzasca, Lago d'	1857	40	?	?		-31.5	-42.7	(±0.01)	-41.6	(±0.1)	-43.1	(±0.6)	-37.2	(±1.4)
	BA	Baldeggersee	464	66	an	eu		-31.9	-36.0	(±0.3)	-39.5	(±0.4)	-36.6	(±0.6)	-34.5	(±0.8)
	CD	Cadagno, Lago di	1926	21	an	eu		-30.3	-34.2	(±0.4)	-34.9	(±0.6)	-34.8	(±0.7)	-33.3	(±0.7)
	CM	Cama, Lago di	1266	16	?	?		-27.1	-28.9	(±0.5)	-30.9	(±0.2)	-32.1	(±1.2)	-29.8	(±0.8)
	CN	Canovasee	777	10	?	?		-31.1	-37.4	(±0.6)	-37.6	(±0.3)	-37.3	(±0.5)	-36.8	
	DS	Dosso, Lago	1656	6	?	?		-30.6	-34.4	(±0.4)	-36.3	(±0.5)	-35.8	(±0.3)	-32.7	(±1.1)
	ED	Endine, Lago di	335	9	?	?		-34.6	-40.6	(±0.2)	-40.1	(±0.2)	-40.0	(±0.4)	-36.3	(±1.4)
	EN	Engtensee	1852	45	ox	olig		-26.8	-28.8	(±0.2)	-30.6	(±0.2)	-29.4	(±0.5)	-27.9	(±0.4)
	FA	Fälensee	1455	24	?	?	y	-30.7	-38.3	(±0.2)	-37.9	(±0.8)	b.d		b.d	
	GA	Garlate, Lake	198	34	?	?		-28.5	-34.9		-34.8		-33.1		-27.8	
	GE	Gerzensee	602	10	an	eu		n.a	-41.5	(±0.9)	-42.5	(±1.0)	-41.7	(±0.8)	-38.6	
	GF	Greifensee	452	32	an	eu	y	-35.2	-42.3	(±0.3)	-43.1	(±0.1)	-43.3		-36.4	
	GH	Ghirla, Lago di	443	14	?	?	y	-30.8	-35.2	(±0.8)	-37.4	(±1.1)	-36.4	(±1.0)	-35.5	(±0.4)
	GL	Glattalpsee	1848	23	?	?		-27.2	-27.9	(±0.1)	-29.3	(±0.7)	-27.3	(±0.7)	-26.3	(±1.0)
	GS	Grimensee	1909	85	?	?		-26.6	-30.3	(±0.03)	-28.2	(±0.2)	-29.2	(±0.0)	b.d	
	HB	Hinterburgsee	1523	12	an	eu	y	-35.7	-42.8		-44.5		-49.7		-42.8	
	HS	Hinterer Schwendisee	1160	4	?	?	y	-33.3	-44.8	(±0.5)	-43.4	(±0.4)	-42.3	(±1.4)	-36.7	(±0.3)
	IF	Iffigsee	2067	24	ox‡‡	olig‡‡	y	-27.6	-28.5	(±0.6)	-33.7	(±0.2)	b.d		-34.7	
	KT	Klöntalersee	847	46	ox	olig		-28.8	-29.9	(±0.8)	-32.3	(±0.5)	-29.6	(±1.0)	-29.3	(±3.1)
	LD	Ledro, Lago di	654	47	?	?	y	-32.1	-39.7	(±0.0)	-41.3	(±0.4)	-35.9	(±1.2)	-36.0	(±0.9)
	LN	Lungerensee	693	68	ox	mes		-28.7	-30.6	(±0.7)	-31.0	(±0.4)	-29.8	(±1.0)	-30.4	(±0.4)
	LL	Lake Lugano	271	286	an	mes	y	-30.8	-36.9	(±0.3)	-36.9	(±0.4)	-39.1	(±0.8)	-34.8	(±0.3)
	LZ	Lauerzersee	447	13	an	mes		-31.3	-34.5	(±0.5)	-36.0	(±0.2)	-33.6	(±0.4)	-32.7	(±0.9)
	MG	Mognola, Lago di	2005	(30)‡	?	?	(y)	-27.9	-29.3	(±0.3)	-30.2	(±0.4)	-29.7	(±0.5)	-29.9	(±1.1)
	NB	Lac de Neuchâtel	434	152	ox	mes	(y)	-30.8	-33.7		-34.8		-35.1		-32.4	
	OE	Oeschinensee	1580	56	?	?	y	-27.5	-27.3	(±0.1)	-29.5	(±0.7)	-29.3	(±0.7)	-30.3	(±0.4)
	PS	Poschiavo, Lago di	963	85	ox	olig	y	-28.8	-30.3	(±0.2)	-33.9	(±0.4)	-34.5	(±0.8)	-27.4	(±0.9)
	RI	Ritom, Lago	1855	69	?	?		-26.5	-29.5		-28.1		-27.6		-26.3	
	RO	Rotsee	428	16	an	eu	(y)	-32.0	-37.1	(±0.8)	-38.0	(±0.4)	-37.5	(±0.7)	-33.9	(±2.0)
	SI	Silsensee	1800	29	ox	olig		-28.4	-30.3	(±0.2)	-30.4	(±0.5)	-30.5	(±0.8)	-29.2	(±1.1)
SO	Soppensee	598	25	an	eu¶¶	y	-34.0	-40.2	(±0.2)	-42.2	(±0.1)	-40.7	(±0.9)	-37.2	(±0.1)	
SV	Silvaplana, Lago di	1791	78	ox	mes##	y	n.a	-32.1	(±0.2)	-34.0	(±0.0)	-33.6	(±0.7)	-33.8	(±1.2)	
TH	Thunersee	559	215	ox	mes		-27.1	-33.2	(±0.4)	-34.7	(±0.2)	-32.1	(±1.4)	-32.5	(±0.4)	
TR	Trüebsee	1767	7	ox	olig		-24.9					b.d		b.d		
VW	Vierwaldstättersee	599	112	ox	mes	(y)	n.a	-34.3	(±0.4)	-35.6	(±0.2)	-34.3	(±0.3)	-33.9		
ZH	Zürichsee	406	135	an	eu	y	-34.6	-41.1	(±0.2)	-41.7	(±0.1)	b.d	b.d	b.d		
catchment soils	AZ	Alzasca, Lago d'	1857				n	-26.5								
	BA	Baldeggersee	464				n	-29.0								
	CD	Cadagno, Lago di	1926				n	-26.0								
	CM	Cama, Lago di	1266				n	-26.7								
	CN	Canovasee	777				n	-27.2								
	DS	Dosso, Lago	1656				n	-25.2	-26.7		-26.5		-27.4			
	FA	Fälensee	1455				n	-27.2								
	GE	Gerzensee	602				n	-28.5								
	GH	Ghirla, Lago di	443				n	-27.6								
	GL	Glattalpsee	1848				n	-25.4	-24.5	(±0.1)	-24.3	(±0.04)	-26.7	(±0.1)	-27.8	(±0.5)
	HB	Hinterburgsee	1523				n	-26.3‡	-26.9‡	(±1.5)	-27.9‡	(±1.5)				
	HS	Hinterer Schwendisee	1160				n	-27.9								
	IF	Iffigsee	2067				n	-26.3								
	MG	Mognola, Lago di	2005				n	-26.4	-26.6	(±0.3)	-25.2	(±0.1)	-24.5	(±0.8)		
	RO	Rotsee	428				n	-28.5								
	SI	Silsensee	1800				n	-27.6	-26.8	(±0.2)	-26.3	(±0.3)	-26.7	(±0.2)		
	SO	Soppensee	598				n	-28.8								
	TR	Trüebsee	1767				n	-26.6								
	LL	Lake Lugano	670–780				n	-26.6	-28.1	(±0.4)	-26.7	(±0.5)				
	LL	Lake Lugano	340–390				n	-26.8	-28.0	(±0.4)	-26.7	(±0.2)				
river sed.	LL	Lake Lugano	318				n	-27.7	-29.8	(±0.7)	-29.7	(±0.7)				
	LL	Lake Lugano	271				n	-26.2	-26.0	(±0.3)	-27.0	(±0.1)				

Table S1. Lake sites investigated in this study, as well as stable C isotopic composition of brGDGT-derived alkanes (a–f) and total organic carbon (TOC). Abbreviations and key — *bottom water oxygenation. an: seasonally or permanently anoxic; ox: year-round oxenic — †: trophic state based on epilimnetic total N and/or total P concentrations (18); water chemistry data was provided by the Federal Office of the Environment, or taken from literature — #: occurrence of isomer IIIa''; parentheses indicate trace amounts — ¶: average of triplicates (STD<0.15 ‰) — ‡‡: based on a single water sampling (Oct. 2007) — ¶¶: (19) — ##: (20)— ‡: (15).

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Chapter 5

5.1. Conclusions

The main purpose of this thesis was to study the bacterial sources of brGDGTs within lake systems, in order to improve their applicability as lacustrine paleo T proxies. We were able to gain constraints on the putative metabolism and ecology of the as yet unidentified aquatic source organisms, leading to the following conclusions:

At least two distinct groups of brGDGT-producing bacteria are active in freshwater lakes and occupy distinct ecological niches. The first one is restricted to the oxygenated water column and comprises aerobic heterotrophs that are involved in the degradation of particulate organic matter. The second group resides in the vicinity of the redox transition zone (RTZ) under hypoxic/anoxic conditions.

The C6-methylated brGDGT isomer IIIa' is preferentially (or exclusively) produced by an aerobic bacterial community, whereas production of the novel C5/C6-methylated isomer IIIa'' must be solely due to anaerobic/microaerophilic source organisms. The remaining 14 brGDGT core lipid types are either shared between both aerobic and anaerobic communities, or are produced by facultative aerobes that are present in both oxic and anoxic aquatic environments.

Consistent with previously reported data from the terrestrial realm, members of the phylum Acidobacteria represent a plausible biological source of brGDGTs in lacustrine environments, as their abundance and vertical niche-differentiation in the water column corresponds well with concentration profiles of individual brGDGTs. Yet, the contribution of brGDGTs produced by other bacterial phyla cannot be excluded.

Although brGDGT-producers are present in surface water, aquatic in situ-production appears to take place predominantly in hypolimnetic water (i.e., below the thermocline), as well as the redox transition zone.

These findings considerably affect the interpretation of brGDGT signals in ancient lake sediments, because they suggest that

(i) a variety of climate-independent ecological effects (e.g. eutrophication leading to changes in the redox regime) may alter the composition of brGDGTs preserved in the sediment record over time,

(ii) lake-derived (autochthonous) brGDGTs are more likely to reflect the water T of the hypolimnion rather than surface water- or mean air T,

(iii) differential mixing of deep water- and soil-derived brGDGTs within the sediments may lead to large offsets in the T estimates derived from brGDGTs records.

These issues can be addressed by employing the stable C isotopic composition of brGDGTs as an indicator for provenance (aquatic vs. terrestrial) and redox regime (presence of a methane). This allows us to identify sediment records that are dominated by brGDGT produced in hypoxic bottom waters, providing insight into climate-driven long-term variations of deep lake temperatures.

5.2. Implications

In light of the fairly complex relationship between MAT and brGDGT distributions in lake sediments, it is remarkable that strong empirical correlations are nevertheless found in large environmental data sets (e.g., Loomis et al., 2014). It further appears that plausible brGDGT-derived paleo MAT estimates, which are in agreement with independent proxy data, are most frequently reported from the African continent (Loomis et al., 2012, 2015; Sinninghe Damsté et al., 2012; Kelly et al., 2014; Russell et al., 2017). Assuming the predominance of deep water-derived brGDGTs also in these lakes, our findings raise the question as to how the hypolimnetic-/bottom water T is related to MAT, and how this relationship is influenced by the overall climate regime of the study site(s).

Generally, the T distribution within the hypolimnion is shaped during convective overturning of the water column, when seasonal cooling of surface water terminates thermal stratification (Michalski and Lemmin, 1995; Ambrosetti and Barbanti, 1999). However, with the exception of very shallow lakes that eventually freeze to the bottom, lake bottom water cannot be much cooler than 4°C (the temperature at which water has its greatest density), regardless of whether winter air temperatures at the lake site are for example 0 °C or -10 °C on average. Lakes of warmer climates, on the other hand, mix at temperatures well above 4 °C (Lewis, 1996; Gulati et al., 2017) and, hence, a more linear relationship between bottom water T and MAT can be expected. Indeed, this is supported by recent numerical modeling, predicting that hypolimnetic water of cool-temperate lakes (MAT < 10 °C; max. depth > 4 m) will be unaffected by a future 2.5 °C increase in MAT, whereas in warmer climates (MAT > 10°C), bottom water T is expected to rise concomitantly with MAT and average surface water T (Butcher et al., 2015). Moreover, the weaker seasonality at lower latitudes produces smaller vertical water T gradients (Lewis, 2010) and, as a consequence, biases induced by differential contribution of soil-, surface-, and deep water-derived brGDGTs are probably less pronounced than in high latitude lakes. I therefore argue that differential brGDGT sources are particularly problematic in cool-temperate climates, whereas they are probably much less of a concern in the tropics.

Another issue is that current only one lacustrine brGDGT calibration distinguishes between C5- and C6-methylated brGDGT isomers (Russell et al., 2017) and, hence, differential effects of environmental variables on the abundances within either of these groups may have been a considerable source of error in previous studies. One promising approach in this context may be the newly proposed MBT'_{6me} index that is based solely on the tetramethylated- and C6-methylated brGDGTs (Eq. 5). Indeed, in Lake Lugano, we observed strong seasonal variations in the MBT'_{6me} values

derived from settling particles at the base of the epilimnion (20 m; Fig. S9 Chapter 4.1). Therefore, a global survey of C5- and C6 isomers in lacustrine setting could be an important step towards disentangling the effects of T and other environmental parameters on different brGDGT isomers.

$$MBT'_{6me} = \left(\frac{Ia + Ib + Ic}{Ia + Ib + Ic + IIa' + IIb' + IIc' + IIIa'} \right) \quad (\text{Eq. 5; Dang et al., 2016})$$

A more general problem concerning proxy calibration is, in my opinion, the use of fractional abundances in multiple linear regression (MLR) models (see section 1.2), which have often been used in previous studies. The initial step of standardization (relative to the sum of all brGDGTs) generates a linear dependency between the individual compounds, and is based upon the assumption that all brGDGTs share a common biological source. As we have shown here, however, at least some of the brGDGT isomers derive from distinct groups of organisms, which may have distinct physiological responses to ambient T. Future studies should therefore aim at constraining the number of brGDGTs considered in proxy indices to the ones that derive from the same/similar biological source(s) and/or ecological niche(s) (e.g. by experimental work), which will hopefully provide a more appropriate basis for the standardization of brGDGT abundances prior to statistical analyses.

Given the apparent intricacies associated with the use of brGDGTs as lacustrine paleo T indicators, it is important to consider alternative lipid-based proxies. In fact, the TEX_{86} index of archaeal GDGTs, as well as the unsaturation ratio(s) of long chain alkenones (LCAs) from haptophyte algae, are also promising tools for paleolimnological reconstructions (see section 1.2). However, TEX_{86} and LCAs both suffer from similar problems as the brGDGTs, mostly related to genetically distinct source organisms at different study sites (Toney et al., 2012; Auguet and Casamayor, 2013), and the confounding effects of hydrochemical parameters on lipid compositions (e.g. pH and salinity; Toney et al., 2011). This clearly illustrates that lacustrine molecular T proxies are not free of taxonomic- and ecological interferences, which likely arise from the heterogeneity among spatially separated lake basins, and the microbial communities they host. In order to improve our ability to reconstruct past continental climates, it seems necessary to equally advance our knowledge on various lipid classes, including both GDGTs and LCAs, as well as 'long chain diols' (LCDs) produced by algae of the Eustigmatophyceae (Villanueva et al., 2014), whose proxy potential in freshwater systems is just being explored (Rampen et al., 2014). The conjunctive application of multiple lipid proxies would provide a means of independent control, which will likely increase the overall confidence in lacustrine paleo T estimates in the future.

Finally, another specific rationale for investigating the biological sources of the branched GDGTs stems from the uniqueness of their structure, which combines typical archaeal traits (membrane-spanning, ether-bound lipids) with a bacterial sn-1,2-glycerol stereochemistry (see section 1.2). Isolation, genome sequencing and phylogenomic analysis of the lipid-biosynthetic genes from

brGDGT-producers, for instance, may help to shed more light on the ‘lipid divide’ between bacteria and archaea, which still is an unsolved conundrum of evolutionary microbiology (e.g. Koga, 2014; Villanueva et al., 2017).

5.3. Future work

Identifying and cultivating the enigmatic group of brGDGT-producing microbes would be a significant achievement with impact in the fields of paleoclimate research and environmental microbiology. Our work has provided valuable clues on the ecology and lifestyle of these organisms, and I encourage future work to address the following research questions:

(i) What is the metabolism of brGDGT producers thriving at the RTZ? What are their substrate requirements, and what is the role of dissolved oxygen and other electron acceptors?

(ii) Are there differences in taxonomy and metabolic lifestyles between brGDGT producers in soils vs. aquatic habitats? And can these differences possibly explain the contrasting brGDGT signatures expressed in these environments?

(iii) What controls environmental brGDGT composition: Physiological adaptation to ambient T of individual cells (i.e., homeostasis), or community level responses that involve multiple species or groups of brGDGT-producers?

To address the above questions, future work should expand on the ^{13}C stable isotope probing experiments we have performed in this study (Chapter 4.1) by including molecular biological methods (i.e., sequencing of the ^{13}C -labelled DNA fraction; e.g. Beck et al., 2013). This will allow linking lipid production patterns and microbial diversity changes, which in turn may yield robust pieces of evidence for the phylogenetic affiliation of brGDGT-producing environmental microbes. For this purpose, Lake Lugano seems an ideal model system because it hosts different communities of brGDGT-producers, which can be sampled and studied separately under different experimental conditions.

Samples — Our results suggest that the redox transition zone (RTZ) in stratified lakes is a ‘hotspot’ for brGDGT production. SPM sampling should therefore center around the oxycline, but also include samples from fully oxic- and anoxic waters. I further suggest to conduct parallel incubations of soil- and lake sediment slurries, to investigate differential patterns of brGDGT production in terrestrial- vs. aquatic- vs. sedimentary habitats.

Microaerophilic brGDGT production — The C source preference of the RTZ community should be investigated in greater detail to answer the question about the C-metabolic lifestyle of the low-oxygen-adapted brGDGT producers. Because it is not possible to maintain a constantly low O_2 tension in batch experiments, the use of a chemostat (i.e., bioreactor) is required. The RTZ community may also be subjected to fully oxic conditions, to test whether brGDGT IIIa” is indeed restricted to low-oxygen environments.

Organic carbon substrates — A larger number of different substrates should be tested to obtain more detailed information on the metabolic capabilities of the heterotrophic brGDGT producers. Besides glucose and other low-molecular-weight C compounds (e.g. short-chain fatty acids, amino- and ketoacids), more complex carbohydrates and biopolymers (e.g. chitin) may be included.

Temperature control on brGDGT distribution — T responses of brGDGTs have been tested in peat (Huguet et al., 2013, 2014), but not yet in aquatic samples. Incubation of suspended biomass under different T may (i) provide proof-of-concept that aquatic brGDGT production is indeed T-dependent, (ii) identify brGDGTs that are most indicative of T variations, and (iii) establish an experimental brGDGT–water T calibration.

pH-control on brGDGT distribution — brGDGT distributions are notoriously sensitive to changes in pH (Weijers et al., 2007; De Jonge et al., 2014). Due to photosynthetic draw-down of CO₂, lake surface water pH in eutrophic lakes increases dramatically during the high-productivity season, resulting in a strong vertical pH gradient in the water column. As this is likely to modulate the brGDGT distribution in the sediments, it is crucial to investigate the effect of pH on the brGDGT-producing bacterial community in separate experiments.

Alternative electron acceptors — After oxygen, nitrate (NO₃⁻) is the preferred e-acceptor in anaerobic respiration (e.g. Lovley and Coates, 2000), and is depleted already ~20 m below the oxycline in Lake Lugano (i.e., at the ‘nitracline’). It seems therefore possible that brGDGT-producers thriving at the RTZ are heterotrophic nitrate reducers. This should be addressed by NO₃⁻-amended anaerobic experiments.

Methane derived carbon — Also the putative (indirect) uptake of methane-derived C compounds may be investigated experimentally by amendment of the RTZ community with ¹³CH₄, and subsequent ¹³C analysis of brGDGTs and methanotroph-specific fatty acids, as well as sequencing of the heavy DNA fraction.

Autotrophic production — Previous studies in soil (Oppermann et al., 2010) and peat (Huguet et al., 2017) have largely ruled out brGDGT production by autotrophic microorganisms. For lacustrine systems, however, such evidence is missing. It is worthwhile to address this aspect in future experimental work.

Microbial diversity in lake sediments — Finally, the link between brGDGT composition and microbial diversity should be explored on larger spatial scales by an inter-lake comparison. It would be particularly interesting whether the presence of brGDGT IIIa” in sediments can be correlated to the occurrence of specific acidobacterial sequences (e.g. affiliated with subdivision 21), as we have observed in the water column of Lake Lugano (Chapter 4). As such, our current survey of Alpine lake surface sediments provides an excellent ‘road map’ for targeted sequencing work in the future.

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