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Application of microbial membrane tetraether lipids in speleothems

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Glycerol dialkyl glycerol tetraethers (GDGTs), which are derived from microbial membranes, occur widely in soils, peats, lake sediments, marine sediments, hot springs, and other environments. GDGTs respond sensitively to environmental changes and have become an important tool in paleoenvironmental reconstruction. However, their application in speleothems, one of the most important archives of the study of past climates, has been quite limited. In this paper, we review the recent progress in the study of GDGTs in speleothems, from their sources to their distribution characteristics and paleoenvironmental applications. GDGTs in speleothems have been shown to come from dripping water, and cave systems. The distribution of GDGTs in speleothems shows that archaeal isoprenoid GDGTs (isoGDGTs) dominate over bacterial branched GDGTs (brGDGTs). The proxies based on isoGDGTs (TEX₈₆) and brGDGTs (MBT/CBT) are correlated with temperature, which may offer promising potential methods for reconstructing terrestrial palaeotemperature. Ultimately, we discuss the difficulties and problems to be solved in studying speleothem GDGTs and the possible future directions for this field.

KEYWORDS

speleothems, GDGTs, Paleoenvironmental reconstruction, TEX₈₆, MBT/CBT

1. Introduction

At present, the issue of global change is attracting increasing attention. The frequent occurrence of extreme climate events has jeopardized people's lives and productivity and caused huge social and economic losses. Therefore, it is essential to study the principles of historical climate change to predict future climate change. The wide distribution of stalagmites and their utility in accurate high-resolution dating have made them an important archive in the field of paleoenvironment research. A series of advances have been made in stalagmite analysis (Wang et al., 2001; Hu et al., 2008; Cai et al., 2010; Cheng et al., 2016; Zhu et al., 2017; Tan et al., 2018; Zhang et al., 2018; Zhang et al., 2021), and environmental proxies based on stalagmites such as carbon and oxygen isotopes (Dorale et al., 1998; Wang et al., 2001), trace elements (Roberts et al., 1998; Tan et al., 2014; Zhang et al., 2018), and magnetism (Zhu et al., 2017) have been used extensively in the reconstruction of paleoenvironments. However, most of these studies have focused on inorganic proxies, which are often affected by many factors (McDermott, 2004).

Carbonate $\delta^{13}\text{C}$ is affected by temperature, soil moisture, cave system openness, and other factors (Hendy, 1971; Genty et al., 2003). As one of the most important proxies of stalagmites, the interpretation of $\delta^{18}\text{O}$ remains controversial. Some studies indicate that $\delta^{18}\text{O}$ reflects the monsoon intensity or monsoon rainfall amount (Wang et al., 2001; Cheng et al., 2016), whereas other studies suggest that $\delta^{18}\text{O}$ indicates the changes in the relative contributions of different water vapor sources (Maher, 2008; Maher and Thompson, 2012). Therefore, a comprehensive comparison of multiple proxies is important to fully understand their real climatic significance.

In addition to inorganic proxies, the organic matter in stalagmites also records environmental information, and the rich variety of lipid biomarkers, a class of organic matter preserved in stalagmites can provide more evidence for paleoenvironment research. Lipid biomarkers are derived from specific organisms. During the degradation of organic matter, the carbon skeleton is retained, and the information on the biological precursors and the environment where the source organism inhabits is recorded; this information can be used in paleoenvironmental reconstructions (Meyers and Ishiwatari, 1993; Castañeda and Schouten, 2011). Until recently, the most well-studied biomarkers include long-chain *n*-alkanes of leaf wax (Castañeda et al., 2016), fatty acids (Wang et al., 2018), long-chain alkenones (U^k_{37}) (Abrantes et al., 2005), glycerol dialkyl glycerol tetraethers (GDGTs) (Weijers et al., 2007a), which have been extensively used in paleo-reconstruction in a variety of archives.

Among lipids, GDGTs derived from archaea and some bacteria are relatively stable. They are resistant to degradation in sediments, and have been detected as far back as Jurassic (Kuypers et al., 2001; Schouten et al., 2003; Jenkyns et al., 2012). GDGTs are found ubiquitously in soils, peatlands, lake sediments, marine sediments, hot springs, and speleothems (Weijers et al., 2006a,b, 2007b; Yang et al., 2011, 2014; Schouten et al., 2013; Zhang et al., 2013; De Jonge et al., 2014; Sinninghe Damsté et al., 2014; Dang et al., 2016). Over the last decade, GDGTs have become a hotspot of bio-organic geochemical research. Researchers have analyzed GDGTs in marine sediments (Wuchter et al., 2004; Zhang et al., 2011), lake sediments (Powers et al., 2010; Sinninghe Damsté et al., 2012a; Hu et al., 2015), peatlands (Zhou et al., 2011; Wang et al., 2017), and loess (Gao et al., 2012; Jia et al., 2013; Tang et al., 2017; Wang et al., 2020) around the world, and successfully reconstructed temperature changes in different regions, such as tropical Africa (Weijers et al., 2007a), Amazon basin in South America (Bendle et al., 2010), Antarctic coast (Pross et al., 2012), and China (Yang et al., 2014; Dong et al., 2015; Tang et al., 2017; Lu et al., 2019). The results of these studies provide important support for understanding climate change across the globe.

The use of proxies in the study of cave paleoenvironment is an important research area and the GDGTs preserved in stalagmites have the potential to provide a terrestrial organic paleothermometer independent of isotopic records (Yang et al., 2011; Blyth and Schouten, 2013). However, there have been relatively few studies of stalagmite GDGTs, and most of these studies have focused on the provenance of GDGTs in stalagmites, as well as the relationship between GDGTs and environmental variables (Yang et al., 2011; Blyth and Schouten, 2013; Blyth et al., 2014, 2016; Baker et al., 2016, 2019; Zhang et al., 2020). Paleoenvironmental reconstructions have only been determined in Mawmluh Cave (India) and Jiangjun Cave (China) (Figure 1; Huguet et al., 2018; Wassenburg et al., 2021). Besides, 5-methylated and 6-methylated bacterial branched GDGT (brGDGTs) isomers have been rarely reported (Zhang et al., 2020).

In this paper, we review the sources and distribution characteristics of stalagmite GDGTs based on recent studies and the use of GDGT-based proxies in stalagmites to provide references for future paleoenvironmental reconstructions based on stalagmite GDGTs.

2. Structures and biological sources of GDGTs

GDGTs are the core membrane lipids of archaea and some bacteria (Schouten et al., 2013) and can be divided into two groups: archaeal isoprenoid GDGTs (isoGDGTs) and bacterial branched GDGTs (brGDGTs) (Figure 2). The configuration of the glycerol backbone in archaeal isoGDGTs is 2,3-di-*O*-alkyl-*sn*-glycerol, and its alkyl chain contains 0–8 cyclopentane moieties. Crenarchaeol and crenarchaeol regioisomer (denoted by crenarchaeol') contain a unique cyclohexane moiety and were thought to be specifically produced by Thaumarchaeota (Pitcher et al., 2011a; Sinninghe Damsté et al., 2012b; Elling et al., 2017). The brGDGTs have 1,2-di-*O*-alkyl-*sn*-glycerol configuration, and its alkyl chain contains various numbers of methyl groups and cyclopentane moieties (Weijers et al., 2006b). BrGDGTs are biosynthesized by some bacteria, among which one strain of Acidobacteria, *Solibacter usitatus*, was capable of producing 5-methylated and tetramethylated brGDGTs (Sinninghe Damsté et al., 2018; Chen et al., 2022; Halamka et al., 2022). The presence of ether bonds confers good thermal stability on GDGTs. In general, "GDGTs" refers to core lipid GDGTs (CL-GDGTs), whose structures include glycerol backbones and alkyl chains. However, in living cells, GDGTs exist in the form of intact polar lipid GDGTs (IPL-GDGTs), which have glycerol backbones at both ends and polar head groups composed of phosphoric acid and sugar moieties (Sturt et al., 2004; Lipp et al., 2008; Peterse et al., 2011). The polar head groups are rapidly lost after cell death to form CL-GDGTs, so IPL-GDGTs indicate living biomass (Lipp et al., 2008; Tierney et al., 2012).

2.1. isoGDGTs

Many archaea can synthesize isoGDGTs observed in the environment (Schouten et al., 2007a). Archaea were initially believed to synthesize isoGDGTs to resist the stresses of extreme environments, such as high temperatures, high salinity, and extreme acidity (Schouten et al., 2013). However, numerous recent studies show that isoGDGTs were found not only in extreme environments, but also widely distributed in normal environments such as oceans, soils, and lakes (Schouten et al., 2013). GDGT-0 is the compound most commonly found in cultured archaea other than halophiles, and therefore GDGT-0 may be prevalent in uncultured archaea living in the environment (Blaga et al., 2009; Weijers et al., 2009). GDGT-1–4 commonly occur in Euryarchaeota, Thaumarchaeota, and Crenarchaeota, and at especially high abundances in anaerobic methanotrophic archaea group 1 (ANME-1; Blumenberg et al., 2004). GDGT-5–8 usually occur in high-temperature environments, such as hot springs, and some cultures of thermophilic Crenarchaeota and Euryarchaeota (Pearson et al., 2004; Pearson et al., 2008; Zhang et al., 2008; Burgess et al., 2012). GDGT-5–8 have only been detected in peat environments and acid mine drainage and may be contributed by the acidophilic archaea in these environments (Naafs et al., 2018; Pei et al., 2019; Yang et al., 2019).

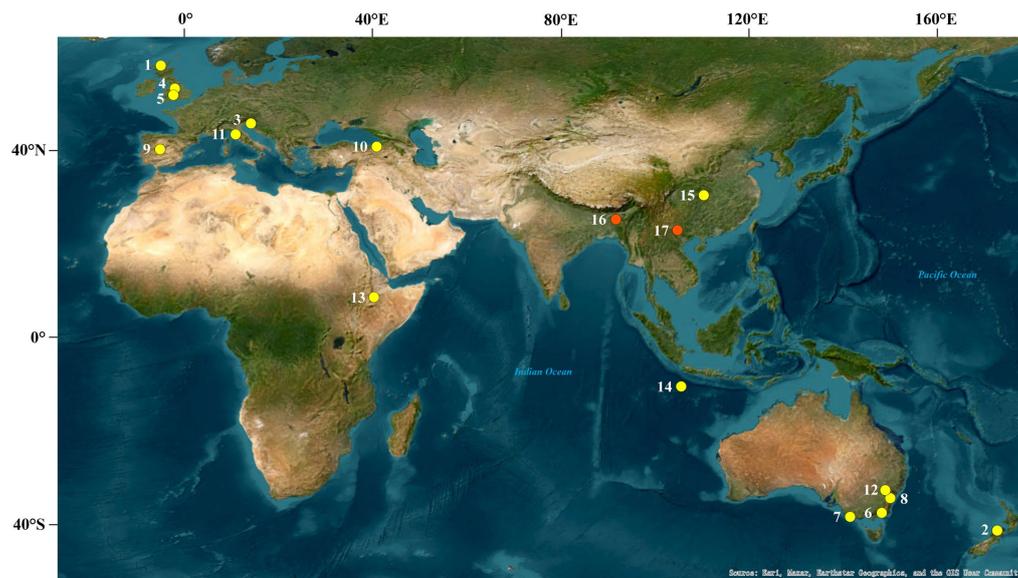


FIGURE 1

Global cave locations of published GDGT-based studies (1, Uamh am Tartair, Scotland; 2, Nettlebed Cave; 3, Postojna Cave; 4, Pooles Cavern; 5, Lower Balls Mine; 6, Buchan Caves; 7, Bats Ridge Cave System; 8, Wombeyan Caves; 9, Eagle Cave; 10, Turkey; 11, Tuscany, Italy; 12, Cathedral Cave and Gaydon Cave; 13, Achere Cave and Rukessia Cave; 14, Christmas Island; 15, Heshang Cave; 16, Mawmluh Cave; 17, Jiangjun Cave. The yellow dots 1–15 are sites of modern-process studies: Yang et al., 2011; Blyth and Schouten, 2013; Baker et al. 2019; and the red dots 16–17 are sites of paleoclimatic studies: Huguet et al., 2018; Wassenburg et al., 2021).

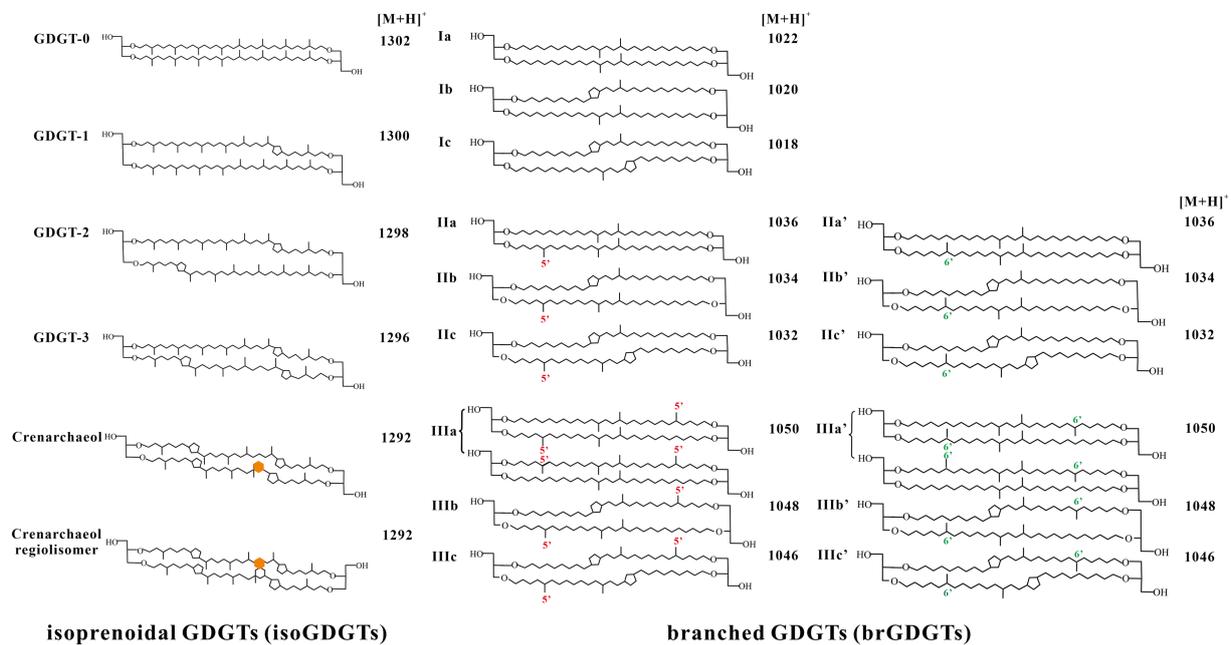


FIGURE 2

Structures of archaeal isoGDGTs and bacterial brGDGTs.

Crenarchaeol was thought to be a biomarker for ammonia-oxidizing Thaumarchaeota (Pitcher et al., 2011b; Sinninghe Damsté et al., 2012b; Elling et al., 2017) and was initially thought to be derived from marine Group I archaea (Hoefs et al., 1997; Delong et al., 1998). Subsequent studies show that crenarchaeol was abundant in diverse settings and can be produced by pure cultures of several strains of

Thaumarchaeota. Sinninghe Damsté et al. (2012b) considered that crenarchaeol specifically containing a cyclohexane moiety is formed by microorganisms to adapt to low-temperature environments because it changes the structure of the cell membrane to increase its fluidity. However, archaea with high crenarchaeol content have since been detected in hot springs (Schouten et al., 2007b). The isoGDGTs

detected by de la Torre et al. (2008) and Pitcher et al. (2010) in thermophilic Thaumarchaeota were mainly crenarchaeol, confirming that crenarchaeol with a cyclohexane moiety is not restricted to low-temperature environments. The formation mechanism of the cyclohexane moiety remains to be determined. Crenarchaeol in the subsurface waters of oceans and lakes may be derived from Thaumarchaeota Group I.1a, which prefers micro-anaerobic environments (Pitcher et al., 2011b; Schouten et al., 2012, 2013).

As well as the marine water column, the most likely biological sources of crenarchaeol in soils (Timonen and Bomberg, 2009) and lake sediments (Buckles et al., 2013) are Thaumarchaeota Group I.1b (Xie et al., 2015) because Group I.1b is most widespread in these environments. Crenarchaeol' (a regioisomer of crenarchaeol) is also thought to be derived from Thaumarchaeota and has been detected in high proportions in soil group I.1b Thaumarchaeota (Sinninghe Damsté et al., 2012b).

2.2. brGDGTs

The alkyl chain of brGDGTs has a methyl branched-chain structure rather than the isoprenoid structure of the isoGDGT alkyl chain, and the glycerol skeleton configuration also differs from that of isoGDGTs. Therefore, these compounds are called "branched" GDGTs. BrGDGTs are derived from some Acidobacteria and possibly by other unknown bacteria (Sinninghe Damsté et al., 2011; Chen et al., 2022). BrGDGTs have been found in soils (Weijers et al., 2007c), lakes (Loomis et al., 2011; Tierney et al., 2012), rivers (Hopmans et al., 2004), and marine sediments (Zhu et al., 2011). To date, more than 15 brGDGT compounds have been detected in different environments around the world, and Acidobacteria may be a biological source of at least the 5-methyl brGDGTs (Chen et al., 2022).

The analysis of peat profiles in Sweden showed that the high content of brGDGTs was probably produced by the major subdivisions of Acidobacteria (Weijers et al., 2009). As peat becomes more anaerobic, the content of IPL-brGDGT increases significantly, indicating that the bacteria producing brGDGTs are probably anaerobic or facultatively anaerobic (Liu et al., 2010; Peterse et al., 2011). However, brGDGTs have also been detected in aerobic environments, such as surface peat and surface soil (Weijers et al., 2006a; Liu et al., 2010), indicating that the bacteria producing brGDGTs in the environment may be able to adapt to a wide range of oxygen concentrations. The content of brGDGTs in soils decreases as pH increases (Peterse et al., 2010) and is relatively high in acidic soils, whereas the contents of some groups of Acidobacteria, such as subdivision 1, decrease significantly at high pH (Jones et al., 2009). The contents of IPL-GDGTs in soils do not differ significantly across the seasons (Weijers et al., 2011), which is consistent with the slow growth of Acidobacteria. BrGDGTs are also relatively stable and not easily degraded, and the high contents of brGDGTs in soil and peat may be the result of their long-term accumulation (Yang et al., 2014).

2.3. OH-GDGTs

In addition to the conventional GDGTs discussed above, GDGTs with 1–2 hydroxyl groups in the alkyl chain, hydroxyl GDGTs (OH-GDGTs), occur in marine sediments (Huguet et al., 2013), lake

sediments (Wang et al., 2017), and acid soils (Man et al., 2023). They are designated OH-GDGT-0, OH-GDGT-1, and OH-GDGT-2 according to the number of cyclopentane moieties. It has been demonstrated that pure cultured Thaumarchaeota Group I.1a can produce OH-GDGTs, whereas Group I.1b cannot (Sinninghe Damsté et al., 2012b). Some methanogenic archaea, such as the thermophilic methanogenic archaea *Methanococcus thermolithotrophicus*, can produce OH-GDGT-0, and the anaerobic methane-oxidizing archaea ANME-2d can synthesize OH-GDGT-2–3 (Kurth et al., 2019).

Huguet et al. (2013) investigated lake and marine sediments worldwide and found that OH-GDGTs occur predominantly in low-temperature regions and that their relative content correlates well with temperature. A subsequent study of OH-GDGTs showed that the number of cyclopentane moieties in OH-GDGTs (the index RI-OH) correlates positively with temperature (Lü et al., 2015). In the sediments of Qinghai Lake, the relative content of OH-GDGTs was consistent with relative changes in the abundance of crenarchaeol and *amoA* (a functional gene of ammonia oxidation in Archaea and bacteria), suggesting that OH-GDGTs are derived mainly from Thaumarchaeota (Wang et al., 2017). The composition of OH-GDGTs in peat may be affected by methanogenic archaea. The RI-OH index of OH-GDGTs correlates significantly with the ratio of GDGT-0/crenarchaeol, and the relative abundance of OH-GDGT-0 increases gradually with the increasing depth of peat (Yang et al., 2019).

3. Extraction and analytical methods for stalagmite GDGTs

3.1. Pretreatment of samples

The organic carbon content preserved in stalagmites accounts for about 0.01–0.3% of the total carbon (Blyth et al., 2013; Li et al., 2014; Quiers et al., 2015), and the content of lipids in stalagmites is relatively low (Huang et al., 2007). Therefore, the extraction of GDGTs requires large samples, and external pollution must be reduced as much as possible during their extraction. All glassware should be cleaned using ultrapure water. After drying, the glassware should be heated in a muffle furnace at 500°C for 6 h to remove all organic matter from the vessel. Other materials used in the experiment, such as filter film and hydrochloric acid, should be treated with dichloromethane (DCM). The stalagmite samples are cut along the growth axis and then divided into small pieces according to the growth level (to ensure high-accuracy dating). The thin surface layer of the massive stalagmite sample is removed, washed using ultrapure water, and dried. The residual organic pollutants on the surface are removed using ultrasound in DCM before analysis (Blyth et al., 2006; Blyth and Schouten, 2013; Zhang et al., 2020).

3.2. Extraction of GDGTs from stalagmites

The early experimental extraction techniques required about 100 g of powdered speleothem sample to extract the lipids (Xie et al., 2003). With the application of acid digestion technology, more organic matter can be released when only limited stalagmite samples are available (Blyth et al., 2006; Huang et al., 2008). In general, GDGTs can be extracted from approximately 5–20 g of stalagmite samples (Blyth and Schouten, 2013). Wang et al. (2012) tested different acid

digestion conditions and found that a 5 g stalagmite sample was sufficient for lipid analysis using 3 mol/l hydrochloric acid and heating at 130°C for 3 h. After the stalagmite sample is digested, liquid–liquid extraction is performed using DCM–MeOH (9:1, v/v) reagent (30 ml × 6). The collected total lipid extracts are concentrated to 1–2 ml, filtered through 0.45 μm polytetrafluoroethylene (PTFE) membrane, and dried under nitrogen before their preparation for analysis (Yang et al., 2011; Wang et al., 2018; Zhang et al., 2020).

3.3. Analytical method for stalagmite GDGTs

In recent years, many studies have shown that some brGDGT may contain one or more isomers, that coelute in the chromatogram obtained by the traditional liquid chromatography method. These isomers are often treated as one compound because they cannot be easily separated. These isomers can interfere with the study of brGDGT distributions, proxies, and their paleoenvironmental applications (Zech et al., 2012). With improvements in HPLC methods, De Jonge et al. (2014) successfully used four silica gel columns to separate all 5-methyl and 6-methyl isomers of GDGT-III and GDGT-II. Hopmans et al. (2016) optimized the liquid chromatography method and separated 15 brGDGT compounds using only two silica gel columns in series. Further study showed the separation effect of the method is better than the previous studies. Isomers have even been found for Ia and Ib (Yang et al., 2015).

Yang et al. (2011) first analyzed the contents of isoGDGTs and brGDGTs in stalagmites following Hopmans et al. (2000). The method used does not separate 5-methyl from 6-methyl brGDGTs. The specific analytical method followed here is described by Zhang et al. (2020). The GDGTs are analyzed using high-performance liquid chromatography–mass spectrometry (HPLC–MS). The dried extracts were resolved in *n*-hexane: isopropanol (9:1, v/v) and a known amount of C₄₆ GTGT (glycerol trialkyl glycerol tetraether; Huguet et al., 2006) internal standard was added. The injection volume of each sample is 10–30 μl (selected according to the concentration of the sample).

Separation of 5- and 6-methylated brGDGTs was performed by using two silica columns in tandem (BEH HILIC column, 150 mm × 2.1 mm, 1.7 μm, Waters or Thermo Finnigan silica column, 150 mm × 2.1 mm, 1.9 μm) (Yang et al., 2015; Hopmans et al., 2016). The liquid chromatography methodologies proposed by Yang et al. (2015) and Hopmans et al. (2016), respectively, differ in the solvent and elution gradient. The former used ethyl acetate and *n*-hexane while the latter used isopropanol and *n*-hexane. The APCI and MS conditions vary among laboratories, depending on the instrument brand used. GDGTs were generally detected with single ion monitoring (SIM) at *m/z* 1302, 1300, 1298, 1296, 1292, 1050, 1048, 1046, 1036, 1034, 1032, 1022, 1020, 1018, and 744 (Yang et al., 2015).

4. Distribution and sources of stalagmite GDGTs

4.1. Distribution of GDGTs in stalagmites

Stalagmites are connected to the surrounding environment by dripping water and receive organic matter from the overlying soil,

vegetation, percolation system, and the interior of the cave, so a variety of chemical signals can be preserved within them. GDGT compounds are widely distributed in stalagmites, and the content of GDGTs varies clearly across different periods (Yang et al., 2011). Previous studies have shown that GDGTs can generally be extracted from 5–20 g stalagmite samples, but the content of GDGTs in some stalagmites is below the limit of detection.

The content of archaeal isoGDGTs in stalagmites is generally higher than that of bacterial brGDGTs (Figure 3). The data from 33 stalagmite samples collected from 16 sites in Europe, Africa, and Oceania showed that the speleothem GDGTs were dominated by archaeal isoGDGTs. The content of crenarchaeol was high, whereas the contents of crenarchaeol' and crenarchaeol, brGDGT-Ic, -Iic, -IIIc, and -IIIb were relatively low (Blyth and Schouten, 2013). The distribution of specific compounds also varied greatly among stalagmites from different regions. The contents of GDGTs in stalagmites from Heshang Cave ranged from 0.15 to 4.19 ng/g dry weight of stalagmite. The isoGDGTs were dominated by crenarchaeol, which reached 0.07–0.94 ng/g dry weight of stalagmite, accounting for 48% of the total weight of the isoGDGTs. However, the contents of GDGT-0–3 and crenarchaeol' were relatively low. The contents of bacterial brGDGTs ranged from 0 to 0.67 ng/g stalagmite dry weight (Yang et al., 2011), and they were dominated by Ila' (Figure 3C). The content of brGDGTs without cyclopentane rings was higher than the content of brGDGTs containing cyclopentane rings (Yang et al., 2011). Similarly, the main isoGDGT of stalagmites in Australian caves was also crenarchaeol, and the content of GDGT-3 was higher than that of GDGT-1 or GDGT-2. The main brGDGTs were Ib and Iib, but the content of brGDGTs containing cyclopentane rings was higher than the content of those without cyclopentane rings (Baker et al., 2016).

4.2. Sources of GDGTs in stalagmites

The two main sources of the organic matter preserved in caves are the roof environment (e.g., vegetation and soil) and inside the cave (dripping water and cave microorganisms) (Xie et al., 2003; Huang et al., 2007; Yang et al., 2011; Blyth et al., 2014; Wang et al., 2018). To examine the sources of stalagmite GDGTs, the stalagmites, dripping water, and overlying soils have been analyzed for GDGTs and GDGT distribution among them differ significantly.

4.2.1. Archaeal isoGDGTs

Yang et al. (2011) analyzed the contents of GDGTs in stalagmites, the surface of weathered dolomite, dripping water, and overlying soil samples from Heshang Cave in China. The distribution of stalagmite isoGDGTs in Heshang Cave was similar to those of the dripping water, both of which were dominated by crenarchaeol, whereas the overlying soil isoGDGTs were dominated by crenarchaeol and GDGT-0. No OH-GDGTs were detected in the cave or overlying soil. Neither the dripping water nor stalagmites inherited the distribution pattern of the soil archaeal isoGDGTs. Furthermore, the distribution of archaeal isoGDGTs in the surrounding rocks was similar to that in the stalagmite, indicating that archaeal isoGDGTs can grow on the surfaces of stalagmites *in situ*. Therefore, the archaeal isoGDGTs of the stalagmites in Heshang Cave were thought to be mainly contributed by microorganisms growing on the surfaces of the

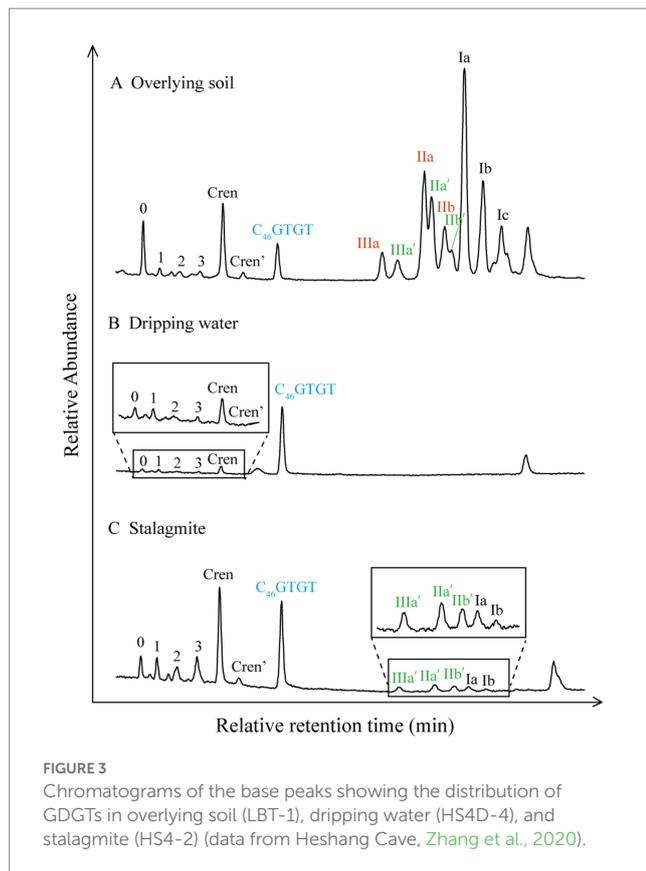


FIGURE 3
Chromatograms of the base peaks showing the distribution of GDGTs in overlying soil (LBT-1), dripping water (HS4D-4), and stalagmite (HS4-2) (data from Heshang Cave, Zhang et al., 2020).

stalagmites and in dripping water (Yang et al., 2011). Ding et al. (2018) first reported GDGTs in a groundwater system, and the CL-GDGTs correlated strongly with the IPL-GDGTs, indicating the presence of a living archaeal community in the groundwater system.

Crenarchaeol and GDGT-0 in soil samples are mainly from Thaumarchaeota Group I.1, and the archaea in underground environments are usually phylogenetically closer to those in soil, belonging to Thaumarchaeota Group I.1a and I.1b (Weidler et al., 2007; Zhao et al., 2017). Therefore, Thaumarchaeota may be the main source of stalagmite isoGDGTs. Because the microbial abundance in the dripping water in caves is low, it is impossible to use the Bligh-Dyer extraction method to extract IPL-GDGTs, which represent the biomass of living archaea. Therefore, it can only be inferred that the crenarchaeol in dripping water is derived from the living archaea in groundwater (Yang et al., 2011).

A comparison of stalagmites and soils in five caves in the UK and Australia showed significant differences in the distribution of GDGTs, and therefore, the soil was not the main source of the stalagmite isoGDGTs either (Blyth et al., 2014). A recent irrigation experiment showed that the GDGT components in dripping water included a high proportion of GDGT-0, which differed significantly from the GDGT compositions in soil and stalagmites. This indicated that soil and dripping water were not the main sources of the isoGDGTs in the stalagmites (Baker et al., 2016). There are rich species of archaea in caves (Wu et al., 2015), and the presence of Thaumarchaeota in caves has been detected in DNA studies (Gonzalez et al., 2006; Zhao et al., 2017). This provides further evidence for the *in situ* growth of stalagmite isoGDGTs.

4.2.2. Bacterial brGDGTs

High abundances of bacterial brGDGTs are generally present in soils. If the brGDGTs preserved in stalagmites are derived from soil, their distributions should be consistent with their characteristic distribution in soils. Studies have shown that the concentrations of bacterial brGDGTs in soils are generally higher than the concentrations of archaeal isoGDGTs (Figure 3). However, this is not the case in stalagmites (Yang et al., 2011; Zhang et al., 2020). The distribution of bacterial brGDGTs in stalagmites from Heshang Cave was similar to that in weathered rock samples, which are usually dominated by brGDGT-IIb. However, the soil was dominated by brGDGTs IIa, IIb, and Ib, indicating that soil was not the main source of bacterial brGDGTs in the stalagmites (Yang et al., 2011). Huguet et al. (2018) analyzed the stalagmite GDGTs in Mawmluh Cave and showed that the lower abundances of bacterial brGDGTs in the stalagmites were probably produced in the cave because the thin layer of overlying soil was poorly developed, and the content of brGDGTs in the soil was higher than the content of isoGDGTs. Therefore, the soil is unlikely to be the main source of stalagmite bacterial brGDGTs.

Other studies have shown that bacterial brGDGTs can be produced *in situ* in aquatic environments (Sinninghe Damsté et al., 2009). Given the significant differences between stalagmites and soils, we can speculate that the bacterial brGDGTs in stalagmites are derived mainly from dripping water or the microorganisms growing on the surfaces of the stalagmites. A 5-year program that monitored the dripping-water bacterial community in Heshang Cave revealed that the bacteria showed ecological adaptability in the oligotrophic environment of dripping water, surviving on the poor nutrients in the water or obtaining essential nutrients from minerals (Yun et al., 2015). Barton et al. (2014) also found that the dripping water in the cave provided the carbon and energy required for the survival and growth of the microbial communities in the cave, which supports the possibility that bacterial brGDGTs are produced in dripping water or inside the cave.

5. Application of GDGT-based proxies in stalagmites

5.1. TEX₈₆

As the temperature increases, the number of cyclopentane rings in isoGDGTs also increases (Uda et al., 2001). Therefore, Schouten et al. (2002) established the tetraether index of 86 carbon atoms (TEX₈₆) proxy (Table 1) to characterize the relative numbers of cyclopentane moieties in isoGDGTs. TEX₈₆ has a good linear relationship with sea surface temperature. Subsequent pure culture experiments have confirmed that the number of cyclopentane moieties in isoGDGTs produced by Thaumarchaeota changes with temperature (Wuchter et al., 2004). The TEX₈₆ proxy is basically unaffected by salinity, and the upper limit of its response to temperature is 38.6°C. The large temperature response range of the TEX₈₆ proxy also makes it better than U^k₃₇ for quantitative paleotemperature reconstruction (especially in high-temperature equatorial seas). When the temperature exceeds 30°C, the response of U^k₃₇ becomes insensitive to temperature (Pelejero and Calvo, 2003). Therefore, TEX₈₆ is used as a thermometer for aquatic settings and has been widely used in marine and terrestrial paleoenvironmental reconstruction.

Blyth and Schouten (2013) investigated the GDGTs of several cave stalagmites around the world and found that TEX₈₆ correlated

significantly with the measured temperature. They established calibration equations, one for the surface mean annual air temperature (MAAT; $R^2=0.78$, $p<0.001$, $n=30$) and one for cave MAAT ($R^2=0.68$, $p<0.001$, $n=19$; Figures 4A,B, Table 2). The standard errors were 2.3 and 2.0°C, respectively. The TEX_{86} values for stalagmite samples from Heshang Cave were 0.60–0.68, which was lower than that of soil. There is a good negative correlation between TEX_{86} and $\delta^{18}O$ in stalagmites and the value of TEX_{86} increases as $\delta^{18}O$ decreases. Because the air temperature increases with the strengthening of monsoon, TEX_{86} can record the temperature changes in a cave (Yang et al., 2011). Blyth et al. (2014) found that the TEX_{86} values for most stalagmites in their study area were lower than the soil TEX_{86} and had a good linear relationship with the surface MAAT ($R^2=0.93$, $p<0.001$). Baker et al. (2019) updated the calibration equations for TEX_{86} and MAAT based on the data of Blyth and Schouten (2013) combined with data from Yang et al. (2011) and two unpublished datasets ($R^2=0.84$, $p<0.001$; $R^2=0.82$, $p<0.001$; Figures 4C,D, Table 2). The calibration equations were successfully applied to the cave air temperature reconstruction for Jiangjun Cave (Wassenburg et al., 2021).

The relationship between TEX_{86} and temperature could be biased by the following factors. The first is the input signal from the overlying soil. The soil is leached by rain, and a small amount of archaeal isoGDGTs is brought into the stalagmites through the seepage system, which could affect the TEX_{86} value directly. Because the input from the external soil (indicated by a high branched and isoprenoidal tetraether [BIT] index, see below) causes TEX_{86} to deviate (Weijers et al., 2006a), Blyth and Schouten et al. (2013) tried to remove samples with BIT >0.5 to improve the accuracy of the calibration equation. However, this did not significantly improve the relationship between TEX_{86} and surface MAAT ($R^2=0.77$, $p<0.001$, $n=27$) or cave MAAT ($R^2=0.70$, $p<0.001$, $n=16$; Table 2). The standard errors were 2.2 and 1.9°C, respectively, and had decreased by only 0.1°C. Therefore, more samples are required to confirm this calibration method.

The second factor biasing TEX_{86} is the differences in archaeal communities. The archaea in caves may differ from those in soil or marine environments and may respond differently to temperature. Therefore, further microbial research is required. The third factor

is the differences in regional microclimates. The measured surface temperatures used in previous studies are generally obtained from the nearest meteorological station. Because there is a certain distance between the sampling point and the meteorological station, the temperature in the area directly above the cave may be somewhat different from the meteorological data (Yang et al., 2011; Blyth et al., 2016). Besides, due to the low growth rate of stalagmites, TEX_{86} from stalagmites may record an average of temperature for multiple years.

Finally, TEX_{86} from stalagmites may record temperature signals biased towards the growing season. Herfort et al. (2006) found that TEX_{86} in surface sediments from the North Sea was influenced by isoGDGT production in a specific season of the year (Herfort et al., 2006). The TEX_{86} -derived temperatures were strongly biased towards the summer temperature in Tibetan lakes (Günther et al., 2014). Leider et al. (2010) also found that TEX_{86} in the surface sediments was best correlated with summer sea surface temperatures of the Mediterranean Sea. Zhang et al. (2022) observed the seasonal bias of TEX_{86} -derived temperatures in high latitudes and high elevations where freezing occurred. Owing to varying water inputs across different seasons, the growth of stalagmites may have hiatus in dry seasons and the temperature signal recorded is expected to be primarily derived from the growing season. Therefore, TEX_{86} proxy in stalagmites may also be affected by seasonal factors, despite that there is no conclusive evidence yet.

5.2. BIT

According to early studies, brGDGTs are derived mainly from terrestrial soil and peat, whereas crenarchaeol occurs predominantly in marine environments. Hopmans et al. (2004) proposed the branched and isoprenoid tetraether (BIT) index (Table 1) to measure the relative contributions of terrestrial organic matter in marine environments. The principle underlying the BIT proxy is that crenarchaeol represents the contribution of the marine environment and brGDGTs represent the contribution of terrestrial soil, so a BIT value is close to 0 in an open

TABLE 1 GDGT proxies are calculated using the following equations:

Proxies	Equations	References
TEX_{86}	$(GDGT-2 + GDGT-3 + Crenarchaeol) / (GDGT-1 + GDGT-2 + GDGT-3 + Crenarchaeol)$	Schouten et al. (2002)
BIT	$(Ia + Ia' + IIIa + IIIa') / (Ia + Ia + Ia' + IIIa + IIIa' + Crenarchaeol)$	Hopmans et al. (2004)
MBT	$(Ia + Ib + Ic) / (Ia + Ib + Ic + IIa + IIa' + IIb + IIb' + IIc + IIc' + IIIa + IIIa' + IIIb + IIIb' + IIIc + IIIc')$	Weijers et al. (2007b)
MBT'	$(Ia + Ib + Ic) / (Ia + Ib + Ic + IIa + IIa' + IIb + IIb' + IIc + IIc' + IIIa + IIIa')$	Peterse et al. (2012)
MBT' _{SME}	$(Ia + Ib + Ic) / (Ia + Ib + Ic + IIa + IIb + IIc + IIIa)$	De Jonge et al. (2014)
MBT' _{6ME}	$(Ia + Ib + Ic) / (Ia + Ib + Ic + IIa' + IIb' + IIc' + IIIa')$	
CBT	$-\log[(Ib + IIb + IIb') / (Ia + IIa + IIa')]$	Weijers et al. (2007b)
CBT _{SME}	$-\log[(Ib + IIb) / (Ia + IIa)]$	De Jonge et al. (2014)
CBT _{6ME}	$-\log[(Ib + IIb') / (Ia + IIa')]$	
IR' _x	$x' / (x + x')$	De Jonge et al. (2014)
IR _{6ME}	$(IIa' + IIb' + IIc' + IIIa' + IIIb' + IIIc') / (IIa + IIb + IIc + IIIa + IIIb + IIIc + IIa' + IIb' + IIc' + IIIa' + IIIb' + IIIc')$	Dang et al. (2016)

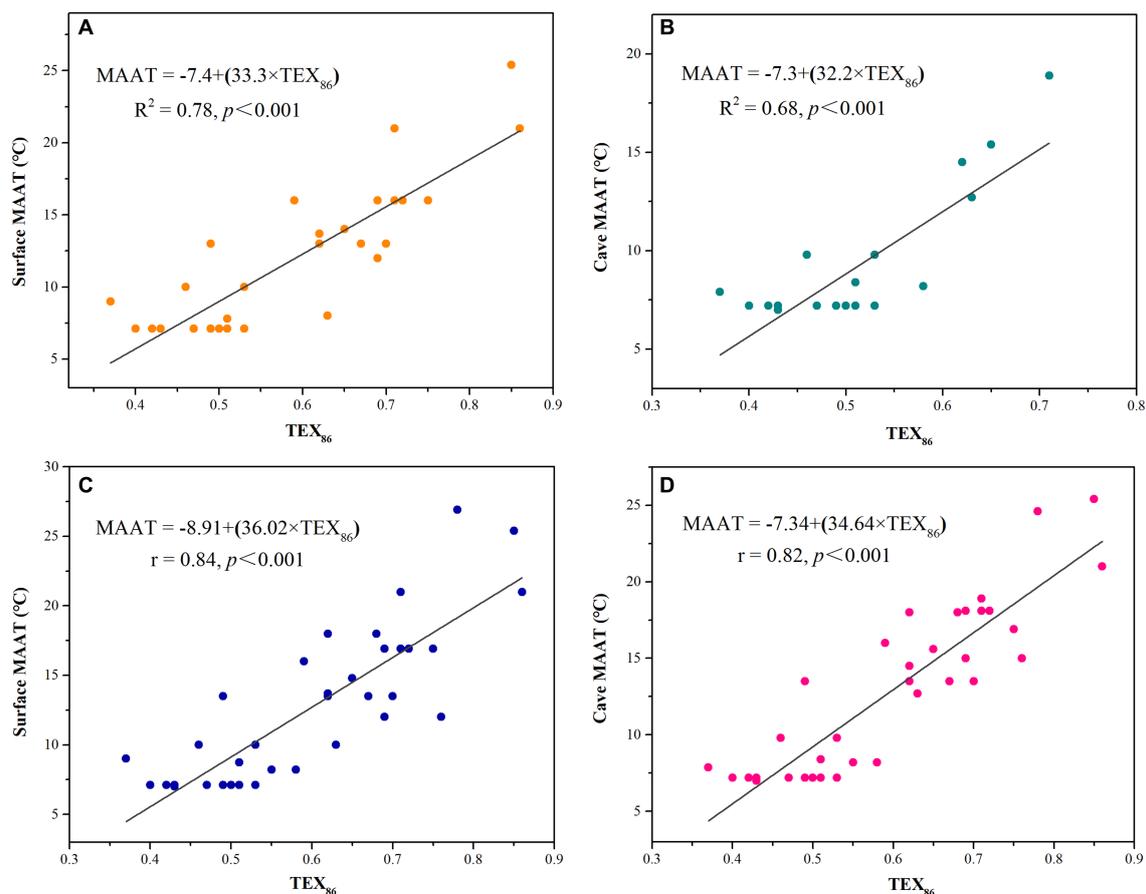


FIGURE 4

Relationships between TEX_{86} , surface MAAT, and cave MAAT (data A and B from Blyth and Schouten, 2013; data C and D from Yang et al., 2011; Blyth and Schouten, 2013; Baker et al., 2019).

marine environment and close to 1 in terrestrial soil and peat (Schouten et al., 2013). The BIT proxy has been increasingly used to trace the migration of terrestrial organic carbon.

According to the stalagmite data published to date, the BIT value of stalagmites is generally lower than that of soil (Figure 5B). The BIT values of the stalagmites in Heshang Cave are 0.21–0.58, and the value of the overlying soils is >0.9 (Yang et al., 2011). The BIT value of Mawmluh Cave is <0.35 (Huguet et al., 2018), and the BIT values of stalagmites in Australia are <0.1 (Blyth and Schouten, 2013). These data indicate that there is no significant soil input signal in stalagmite GDGTs and that they are more likely to be generated *in situ* than to be derived from the soil.

Huguet et al. (2018) showed that the BIT values of the stalagmites in Mawmluh Cave were relatively low (BIT <0.35). The BIT value decreased to ~ 0.1 during the Holocene, indicating the high contribution of archaea in the cave. During the weak monsoon period, the retention time of dripping water in the surface soil and seepage system increases, and the relative contributions of the microorganisms in the soil or seepage system to GDGT production increase. In contrast, the higher rate of dripping water during the period of strong monsoon reduces the GDGTs produced *in situ* by microorganisms in caves, so the BIT correlates positively with $\delta^{18}\text{O}$ ($R^2 = 0.49$, $p < 0.005$) (Huguet et al., 2018).

5.3. MBT/CBT

Weijers et al. (2007c) proposed the methylation index of branched tetraethers (MBT; Table 1) and the cyclization ratio of branched tetraethers (CBT; Table 1) to describe the relative changes in the number of methyl groups and the number of cyclopentane rings in bacterial brGDGTs, respectively. A negative correlation was detected between MBT and MAAT, which was also affected by soil pH. The CBT proxy, which represents the relative number of cyclopentane rings, is related to the soil pH. Because the pH can be calculated from CBT, the combination of MBT and CBT (MBT/CBT) can jointly reconstruct MAAT, or CBT can be used for pH reconstruction alone (Weijers et al., 2007c).

The MBT and CBT values for stalagmites are generally lower than those for the overlying soil (Figures 5C,D). However, pH data were not available at all sampling sites in the published data, which limited the application of the CBT proxy in stalagmites. Nevertheless, the pH of Buchan soil was 6.8–7.5, whereas the pH of the dripping cave water was 6.9–8.0, which is slightly alkaline. The CBT-derived pH of stalagmite was 8.0, suggesting that CBT values reflect pH changes (Blyth and Schouten, 2013). Other studies have found that the CBT proxy in caves overestimates pH (Blyth et al., 2014; Zhang et al., 2020). Because the equation is based on soil and the instantaneous pH of

TABLE 2 Correlations of TEX₈₆ and MBT/CBT based on stalagmite GDGTs.

Proxies	Equations	R ² /r	p	Standard error (°C)	References
TEX ₈₆	Surface MAAT = -7.4 + (33. × TEX ₈₆)	R ² = 0.78	<0.001	2.3	Blyth and Schouten (2013)
	Cave MAAT = -7.3 + (32.2 × TEX ₈₆)	R ² = 0.68	<0.001	2	
TEX ₈₆ (BIT < 0.5)	Surface MAAT = -7.1 + (32.1 × TEX ₈₆)	R ² = 0.77	<0.001	2.2	
	Cave MAAT = -9.2 + (35.2 × TEX ₈₆)	R ² = 0.70	<0.001	1.9	
TEX ₈₆ (surface-cave data sets)	Surface MAAT = -8.1 + (34.2 × TEX ₈₆)	R ² = 0.65	<0.001	2.5	
	Cave MAAT = -8.3 + (34.4 × TEX ₈₆)	R ² = 0.73	<0.001	1.9	
MBT/CBT	Surface MAAT = 5.4 + (27.1 × MBT) - (4.8 × CBT)	R ² = 0.73	<0.001	2.7	
	Cave MAAT = 6.1 + (21.3 × MBT) - (5.4 × CBT)	R ² = 0.56	<0.001	2.5	
MBT/CBT (surface-cave data sets)	Surface MAAT = 6.0 + (20.9 × MBT) - (4.5 × CBT)	R ² = 0.44	0.031	3.2	
	Cave MAAT = 6.6 + (20.2 × MBT) - (5.8 × CBT)	R ² = 0.56	0.007	2.7	
TEX ₈₆ (unscreened data)	Surface MAAT = -8.91(±2.39) + 36.02(±3.93) × TEX ₈₆	r = 0.84	-	±0.2	Baker et al. (2019)
	Cave MAAT = -7.34(±2.71) + 34.64(±4.16) × TEX ₈₆	r = 0.82	-	±1	
TEX ₈₆ (screened data)	Surface MAAT = -7.72(±2.64) + 34.04(±4.36) × TEX ₈₆	r = 0.81	-	±0.2	
	Cave MAAT = -4.09(±3.09) + 34.04(±4.36) × TEX ₈₆	r = 0.75	-	±1	

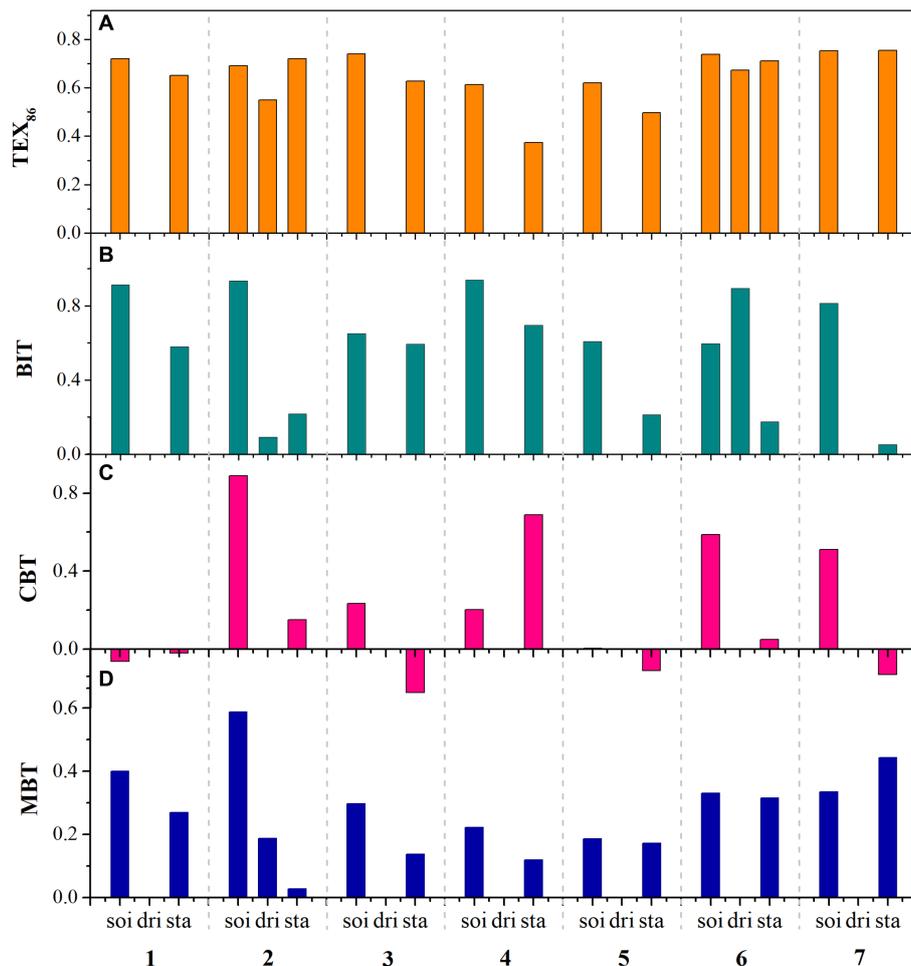


FIGURE 5 Comparison of GDGT proxies for soils (soi), dripping water (dri), and speleothems (spe) (1, Heshang Cave, Yang et al., 2011; 2, Heshang Cave, Zhang et al., 2020; 3, Wombeyan Caves; 4, Pooles Cavern; 5, Lower Balls Mine; 6, Cathedral Cave; 7, Gaden Cave; data 3–7 from Blyth et al., 2014).

sampling cannot represent the long-term changes in dripping water, it will be necessary to develop a CBT–pH calibration equation for the cave environment to improve its application in the future.

Based on the MBT/CBT calibration equation of Weijers et al. (2007b), Yang et al. (2011) calculated that the temperature range under which the stalagmites in Heshang Cave formed was 1.6–7.5°C, which is significantly different from the temperature in the study area and much lower than the temperature calculated with TEX_{86} . These results indicate that brGDGT-producing bacteria in caves respond differently to temperature than those in soils. There was a significant correlation between stalagmite MBT and MAAT outside the cave ($R^2=0.62$) but a weak correlation between stalagmite MBT and MAAT inside the cave ($R^2=0.38$). Blyth and Schouten et al. (2013) conducted a multiple regression analysis of stalagmite CBT, MBT, and measured MAAT. Two sets of calibration equations were obtained (surface MAAT, $R^2=0.73$, $p<0.001$, standard error=2.7°C; cave MAAT, $R^2=0.56$, $p=0.003$, standard error=2.5°C; Table 2). In a multiple regression analysis, the relationship between stalagmite CBT, MBT, and measured MAAT was significantly improved, and the calibration standard error was smaller than that reported in soil ($R^2=0.77$, standard error=5°C) (Weijers et al., 2007b) or a lake ($R^2=0.62$, standard error=2.5°C) (Sun et al., 2011).

Since the GDGTs in stalagmites are dominantly derived from dripping water and cave systems, it is more logical to see that GDGT-derived proxies show a better correlation with the cave MAAT. The cave MAAT is usually close to the surface MAAT. However, published work indicated both TEX_{86} and MBT/CBT from caves are more closely related to the surface MAAT. This may be ascribed to limited stalagmite GDGTs data and less cave MAAT data (compared to more surface MAAT data). Blyth and Schouten et al. (2013) found both TEX_{86} and MBT/CBT proxy are better correlated with cave MAAT, if the cave and surface MAAT data were both observed (Table 2). Therefore, more stalagmite GDGTs data and cave monitoring data around the world are needed to improve the accuracy of calibration in the future.

In terms of correlation and standard error, MBT/CBT is not as applicable as the TEX_{86} proxy, which may be because more-complex factors influence the brGDGTs in stalagmites. In fact, the MBT and CBT proxies for brGDGTs in alkaline environments, such as soil, are influenced by factors other than temperature and pH (Yang et al., 2014; Dang et al., 2016).

5.4. 5-methylated and 6-methylated brGDGT isomer ratio (IR) proxies

De Jonge et al. (2014) found that the presence of 6-methyl brGDGTs was one of the reasons for the bias in the MBT(′)/CBT proxy, and that the higher levels of IIa′ and IIIa′ in arid areas led to lower MBT′. The relative abundance of 6-methyl brGDGTs is mainly controlled by pH, and after 6-methyl brGDGTs were excluded from the MBT′ proxy, MBT′_{5ME} correlated more closely with temperature. The four proxies MBT′_{5ME}, MBT′_{6ME}, CBT′_{5ME}, and CBT′_{6ME} were then derived (Table 1). It has also been proposed CBT′, thereafter the accuracy of brGDGT-based proxies in temperature and pH reconstructions was improved to a greater extent (De Jonge et al., 2014; Yang et al., 2015).

De Jonge et al. (2014) proposed the use of an isomer ratio (IR; Table 1) representing the relative amounts of methyl isomers in brGDGTs: After separating the 5-methyl and 6-methyl isomers of bacterial brGDGTs. Yang et al. (2015) showed that the 6-methyl brGDGTs in soils from an altitude transect in Shennongjia correlated with the soil pH, and therefore proposed that $IR_{IIIa'}$ and $IR_{IIa'}$ be used to reconstruct pH. Dang et al. (2016) investigated the brGDGTs in soils with a gradient of different water contents (0–61%) along the coast of Qinghai Lake, and they found that the soil water content strongly affected 6-methyl brGDGTs, whereas 5-methyl brGDGTs were controlled by temperature. This causes a deviation in the temperatures reconstructed using the MBT′/CBT proxy in arid and semi-arid areas. Therefore, IR_{6ME} can be used to specify the use of proxies: when $IR_{6ME}<0.5$, MBT′/CBT can be used as a proxy for temperature, and when $IR_{6ME}>0.5$, MBT′ correlates well with the hydrological conditions in the region (Dang et al., 2016). However, the number “0.5” is just an empirical or statistical value. There might be a range within which the MBT is both controlled by temperature and precipitation.

Zhang et al. (2020) tested 5-methyl and 6-methyl brGDGTs from stalagmites using the new method and found that 6-methyl brGDGTs were the main isomer type in stalagmites, whereas 5-methyl brGDGTs were largely below the limit of detection. However, 5-methyl brGDGTs were dominant in the surface soil and the soil profile (Figure 3). The MBT′_{5ME} and MBT′_{6ME} proxies of soil and stalagmites were significantly different, and the pH of the stalagmite reconstructed with CBT′_{5ME} was significantly higher than that of the soil, confirming again that the stalagmite GDGTs were not inherited from the overlying soil. Furthermore, the IR value of stalagmites is stable at about 1, which is also quite different from that of soil. The environmental information recorded by 5-methyl and 6-methyl bacterial brGDGTs in stalagmites remains unclear, and the application of proxies established using the new method in reconstructing the paleoclimates of stalagmites has not yet been reported. Moreover, a study of 102 surface sediments from 37 lakes in China found that they contained 7-methyl isomers as well as 5-methyl and 6-methyl isomers of brGDGT compounds (Ding et al., 2016). These results suggest there may be many unidentified compounds in GDGTs, with great potential utility in paleoenvironmental reconstruction.

5.5. Applications of stalagmite GDGTs in the paleoenvironment

So far, stalagmite GDGTs have been used for paleoenvironmental reconstructions in limited works by using. Huguet et al. (2018) first applied GDGTs to the paleoenvironmental reconstruction from stalagmites in Mawmluh cave and found that it could provide both temperature and moisture information. Over the 22 to 6 ka period, the gradual increase in monsoon strength was related to the higher TEX_{86} temperatures. The highest TEX_{86} -derived temperatures (19–20°C, standard error=1.9°C) in KM-1 were observed at ~6 ka, which was consistent with the surface and cave MAAT, whereas the temperature of the last glacial maximum (LGM) was about 4°C lower. The GDGT proxies supported the hypothesis of a shift in paleohydrologic conditions at the end of the LGM and before the onset of the Holocene (Huguet et al., 2018). Wassenburg et al. (2021) used TEX_{86} -derived

temperatures to deconvolve the effects of temperature in stalagmite $\delta^{18}\text{O}_{\text{cc}}$. The structure of the TEX_{86} -derived temperatures was similar to $\delta^{18}\text{O}_{\text{cc}}$ record in Jiangjun Cave. Thus, GDGTs can provide a quantitative estimate of temperature and is an important addition to stable isotope records in stalagmites (Huguet et al., 2018).

6. Summary and prospects

(1) Identifying the biological sources of stalagmite GDGTs. At present, the GDGTs in stalagmites have been shown to come from dripping water, *in situ* production within a cave, and their proportions in stalagmites remain unclear. In cave systems, both archaeal isoGDGTs and bacterial brGDGTs may originate from *in situ* production in the cave, but the contributions from dripping water cannot be excluded. The biological sources of GDGTs in stalagmites remain unclear, and their clarification requires the use of microbiological and molecular biological techniques. Resolving this issue will extend our understanding of the environmental information recorded by stalagmite GDGTs.

(2) Establishing regional calibration equations. Both TEX_{86} and MBT/CBT have potential utility as proxies for temperature in stalagmites, and the calculated temperature correlates strongly with the measured temperature. However, the input signal of the overlying soil, the differences in the archaeal communities in caves, and the microclimate at the top of the cave may affect the accuracy of reconstructions. More modern regional monitoring efforts in the future could significantly reduce these uncertainties and improve the accuracy of temperature reconstructions.

(3) Improving extraction and analytical methods. Stalagmite samples are relatively precious, and the content of organic matter is less than 0.3% of the total carbon. Therefore, the lipid content in stalagmites is usually low, and a large number of samples is required to ensure their instrumental detection. Consequently, the high-resolution detection of GDGTs in these samples is difficult. More-efficient methods for GDGT analysis must be established to reduce the amount of samples required, while still obtaining high-resolution paleoclimatic records.

(4) Focusing on new compounds in stalagmite GDGTs. The distributions of 5-methyl and 6-methyl brGDGT compounds separated using the new method in dark caves and oligotrophic environments differ from those in soil, and the environmental factors operating may also be different. However, 5-methyl and 6-methyl bacterial brGDGTs and their related proxies have only been reported in Heshang Cave.

(5) More-extensive paleoenvironment research on stalagmite GDGTs. Stalagmite GDGTs have great potential utility in the quantitative reconstruction of paleoclimates, especially paleotemperatures. However,

recent studies have focused mainly on modern processes (paleoclimate reconstruction has only been undertaken in two caves, Mawmluh Cave and Jiangjun Cave). In the future, more-extensive studies of the paleoclimates represented by GDGTs in caves are required to provide supplementary information on the relationships between temperature, pH, and humidity and other proxies, such as isotopes, to better understand and interpret monsoonal changes.

Author contributions

All authors listed have made a substantial and direct contribution to the work and approved it for publication.

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