Dear Biogeosciences Editor,

First, on behalf of my coworkers, I really thank you to give me one month extension to revise our manuscript. After three month hardworking, we finally resubmit our revised manuscript including new data, new method and new discussion and conclusion.

Generally speaking, there is no much work to do for reviewer 1 and 2. However, the reviewer 3 provided several concerns, particularly for new compounds 6-methyl brGDGTs, which were not reported in our original manuscript. 6-methyl brGDGTs did not report until the year of 2013 by de Jonge et al. In most studies about GDGTs, only one LC column was used and therefore 5-methyl brGDGTs co-eluted with 6-methyl brGDGTs, resulting in mis-identification in most previous studies. These coelution may also cause misinterpretation of brGDGT indices and environmental factors such as temperature, precipitation and soil pH.

Considering these issues, we used tandem LC columns, and re-analyze all our samples. This explains why it takes us three months to finish revision. Fortunately, we identified a series of 6-methyl brGDGTs in our samples. Thus we re-interpreted our data. We also proposed two new indices to estimate MAT and soil pH. Our new data also suggest the position of methyl group of brGDGTs play an important role for bacteria to adapt ambient pH changes.

We believe our revised manuscript has improved significantly. Please see our revised manuscript for details. We are looking forward to hearing from you.

Regards

-Yunping

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**Anonymous Referee #3**

REPLY TO REFEREE COMMENTS

RESPONSE

On behalf of my co-authors, we greatly appreciate reviewer 3 to supply many useful comments. Over the past three months, we followed her/his suggestions, rerun our samples with new HPCL-$MS^n$ system, re-analyzed the samples and rewrote our manuscript. Here we try our best to address those comments. Generally, there are two major concerns. The first one, also the most important, is the issue about new brGDGTs, called 6-methyl brGDGTs, which was not reported in our original manuscript. The second concern is about the novelty of our work.

The 6-methyl brGDGTs is very new biomarkers and were identified and reported after 2013. Similar to our original method, many publications also used a single LC column which cannot resolve 5-methyl brGDGTs and 6-methyl brGDGTs. As the result, they misidentified them as one compounds (5-methyl brGDGTs). So far, only very limited researchers such as de Jonge et al. (2013; 2014a, 2014b) and Yang et al. (2015) used advanced chromatography to separate 6-methyl brGDGTs from 5-methyl brGDGTs. In the revised manuscript, we used two tandem LC columns to rerun our soil samples. With this new approach, 5-methyl and 6-methyl brGDGTs were well separated, and the number of identified brGDGTs increased from 9 to 15, including six 6-methyl brGDGT compounds. We organized our new data, re-plot figures and performed statistical analyses. Based on these, we rewrote most parts of our manuscript (which are highlighted). This is a tough work, but we are happy to see much improved accuracy of brGDGTs indices. Furthermore, we found that two new indices based on 5-methyl and 6-methyl GDGTs, called $MBT_{5/6ME}$ and IBT, are useful for reconstruction of MAT and soil pH in the QTP. Please see the revised manuscript for details.

For the second concern, we believe the revised manuscript contains many interesting points. First, our work is the first time to report 6-methyl brGDGTs in the QTP. Several studies about QTP mis-assigned the co-eluted peaks of 5-methyl and 6-methyl to one compound, which like led to large scatter of the brGDGTs-MAT calibration in the QTP. With our new data, both correlation coefficients and RMSE were significantly improved. Second, global calibrations always overestimate MAT in cold regions, and therefore the regional calibration is required. As the third pole, the QTP has cold and dry climate, providing ideal locations to evaluate relationship between brGDGTs' distributions and environmental parameters. Our studies introduced two new indices, $MBT_{5/6ME}$ and IBT, which are useful for the reconstruction of MAT and soil pH, respectively, in the QTP. These quantitative proxies are valuable to understand climate evolutions in QTP. Finally, our data also reveal that the position of methyl groups of brGDGTs play an important role for soil bacteria to adapt ambient pH change. This is also the first time report.

Overall, with application of advanced analytical technique, our new data made much improvement in accurate reconstruction of temperature and soil pH, which is useful not only for QTP, but also for other cold regions.

Other minor issues.

p. 483, l.16. Peterse et al 2012 is about GDGTs, not the methods mentioned here. Replace with references where these caveats are discussed.

Response: this is a good comment. We delete this reference and replaced with new references. We rewrote as "Microfossil assemblages based on pollen, diatom or chironomid are commonly used paleothermometers, but they are also influenced by precipitation, salinity, nutrient or other environmental factors (Keatley et al., 2009; Meriläinen et al., 2000; Seppä & Birks, 2001).

p.483, l. 17. 'In addition' sounds strange as the sentence discusses a positive application rather than a caveat.

Response: we deleted "in addition' in the revised manuscript.

p. 483, l.26 : : :.lakes, although they: : :

Response: We changed this sentence into “A major limitation of UK’37 is that long-chain alkenones are not always present in lakes, although they were reported in some QTP lakes” in the revised manuscript.

p. 484, l. 7. According to their chemical structure: : :

Response: This sentence is related to isoprenoids and branched GDGTs. After separation of 5-methyl and 6-methyl brGDGTs, we thought it is better to focus on these new compounds. So we deleted all contents about isoprenoid GDGTs in the revised manuscript.

p. 484, l. 10-15. A more nuanced BIT distribution can be found in Schouten et al. 2013, OG where endmembers are different than based on older literature.

Response: As mentioned above, we deleted all contents about isoprenoid and BIT index since new 6-methyl brGDGTs was identified with our new method.

p. 485. Can all these calibration equations not simply be summarized in a Table ?

Response: we also want to put all equations in one table, but it seems difficult since there are over ten equations in our work. So we still keep them separately.

p. 485. l.8. It is impossible to say if proxies are successful, what you can say is that they have been applied. Furthermore, the lake situation is quite different from that of soils, i.e. different calibrations are used because of in situ production (e.g. Tierney 2010, Pearson 2011, etc.). This is different from a ‘regional calibration’,i.e. the MBT/CBT is not used but rather transfer functions of individual GDGT concentrations. This should be made clear in the introduction right here, and not just briefly at p. 486.

Response: We changed “successfully” into “increasingly”. It is true there are more than one calibrations. So we changed “a regional calibration’ into “regional calibrations”.

p. 485, l. 20. Blyth and Schouten was not using soils but stalagmites.

Response: We deleted this reference in the revised manuscript.

p. 487, l. 7. In the Suppl. Table the range in MAT is much wider than simply 0.1 C.
Importantly, how many weather stations are on the QTP and how were the temperature corrections for adiabatic laps rates done ?

Response: Good comments. We added these information in the revised manuscript. From line 145 to 153, we added sentences as “There are about 70 meteorological stations in the QTP, mainly distributed in the eastern part and northern border of the QTP. Thus, direct observation data on temperature and precipitation at our sampling sites are generally lacking. In this study, we use the WorldClim dataset (Hijmans et al., 2005) to interpolate annual, seasonal and monthly mean precipitation and temperature (Table S1). The local climate is dry and cold. The MAT of our sampling sites ranges from -5.5 to 7.6 °C with a vertical lapse rate of 0.487 °C/100 m to 0.699 °C/100 m (Cheng et al., 2012). The vertical lapse rate of air temperature decreases from north to south of the QTP. The mean annual precipitation (MAP) at different altitudes varies from ca. 85 mm to ca. 495 mm.”.

p. 489. l. 10. I see there has been no correction for the difference in ionization efficiency of the internal standard (a C46 GTGT) compared to GDGTs. This can be quite variable and substantial (see Huguet et al., 2006). Hence, you do not have real absolute concentrations but estimated based on the (likely incorrect) assumption that the internal standard has the same response factor as GDGTs. Please note this caveat.

Response: this is true that ionization efficiency is different between internal standard C46 GDGTs and brGDGTs. So far, no authentic standard is available and C46 GDGTs has been regarded as the best internal standard for GDGT analysis. Also, the quantification of GDGTs without response factor correction is very popular among organic geochemists. Considering that all brGDGTs proxies are based on relative abundance rather than absolute concentrations, we only used peak areas to calculate the brGDGT ratios. In the revised manuscript, we added the sentence as "Since we assume same response factors among different brGDGTs and C46 GDGTs, our study can be only regarded as semi-quantification." (Section 2.3)

p. 490, l. 15. Please refer to the recent compilation of Schouten et al 2013 which showed that the average BIT value is 0.90+/- 0.14. p. 490. l. 20. I do not know of any recent studies who use the BIT in lakes to trace soil OM because of the now well-known large in situ production in lakes. Hence this 'warning' is unnecessary. p. 490. L. 2. Cite also Kim et al. 2010 L&O who found lower BIT values at pH>7 in French soils. Your Fig. 3a looks exactly like their Fig. 6.
Response: As mentioned above, since six 6-methyl brGDGTs were identified from previously co-eluted 5-methyl brGDGTs, the traditional BIT definition is not applicable. So we deleted all contents related to BIT in the revised manuscript.

p. 492. I wonder in how far the RDA results are affected by the limited number of soils and also the specific range in pH, temperature and MAP of QTP. Compared to the different global data sets the range in temperature is relatively small (<10 C). In particular, the global calibrations shows at MAT<15 C a pronounced heterogeneity. Can it therefore not be expected that MAP plays a more important role than MAT ?
Response: this is a good suggestion. In the revised manuscript, we performed RDA and pRDA based on new data (including 5-methyl and 6-methyl brGDGTs). Our results showed soil pH is the most important contributor to relative abundance of brGDGTs, followed by MAT, while MAP is the least important one. This result is consistent with the recent study of de Jonge et al. (2014), but contrast with our previous result for the same samples, highlighting the importance of separating 5-methyl and 6-methyl brGDGTs to evaluate brGDGT indices. We rewrote as "Our RDA result shows that MAT and pH have a significant independent effect on the brGDGT distribution in the QTP soils, however, no significant correlation was observed between MAP and brGDGTs ($p > 0.05$; Table 1). Soil pH explaining up to 60.1% of the total variables is the largest contributor to the variance, followed by MAT (up to 16.4%) and MAP (up to 10.8%). " in section 3.2

p. 493. l. 3. Please perform a statistical test to see if the slopes are significantly different between the different calibrations.
Response: After re-calibration based on 5-methyl and 6-methyl brGDGTs, the equation 13 and 15 showed same slope (1.49). In the revised manuscript, we discussed CBT'-pH (De Jonge et al., 2014) and new defined CBT"-pH (this study) in details. Please see section 3.3.2 (line 319-344).

p. 494, l. 15. I think the smaller RSME of your QTP calibration compared to the global calibration is artificial. This is because the temperature range of your data set is much smaller than that of the global calibration (ca. 10 C versus 30 C). So, an error of 2.4 C on a range of 10 C is worse than that of 5 C RSME on a scale of 30 C. This should be discussed more fairly.
Response: this is correct. The lower RMSE of regional calibration is due to its narrow temperature range. We already realized this point and discussed it in section 3.2 from line 292 to 304 in the revised manuscript.

p. 495. l. 26. Perhaps a better way to test this is to see statistically if the correlations for QTP are significantly different from the global correlation or the Yang et al. calibration. Note also that interlaboratory differences between labs may play a big role in the added scatter with the Yang et al 2014 data set mostly coming from a single lab. p. 496, l. 8-17. This part is not clear to me. First

I wonder if the slightly better correlation is really statistically significant or just because the temperature range seems larger for this season. I also do not understand on what data the conclusion is based that "more variation in brGDGT compositions occurs in winter". And I also do not see the connection with soil respiration as stated in the next sentence. This should be clarified.
Response: Very good comments. As mentioned above, after separation of 5-methyl and 6-methyl brGDGTs with improved chromatography, CBT is no longer an important factor on MAT. So we deleted the equation 12 and 13 and related discussion from our original manuscript. Instead, we discussed several new indices such as $MBT_{5ME,}$ IBT and CBT". Please see section 3.3 for details.

Table 1. Considering the uncertainty in the quantification of branched GDGTs (see previous comment), the numbers should not be reported with a decimal point.
Response: we made correction in the revised manuscript. As mentioned above, we no longer discussed absolute concentrations of brGDGTs.

We thank Huan Yang for his useful comments, which great improve our manuscript. Here, on behalf of my coauthor, I answer the comments as follows:

This paper provides valuable soil GDGT dataset from a cold and dry region, the Qinghai-Tibetan plateau (QTP), which is a hotspot for studying the paleoclimate change. This manuscript is generally well written. However, several issues should be addressed before it can be accepted for publication in Biogeosciences.

Introduction Developing or testing new proxies is of significant importance in paleoenvironment reconstruction. However, I am not quite clear about the purpose of the new calibration of MBT/CBT in this paper because there is no loess-paleosol sequence in the QTP. Paleoclimatic reconstruction in the QTP largely depends on the lacustrine or fluvial-lacustrine sediments, where the soil MBT/CBT calibration does not fit for the paleotemperature reconstruction. The authors therefore should re-consider the purpose of this research.
**Response:** it is surely true that loess-paleosol sequence and lake sediments are valuable archives for paleoenvironmental reconstruction. However, understanding composition and distribution of brGDGTs in soils is of importance too since brGDGTs in lakes are mainly derived from terrestrial inputs. So if we want to develop brGDGTs paleo-proxies, we need to know the source of brGDGTs and compare GDGTs indices between aquatic and terrestrial environments. Another major merit of our work is with application of new chromatography method, we successfully identified 6-methyl brGDGTs, which are first reported in the QTP. This finding is helpful to explain lower correlation coefficients in earlier studies. We also developed new indexes based on those new compounds. We added these contents in the revised manuscript. Please see discussion and conclusion parts.

P487 Line 5 ‘The local climate is dry and cold with MAT of 0.1 _C and MAP of 317 mm’. I think the MAT can vary in a large amplitude due to a wide range of elevation (3066 to 5418 m). It is not easy to obtain the exact meteorological information for each sampling site as few meteorological stations were established in the QTP.
**Response**: This is true. The air temperature decreases with elevation at a rate of 6 $^{o}$C/1000 m. However, since our soil samples cover a large area of the QTP, other factors on air temperature should be considered such as locations and vegetation cover. In our studies, we added these contents in the section of “material and method” as “There are about 70 meteorological stations in the QTP, mainly distributed in the eastern part and northern border of the QTP. Thus, direct observation data on temperature and precipitation at our sampling sites are generally lacking. In this study, we use the WorldClim dataset (Hijmans et al., 2005) to interpolate annual, seasonal and monthly mean precipitation and temperature (Table S1). The local climate is dry and cold. The MAT of our sampling sites ranges from -5.5 to 7.6 °C with a vertical lapse rate of 0.487 °C/100 m to 0.699 °C/100 m (Cheng et al., 2012). The vertical lapse rate of air temperature decreases from north to south of the QTP. The mean annual precipitation (MAP) at different altitudes varies from ca. 85 mm to ca. 495 mm.”

P488 Testing the method only in Chinese loess plateau is far not enough to support its applicability in the QTP. How the final calibration looks like will be largely determined by these environmental parameters. The meteorological data in the QTP, though limited, can be obtained from a number of literatures and websites. The authors should clearly show that the method used is applicable to the QTP, especially because the QTP has a very complex landscape.
**Response**: this is true. Considering complex environments, local or regional calibrations are needed. This is the reason we conduct GDGTs study in the QTP. We added related contents in our material and method part. Please see line 136 to 213 for details.

P488 Line 1-5 It is really not clear which season or months are the most humid or most warmest

quarters in the QTP. Please be more specific.
**Response**: good suggestion. After separation of 5-methyl and 6-methyl brGDGTs, we found there is no apparent seasonal bias with brGDGT indexes. We attribute this to strong correlation between seasonal temperature and annual temperature. Please see section 3.3.3 for details.

P488 Line 17-20 Huguet et al. (2006) should be cited because these authors proposed this internal standard.
**Response:** we cited this reference in the revised manuscript.

P489 Line 3 The authors used a different elution gradient than the previous studies. An increase in the amount of B phase from 10% to 80% in 45 min seems to be a little bit large for GDGTs separation, and in turn the separation of GDGTs will be inevitably affected. The authors need to show the chromatogram of GDGTs.
**Response:** we used the new method as suggested by the reviewer 3 and rewrote analytical part (line 169 to 199)

P490 Line 6 It is not reasonable to compare the concentration of GDGTs measured with different LC-MS because each instrument has its own response factor. The GDGT concentrations for two data sets both have wide ranges, and it seems to be a little bit far-fetched to say one data set is lower than another unless you perform an independent t-test on these datasets.
**Response:** good suggestion. In the revised manuscript, we did not present and discuss the absolute concentrations. Instead we focus on relative abundance.

P490 Line 11-13 'iGDGTs are mainly of an aquatic archaea origin' This statement is not right. In some soils, iGDGTs can be very abundant and they mainly derive from soil Thaumarchaeota. This part needs to be re-written.
**Response:** we accept this comment. And delete these statament in the revised manuscript.

P490 Line 15-20 The authoras omitted some important references, e.g. Yang et al.(2012, 2014) and Xie et al. (2013) because these papers investigated Chinese soils in a larger area and found low BIT values are widespread in alkaline and dry soils.
P490 Line 15-20 'All these results support the existence of in-situ production of iGDGTs in the QTP soils'. It seems to be not necessary to conclude like this because iGDGTs have no other sources other than soils.
P490 Line 20-23 Soil moisture appears to be an important factor controlling the BIT in soils. The authors should specify the relationship between soil moisture and BIT and then reach the conclusion.
**Response:** For above four comments, as we mentioned in "response to reviewer 3", since we achieved separation of 5 and 6-methyl brGDGTs, traditionally defined BIT is no longer meanful. So we did not discuss the BIT index as well as iGDGTs in the revised manuscript.
P490 Line 24 'favorite' changed to 'favorable '
**Response**: we rewrote the abstract and made changes as well.

P492 Line 3-5 'when all three variables are considered to the dataset'. This sentence is unclear. Pls rephrase.
**Response**: we rephrase this sentence in our revised manuscript.

P492 Line 15-20 'In some semiarid and arid regions, a strong correlation between MAP and MBT was observed (Dirghangi et al., 2013; Menges et al., 2014). I didn't see very strong correlations between MAP and MBT in above two papers. Instead, the relationship is weak. The authors should at least provide the R2 and Pearson coefficient to demonstrate there are strong correlations between

MAP and MBT. In fact, the MBT' has a much stronger correlation with soil pH than with MAP in Menges et al. (2014). ' a possible relationship between MAP and MBT' may be more appropriate. P493 Line 9-10 'Compared to soil pH, temperature and precipitation have much weaker influence on CBT ($r2 = 0.44$ and 0.03; Fig. 5)' The R2 does not correspond to theprevious environmental parameter. Please revise.

**Response**: we accept this comment and made correction. Actually, as mentioned above, after successful separation of 5-methyl and 6-methyl brGDGTs, MAP became the least important factor on brGDGT distributions. So we focus on the relationship between brGDGT and MAT/soil pH in the revised manuscript. Please see result and discussion part for details.

P494 Line 5-15 It seems to be not logical to develop a calibration of MBT, CBT, and MAT or MAP here because MBT has no strong correlation with either MAT or MAP in the QTP. The recent calibrations of MBT/CBT all depend on the strong correlation between MBT and temperature.

P496 Line 1-2 It is not reasonable to say 'our new calibration has successfully extended the minimum applicable threshold from 5 to -5 _C 'here due to the following reasons. First, the Eq.(12) only provided the RMSE for the total dataset. However, this RMSE may be largely determined by the GDGT data compiled from other publications because your dataset comprises only a relatively small proportion of the total dataset. I am not clear about the performance of this new calibration for MAT reconstruction inthe QTP alone. In most cases, the aim of developing new calibrations is to reduce thescatter and to improve the accuracy of paleoenvironmental reconstruction. The authors should provide the residual errors generated by the Eq. (12), Chinese calibration (Yang et al., 2014) and the Peterse et al(2013) global calibration to compare whether the new calibration has a better performance.

P496 Line13-15 'The reason for the slight bias of MBT-CBT towards winter season is that although more amounts of bGDGTs are produced in summer, more variation in bGDGT compositions occurs in winter'. This sentence is not logical' Did the authors analyze the correlation between winter T and MAT? In fact, these two parameters were highly correlated in Chinese region (Yang et al., 2014).

Figure 4 The authors should add MBT and CBT to the RDA triplot to show the relationship of them with environmental variables.

**Response:** we accepted above four comments. We gave detailed answers to similar comments in the "response to reviewer 2 and 3". So here, we just explain them in brief. With separation of 5-methyl and 6-methyl brGDGTs, MAP became least important factor on brGDGTs in QTP. Meanwhile, we found with new data, modified MBT index such as $MBT_{5ME}$ can well estimate MAT, while modified CBT such as CBT" and $CBT_{5ME}$ have strong correlations with soil pH. In addition, there is no apparent seasonal bias based on new brGDGT data. We rewrote these points in the revised manuscript. Please see result and discussion part.

We really appreciate Dr. Zech to provide valuable comments. We are also grateful to his acknowledge for the merits of our manuscript. Since the reviewer 3 pointed out one critical concern about the analytical method, we used tandem LC columns to rerun our samples where a series of new brGDGTs, namely 6-methyl brGDGTs, were identified (see Response to reviewer 3 for details). So we finished the section of result and discussion according to the new data, and thus made lots of changes in the revised manuscript. And some problems from Dr. Zech no longer exist. Here, we answer those comments point by point.

General comments:
The manuscript of Ding et al. provides new, interesting GDGT data from the Qinghai-Tibetan Plateau. It is well structured and written (but I am not a native speaker and didn't focus on language), and appropriate references are cited. I recommend publication in BG, although I think more statistical analyses could possibly improve the manuscript:

- The authors show that the MAP (mean annual precipitation) is better correlated with MBT and CBT (R2 0.5) than the MAT (mean annual temperature, R2 0.36, page 494). Then in the next step, when they present a recalibration for all Chinese soils, they do this ONLY for MAT. Why not also for MAP? Why not for an Aridity Index?
- Although the isoprenoid GDGTs are measured as well, they are not fully included in the statistical analyses and discussion. The Ri/b and BIT have been shown to correlate with environmental conditions (aridity), so why not testing respective correlations and including the isoprenoid GDGTs and an Aridity Index in the statistical analyses?

Response: after we successfully separated 5-methyl and 6-methyl brGDGTs, we found that precipitation is no longer important on $MBT_{5ME.}$ Soil pH is the most important factor on brGDGTs' distributions, followed by MAT, while MAP is the least important one. This result is consist with recent finding of de Jonge et al. (2014) who also separated 5-methyl and 6-methyl brGDGTs. Given these facts, we do not pay much attention to MAP.

Specific suggestions:
The molecular structures of the GDGTs have already often been published in manuscripts. I would put Fig. 1 in the appendix.

**Response**: It is true the structures of traditional GDGTs have been shown in many papers. However, we identified several 6-methyl brGDGTs, which were not reported in most early studies, so we still show the chemical structures of GDGTs.

Figures 6 and 7 are not really necessary. As all the seasonal parameters are not improving the correlations much, I would keep only Fig 6a, 7a. I would also delete fig.8b.

**Response:** This is a good comment. In the revised manuscript, we deleted all figures about seasonal parameters, but gave brief discussion in the section 3.3.3

Minor specific suggestions:
On page 482, line 4, you may want to write " : : : are NOVEL proxies", or ": : : are POTENTIALLY useful proxies". There are many remaining unknowns and uncertainties related to GDGTs.

Response: we accepted this suggestion.

On page 484, lines 22 and 23, check your reference to the equations.
Response： We checked and made correction.

In line 24: I think the extended dataset are 278 soils? But n in the following equations is only 176? Maybe clarify this in the text (I am wondering whether it is statistically justified to exclude the missing samples and whether this causes a bias in the calibration!?)
Response: they have total 278 samples, but some has no detectable GDGTs, reducing number to 176.

On page 485, double check equation 5 (MAT on the right should probably be MBT?)
Response: we checked and confirm it.

In line 7, you might want to write ": : : the MBT-CBT proxy has been INCREASINGLY used“, not SUCCESSFULLY.
**Response**: we accepted this suggestion and made change in the revised manuscript.

On page 496, line 14: "The reason : : : IS ...“ sounds too confident for my taste. Better " : : : MIGHT BE : : :“?
**Response**: we changed “is” into “might be” in the revised manuscript.

# Distribution of branched glycerol dialkyl glycerol tetraethers in surface soils of Qinghai–Tibetan Plateau: implications of brGDGTs-based proxies in cold and dry regions

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**Abstract.** The methylation index of branched tetraethers (MBT) and cyclization ratio of branched tetraethers (CBT) based on the distribution of branched glycerol dialkyl glycerol tetraethers (brGDGTs) are useful proxies for the reconstruction of mean annual air temperature (MAT) and soil pH. Recently, a series of 6-methyl brGDGTs were identified which were previously co-eluted with 5-methyl brGDGTs. However, little is known about the distribution of 6-methyl brGDGTs in Qinghai-Tibet Plateau (QTP), a critical region of the global climate system. Here, we investigated 30 surface soils covering a large area of QTP, among which 6-methyl brGDGTs were the most abundant components (average 53±17 % of total brGDGTs). The fractional abundance of 6-methyl brGDGTs showed a good correlation with soil pH, while the global MBT'$_{5ME}$ calibration overestimates MAT in cold regions like QTP. We therefore propose a MBT$_{5/6}$ index including both 5- and 6-methyl brGDGTs, presenting a strong correlation with MAT in QTP: $\mathrm{MAT} = -20.14 + 39.51 \times \mathrm{MBT}_{5/6}\ (n = 27, r^2 = 0.82; \mathrm{RMSE} = 1.3\ °\mathrm{C})$. Another index, namely IBT based on carbon skeleton isomerism of 5-methyl to 6-methyl brGDGTs, is dependent on soil pH: $\mathrm{pH} = 6.77 - 1.56 \times \mathrm{IBT}\ (n = 27;\ r^2 =$

$0.74$, RMSE $= 0.32$). Our study suggests that changing the position of methyl group of brGDGTs may be another mechanism for some soil bacteria to adapt ambient pH change besides well-known cyclization.

## 1. Introduction

The Qinghai–Tibetan Plateau (QTP), with an area of over 2.5 million km$^2$ and an average elevation of over 4000 meters above sea level (a.s.l.), is the world highest and largest mountain plateau. The uplift of the QTP since early Cenozoic profoundly influences regional and global climates such as the evolution of Asian monsoon which affects lives of over two billion people (An et al., 2001; Li, 1991; Lin et al., 2008; Wang et al., 2008). A number of studies have showed that the QTP is a highly sensitive area for global climate change (e.g., Kang et al., 2010; Liu & Chen, 2000; Qiu, 2008; Yao et al., 2007). The record of 97 meteorological stations located over 2000 meters a.s.l. in China reveals that winter temperature rise is 0.32 °C per decade in the QTP since 1950s, approximately three times the global warming rate (Liu & Chen, 2000). However, the history of instrumental measurement is too short to fully record the evolution of the QTP climate. The reconstruction of the QTP temperature beyond instrumental measurement is challenging because few quantitative proxies are available. Microfossil assemblages based on pollen, diatom or chironomid are commonly used paleothermometers, but they are also influenced by precipitation, salinity, nutrient or other environmental factors (e.g., Keatley et al., 2009; Meriläinen et al., 2000; Seppä & Birks, 2001). The $\delta^{18}$O value of ice core in the QTP shows a good correlation with northern hemisphere temperature (Thompson et al., 1997; Yao et al., 2002). Unfortunately, ice core with a long term, continuous record is lacking in most QTP.

Over the past decades, two molecular proxies have been developed for estimation of continental temperature. The first one, namely UK’37, is based on the distribution of haptophyte-derived long-chain alkenones. This proxy was originally proposed for paleoceanography (Brassell et al., 1986; Prahl et al., 1988), but was found applicable for reconstruction of lake surface temperature (e.g., Liu et al., 2006; Zink et al., 2001). A major limitation of UK’37 is that long-chain alkenones are not always present in lakes, although they were reported in some QTP lakes (e.g., Chu et al., 2005; Liu et al., 2011; Liu et al., 2006). In addition, salinity influences the compositions of long-chain alkenones in lakes (Liu et al., 2011). Besides UK’37, the methylation index of branched tetraethers (MBT) and cyclization ratio of branched tetraethers (CBT) can be also used to infer past continental temperature based on the distribution of branched glycerol dialkyl glycerol tetraethers (brGDGTs) (Weijers et al., 2007b):

$$\mathrm{MBT} = \frac{\mathrm{Ia+Ib+Ic}}{\mathrm{Ia+Ib+Ic+IIa+IIb+IIc+IIIa+IIIb+IIIc+IIa'+IIb'+IIc'+IIIa'+IIIb'+IIIc'}} \qquad (1)$$

$$\mathrm{CBT} = -\log\frac{\mathrm{Ib+IIb+IIb'}}{\mathrm{Ia+IIa+IIa'}} \qquad (2)$$

where roman numbers denote relative abundance of compounds in Fig. 1. It should be pointed out that the Eq. 1 and 2 are rewritten from original definitions because the peaks previously identified as pure 5-methyl brGDGTs (Weijers et al., 2007b) are actually mixtures of 5-methyl and 6-methyl isomers (De Jonge et al., 2013).

So far, only two species of Acidobacteria were identified to produce brGDGTs (Sinninghe Damsté et al., 2011), but the ubiquitous occurrence of brGDGTs in soils/peats, lakes and marginal seas suggest that other biological sources are likely (Schouten et al., 2013 and references therein). By analyzing globally distributed soils, Weijers et al. (2007b) found that the MBT is controlled by mean annual air temperature (MAT) and to less extent by soil pH, whereas CBT only relates to soil pH. Such relationship was corroborated by the subsequent study of Peterse et al. (2012) who proposed a simplified format of MBT (or MBT’) based on seven quantifiable brGDGTs.

Since the advent, the MBT(MBT’)-CBT paleotemperature proxy has been increasingly used for lakes (e.g., D'Anjou et al., 2013; Loomis et al., 2012; Sun et al., 2011), paleosol-loess sequences (e.g., Peterse et al., 2011; Zech et al., 2012), peat (Ballantyne et al., 2010) and marginal seas (e.g., Bendle et al., 2010; Weijers et al., 2007a; Zell et al., 2014). However, a relatively large scatter in global MBT/CBT–MAT calibrations (about 5 $^{o}$C for root mean square error; RMSE) suggests that other factors besides temperature may influence brGDGTs-based indices (Peterse et al., 2012; Weijers et al., 2007b). In arid and semiarid areas such as western United States where precipitation is the ecological limiting factor, mean annual precipitation (MAP) rather than MAT is the most important factor that affects brGDGT compositions (Dirghangi et al., 2013; Menges et al., 2014). The updated global calibration of MBT'-CBT indices (Peterse et al., 2012) also shows a weak correlation with MAT for those soil samples from arid regions (MAP < 500 mm). Some studies suggest that regional calibrations are needed to improve accuracy of the GDGTs-based proxy (e.g., Loomis et al., 2012; Pearson et al., 2011; Shanahan et al., 2013; Zink et al., 2010).

Another factor to cause the relatively large scatter of the MBT/CBT-MAT calibration is analytical error. By applying advanced analytical techniques, De Jonge et al. (2013) identified a series of novel 6-methyl brGDGTs which previously co-eluted with 5-methyl GDGTs that were used to calculate the brGDGTs proxies. The successful separation of 5- and 6-methyl brGDGTs resulted in a set of new brGDGT proxies, which were used to recalibrate traditionally defined MBT-CBT indexes (De Jonge et al., 2014):

$$\mathrm{MBT'_{5ME}} = \frac{\mathrm{Ia+Ib+Ic}}{\mathrm{Ia+Ib+Ic+IIa+IIb+IIc+IIIa}} \tag{3}$$

$$\mathrm{MAT} = -8.57 + 31.45 \times \mathrm{MBT'_{5ME}}$$

$$(n = 222, r^2 = 0.66; \mathrm{RMSE} = 4.8\ ^\circ\mathrm{C}, \mathrm{P} < 0.001) \tag{4}$$

$$\mathrm{CBT_{5ME}} = -\log \frac{\mathrm{Ib + IIb}}{\mathrm{Ia + IIa}} \tag{5}$$

$$\mathrm{pH} = 7.84 - 1.73 \times \ \mathrm{CBT_{5ME}}$$

$$(n = 221, r^2 = 0.60; \mathrm{RMSE} = 0.84, \mathrm{P} < 0.001) \tag{6}$$

$$\mathrm{CBT'} = \log \frac{\mathrm{Ic + IIa' + IIb' + IIc' + IIIa' + IIIb' + IIIc'}}{\mathrm{Ia + IIa + IIIa}} \tag{7}$$

$$\mathrm{pH} = 7.15 + 1.59 \times \mathrm{CBT'}$$

$$(n = 221, r^2 = 0.85; \mathrm{RMSE} = 0.52, \mathrm{P} < 0.001) \tag{8}$$

For the QTP, several studies have reported GDGTs in lakes, mountains, hot springs and paleo-soils (e.g., Günther et al., 2014; He et al., 2012; Liu et al., 2013; Wang et al., 2012; Wu et al., 2013; Xie et al., 2012). Wang et al. (2012) analyzed GDGTs in surface sediments of the Lake Qinghai and surrounding soils, showing that brGDGTs-inferred MAT and soil pH were consistent with measured values. In contrast, Wu et al. (2013) found that brGDGTs-derived MAT was higher than instrumentally measured MAT in Kusai Lake sediments from the QTP. Based on the distributions of GDGTs in surface sediments of the QTP lakes, Günther et al. (2014) developed the local calibration of MBT'-CBT ($r^2$ = 0.59; RMSE = 1.2 ºC). However, there are only 9 lake sediments included in Günther et al. (2014). For the application of MBT-CBT indices in lakes, brGDGTs in lake sediments must be exclusively derived from inputs of surrounding soils. However, in-situ production of brGDGTs occurs in various lakes (e.g., Blaga et al., 2009; Blaga et al., 2010; Fietz et al., 2012; Pearson et al., 2011; Sinninghe Damsté et al., 2009; Tierney & Russell, 2009). Furthermore, the 6-methyl brGDGTs were not reported in any QTP studies, which may explain the relatively low $r^2$ value of the MBT/CBT-MAT calibration (e.g., Günther et al., 2014). Given these facts, a direct investigation of soils with improved chromatography is needed to understand environmental influences on the brGDGT distributions in the QTP.

Here, we analyzed all 5- and 6-methyl brGDGTs in 30 surface soils from the QTP. Our main objectives are to (1) determine the relative abundance and distribution of 5- and 6-methyl brGDGTs in the QTP soils; (2) evaluate the effect of recently identified 6-methyl brGDGTs on soil pH in the QTP; and (3) test whether global brGDGTs-MAT calibration is applicable in the QTP and thereby understand the influence of temperature, precipitation and soil pH on 5- and 6-methyl brGDGTs in the QTP.

## 2. Materials and methods

### 2.1. Sampling

A total of 30 surface soil samples (0-10 cm) were collected during two fieldworks in 2011 and 2012, which cover a large area of the QTP (84.64°~101.20° E; 28.24 °~37.45° N) (Fig. 2). Sampling sites are typical alpine meadow, alpine steppe or alpine meadow steppe. The extremely dry winter results in the lack of persistent snow cover in most sampling sites. The soil samples were air-dried and passed through a 2 mm mesh to remove large gravels. Fine roots (if present) were picked up by steel tweezers. The detailed information on the sampling sites and environmental variables are listed in the supplementary material (Table S1).

### 2.2 Climate data

There are about 70 meteorological stations in the QTP, mainly distributed in the eastern part and northern border of the QTP. Thus, direct observation data on temperature and precipitation at our sampling sites are generally lacking. In this study, we use the WorldClim dataset (Hijmans et al., 2005) to interpolate annual, seasonal and monthly mean precipitation and temperature (Table S1). The local climate is dry and cold. The MAT of our sampling sites ranges from -5.5 to 7.6 °C with a vertical lapse rate of 0.487 °C/100 m to 0.699 °C/100 m (Cheng et al., 2012). The vertical lapse rate of air temperature decreases from north to south of the QTP. The mean annual precipitation (MAP) at different altitudes varies from ca. 85 mm to ca. 495 mm. The integrated maps are derived from monthly temperature and precipitation values gathered from thousands of weather stations around the world from 1950 to 2000 (47,554 locations for precipitation and 24,542 locations for temperature). The original point data was splines interpolated using latitude and longitude at a fine resolution, making it possible to obtain a reasonable estimation of climatic conditions at individual sites. The WorldClim GIS data used contain annual average of 6 climate variables at a 30 arc seconds resolution (~1 km resolution; http://www.worldclim.org/current.htm). Besides MAT and MAP, additional four climate variables were also used to evaluate the relationship between climate and 5- and 6-methyl brGDGTs indices, including Mean Temperature of Wettest Quarter (MWQT), Mean Temperature of Driest Quarter (MDQT), Mean Temperature of Warmest Quarter (MWQT'), Mean Temperature of Coldest Quarter (MCQT). A total of 30 sites from QTP cold and dry regions (Table S1) were extracted by 6 climate variables using Arcgis 9.3.

## 2.3 Soil pH and brGDGT analyses

For pH measurement, soils were mixed with deionized water in a ratio of 1/2.5 (g/ml). The soil pH values were determined by a pH meter with a precision of ±0.01 pH. The pH was reported as an average of three duplicate measurements for each sample with standard deviation of ±0.05.

The detailed procedure for lipid extraction was described by Wu et al. (2014). About 6 g dry soils were mixed with 600 ng $C_{46}$ GDGT (internal standard) (Huguet et al., 2006) and ultrasonically extracted with 20 ml dichloromethane (DCM)/methanol (3:1 v:v) for 15 min (3×). The combined extracts were concentrated to near dryness by a rotary evaporator and transferred to small vials. The concentrated extracts were completely dried under a mild stream of $N_2$ and re-dissolved in DCM. The total extracts were separated into two fractions by 5 ml hexane/DCM (9:1 v:v) and 5 ml DCM/methanol (1:1 v:v), respectively, on silica gel columns. The latter fraction containing brGDGTs was dissolved in 300 μl hexane/EtOAc (84:16,v/v).

The GDGTs were analyzed on an Agilent 1200 High Performance Liquid Chromatography-atmospheric pressure chemical ionization-triple quadruple mass spectrometry (HPLC-APCI-$MS^2$) system (Yang et al., 2015). The injection volume was 10 μl. The separation of 5- and 6-methyl brGDGTs was achieved with two silica columns in sequence (150 mm × 2.1 mm; 1.9 μm, Thermo Finnigan; USA) at a constant flow of 0.2 ml per min. The solvent gradient was: 84% A (hexane) and 16% B (EtOAc) for 5 min, then increasing the amount of B from 16% at 5 min to 18% at 65 min, and then to 100% B in 21 min. The column was flushed with 100% B for 4 min, and then back to 84/16 A/B to equilibrate it for 30 min. The APCI and MS conditions were: vaporizer pressure of $4.2 \times 10^5$ Pa, vaporizer temperature of 400 °C, drying gas flow of 6 L $min^{-1}$,temperature of 200 °C, capillary voltage of 3500 V, and corona current of 5 μA (3.2 kV). Peak integration was carried out using Agilent MassHunter. Samples were quantified based on comparisons of the respective protonated-ion peak areas of each GDGT to the internal standard in selected ion monitoring (SIM) mode. The protonated ions were m/z 1050, 1048, 1046, 1036, 1034, 1032, 1022, 1020, 1018 and 744 ($C_{46}$ GDGTs). Since we assume same response factors among different brGDGTs and $C_{46}$ GDGTs, our study can be only regarded as semi-quantification.

### 2.4 Statistical analyses

In order to assess the relationship of 5- and 6-methyl brGDGT distributions with environmental variables such as temperature, precipitation and soil pH, we performed redundancy analysis (RDA) (van den Wollenberg, 1977), a constrained form of the linear ordination method of principal components analysis (PCA). Species (fractional abundance of 15 brGDGTs) were centered and standardized with zero average and unit variance before RDA. The significance of the explanatory variances within a 1% confidence interval was tested with 999 unrestricted Monte Carlo permutations. Subsequently, a series of partial RDAs (pRDA) were performed to constrain the unique and independent influence of individual environmental parameter alone, as well as compared to all other parameters. All statistical analyses were performed with the CANOCO version 4.5 software (Wageningen UR, USA).

## 3. Results and discussion

### 3.1 brGDGTs abundance in the QTP soils

All soil samples except for P790, P840 and P855 contain detectable amounts of brGDGTs. Consequently, 27 soils were used to calibrate brGDGTs' indices in this study. With the application of two silica LC columns in tandem, 5-methyl and 6-methyl brGDGT isomers were successfully separated, increasing the number of detectable brGDGT compounds from 9 (Peterse et al., 2012; Weijers et al., 2006) to 15 (Fig. 1). There were three tetra-methylated brGDGTs (Ia, Ib and Ic), six penta-methylated brGDGTs (IIa, IIb, IIc, IIa', IIb', IIc') and six hexa-methylated brGDGTs (IIIa, IIIb, IIIc, IIIa', IIIb', IIIc'). The mean fractional abundance of 5-methyl brGDGTs ($f_{5ME}$) and 6-methyl brGDGTs ($f_{6ME}$) was shown in Fig. 3. The 6-methyl brGDGTs accounted for average 53% of the total amount of brGDGTs, which were dominated by IIa' and IIIa'. Such composition of brGDGTs is different from that of the global soils (239 soils) that 5-methyl brGDGT (Ia and IIa) are usually the most abundant isomers and 6-methyl brGDGTs only comprise on average 24% of the total amounts of brGDGTs (De Jonge et al., 2014), suggesting that the brGDGT-producing bacteria may change their membrane lipids to adapt environmental conditions. So far, two species of Acidobacteria are only identified biological sources for brGDGTs, but they only produce tetra-methylated brGDGTs (Sinninghe Damsté et al., 2011). In our study, the majority of the QTP soils are weak alkaline (6.2~8.4 pH unit), which may favor thriving of non-Acidobacteria and thereby lead to the higher proportion of 6-methyl brGDGTs.

### 3.2 Environmental control on brGDGT distributions in QTP

A number of studies have demonstrated that temperature, precipitation and pH are the most important factors that affect the brGDGT distributions in soils (e.g., De Jonge et al., 2014; Dirghangi et al., 2013; Peterse et al., 2012; Weijers et al., 2006; Weijers et al., 2007b; Yang et al., 2015; Yang et al., 2014). In order to evaluate the contribution of these parameters to 5- and 6-methyl brGDGT distributions in the QTP, a RDA was performed (Fig. 4). The first component explains 65.2% of the variance, mainly reflecting the variation in soil pH and to less extent MAP. Soil pH presents strong positive relationships with fractional abundance of brGDGTs IIIa', IIb', IIb, and negative relationships with that of IIIa, IIa, Ia. The second component of the RDA plot explains 6.1% of total variance, mainly reflecting the variation in MAT and MAP. The brGDGT-IIIa', IIIa, IIa, IIb, IIc show negative relationships with MAT (in the lower part of RDA), whereas brGDGT-IIa, Ia, Ib and Ic present positive relationships with MAT (in the upper part of RDA). These results support a physiological mechanism that soil bacteria change the number of methyl branches of brGDGTs with temperature in order to maintain acceptable fluidity of their membranes (Weijers et al., 2007b).

Our RDA result shows that MAT and pH have a significant independent effect on the brGDGT distribution in the QTP soils, however, no significant correlation was observed between MAP and brGDGTs ($p > 0.05$; Table 1). Soil pH explaining up to 60.1% of the total variables is the largest contributor to the variance, followed by MAT (up to 16.4%) and MAP (up to 10.8%). The predominant influence of soil pH on brGDGT distributions was also observed in global soil dataset (De Jonge et al., 2014; Peterse et al., 2012) and Chinese soils (Yang et al., 2015; Yang et al., 2014). In order to estimate the independent, marginal effect of MAT, MAP and pH, partial RDA (pRDA) was performed. The explained variance of pH still remains high (39.9%), indicating that brGDGT distributions are indeed linked to soil pH, whereas MAT contribute to a smaller amount (10.6%) of the variance (Table 2). Similar to the result of RDA, pRDA also showed minor contribution of MAP (2.0%) to brGDGT distributions. The comparison between RDA and pRDA suggests a decreasing contribution of these three environmental variables (pH, MAT, MAP) when they are considered as a unique contribution (Table 2). Thus, there is a “synergistic effect” (an “antagonistic action”) when MAP and pH (MAT and pH) are considered as covariables, resulting in a positive joint effect of 20.4% for total contribution of pH+MAT+MAP to brGDGT distributions in the QTP soils.

### 3.3 Evaluation of brGDGT-based proxies in the QTP

Since the identification of 6-methyl brGDGTs (De Jonge et al., 2013), a set of new brGDGT indices such as MBT’$_{5ME}$ and CBT$_{5ME}$ have been proposed in order to reduce uncertainty of reconstructed MAT and soil pH (De Jonge et al., 2014; Weijers et al., 2007a; Yang et al., 2015). However, even with application of the MBT$_{5ME}$-MAT recalibration and the multiple regression, relatively large scatter still exists for those samples from cold regions (De Jonge et al., 2014). Therefore, further calibrations of brGDGT-derived proxies are needed.

#### 3.3.1 MAT calibration in cold and dry regions of the QTP

Consistent with the finding of De Jonge et al. (2014), our result shows that CBT$_{5ME}$ no longer contributes significantly to MAT after the exclusion of 6-methyl brGDGTs (*p* = 0.51; *n* = 27). Therefore, we use MBT’$_{5ME}$ only to calibrate MAT. Considering that limited samples from cold regions were included in previous studies (Peterse et al., 2012; Weijers et al., 2007b), we added our QTP data into the global soil dataset (De Jonge et al., 2014), resulting in a new calibration of MBT’$_{5ME}$-MAT:

$$\mathrm{MAT} = -10.07 + 33.50 \times \mathrm{MBT'}_{5\mathrm{ME}}$$

$$(n = 249, r^2 = 0.70; \mathrm{RMSE} = 4.7\ °\mathrm{C}, \mathrm{P} < 0.001) \qquad (9)$$

The correlation coefficient of Eq. 9 ($r^2$ = 0.70) is slightly higher than the previous global calibration ($r^2$ = 0.66; Eq. 4), while its RMSE (4.7 °C) is similar to the previous calibration (4.8 °C; Eq. 4) (De Jonge et al., 2014). Furthermore, the comparison of our estimated MAT and actual MAT ($\Delta$MAT = MAT$_{est}$ – MAT$_{act}$) showed an apparent overestimation (average 2.8 °C; Fig. 5). Therefore, the simple extension of dataset is not successful in improving accuracy of the MBT’$_{5ME}$-MAT proxy at the global scale.

Alternatively, we conducted a regional calibration of MBT’$_{5ME}$ versus MAT based on 27 QTP soils, and a new equation of MBT'$_{5ME}$-MAT was expressed:

$$\mathrm{MAT} = -10.82 + 28.36 \times \mathrm{MBT'}_{5\mathrm{ME}} \quad (n = 27, r^2 = 0.65; \mathrm{RMSE}=1.8\ °\mathrm{C}, \mathrm{P}<0.001) \quad (10)$$

The slope of Eq. 10 (28.36) is distinct difference from that of global surface soils (33.50; Eq. 4). Meanwhile, its RMSE value (1.8 °C) is substantially smaller than that of De Jonge et al. (2014) (4.8 °C). This reduced uncertainty in reconstructed MAT is attributed to smaller spatial heterogeneity of soils, similar vegetation types (e.g., alpine meadow) and a narrower MAT range (-5.5~7.6°C) in the QTP. Usually, the regional calibration has higher $r^2$ values than the global one due to its smaller size of dataset and smaller spatial heterogeneity (Loomis et al., 2014; Yang et al., 2014). However, our calibration for the QTP has a slightly lower $r^2$ value (0.65) than the global one (0.70; Eq. 9), suggests that the calibration based on MBT'$_{5ME}$ alone is not superior to the traditional MBT calibration. The RDA result reveals that similar to 5-methyl brGDGTs, 6-methyl brGDGTs also significantly correlate with MAT (Fig. 4). Thus, we propose a new brGDGT index (MBT$_{5/6}$) including 5-methyl brGDGTs used in the traditional definition and two dominant 6-methyl brGDGTs (IIa' and IIIa'), expressed as:

$$\mathrm{MBT}_{5/6} = \frac{\mathrm{Ia} + \mathrm{Ib} + \mathrm{Ic} + \mathrm{IIa'}}{\mathrm{Ia} + \mathrm{Ib} + \mathrm{Ic} + \mathrm{IIa} + \mathrm{IIb} + \mathrm{IIc} + \mathrm{IIIa} + \mathrm{IIIa'}} \quad (11)$$

Based on data of the QTP soils, the linear correlation of MAT and MAT$_{5/6}$ was established as (Fig. 6): $\mathrm{MAT} = -20.14 + 39.51 \times \mathrm{MBT}_{5/6}$

$$(n = 27, r^2 = 0.82; \mathrm{RMSE} = 1.3\ °\mathrm{C}, \mathrm{P} < 0.001) \quad (12)$$

This calibration has substantially higher $r^2$ (i.e. 0.82) and lower RMSE (i.e. 1.3 ºC) than Eq. 9 (i.e., 0.70 for $r^2$ and 4.7 ºC for RMSE) and Eq. 11 (i.e., 0.65 for $r^2$ and 1.8 ºC for RMSE), supporting that the inclusion of 5-metyl and 6-methyl brGDGTs is essential for improved accuracy of MAT reconstruction. However, this result is different from the finding from the Mount Shennongjia (central China) that 6-methyl brGDGTs are regarded as the interference, leading to a larger scatter of the MBT'-MAT proxy (Yang et al., 2015). Nevertheless, these differences highlight the importance of regional calibrations of brGDGT proxies.

### 3.3.2 Effect of soil pH on position of methyl group(s) of brGDGTs

It is generally accepted that the proton permeability of the cell membrane plays a crucial role in maintaining pH gradient across the membrane of bacteria and archaea (Konings et al., 2002). Weijers et al. (2007b) observed a strong correlation between relative abundance of cyclopentane moieties of brGDGTs and soil pH, and hypothesized that, some soil bacteria can change the methyl groups of brGDGTs into cyclopentyl groups with ambient pH rise, which will loosen the packing of the membrane lipids, enabling more water molecules to get trapped.

Following the approach of De Jonge et al. (2014), we got the following correlation between soil pH and CBT' which is a modified format of originally defined CBT (Weijers et al., 2007b):

$$\mathrm{pH} = 7.01 + 1.49 \times \mathrm{CBT}' \qquad (n = 27, r^2 = 0.78, \mathrm{RMSE} = 0.30\ \mathrm{pH\ unit}) \qquad (13)$$

The Eq. 12 has slightly lower $r^2$ and substantially lower RMSE compared with the global calibration of pH-CBT' ($n$ =221, $r^2$ = 0.85, RMSE = 0.52) (De Jonge et al., 2014), suggesting that both global and regional calibrations are applicable for soil pH reconstruction.

We noted that some non-cyclopentyl brGDGTs such as Ia, IIa and IIIa show negative correlations with soil pH, while other brGDGTs show positive correlations with soil pH in the RDA (Fig. 4). Based on these facts, we put all positively correlated brGDGTs on the numerator and all negatively correlated brGDGTs on the denominator to build a new CBT index (or CBT"):

$$\mathrm{CBT}'' = \log \frac{\mathrm{Ib+Ic+IIb+IIc+IIIb+IIIc+IIa'+IIb'+IIc'+IIIa'+IIIb'+IIIc'}}{\mathrm{Ia+IIa+IIIa}} \qquad (14)$$

A linear correlation between soil pH and CBT" was established based on 27 QTP soils:

$$\mathrm{pH} = 6.93 + 1.49 \times \mathrm{CBT}'' \qquad (n = 27, r^2 = 0.80, \mathrm{RMSE} = 0.29\ \mathrm{pH\ unit}) \qquad (15)$$

The similar $r^2$ and RMSE between Eq. 13 and 15 was attributed to minor amounts of brGDGTs Ib, IIb, IIc, IIIb and IIIc (average 8% of total brGDGTs; Fig. 3) which were excluded from the CBT' index but included in our CBT" index.

The fractional abundance of 6-methyl brGDGTs showed strong positive correlations with soil pH in both the QTP ($r^2$ = 0.74; Fig. 8) and global soil dataset (0.41 < $r^2$ < 0.72; De Jonge et al., 2014). This is apparent contrast with the previous assumption that non-cyclopentyl moieties (such as IIa' and IIIa') negatively correlate with soil pH. Unlike 6-methyl brGDGTs, some 5-methyl brGDGTs did not show positive correlations with soil pH (de Jonge et al., 2014). Thus, we hypothesize that besides cyclization, the position of methyl group(s) of brGDGTs also influences cell membrane fluidity. In order to test this hypothesis, we define a new index about carbon skeleton Isomerism of Branched Tetraethers (or IBT) as the abundant ratio of non-cyclopentyl 6-methyl to 5-methyl brGDGTs:

$$\mathrm{IBT} = -\log\frac{\mathrm{IIIa'}+\mathrm{IIa'}}{\mathrm{IIIa}+\mathrm{IIa}} \qquad (16)$$

We performed a linear regression of IBT versus soil pH based on 27 QTP soils (Fig. 8), yielding an equation as:

$$\mathrm{pH} = 6.77 - 1.56 \times \mathrm{IBT} \qquad \left(\mathrm{n} = 27;\ r^2 = 0.74, \mathrm{RMSE} = 0.32\right) \qquad (17)$$

Meanwhile, the linear correlation of $\mathrm{CBT_{5ME}}$ and soil pH was also established:

$$\mathrm{pH} = 7.98 - 1.12 \times \mathrm{CBT_{5ME}} \qquad \left(\mathrm{n} = 27;\ r^2 = 0.66, \mathrm{RMSE} = 0.37\right) \qquad (18)$$

For the regional calibration, the IBT index has higher $r^2$ and lower RMSE than traditionally defined $\mathrm{CBT_{5ME}}$ index, supporting that the carbon skeleton isomerism of brGDGTs (i.e., changing the position of methyl group) is indeed a physiological mechanism of brGDGTs-producing bacteria to adapt soil pH change.

### 3.3.3 Seasonality of brGDGTs proxies in the QTP

The QTP is under strong influence of Asian Monsoon, characterized by warm/humid summer (June to August) and dry/cold winter (December to February) (An et al., 2001; Qiu, 2008). In order to examine if there is a seasonal bias on brGDGT distributions, we analyze the correlation coefficients of 5- and 6-methyl brGDGTs proxies (i.e., $\mathrm{MBT'_{5ME}}$, $\mathrm{MBT_{5/6}}$, $\mathrm{CBT_{5ME}}$ and IBT) versus annual and seasonal air temperature (Table S2). Overall, there is no apparent seasonal bias for $\mathrm{MBT'_{5ME}}$ and $\mathrm{MBT_{5/6}}$. This is likely attributed to significant correlation between seasonal temperature and MAT in the QTP ($r^2 > 0.80$, $p < 0.0001$). In addition, In addition, no significant correlation was observed between the CBT indices/IBT and MAT/seasonal temperature ($-0.3 < r < 0.3$; Table S2), suggesting minor influence of air temperature on these indices.

Our results are consistent with that of Weijers et al. (2011) who found no significant seasonal bias in MBT-CBT indices in mid-latitude soils. Therefore, the reconstruction of MAT based on the 5- and 6-methyl brGDGTs proxies is doable in the QTP.

## 4. Conclusions

By applying improved chromatography, we successfully separated 5- and 6-methyl brGDGTs in the surface soils from the Qinghai-Tibet Plateau (QTP), a cold and dry region. This is the first time to report 6-methyl brGDGTs in the QTP, providing an opportunity to optimize brGDGTs' proxies in this critical region. Three conclusions were reached based on brGDGT data in 27 surface soils. Firstly, the 6-methyl brGDGTs are widely distributed in the QTP soils accounting for average 53% of total amounts of brGDGTs. Secondly, soil pH is the most important contributor to the variance of brGDGTs, followed by MAT, while MAP has no significant effect on brGDGTs' distributions. Thirdly, two new indices including recently identified 6-methyl brGDGTs were proposed to estimate MAT and soil pH, respectively. The first one, namely $MBT_{5/6}$, is useful for the MAT reconstruction in cold and dry regions (like QTP) with an improved RMSE of 1.3 °C. The second one, namely IBT, is allowed to estimate soil pH with an RMSE of 0.32. Our study demonstrates that besides previously reported cyclization, isomerization of 5-methyl to 6-methyl brGDGTs (expressed as IBT) is another strategy for brGDGTs-producing bacteria to adapt ambient pH change.

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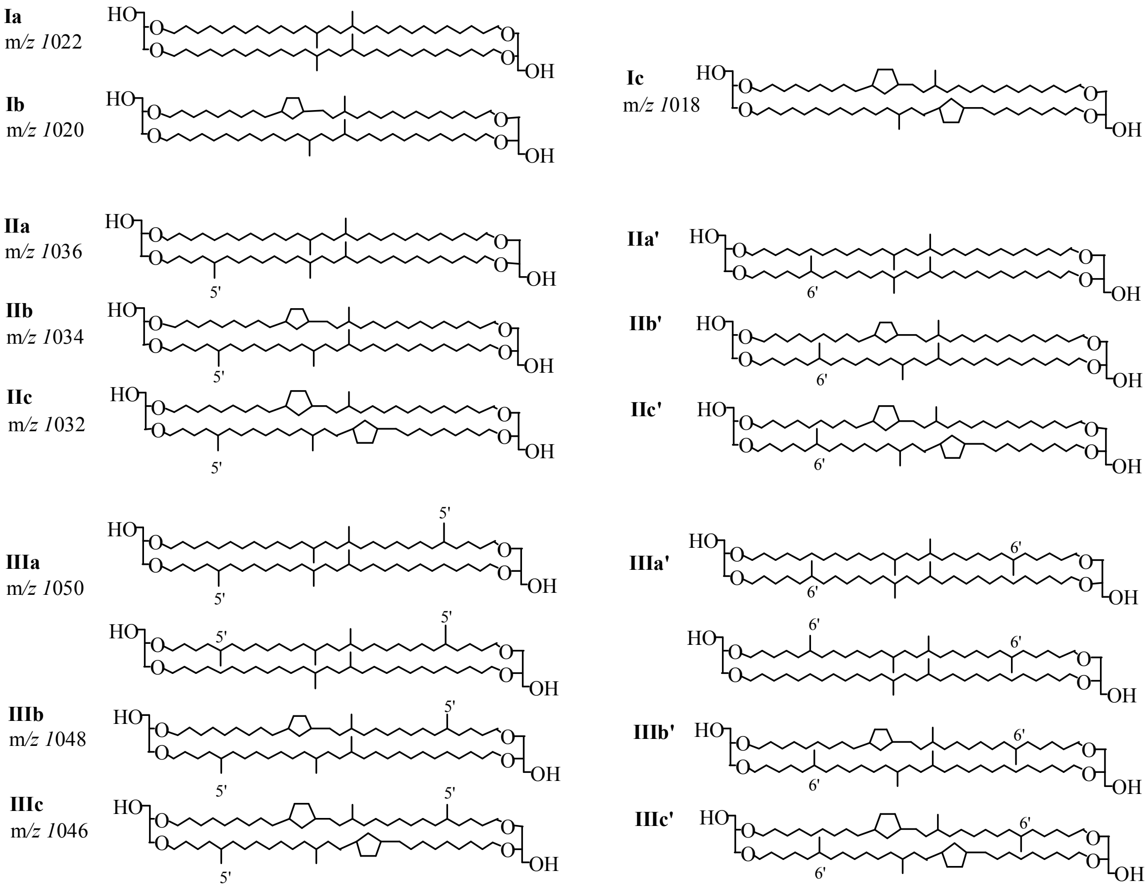

**Fig. 1.** Molecular structures of 5- and 6-methyl branched GDGTs used in this study. The compounds that have one or two methyl groups at the α6 or ω6 position are defined as 6-methyl brGDGTs, while the compounds that have one or two methyl groups at the α5 or ω5 position are defined as 5-methyl brGDGTs.

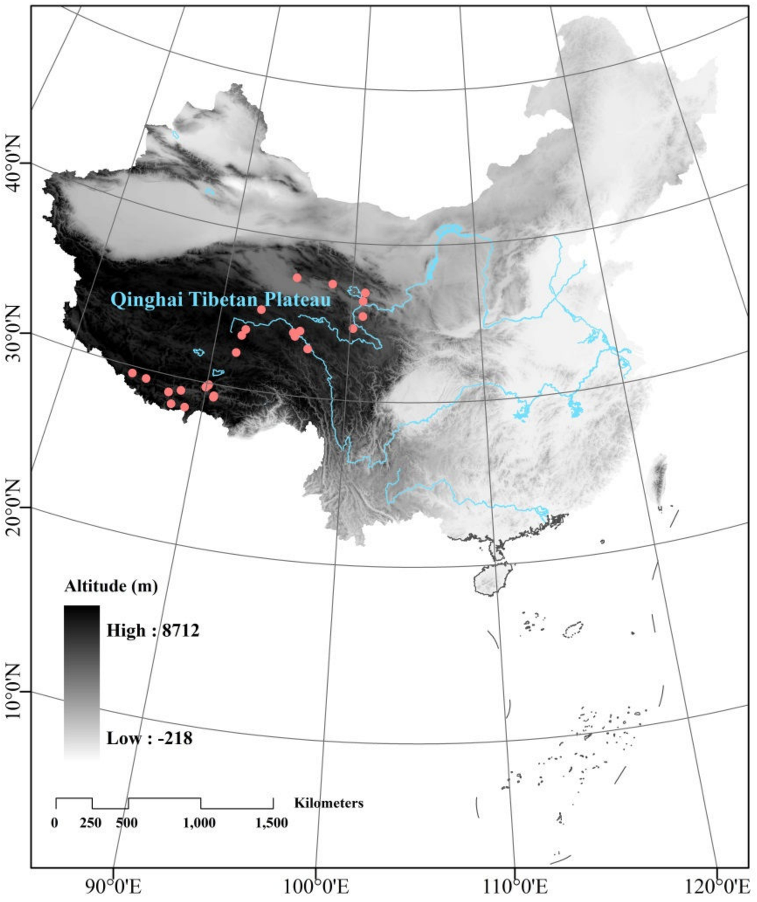

**Fig. 2**. Locations of soil sampling sites (n = 30) in the QTP (Pink solid circles).

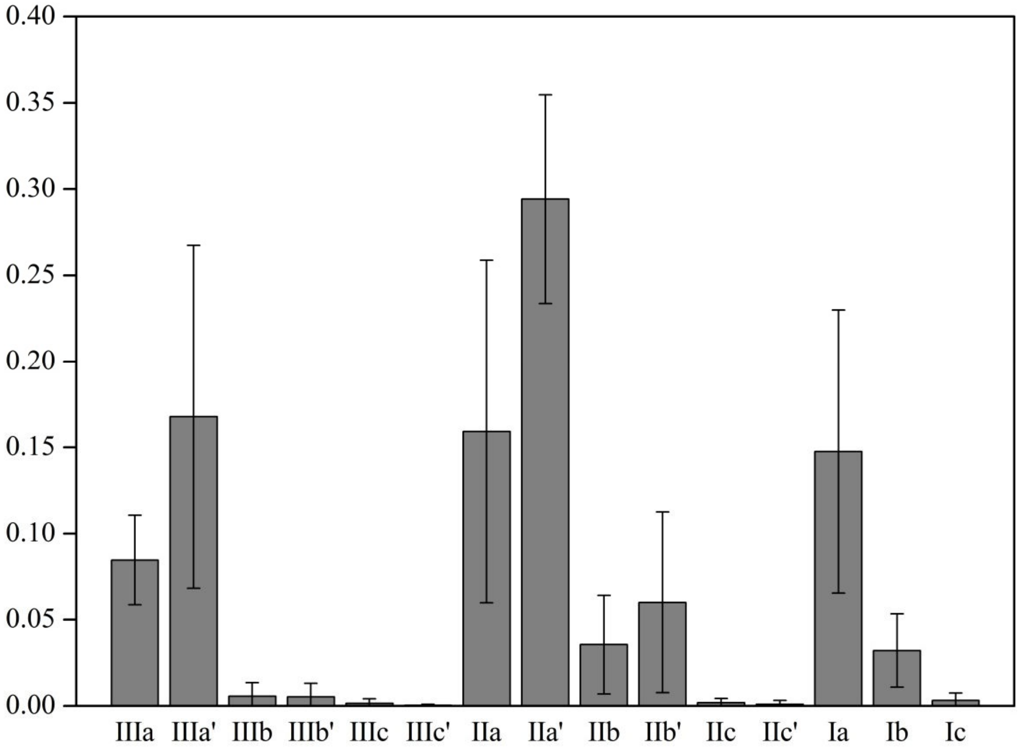

**Fig. 3**. Average (*n* = 27) fractional abundance of brGDGTs in surface soils of the QTP.

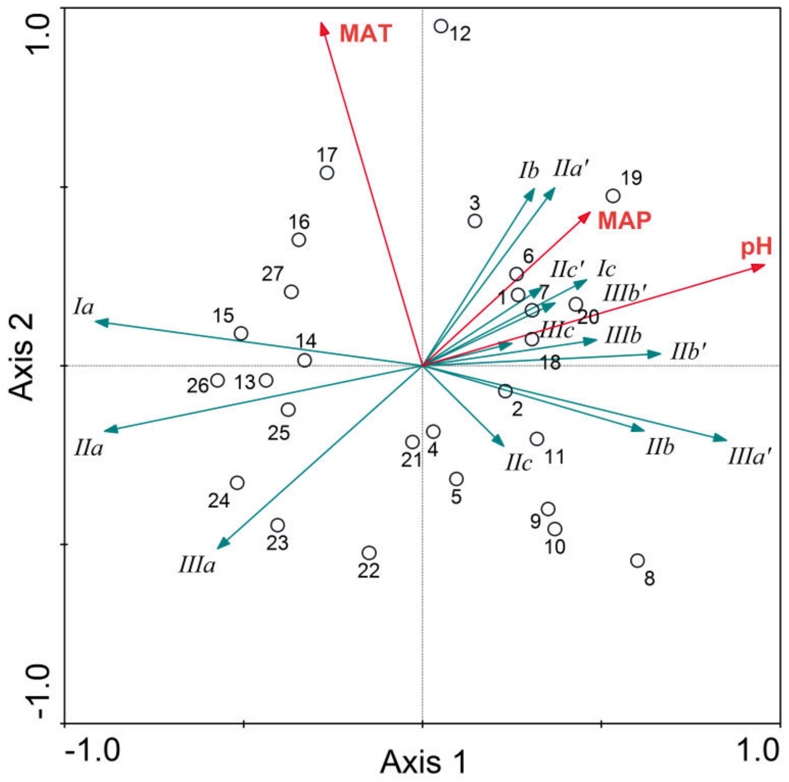

**Fig. 4**. RDA triplot showing the relationship between 5- and 6-methyl brGDGTs%, MAT, MAP and soil pH from the QTP. Numbers in the plot correspond to the soils in supplementary material (Table S1). The first and second axis explained 65.2% and 6.1% of the variance, respectively.

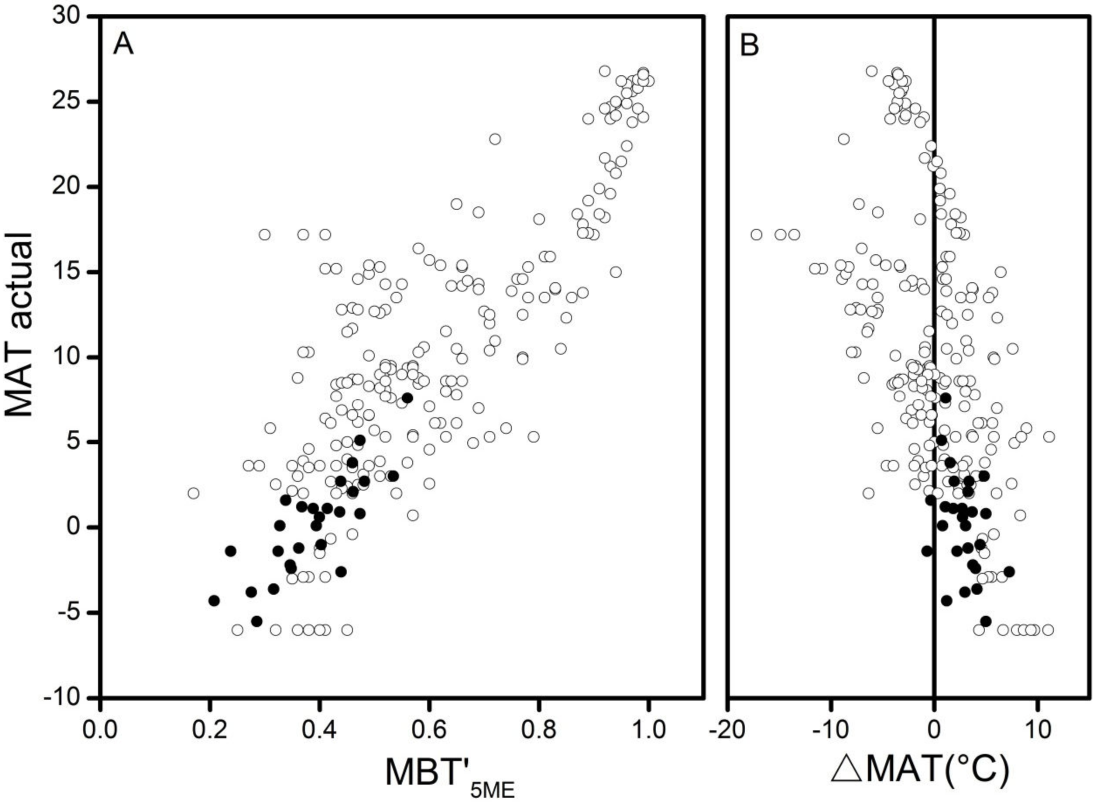

**Fig. 5.** A) Scatterplot of MBT'$_{5ME}$ with actual MAT; B: difference between estimated MAT and actual MAT (ΔMAT). Solid and empty circles represented soils in this study and global soils (de Jonge et al., 2014), respectively.

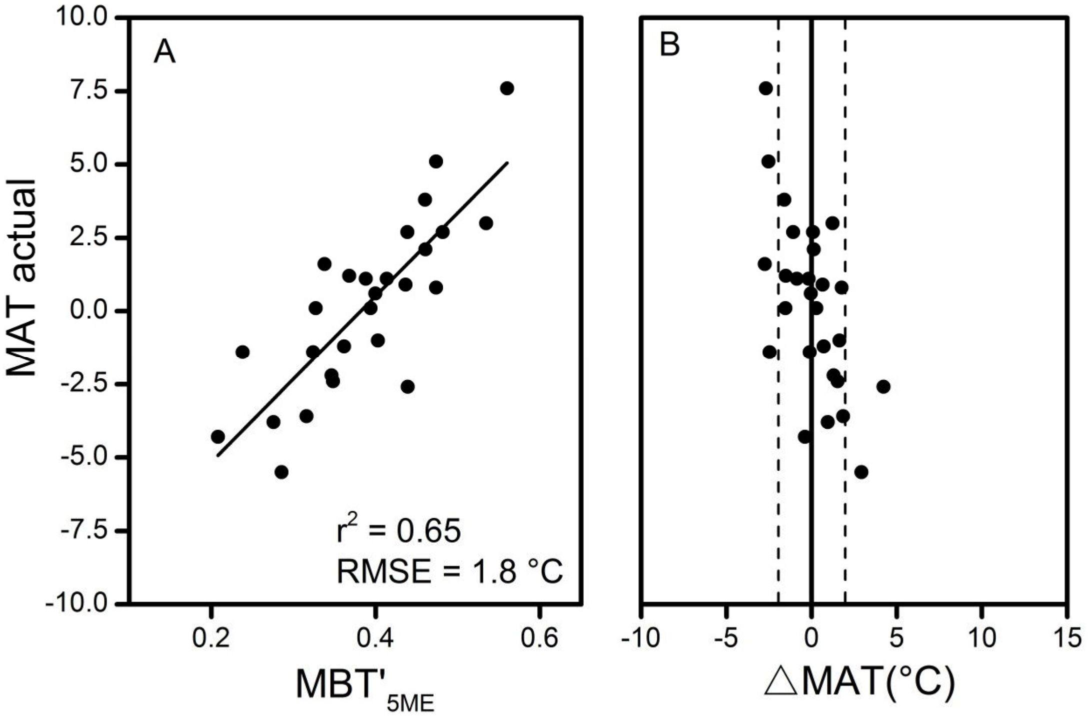

**Fig. 6.** A) Linear regression of MBT'$_{5ME}$ with actual MAT; B) difference between estimated MAT and actual MAT (ΔMAT). Data are from this study for 27 surface soils of the QTP.

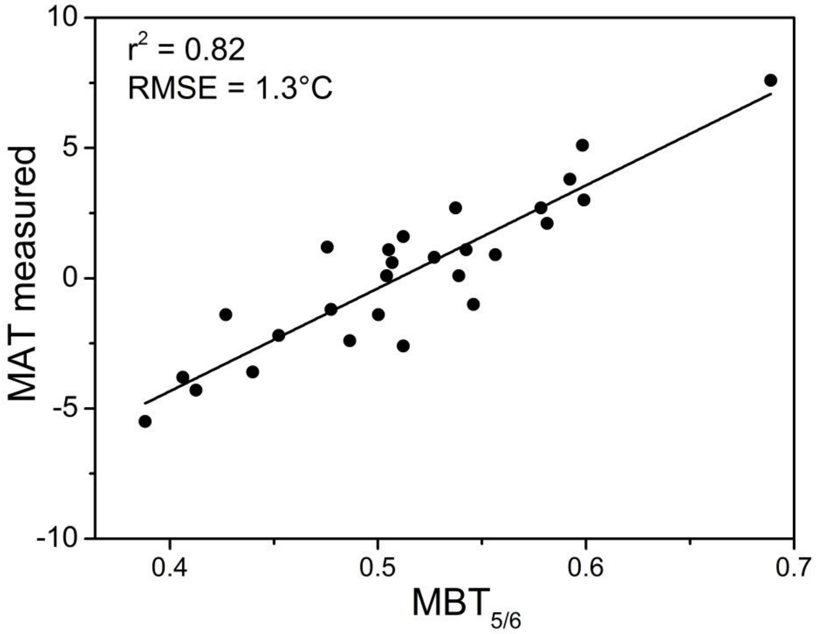

**Fig. 7**. Linear regression plot of $MBT_{5/6}$ versus MAT in the QTP.

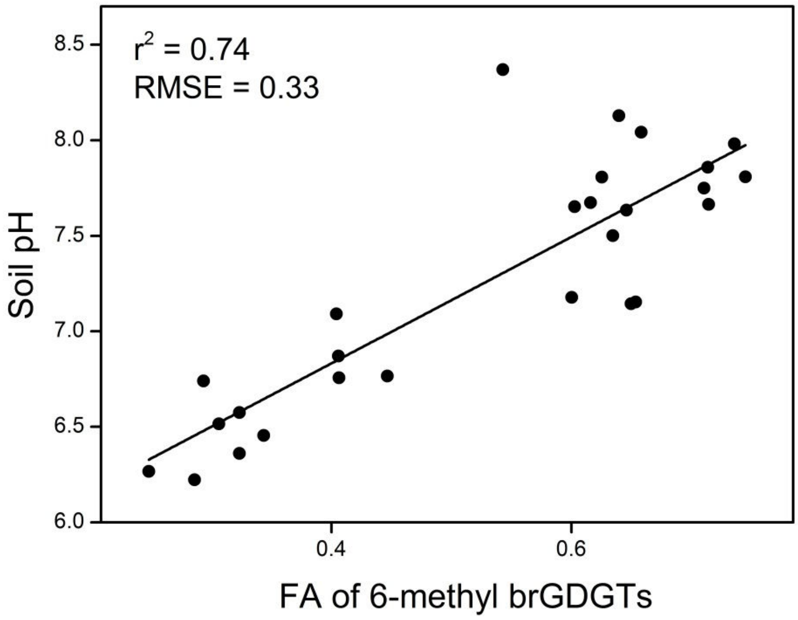

**Fig. 8**. Plots of fractional abundance of 6-methyl brGDGTs of the total amount of brGDGTs ($f_{6ME}$) versus soil pH in the QTP.

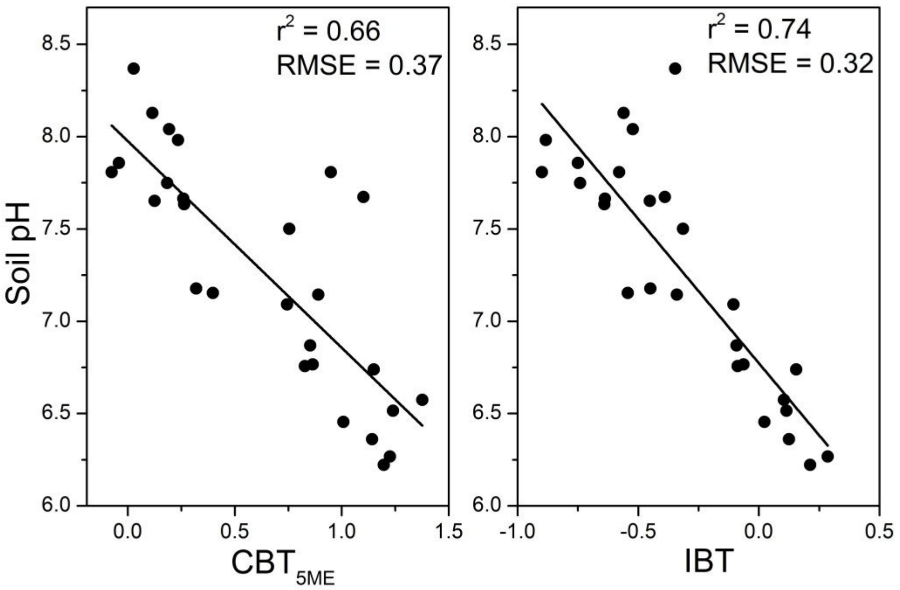

**Fig. 9**. Scatterplots of A) soil pH versus CBT$_{5ME}$ and B) soil pH versus IBT based on 27 soil samples in the QTP.

**Table 1**: Results of RDA and partial RDA (pRDA) showing the total and unique contributions of soil pH, MAT and MAP to the variance in brGDGT distributions in the QTP soils.

| Variables | Total contribution (%) | | Unique contribution (%) | |
|---|---|---|---|---|
| | *p* Value | Max eigenvalues* | *p* Value | Eigenvalues |
| pH | 0.001 | 60.1 | 0.001 | 39.9 |
| MAT | 0.001 | 16.4 | 0.001 | 10.6 |
| MAP | 0.179 | 10.8 | 0.172 | 2.0 |
| All variables | 0.001 | 72.9 | | |
| Joint effects | | | | 20.4 |
| MAT + pH | 0.001 | 56.4 | | |
| MAT + MAP | 0.001 | 12.8 | | |
| MAP + pH | 0.001 | 62.1 | | |

*The first environmental variable which has been selected into the analysis has the maximum eigenvalues (explained variances), there are 6 sequences with different arrangement of pH, MAT and MAP. However, no matter which sequence has been selected for RDA, the total variables contribution is invariant.

## Supplementary Material

**Table S1**. Summarization of the measured environmental variables and soils information in the QTP

The MAT, MWQT, MDQT, MWQT’ and MCQT are in °C, while the MAP is in mm year$^{-1}$.

Mean Temperature of Wettest Quarter (MWQT), Mean Temperature of Driest Quarter (MDQT), Mean Temperature of Warmest Quarter (MWQT’), Mean Temperature of Coldest Quarter (MCQT)

| No. | ID | LAT ( N ) | LONG (E) | ALT/m | Primary vegetation | pH | MAT | MWQT | MDQT | MWQT' | MCQT | MAP |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | P756 | 35.35 | 100.93 | 3366 | alpine and sub-alpine meadow | 7.75 | 1.1 | 10.7 | -9.9 | 10.7 | -9.9 | 449 |
| 2 | P760 | 34.54 | 100.22 | 3728 | alpine and sub-alpine meadow | 7.50 | -1 | 8.6 | -12.1 | 8.6 | -12.1 | 495 |
| 3 | P765 | 33.03 | 96.92 | 3825 | plain grassland | 7.63 | 3 | 11.6 | -5.8 | 11.6 | -6.6 | 485 |
| 4 | P773 | 33.95 | 95.70 | 4152 | alpine and sub-alpine meadow | 7.14 | -1.4 | 8.4 | -11.3 | 8.4 | -12.2 | 414 |
| 5 | P775 | 34.10 | 96.20 | 4366 | alpine and sub-alpine meadow | 7.18 | -2.6 | 7.1 | -12.4 | 7.1 | -13.2 | 410 |
| 6 | P782 | 36.86 | 101.02 | 3066 | alpine and sub-alpine meadow | 7.81 | 1.6 | 11.7 | -8.6 | 11.7 | -9.8 | 401 |
| 7 | P787 | 37.28 | 98.39 | 3425 | desert grassland | 7.98 | 0.8 | 12 | -10.3 | 12 | -11.3 | 194 |
| 8 | P790 | 37.45 | 95.50 | 3181 | Desert | 7.96 | 2 | 13.9 | -4.8 | 13.9 | -10.7 | 85 |
| 9 | P796 | 35.13 | 93.04 | 4733 | alpine and sub-alpine meadow | 8.04 | -5.5 | 4.5 | -14.6 | 4.5 | -15.7 | 273 |
| 10 | P801 | 33.73 | 92.10 | 4697 | alpine and sub-alpine plain grass | 7.66 | -3.8 | 6.4 | -13.5 | 6.4 | -14.4 | 305 |
| 11 | P802 | 33.32 | 91.88 | 4836 | alpine and sub-alpine plain grass | 7.65 | -4.3 | 5.9 | -13.8 | 5.9 | -14.8 | 320 |
| 12 | P804 | 32.19 | 91.68 | 4632 | alpine and sub-alpine meadow | 7.67 | -2.2 | 7.5 | -11.6 | 7.5 | -12.6 | 370 |
| 13 | P816 | 29.28 | 90.64 | 3630 | alpine and sub-alpine plain grass | 7.81 | 7.6 | 15.3 | -0.8 | 15.3 | -0.8 | 436 |
| 14 | P817 | 29.19 | 90.62 | 4848 | alpine and sub-alpine meadow | 6.52 | 0.9 | 9.1 | -7.7 | 9.1 | -7.7 | 248 |
| 15 | P818 | 29.20 | 90.62 | 4721 | alpine and sub-alpine meadow | 6.74 | 1.1 | 9.2 | -7.4 | 9.2 | -7.4 | 250 |
| 16 | P819 | 29.21 | 90.64 | 4544 | alpine and sub-alpine meadow | 6.46 | 2.1 | 10.2 | -6.5 | 10.2 | -6.5 | 263 |
| 17 | P820 | 29.22 | 90.63 | 4333 | alpine and sub-alpine meadow | 6.87 | 3.8 | 11.8 | -4.7 | 11.8 | -4.7 | 297 |
| 18 | P821 | 29.23 | 90.63 | 4148 | alpine and sub-alpine meadow | 7.09 | 5.1 | 12.9 | -3.4 | 12.9 | -3.4 | 332 |
| 19 | P831 | 28.24 | 88.77 | 4745 | alpine and sub-alpine plain grass | 7.86 | 0.1 | 7.5 | -7.1 | 8 | -8.2 | 294 |
| 20 | P833 | 28.26 | 87.79 | 4225 | alpine and sub-alpine meadow | 8.37 | 2.7 | 10.3 | -4.2 | 10.3 | -5.3 | 457 |
| 21 | P840 | 28.95 | 87.44 | 5310 | alpine and sub-alpine plain grass | 7.06 | -2.6 | 5.4 | -10.2 | 6.1 | -11.2 | 212 |
| 22 | P848 | 29.55 | 84.64 | 4595 | bare rocks | 8.13 | 0.6 | 6.9 | -3 | 7.5 | -6.5 | 297 |
| 23 | P851 | 29.44 | 85.67 | 4985 | alpine and sub-alpine plain grass | 7.15 | -1.4 | 5.8 | -8.3 | 6.4 | -9.2 | 236 |
| 24 | P855 | 29.21 | 88.27 | 3989 | bare rocks | 7.69 | 5 | 12.5 | -3.7 | 13.2 | -3.7 | 401 |
| 25 | P858 | 29.90 | 90.13 | 5418 | alpine and sub-alpine meadow | 6.77 | -3.6 | 4.5 | -11.3 | 5.1 | -12.5 | 221 |
| 26 | P859 | 29.89 | 90.12 | 5225 | alpine and sub-alpine plain grass | 6.36 | -2.4 | 5.7 | -10.1 | 6.3 | -11.2 | 228 |
| 27 | P860 | 29.87 | 90.12 | 5065 | alpine and sub-alpine meadow | 6.22 | -1.2 | 6.8 | -8.8 | 7.4 | -10.1 | 238 |
| 28 | P861 | 29.84 | 90.06 | 4845 | alpine and sub-alpine meadow | 6.58 | 0.1 | 7.9 | -7.5 | 8.6 | -8.9 | 250 |
| 29 | P862 | 29.80 | 90.03 | 4627 | alpine and sub-alpine meadow | 6.27 | 1.2 | 9 | -6.3 | 9.7 | -7.7 | 262 |
| 30 | P863 | 29.71 | 89.96 | 4425 | alpine and sub-alpine plain grass | 6.76 | 2.7 | 11.1 | -6.3 | 11.1 | -6.3 | 283 |

**Table S2**. Correlation coefficients between the 5- and 6-methyl brGDGTs derived proxies and temperature variables in the QTP.

| | MBT’$_{5ME}$ | MBT$_{6ME}$ | MBT$_{5/6}$ | CBT$_{5ME}$ | CBT$_{6ME}$ | CBT’ | IBT | CBT’’ |
|---|---|---|---|---|---|---|---|---|
| MAT | **0.811** | 0.309 | **0.910** | 0.134 | 0.152 | -0.142 | -0.034 | -0.135 |
| MWQT | **0.756** | 0.132 | **0.823** | 0.023 | 0.061 | 0.035 | 0.154 | 0.035 |
| MDQT | **0.713** | 0.385 | **0.799** | 0.148 | 0.182 | -0.237 | -0.157 | -0.221 |
| MWQT’ | **0.765** | 0.169 | **0.839** | 0.053 | 0.099 | 0.002 | 0.123 | 0.002 |
| MCQT | **0.763** | 0.423 | **0.871** | 0.203 | 0.215 | -0.278 | -0.187 | -0.264 |

The bold represents significant correlation as $p < 0.01$.