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Title: Multiple environmental and ecological controls on archaeal ether lipid distributions in saline ponds

Article Type: Research paper

Keywords: isoGDGTs; archaeal diethers; ACE; TEX86; mcrA gene; saline ponds

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Abstract: The distribution of archaeal ether lipids is thought to be governed by salinity in hypersaline and marine environments due to differences in their biological sources. However, little is known about their source and relationship with salinity in lacustrine environments. To explore this, we analyzed the distribution of specific archaeal lipids (isoprenoid GDGTs and diethers) of 11 ponds along a salinity gradient (0.8 ‰ to 279 ‰) in Inner Mongolia, NE China. Functional gene mcrA and compound-specific stable carbon isotope data were used to explore the biological source(s) of archaeal diether and tetraether lipids. Both lipid- and DNA-based methods showed methanogens to be abundant in ponds, which is consistent with the dominance of GDGT-0 relative to other isoprenoid GDGTs as well as the occurrence of archaeol (C20/C20 DGD), hydroxyarchaeol and the mcrA gene. However, the occurrence of the extended archaeol (C20/C25 DGD) also suggests the presence of Halobacteriales, non-halophilic methanogens and halophilic methanogens. This means that the biological sources of archaeal diethers and tetraethers are complicated. We subsequently evaluated the applicability of TEX86 temperature and ACE salinity proxies in these settings; however, due to the mixed sources of archaeal tetraethers and diethers, these proxies are not applicable in these ponds and it appears that salinity is not the dominant control on archaeal ether lipid distributions in these saline ponds.

Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:

Data will be made available on request

Reviewer #1: The manuscript by Li et al. is much improved over the previous version. Although the methodological issues were not resolved, I appreciate that the authors scaled back on their original conclusions. However, some criticisms have not been sufficiently addressed. Below I highlight the remaining issues as replies to the authors. The manuscript could benefit from careful language editing, especially in the revised sections.

RE : We really appreciate your kindness and patience for helping us to improve our manuscript and we have revised accordingly. Many thanks.

There is extensive literature (cited by the authors) on similar lake and saline pond environments that comes to similar and/or diverging conclusions. The present study is thus only confirmatory and may appeal only to a niche audience. To improve the novelty of the study, whole community sequencing or sequencing of the *mcrA* genes would have been beneficial. A major problem with using only qPCR of *mcrA* genes is the lack of a reference frame. Without similar qPCR analyses on halophiles or general archaeal sequences this information does not suffice to state that methanogens are important sources of archaeal lipids relative to an unknown number of other archaea. This caveat should be acknowledged in the manuscript and is highlighted by the fact that gene abundance is 10x higher in one lake (IM5) relative to three others (IM3, IM6, IM8). How do the authors define high methanogen abundance? Are all three lakes high in abundance or only IM5? What then, is the basis for the conclusion that methanogen abundance is high in these lakes?

Re: Yes, whole community sequencing and sequencing of the *mcrA* genes are a good way to increase the novelty of our study and we will consider this in the future study. It's a shame that we did not have the general archaeal sequence data, so it is inaccurate to evaluate that the dominance of methanogens in archaea community from these ponds; we revised the text accordingly.

Reply: The issues raised in my initial comment are still relevant for the present manuscript. I do not think one can simply correlate *mcrA* abundance and lipid abundance or isotopes without any form of normalization. Higher *mcrA* abundances could be caused by higher total biomass in the low-salinity pools, which is entirely plausible. I do consider it likely that acetogenic/hydrogenotrophic methanogens are more abundant in low-salinity ponds but in my opinion the correlation you present for methodological reasons cannot be used to support this point.

RE: sorry, the relative abundance of *mcrA* gene to the archaeal 16S rRNA gene of pond surface sediments was not shown in earlier version. In the present version, we have added the archaeal 16S rRNA gene copies number from 7 pond surface sediments in section 3.4 and Table S3. In Line 390, we added “ The archaeal 16S rRNA gene copies number from these seven pond surface sediments are listed in Table S3, the archaeal 16S rRNA gene from IM8 is higher than the other three ponds. While ponds IM3, IM6, and IM8 all contain similar abundances of the *mcrA* gene, with an average of 65 copy numbers per ng DNA, the relative abundance of *mcrA* gene to the archaeal 16S rRNA gene in IM5 is higher than the other three ponds (Fig. 4).”

However, there are other opportunities to increase the novelty of the study. The authors could analyze the carbon isotopic composition of hydroxyarchaeol and GDGT-derived biphytanes to further constrain their origin. This would nicely complement the archaeol-13C dataset. The d13C of biphytanes derived from crenarchaeol and other GDGTs would likely differ if they originate from distinct sources. The

1 authors should further use more precise taxonomy, e.g., stating the exact lineages of halophilic or
2 methanogenic archaea that are relevant. Classically, most extreme halophiles were located in the
3 Halobacteriales, but there are many halophiles outside this lineage and thus the label "halophilic
4 archaea" is too imprecise to be of any use. I also want to highlight the new study by Bale et al.
5 (Frontiers in Microbiology) on the lipids of halophiles which may help interpret the present data.
6

7
8 Re: The carbon isotopic composition analysis of GDGTs and OH-AR is a helpful way to constrain the
9 biological source. However, in our study the abundance of OH-AR is too low to be detectable, and we
10 will consider it in the future study. We have revised the halophilic archaea to be more specific in the
11 manuscript and cited Bale et al. (2019).
12

13 Reply: The remaining option that you have not addressed is ^{13}C analysis of biphytanes, which could
14 shed light on GDGT sources.
15

16 **RE:** yes, thanks for reminding. We have added "Further, the carbon specific analysis of biphytanes
17 derived from crenarchaeol and other GDGTs could shed light on the origin of GDGTs, which will be
18 consider in the future study." in Line 457.
19
20

21
22 Lines 136-139: Since the studied ponds are so shallow the authors should address how important
23 seasonal fluctuations in water level and salinity are for changes in community composition and activity
24 and thus for lipid ratios. The correlation between mcrA gene and lipid abundance would also be
25 affected by different degradation rates of DNA and lipids, especially when analyzing core lipids only.
26
27

28
29 Re: Yes, we only sampled in summer time. Microbial activity of these shallow ponds could be affected
30 by the seasonal fluctuations in water level and salinity which we did not address in this paper. We may
31 include the seasonal variation of DNA and lipids in these shallow ponds in future study to further
32 examine the community composition of microorganism.
33

34 Reply: The caveat of analyzing core lipids only is exacerbated by the seasonal fluctuations. It is unclear
35 when the lipids were produced and thus how their distribution is related to temperature or salinity. This
36 caveat needs to be mentioned specifically in the manuscript. A good place to do this would be in the
37 discussion around lines 409-412 of the original manuscript.
38
39

40 **RE:** In the present version, we have added in Line 432 "Seasonal fluctuation of these shallow ponds
41 such as the water level and salinity could affect the microbial community composition and the
42 distribution of GDGTs, however in the present study we only sampled in summer time and the core
43 lipids analysis only which is not suffice to unravel the time when these lipids were produced. So the
44 seasonal affect and intact polar lipid analysis should be considered in the future to examine the
45 relationship between the temperature and/or salinity."
46
47

48
49 Line 380-434: At some point in this section it should be discussed whether there are Thaumarchaeota in
50 hypersaline environments and whether they change their ring composition in response to salinity. To
51 answer this partially, as far as I know: The highest salinity reported for Thaumarchaeota so far was
52 from brine pools in the red sea at 18.2% (Ngugi et al., 2014, ISME J, doi:10.1038/ismej.2014.137).
53 Then there are salinity experiments by Wuchter et al. (2004, Paleoceanography, doi:10.1029/2004PA001041) and Elling et al. (2015, GCA, doi:10.1016/j.gca.2015.09.004) to be
54 considered.
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1 Re: Thank you for your suggestion; it could be a good way to examine the relationship between the
2 Thaumarchaeota in saline ponds and the salinity. We did calculate the ring index of isoGDGTs
3 proposed by Pearson et al. (2004 AEM), but there is no correlation between salinity and the ring indices
4 of isoGDGTs from these ponds.

5 Reply: I think this finding should be mentioned in the manuscript since part of your discussion revolves
6 around TEX86.
7

8
9 **RE:** We have added in Line 609 "However, in these ponds, there is no correlation between the ring
10 index of isoGDGTs and salinity indicating that the Thaumarchaeota in these saline ponds do not change
11 their ring composition in response to salinity."
12
13

14
15 Line 409-412: It would be interesting to know if the salinities were constant throughout the year or
16 throughout the recent years to evaluate whether GDGTs could be produced during a low salinity season.
17
18

19 Re: It's a shame that we only sampled in the summer time, and we did not conduct the seasonal
20 comparison of pond salinity. According to Zheng et al. (1992, Salt lakes in Inner Mongolia), the
21 salinity of these ponds increases in dry season due to the increased evaporation and decreases in rainy
22 season. The effect of seasonal fluctuation on GDGTs could be performed in the future study.
23
24

25 Reply: See reply to lines 136-139

26 **RE:** yes, we have added in Line 432 in the present version as you suggest .
27
28

29 Line 505-508: Is this correlation shown in a figure?
30
31

32 Re: No, this correlation is not shown in the figures. Only four samples have the mcrA gene abundance
33 data, so significant correlation is not convincing.

34 Reply: If you do not trust the correlation you should not include it in the discussion.

35 **RE:** done, we have deleted this part, and it seems not helpful.
36
37

38 Comment on Table S2: Please note in caption what "Ri/b" and "total Con." refer to.
39
40

41 **RE:** we have added in the caption.
42
43

44 Reviewer #2: The authors have addressed most of my comments, but not all of them have been
45 incorporated into the discussion of the manuscript. As I mentioned previously, I find it very important
46 to raise awareness to the potential problems that may arise when using the ACE index if proper
47 response factors are not applied. Here are my suggested edits for a revised version.
48
49

50 **RE:** We do appreciate your constructive comments that helping us to improve our manuscript. Many
51 thanks.
52
53

54 Line 35 and 640: Change to plural: "non-halophilic methanogens and halophilic methanogens"
55
56

57 **RE:** we have done accordingly in the whole manuscript.
58
59

60 Line 206: Add after the revised sentence: "...and could thus affect the reported ACE values."
61
62

63 **RE:** done.
64
65

Line 543: Change to "archaeal"

RE: done

Line 584: Mention at the end of this paragraph the potential problem of comparing ACE indices from different studies that arises if no standards are used and archaeol and GDGT-0 are not response factor corrected.

RE: we have added “However, for comparing ACE indices from different studies the standard is necessary to correct the response factor between archaeol and GDGT-0.”.

1 Multiple environmental and ecological controls on archaeal ether lipid
2 distributions in saline ponds
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Abstract

The distribution of archaeal ether lipids is thought to be governed by salinity in hypersaline and marine environments due to differences in their biological sources. However, little is known about their source and relationship with salinity in lacustrine environments. To explore this, we analyzed the distribution of specific archaeal lipids (isoprenoid GDGTs and diethers) of 11 ponds along a salinity gradient (0.8 ‰ to 279 ‰) in Inner Mongolia, NE China. Functional gene *mcrA* and compound-specific stable carbon isotope data were used to explore the biological source(s) of archaeal diether and tetraether lipids. Both lipid- and DNA-based methods showed methanogens to be abundant in ponds, which is consistent with the dominance of GDGT-0 relative to other isoprenoid GDGTs as well as the occurrence of archaeol (C_{20}/C_{20} DGD), hydroxyarchaeol and the *mcrA* gene. However, the occurrence of the extended archaeol (C_{20}/C_{25} DGD) also suggests the presence of Halobacteriales, non-halophilic methanogens and halophilic methanogens. This means that the biological sources of archaeal diethers and tetraethers are complicated. We subsequently evaluated the applicability of TEX_{86} temperature and ACE salinity proxies in these settings; however, due to the mixed sources of archaeal tetraethers and diethers, these proxies are not applicable in these ponds and it appears that salinity is not the dominant control on archaeal ether lipid distributions in these saline ponds.

Keywords: isoGDGTs; archaeal diethers; ACE; TEX_{86} ; *mcrA* gene; saline ponds

1. Introduction

Dialkyl glycerol diether (DGD) and glycerol dialkyl glycerol tetraether lipids (GDGTs), membrane lipid constituents of many archaea and certain bacteria, are increasingly used to explore biogeochemistry and paleoclimate in aquatic and terrestrial environments (Schouten et al., 2013). Isoprenoid GDGTs (isoGDGTs), comprising acyclic or ring-containing isoprenoidal biphytanyl carbon chains (see Fig.1), are synthesized by a wide range of Archaea, including Euryarchaeota, Crenarchaeota and Thaumarchaeota (Schouten et al., 2007; Pearson and Ingalls, 2013; Schouten et al., 2013).

GDGT-0, comprising two acyclic biphytanes, is a common isoGDGT with a large variety of biological sources. It occurs in all major groups of cultivated Archaea and likely is also produced by many of the uncultivated Archaea [see Schouten et al. (2013) for a review]. In lacustrine settings, GDGT-0 is often predominantly derived from methanogens and ammonia oxidizing Thaumarchaeota (Blaga et al., 2009; Powers et al., 2010; Sinninghe Damsté et al., 2012a). In addition, uncultured crenarchaeotal groups (group 1.2 or Bathyarchaeota (group 1.3)) and marine benthic group C (MBG-C) might also be a dominant source of GDGT-0 in the lake water column and sediments (Sinninghe Damsté et al., 2009; Buckles et al., 2013). Crenarchaeol, containing four cyclopentane rings in addition to one cyclohexane ring, is believed to be unique to Thaumarchaeota (Sinninghe Damsté et al., 2002; Schouten et al., 2013), although this has been debated for marine environments (Lincoln et al., 2014; Schouten et al., 2014). IsoGDGTs containing 1 to 3 cyclopentane moieties (isoGDGTs 1-3) are non-specific and are commonly found in a wide range of Archaea, including Thaumarchaeota (Wuchter et al., 2006), methanogenic Euryarchaeota (Gattinger et al., 2002; Zhang et al., 2011), anaerobic methane-oxidizing archaea (Pancost et al., 2001) and extremophiles

74 (Lattuati et al., 1998; Ellen et al., 2008).

75 Among the isoGDGT-based proxies used in lakes are the TEX₈₆ (TetraEther
76 indeX of 86 carbons) palaeothermometer and the Archaeol to Caldarchaeol
77 Ecometric (ACE) salinity index. TEX₈₆ was originally developed in marine
78 environments (Schouten et al., 2002); it is based upon the relative distribution
79 of isoGDGTs containing cyclopentane moieties, synthesized by aquatic
80 Thaumarchaeota, and is significantly correlated with sea surface temperature
81 (Schouten et al., 2002; Kim et al., 2008; 2010). Subsequent studies
82 demonstrated that TEX₈₆ in lacustrine sediments is also correlated with lake
83 surface water temperatures (Powers et al., 2004; Blaga et al., 2009; Powers et
84 al., 2010). However, in lakes, the contribution of isoGDGTs derived from soils
85 in the surrounding catchment and/or production by methanogenic archaea
86 within the lake can influence the distribution of sedimentary GDGTs, yielding
87 unreliable TEX₈₆-inferred temperatures (Sinninghe Damsté et al., 2009; Bechtel
88 et al., 2010; Sinninghe Damsté et al., 2012a). Before the TEX₈₆
89 paleothermometer can be applied confidently in certain lacustrine systems,
90 therefore, it is important to constrain the origins of the associated GDGTs. It is
91 likely that application of TEX₈₆ is inappropriate in some saline lakes, but this
92 has not been extensively explored.

93 The ACE salinity index was originally developed for and applied to marine
94 environments and is based upon the relative abundance of archaeol relative to
95 GDGT-0 (Turich and Freeman, 2011). The index is based on the hypothesis that
96 Halobacteriales appear to exclusively/predominantly produce DGDs as core
97 lipids (Teixidor et al., 1993; Kates, 1996; Kamekura and Kates, 1999; Dawson
98 et al., 2012), predominantly C₂₀/C₂₀ DGDs (archaeol) and C₂₀/C₂₅ DGDs
99 (extended archaeol) (Xu et al., 2001; Hezayen et al., 2002; Dawson et al., 2012).
100 In hypersaline environments the shift in archaeal community from marine
101 Euryarchaeota and Thaumarchaeota to halophilic Euryarchaeota (Jiang et al.,

2009), causes isoGDGTs to disappear, while archaeol becomes abundant (Turich and Freeman, 2011). The ACE index has been applied to some lakes (Wang et al., 2013). However, a prerequisite for the application of the ACE index is to constrain the source of archaeol and GDGT-0. For example, GDGT-0 can be synthesized by Thermoplasmatales (Turich and Freeman, 2011), ANME Archaea and methanogens (Koga et al., 1998; Pancost et al., 2000a; 2005; Naeher et al., 2014a). In addition, archaeol could be produced by Methanosarcinales, Methanopyrales, Methanococcales and Thermococcales other than Halobacteriales (Villanueva et al., 2014), which may bias the ACE proxy. Due to the complexity of the archaeal community in lake systems, the ACE index was suggested not to be applicable to tropical ponds (Huguet et al., 2015), Tibetan saline lakes (Günther et al., 2014) and a Chinese hypersaline lake (Li et al., 2016).

For these reasons, it is useful to distinguish the biological sources of archaeal diethers and tetraethers in any given setting. Methanogens are slow growers and sensitive to oxygen, difficult to isolate or culture in laboratory conditions, but the methyl coenzyme M reductase (*mcrA*) gene sequences can be used for the identification of methanogens in a wide range of environments (Luton et al., 2002; Steinberg and Regan, 2008; Ma et al., 2012). Another promising way to examine archaeal ecology is via compound-specific stable carbon isotope analysis ($\delta^{13}\text{C}$) of their lipid biomarkers (Summons et al., 1998; Blumenberg et al., 2004; Naeher et al., 2014a). $\delta^{13}\text{C}$ values of microbial lipids produced by methanogens can be strongly depleted if lipid biosynthesis is linked to the production of isotopically light methane; however, ^{13}C -depletion does not occur in many cases (Pancost and Sinninghe Damsté, 2003).

Previously, we investigated the distribution of branched GDGTs that are derived from bacteria in 11 ponds from Inner Mongolia, NE China, representing a salinity gradient from 0.8 ‰ to 279 ‰, and the surrounding soils

(Li et al., 2017). In this study, we combine biomarker distributions, functional gene abundance and compound-specific stable carbon isotopic compositions to explore the biological source(s) of archaeal diethers and tetraethers lipids, as well as to evaluate the applicability of archaeal ether lipid-based proxies in these saline aquatic settings.

135

136 2. Materials and methods

137

138 2.1. Sampling

139

140 Eleven ponds with a broad salinity range (from freshwater ponds with a
141 salinity of 0.8 ‰ to hypersaline ponds with a salinity of 279 ‰) have been
142 investigated previously, and the sampling information is provided in Li et al.
143 (2017) (Fig. 2 and Table S1). The pH of pond water was measured with a
144 portable pH meter (Table S1), and the salinity is calculated as the sum of the
145 measured ions in the unit of g L⁻¹ (Table S1), as reported in Li et al. (2017). In
146 general, these small (1.5–7.0 km² surface area) and shallow (~0.5 m water
147 depth) ponds are located in New Barag Left Banner of Inner Mongolia in the
148 Northeast China, and all developed in the mid to late Holocene (Zheng et al.,
149 1992; 2002). Local Meteorological Station records indicate that the mean
150 annual air temperature (MAAT) is 0.4 °C, while the mean annual minimum and
151 maximum temperatures are –5.1 and 6.7 °C, respectively; the mean annual
152 precipitation was about 284 mm during the period from 1971 to 2013
153 (<http://cdc.nmic.cn/home.do>). This area is characterized by a semi-arid desert
154 steppe climate, with grassland land use and the vegetation dominated by C3
155 plants, mainly *Leymus chinensis*, *Bromus inermis*, *Elytrigia repens*. The typical
156 soils of the study site are chernozem, castanozem and meadow soil.

157 Surface sediments (n=11; 0–2 cm) from the deepest point in each pond and

1
2 158 soil samples (n=11; 0–2 cm) were collected during a field campaign in August
3
4 159 2013. Water samples were collected above the surface sediments and sealed in
5
6 160 50 mL sterile centrifuge tubes. All samples for lipid analysis were rapidly
7
8 161 frozen and immediately transported to the laboratory and stored at –20 °C until
9
10 162 further analysis. DNA isolation was performed for only seven out of eleven
11
12 163 ponds, due to failure to store the other four samples at low temperature. Surface
13
14 164 sediment samples for DNA analysis were collected in 50 mL sterile centrifuge
15
16 165 tubes and immediately kept on dry-ice, then transported to the lab and stored at
17
18 166 –80 °C for future use.

19 167

20 168 *2.2. Total organic carbon analysis, lipid extraction and isoGDGT analysis*

21 169

22
23
24 170 The method for TOC content determination was reported in Li et al. (2016).
25
26 171 In general, it was measured by subtracting the inorganic carbon content from
27
28 172 the total elemental carbon content, determined by using a Carlo Erba EA1108
29
30 173 Elemental Analyzer and modified Coulomat 702 analyser at the University of
31
32 174 Bristol. All TOC measurements were performed in duplicate.

33
34 175 Lipid extraction was determined according to the procedures in Li et al.
35
36 176 (2017). Briefly, surface sediment and soil samples were freeze dried and ground
37
38 177 to powder by using a mortar and pestle, and ca. 10 g of grounded sediment and
39
40 178 soil samples were extracted in pre-cleaned cellulose extraction thimbles via
41
42 179 Soxhlet apparatus for 24 h with dichloromethane/methanol (2:1, v/v) (Naafs
43
44 180 and Pancost, 2014). An aliquot (50 %) of the total lipid extract (TLE) was
45
46 181 separated over activated alumina column into an apolar and a polar fraction
47
48 182 using Hexane/DCM (9:1, v/v) and DCM/MeOH (1:2, v/v), respectively.

49 183 The polar fraction containing GDGTs was dissolved in hexane/isopropanol
50
51 184 (99:1, v/v) and filtered through 0.45 µm PTFE filters and dried under N₂ prior
52
53 185 to analysis by high performance liquid chromatography with triple quadruple

1
2 186 mass spectrometry (HPLC-MS²) using an Agilent 1200 series, with
3
4 187 auto-injection and ChemStation manager software. Samples were re-dissolved
5
6 188 in hexane/isopropanol (99:1,v/v) and an internal synthetic C₄₆ standard (Huguet
7
8 189 et al., 2006) added for quantification. The separation of isoGDGTs was
9
10 190 achieved with a Thermo Finnigan silica column (150 mm × 2.1 mm, 1.9 µm).
11
12 191 The elution gradient follows the procedure of Yang et al. (2014) with some
13
14 192 modifications. It was first eluted isocratically (5 min) with 80% A and 20% B,
15
16 193 where A = hexane and B = hexane/isopropanol (9:1, v/v), followed by changing
17
18 194 the ratio to 72% A and 28% B from 5 to 45 min and then to 70% A and 30% B
19
20 195 for 1 min, followed by column washing with 100% B for 5 min and then 80% A
21
22 196 and 20% B to equilibrate the column. The flow rate was 0.2 ml/min. The
23
24 197 detection of GDGTs was achieved by using single ion monitoring (SIM) mode
25
26 198 of [M+ H]⁺, targeting *m/z* 744 for the C₄₆ internal standard, *m/z* 653 and 1302,
27
28 199 1300, 1298, 1296, 1292, 1050, 1048, 1046, 1036, 1034, 1032, 1022, 1020 and
29
30 200 1018 for archaeol, isoGDGTs and brGDGTs. The final quantification was
31
32 201 semi-quantitative as we assume the ionization efficiency and the relative
33
34 202 response factor between GDGTs and the C₄₆ internal standard is 1:1. However,
35
36 203 due to the lack of an archaeol standard, the difference in response factor
37
38 204 between archaeol and GDGT-0 is not available. Hence the ratio of archaeol/
39
40 205 GDGT-0 here represents a of ratio of instrumental response rather than of the
41
42 206 absolute abundance and could thus affect the reported ACE values. The
43
44 207 concentration of GDGTs was then normalized to TOC as determined in Li et al.
45
46 208 (2017).
47
48 209

210 2.3. Dialkyl glycerol diether (DGD) and compound-specific stable carbon 211 isotope analysis

212
213 The other aliquot (50 %) of the TLE was separated over an aminopropyl NH₂

1
2 214 bond elute column (Biotage) into neutral, free fatty acid and phospholipid
3
4 215 fractions by elution of DCM/isopropanol (2:1, v/v), 2% acetic acid in ethyl
5
6 216 ether and MeOH, respectively (Zheng et al., 2014). The neutral fraction was
7
8 217 further separated using activated alumina column by elution of hexane/DCM
9
10 218 (9:1, v/v) and DCM/MeOH (1:1, v/v) into the apolar and polar fraction,
11
12 219 respectively. The polar fraction was silylated with N, *O*-Bis(trimethylsilyl)
13
14 220 trifluoroacetamide (BSTFA) and Pyridine (Sigma-Aldrich) at 70 °C for 1 h and
15
16 221 dissolved in hexane prior to analysis by gas chromatography-mass spectrometry
17
18 222 (GC-MS) and high temperature gas chromatography-mass spectrometry
19
20 223 (HTGC-MS) (Kaur et al., 2015).

21 224 The polar fraction containing DGDs was analyzed by GC-MS using a
22
23 225 ThermoQuest Finnigan Trace GC and MS instrument equipped with a
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25 226 non-polar silica CP Sil5-CB column (50 m × 0.32 mm × 0.12 µm) using the
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27 227 following temperature program, 70 °C to 130 °C at 20 °C min⁻¹, ramp to 300 °C
28
29 228 at 4 °C min⁻¹, and held at 300 °C for 25 min. The ionization potential was 70 eV,
30
31 229 and full scan spectra were obtained with the scanning range *m/z* 50-650. The
32
33 230 DGDs were reanalyzed by HTGC-MS using a PerkinElmer Turbomass Gold
34
35 231 MS and fitted with DB1-HT column (15 m × 0.32 mm × 0.12 µm) using the
36
37 232 following temperature program, 50 °C isotherm for 2 min, ramp to 350 °C at
38
39 233 10 °C min⁻¹ and isotherm for 10 min. Semi-quantification was achieved by
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41 234 adding 5α-androstane and 2-hexadecanol as internal standards.

42 235 Compound specific δ¹³C values of archaeol were determined using gas
43
44 236 chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS)
45
46 237 with an IsoPrime 100 IRMS coupled with an Agilent 7693 GC. The fused silica
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48 238 capillary CP-Sil-5 column (60 m × 0.32 mm × 0.10 µm) was used with the
49
50 239 same GC temperature program as the GC-MS. The δ¹³C values are reported
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52 240 relative to the Vienna Pee Dee Belemnite (VPDB) standard, and the analytical
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54 241 error was determined using co-injected standards and was ± 0.5 ‰. The
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instrument stability was monitored by regular analysis of a fatty acid methyl ester standard mixture with a long-term precision of ± 0.3 %.

2.4. DNA extraction, PCR and *mcrA* gene analysis

Genomic DNA was extracted from 0.5 g dry samples with FastDNA[®] Spin Kit for soil (MP, USA). Primer sets *mls* and *mcrA*-rev (Steinberg and Regan, 2009) were used for *mcrA* gene quantitative analysis. Quantification of *mcrA* gene was performed using the Bio-Rad real-time system CFX96 with TAKARA SYBR Premix Ex Taq (Tli RNaseH Plus, DRR420A). The primer set was tested both with positive controls (plasmid with *mcrA* insert) and negative controls (plasmid without *mcrA* insert) to avoid false positive detection and increase positive detection. The standard copy number was achieved from the plasmid containing *mcrA* gene through the clone library construction method according to pMD18-T simple clone system (TAKARA, Dalian, China). A series of ten-fold dilution was used to obtain a standard curve with efficiency close to 100% (95-105%). Real time PCR was conducted in a 20 μ L mixture containing 10 μ L of 2 $\times$ SYBR Green I premixture (DRR420A, TAKARA), 0.5 μ L of 10 μ M each primer, [about](#) 2 μ L of temple DNA (100 ng) and 7 μ L PCR-grade distilled water. The real time PCR was run for 40 cycles with the following conditions: initial denaturing at 95 $^{\circ}$ C for 1 s, annealing at 55 $^{\circ}$ C for 30 s and extension at 72 $^{\circ}$ C for 30 s, and fluorescent capture at 80 $^{\circ}$ C for 20 s with the initial denaturation at 95 $^{\circ}$ C for 30 s.

2.5. GDGT-based proxy calculations

Roman and Arabic numerals refer to the isoGDGT structures indicated in Fig. 1: 0, 1, 2, 3 indicate isoGDGTs containing zero, one, two or three cyclopentane

moieties, respectively; Cren and Cren' refer to crenarchaeol and its regioisomer. TEX₈₆ is calculated following Schouten et al. (2002) and reflects the degree of cyclization of particular isoGDGTs:

$$\text{TEX}_{86} = \frac{(\text{GDGT} - 2 + \text{GDGT} - 3 + \text{Cren}')}{(\text{GDGT} - 1 + \text{GDGT} - 2 + \text{GDGT} - 3 + \text{Cren}')} \quad (1)$$

The ACE index is based upon the relative abundance of archaeol relative to GDGT-0 (Turich and Freeman, 2011):

$$\text{ACE} = \frac{\text{archaeol}}{(\text{archaeol} + \text{GDGT} - 0)} \quad (2)$$

The R_{ib} (Xie et al., 2012) represents the relative abundance of total isoGDGTs to total brGDGTs:

$$\text{R}_{ib} = \sum \text{isoGDGTs} / \sum \text{brGDGTs} \quad (3)$$

The branched versus isoprenoid tetraether (BIT) index is calculated on the basis of Hopmans et al. (2004), reflecting the relative abundance of brGDGTs vs. crenarchaeol.

$$\text{BIT} = \frac{(\text{Ia} + \text{IIa} + \text{IIIa} + \text{IIa}' + \text{IIIa}')}{(\text{Ia} + \text{IIa} + \text{IIIa} + \text{IIa}' + \text{IIIa}' + \text{Cren})} \quad (4)$$

2.6. Statistical analysis

Canonical correspondence analysis (CCA) was performed using the software Canoco for Windows version 4.5. The fractional abundance of isoGDGTs, DGDs and the GDGT-based proxies as response variables and water chemistry parameters as explanatory variables were transferred into the Canoco software. Detrended correspondence analysis (DCA) was first used to measure the lengths of variances; however, all of these lengths were < 3, instead the CCA analysis based on a linear ordination method was selected to process the dataset. The variable significance was assessed using a Monte-Carlo significance permutation test. The significant difference between datasets was determined by a Pearson's coefficient (p -value) < 0.05 in the independent sample t -test by

297 using the SPSS software.

298

299 **3. Results**

300

301 *3.1. Measured pond water parameters and TOC contents*

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303 The pH of the eleven ponds varied from 7.7 to 10, whereas salinity varied
304 from 0.8 ‰ to 279 ‰ (Table S1). The lowest pH value and highest salinity was
305 observed in IM7, whereas other ponds had a similar pH values but lower
306 salinity. The pond water pH and salinity are linearly correlated ($r^2 = 0.5$, $p =$
307 0.02) and samples with high pH tend to have lower salinity. The TOC content
308 of surface sediments varies from 0.2 to 2.7 wt.% and that of soils ranges from
309 0.1 to 5.3 wt.%; the TOC contents of surface sediments increase with the
310 salinity ($r^2 = 0.66$, $p = 0.005$).

311

312 *3.2. Distribution of isoGDGTs from pond surface sediments and soils*

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314 As illustrated in Table S2 and Fig. 3, the isoGDGT distribution of both
315 surface sediments and soils is dominated by GDGT-0. The average fractional
316 abundance of GDGT-0 is 80% in surface sediments and 60% in soils (Fig. 3).
317 The second most abundant isoGDGT is crenarchaeol, with an average
318 proportion of 10 and 30% for surface sediments and soils, respectively. The
319 relative abundance of isoGDGTs-1 to -3 as well as the crenarchaeol
320 regioisomer is generally higher in soils than in surface sediments. However,
321 there is almost no relationship between isoGDGT abundances and pond water
322 salinity; only the fractional abundance of GDGT-1 is slightly correlated with
323 salinity ($r^2 = 0.32$, $p = 0.09$).

324 The isoGDGT-based proxies for both surface sediments and soils are

presented in Table S2. TEX₈₆ values for soils are 0.64 ± 0.05 , and the values of surface sediments are slightly lower with 0.57 ± 0.15 (Table S2). The relative abundance of isoGDGTs to brGDGTs ($R_{i/b}$) in surface sediments ranges from 0.6 to 9.1, with an average of 2.7, and it ranges from 0.2 to 1.9 with an average of 0.8 in soils (Table S2). The ratio of GDGT-0 to crenarchaeol (GDGT-0/Cren) ranges from 3 to 101 in surface sediments and from 0.5 to 40 in soils, and the ratio of crenarchaeol to crenarchaeol regioisomer (Cren/Cren') ranges from 8 to 17 in surface sediments and 11 to 22 in soils. The ACE is higher ($t = 4.188$; $p < 0.001$, shown by the paired sample t -test) in surface sediments than in soils, ranging from 0.14 to 0.97 in surface sediments and from 0.04 to 0.93 in soils (Table S2).

The TOC-normalized concentrations of isoGDGTs are highly variable, ranging from 0.1 to 8 $\mu\text{g/g}$ TOC for surface sediments and 0.05 to 44 $\mu\text{g/g}$ TOC for soils (Table S2). The concentration of isoGDGTs is generally higher in soils compared to the corresponding surface sediments, but with some exceptions (IM1, IM8 and IM10).

3.3. Distribution of DGDs and compound specific $\delta^{13}\text{C}$ values of archaeol

The concentration of DGDs is shown in Fig. 4 and Table S3. The most abundant DGD in surface sediments and soils is archaeol ($\text{C}_{20}/\text{C}_{20}$ DGD, bio-*O*-phytanylglyceroldiether, see Fig. 1 for structure). However, a variety of other isoprenoidal DGDs, including hydroxyarchaeol and extended archaeol ($\text{C}_{20}/\text{C}_{25}$ DGD) are present in many surface sediment samples (Fig. 4 and Table S3). Archaeol is present in all surface sediments but not detected in four soil samples. Its concentrations are variable in both sediments (9–5800 $\mu\text{g/g}$ TOC), and soils (1–430 $\mu\text{g/g}$ TOC, Table S3). Due to the lower abundance of archaeol in soils, we only measured its stable carbon isotopic composition ($\delta^{13}\text{C}$) in pond

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2 353 surface sediments. It ranges from -33 ‰ to -19 ‰, with the lowest value in IM2
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4 354 and the highest value in IM6. In pond samples IM1, IM9 and IM10 the
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6 355 abundance of archaeol was too low to determine its $\delta^{13}\text{C}$ value.

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8 356 Extended archaeol, $\text{C}_{20}/\text{C}_{25}$ dialkyl glycerol diether (DGD) (Fig.1), has a
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10 357 similar structure as archaeol ($\text{C}_{20}/\text{C}_{20}$ DGD) but possesses phytanyl (C_{20}) and
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12 358 sesterpanyl (C_{25}) isoprenoid chains instead of double phytanyl chains (Tindall
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14 359 et al., 1984; Romano et al., 2007). This compound was found in Halobacteriales,
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16 360 as well as *Methanomassiliicoccus luminyensis* (the solo cultured representative
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18 361 of the seventh methanogenic order) and the halophilic methanogen
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20 362 *Methanonatronarchaeia* (Becker et al., 2016; Bale et al., 2019). In these ponds,
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22 363 the $\text{C}_{20}/\text{C}_{25}$ DGD is less abundant in surface sediments compared to archaeol
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24 364 (Fig. 4 and Table S3). It occurs in seven out of eleven surface sediment samples
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26 365 and its concentration ranges from 20–550 $\mu\text{g/g}$ TOC. However, it is present in
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28 366 only one soil sample (IMS6), with a concentration of 65 $\mu\text{g/g}$ TOC (Table S3).

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30 367 Hydroxyarchaeol has a structure similar to that of archaeol but with an
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32 368 additional hydroxyl group on the third carbon of the phytanyl carbon skeleton
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34 369 linked to either the second (*sn*-2-hydroxyarchaeol, Fig.1) or the third
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36 370 (*sn*-3-hydroxyarchaeol, Fig.1) glycerol carbon (Hinrichs et al., 2000a; Pancost
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38 371 et al., 2001). Hydroxyarchaeol is present in seven out of eleven surface
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40 372 sediment samples, but absent in all soil samples (Table S3). The concentration
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42 373 of *sn*-2-hydroxyarchaeol varies from 0.3 to 13 $\mu\text{g/g}$ TOC, and the concentration
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44 374 of *sn*-3-hydroxyarchaeol ranges from 0.6 to 25 $\mu\text{g/g}$ TOC in surface sediments
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46 375 (Table S3), but neither shows a correlation with salinity. As with extended
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48 376 archaeol and hydroxyarchaeol, the carbon isotopic composition could not be
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50 377 determined due to low concentrations.

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52 378 The statistical analysis indicates that none of the fractional abundances of
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54 379 isoGDGTs, isoGDGTs-based proxies or the distribution of DGDs has a
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56 380 significant correlation with pH. However, TEX_{86} and the ratio of
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GDGT-2/GDGT-3 are correlated with pond salinity (correlated with $r^2 = 0.53$, $p = 0.009$ and inversely correlated with $r^2 = 0.5$, $p = 0.014$, respectively). To test this further, we performed RDA to measure the impact of solute species, including Na^+ , K^+ , Mg^{2+} , Ca^{2+} , Cl^- , SO_4^{2-} , HCO_3^- and CO_3^{2-} concentrations, on the distribution of isoGDGTs and DGDs in pond surface sediments (Fig. 5). In the first RDA dataset, the analysis confirms that there is no significant correlation between water chemistry and the distribution of isoGDGTs and DGDs, and only HCO_3^- seems to affect the distribution of these archaeal lipids with the explanation of 45.2% ($p = 0.046$).

3.4. Distribution of *mcrA* gene in pond surface sediments

The *mcrA* was developed to target the gene sequence by determining methanogen gene copy numbers through the use of qPCR. Seven pond surface sediments were analyzed for the methanogen-specific marker *mcrA* gene. The functional molecular marker of methanogenic archaea could only be detected in four samples (IM3, IM5, IM6, and IM 8) (Fig. 4). The archaeal 16S rRNA gene copies number from these seven pond surface sediments are listed in Table S3, the archaeal 16S rRNA gene from IM8 is higher than the other three ponds. Samples-While ponds IM3, IM6, and IM8 all contained similar abundances of the *mcrA* gene, with an average of 65 copy numbers per ng DNA, but the relative abundance of *mcrA* gene to the archaeal 16S rRNA gene in IM5 is higher than the other three ponds (Fig. 4). ~~the concentration in the low salinity sample IM5 was much higher with 1400 copy numbers per ng DNA.~~

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4. Discussion

4.1. Provenance of archaeal lipids

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410 4.1.1. *isoGDGTs*

411 In pond surface sediments and surrounding soils, GDGT-0 is the most
412 abundant component among *isoGDGTs*, consistent with previous studies of
413 lacustrine sediments (Blaga et al., 2009; Tierney et al., 2012; Wang et al., 2012;
414 Naeher et al., 2014b; Li et al., 2016). However, in some soil samples (e.g., IMS
415 7) crenarchaeol was more abundant, as previously observed in other soils
416 (Sinninghe Damsté et al., 2009; Wang et al., 2012; Naeher et al., 2014b; Hu et
417 al., 2016). Similar distribution of *isoGDGTs* between surface sediments and
418 surrounding soils indicating that the main source of organic matter is soil input
419 as these are shallow ponds.

420 GDGT-0 is fairly non-specific and a commonly found lipid in a wide range
421 of archaea (Schouten et al., 2013); in lacustrine environments, the source of
422 GDGT-0 can be highly variable and it can be produced in different layers of the
423 water column and sediment. For example, GDGT-0 can originate from
424 Thaumarchaeota living in the surface water or from Crenarchaeota Group 1.2
425 and MBG-C that are resident in the anoxic lower water column as seen in Lake
426 Challa of equatorial East Africa (Sinninghe Damsté et al., 2009). Furthermore,
427 new evidence has indicated that the uncultured crenarchaeotal groups 1.2 and
428 Bathyarchaeota could also produce GDGT-0 in the anoxic lake settings
429 (Buckles et al., 2013). However, more recently, the *mcrA* gene that related to
430 methane metabolism has been detected in Bathyarchaeota based on the genomic
431 evidence (Evans et al., 2015; Zhou et al., 2018). In Lake Lucerne,
432 Thaumarchaeota are the dominant source of GDGT-0 (Blaga et al., 2011).
433 Methanogenic archaea can also be an important source of GDGT-0 within the
434 water column and sediment (Blaga et al., 2009; Powers et al., 2010; Naeher et
435 al., 2014b). The influence of methanogenic archaea can be explored by the ratio
436 of GDGT-0/crenarchaeol proposed by Blaga et al. (2009) as the methanogens

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2 437 produce GDGT-0 but no crenarchaeol. Consequently, high ratios in these
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4 438 surface sediments (3~102), especially high ratio occurred in IM10, IM3 and
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6 439 IM5 (Table S2), could indicate a strong methanogenic contribution.

7 440 Seasonal fluctuation of these shallow ponds such as the water level and
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9 441 salinity could affect the microbial community composition and the distribution
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11 442 of GDGTs, however in the present study we only sampled in summer time and
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13 443 the core lipids analysis only which is not suffice to unravel the time when these
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15 444 lipids were produced. So the seasonal affect and intact polar lipid analysis
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17 445 should be considered in the future to examine the relationship between the
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19 446 temperature and/or salinity.

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21 447 However, archaea in soils also produce isoGDGTs (Weijers et al., 2006), and
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23 448 substantial input of soil organic matter into these ponds could alter the observed
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25 449 isoGDGT distributions that are produced *in situ* within the ponds. A strong
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27 450 contribution of soil-derived isoGDGTs is consistent with the high BIT indices
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29 451 of these surface sediments (> 0.7). The higher abundance of GDGT-0 in surface
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31 452 sediments compared to soils – as well as higher GDGT-0 to crenarchaeol ratios
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33 453 – suggests that GDGT-0 is not solely derived from surrounding soils, and *in situ*
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35 454 production of GDGT-0 occurs in these ponds (likely by methanogens),
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37 455 consistent with previous findings from different type of lakes (Blaga et al.,
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39 456 2009; Naeher et al., 2014b; Hu et al., 2016).

40 457 This conclusion is supported by the presence of the *mcrA* gene in four out
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42 458 of seven ponds (Fig. 4). However, the concentration of GDGT-0 for IM7 is
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44 459 higher than that in IM3 and IM6, but the IM7 sediment contained no detectable
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46 460 *mcrA*, indicating that high concentrations of GDGT-0 do not necessarily
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48 461 correspond to high *mcrA* gene concentrations (Fig. 4). The high concentration
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50 462 of GDGT-0 in IM7 is also intriguing, given the fact that this is the most saline
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52 463 pond and Halobacteriales are not thought to produce GDGT-0 (Schouten et al.,
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54 464 2013), or there is a possibility that GDGTs could produce during a low salinity
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season. This is consistent with the presence of other archaeal lipids, such as archaeol and hydroxyarchaeol that are phylogenetically widespread (Koga et al., 1993; Sprott et al., 1993). Based on this, we interpret the source of GDGT-0 to be primarily methanogens in this setting, although the contribution from Thaumarchaeota and other Euryarchaeota or other phyla of archaea cannot be excluded. However, GDGT-0 and GDGT-1 have been detected in halophilic methanogens, *Methanonatronarchaeia* (Sorokin et al., 2018), and it could also be a specific biological source of isoGDGTs in these ponds. Further, the carbon specific analysis of biphytanes derived from crenarchaeol and other GDGTs could shed light on the origin of GDGTs, which will be consider in the future study.

4.1.2. DGDs

Archaeol is the most common and ubiquitous compound among the archaeal diethers (De Rosa and Gambacorta, 1988), and is produced by Halobacteriales, Thermophiles, Thaumarchaeota and methanogens (Teixidor et al., 1993; Sprott et al., 1997; Koga et al., 1998; Elling et al., 2017; Bale et al., 2019). It has been detected in a range of environments, including marine subsurface sediments (Lipp et al., 2008; Oba et al., 2015), cold seeps (Pancost et al., 2001; Rossel et al., 2011), peats (Pancost et al., 2011), soils (Lim et al., 2012), and hypersaline environments (Birgel et al., 2014; Li et al., 2016). In this study, relatively high amounts of archaeol are present in all pond surface sediment samples (Fig.4 and Table S3), whereas in soil samples the concentration of archaeol is low or below the detection limit (Table S3). This indicates that archaeol is mainly produced within these ponds; the contribution of Halobacteriales to archaeol may be higher in the more saline ponds, whereas methanogens may show a dominant contribution in the freshwater ponds. However, the contribution of

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2 493 halophilic methanogens cannot be excluded.

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4 494 The occurrence of other lipid biomarkers may also help identify the
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6 495 biological source(s) of archaeol. For example, hydroxyarchaeol including both
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8 496 the *sn*-2-and *sn*-3 isomers, occurs in seven out of eleven surface sediment
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10 497 samples and one soil sample (IMS6) (Table S3). A high abundance of archaeol
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12 498 and hydroxyarchaeol is usually related to the occurrence of methanogens and
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14 499 anaerobic methanotrophs in environmental samples, including marine
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16 500 sediments (Blumenberg et al., 2004; Elvert et al., 2005; Oba et al., 2006; Rossel
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18 501 et al., 2011), lake sediments (Bechtel and Schubert, 2009; Naeher et al., 2014a),
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20 502 peats (Pancost et al., 2011; Zheng et al., 2014). There is some evidence that
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22 503 Halobacteriales (Upasani et al., 1994), Thermoacidophiles (Sprott et al., 1997)
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24 504 and halo(alkali)philic methanogens isolated from hypersaline lakes (Bale et al.,
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26 505 2019) synthesize hydroxyarchaeol. Hydroxyarchaeol is also associated with
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28 506 anaerobic methanotrophic archaea (ANMEs), predominantly ANME-2 and/or
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30 507 ANME-3 (Hinrichs et al., 2000b; Blumenberg et al., 2004; Rossel et al., 2011),
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32 508 but less so ANME-1 (Aquilina et al., 2010). However, *sn*-2-hydroxyarchaeol is
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34 509 found mostly in *Methanosarcinales* and *Methanococcales* (Koga et al., 1998),
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36 510 whereas the *sn*-3-hydroxyarchaeol is found predominantly in *Methanosaeta*
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38 511 *concilii* (Ferrante et al., 1988) and in most strains of *Methanococcales* and
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40 512 *Methanosarcinales* (Koga and Nakano, 2008). Also, *sn*-3-hydroxyarchaeol is
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42 513 less common and abundant than *sn*-2-hydroxyarchaeol in environmental
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44 514 samples (Elvert et al., 2005; Niemann et al., 2005).

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46 515 There is a significant positive correlation between the abundance of
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48 516 *sn*-2-hydroxyarchaeol and *sn*-3-hydroxyarchaeol ($r^2 = 0.77$, $p = 0.009$) in pond
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50 517 surface sediments, suggesting that these two diether isomers are from the same
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52 518 biological source. However, there is no correlation between the concentrations
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54 519 of archaeol and the two hydroxyarchaeol isomers. The occurrence of archaeol
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56 520 and the absence of hydroxyarchaeol in soils, as well as the observation that the
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2 521 archaeol concentrations are much higher than those of *sn*-2-hydroxyarchaeol
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4 522 and *sn*-3-hydroxyarchaeol, suggests that archaeol has an additional source in
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6 523 these settings. Intriguingly, the concentrations of archaeol (C_{20}/C_{20} DGD) and
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8 524 extended archaeol (C_{20}/C_{25} DGD) are strongly correlated ($r^2 = 0.98$, $p < 0.001$,
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10 525 $n = 7$).

11 526 Carbon isotopic compositions can help elucidate the carbon substrates of the
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13 527 archaeol source organisms (Pancost and Sinninghe Damsté, 2003). We could
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15 528 only determine $\delta^{13}C$ values for archaeol in eight pond surface sediments
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17 529 because the concentrations of archaeol from other three samples are too low to
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19 530 do carbon isotopic analysis. The values range from -33‰ to -19‰ (Table S3),
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21 531 falling out of the highly ^{13}C -depleted range commonly associated with archaeal
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23 532 methanotrophy ($\sim -60\text{‰}$ to -130‰ ; see Zhuang et al. (2016) for reference).
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25 533 They are, however, similar or slightly depleted relative to those (avg. -12‰)
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27 534 from other hypersaline environments (Birgel et al., 2014).

28 535 Although the abundances of archaeol and its isotopic composition from
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30 536 pond surface sediments preclude an allochthonous source and an AOM source,
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32 537 it is less clear whether it derives from methanogens or Halobacteriales, or
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34 538 halophilic methanogens. The presence of *mcrA* gene, as discussed above,
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36 539 clearly indicates that methanogens are present in at least some ponds and likely
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38 540 contribute to some of the archaeol observed, but the isotopic compositions are
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40 541 ambiguous. ^{13}C -depletion in archaeal lipids has been invoked as evidence for
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42 542 methanogenesis, but there are diverse controls on their isotopic composition
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44 543 and ^{13}C -depletion is not expected in all cases (Summons et al., 1998). For
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46 544 example, the carbon isotopic composition of inferred methanogen lipids has
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48 545 been shown to be low in some ancient and modern settings (Freeman et al.,
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50 546 1990; 1994), but this is rarely observed in modern environments, including
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52 547 some thought to be characterized by methanogens (Hayes et al., 1987; Schouten
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54 548 et al., 2001; Naeher et al., 2014a). Acetoclastic and hydrogenotrophic
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methanogenesis are the two main methanogenic pathways in lake environments (Liu and Whitman, 2008). Indeed, the mixture of two methanogenic pathways can coexist in lake settings (Chan et al., 2005; Conrad, 2005), and both of them could be the source of archaeol. However, the dominant methanogenic pathway in the cold lake environment is usually the acetoclastic representatives with the contribution of hydrogenotrophic methanogenesis ($f_{H_2} < 10\%$ (Zepp Falz et al., 1999; Nozhevnikova et al., 2007). The hydrogenotrophic methanogens decrease in abundance as the salinity increase in high altitude lake sediments on the Tibetan Plateau, while the trend for acetoclastic methanogens is opposite (Liu et al., 2016). Although the archaeal community structure of these ponds is not available, hydrogenotrophic methanogenesis has a larger kinetic isotope effect than acetotrophic methanogenesis, which results in a more negative $\delta^{13}C$ (Conrad, 2005). Where archaeol of these ponds has high $\delta^{13}C$ values, this has been mainly attributed to acetoclastic methanogens (Pancost et al., 2000b; Pancost and Sinninghe Damsté, 2003; Valentine et al., 2004; Goevert and Conrad, 2009; Naeher et al., 2014a). ~~In this study the abundance of *mcrA* gene is correlated with the $\delta^{13}C$ values of archaeol ($r^2 = 0.96$), with the lowest archaeol $\delta^{13}C$ values associated with higher abundances of *mcrA* gene. Although based on only four samples, this is consistent with a methanogen source.~~ Moreover, archaeol $\delta^{13}C$ values are positively correlated to salinity ($r^2 = 0.47$, $p = 0.009$, Fig. 6a), with low salinity ponds having lower values. Therefore, the biological source of archaeol appears to be a mixture of ^{13}C -depleted acetoclastic methanogens, Halobacteriales and halophilic methanogens, varying in dominance between low and high salinity ponds, respectively. This interpretation is consistent with the presence of extended archaeol. The presence of both hydroxyarchaeol isomers, and diverse GDGTs also suggests a diverse archaeal lipid producing community.

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4.2. Relationship between ether lipids and salinity in these ponds

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579 The ACE index was initially proposed to reconstruct salinity in marine
580 environments by Turich and Freeman (2011), and then applied successfully in
581 inland lakes along the salinity gradient on the Qinghai-Tibetan Plateau (Wang
582 et al., 2013). However, the methanogen-inferred contribution to archaeol and
583 GDGT-0, as well as the possible contribution of GDGT-0 from halophilic
584 archaea could affect the application of ACE index in tropical saline ponds
585 (Huguet et al., 2015) and saline lakes (Günther et al., 2014; Li et al., 2016),
586 leading to a complicated relationship between ACE and salinity.

587 In our study, the ACE values of soils (mean value 0.19) are consistently
588 lower than the surface sediments (mean value 0.68), consistent with a higher
589 salinity in the ponds compared to soils. Moreover, although there is a weak
590 non-linear correlation between salinity and the ACE index of surface sediments
591 ($r^2 = 0.38$, $p = 0.048$, Fig. 6b), ACE values are high at elevated salinities. For
592 example, ACE indices are all higher (> 0.6) in ponds with salinity > 50 ‰ (Fig.
593 6b). However, these values are lower than those observed in marine
594 environments (with a salinity of ~ 35 ‰) (Turich and Freeman, 2011; Huguet et
595 al., 2015) but higher than those in Chaka Salt Lake surface sediments (salinity
596 ~ 320 ‰ with an average ACE of 0.41) (Li et al., 2016). Therefore, the ACE
597 index seems to record some characteristics of these ponds, but it remains
598 unclear how those relationships differ from or align with different types of
599 aquatic environments. However, for comparing ACE indices from different
600 studies the standard is necessary to correct the response factor between archaeol
601 and GDGT-0.

602 We also determined the relationship between DGDs (C_{20}/C_{20} DGD, C_{20}/C_{25}
603 DGD) and salinity. There is no correlation between DGD concentration and
604 salinity in these ponds (Fig. 6c&d). For example, both the highest concentration

of C₂₀/C₂₀ DGD and C₂₀/C₂₅ DGD was found in pond IM1 with a low salinity of 4.2 ‰ (Fig. 4 and Table S3), whereas pond IM7 (highest salinity of 280‰) contained a relatively low concentration of archaeal DGDs (Fig. 4 and Table S3). Also, there is no correlation between salinity and the ratio of archaeol/extended archaeol, indicating that there are various factors control the abundance of archaeal DGDs.

4.4. Reconstructed LST based on TEX₈₆

The application of isoGDGTs in lacustrine environments as a temperature proxy was first investigated by Powers et al. (2004), leading to TEX₈₆ calibration for large lakes (Powers et al., 2005; Tierney et al., 2010). However, TEX₈₆ is often not applicable in lacustrine environments due to the potential contribution from other sources such as methanotrophs or non-aquatic Thaumarchaeota (Blaga et al., 2009; Naeher et al., 2014b). We would expect that to be the case here, given potential contributions of methanogens and halophiles and suppression of Thaumarchaeotal growth at elevated salinity.

However, in these ponds, there is no correlation between the ring index of isoGDGTs and salinity indicating that the Thaumarchaeota in these saline ponds do not change their ring composition in response to salinity.

Allochthonous contribution would also bias TEX₈₆ values (Sinninghe Damsté et al., 2012b); nonetheless, we sought to explore the performance of TEX₈₆ in these ponds and assess under what conditions it appears to lose fidelity. To do this, we converted isoGDGT distributions into TEX₈₆-pond temperatures using several lake calibrations (Powers et al., 2010; Tierney et al., 2010; Castañeda and Schouten, 2011).

TEX₈₆ values for surface sediments show some variability (0.57 ± 0.15 ; Table S2), given the range of environmental conditions, and yield reconstructed

temperatures around 15°C. This is significantly higher than the observed MAAT of 0.4°C and the summer air temperature of 6.7°C. It is possible that this is due to allochthonous inputs, especially since TEX₈₆ values for soils are also high with an average value of 0.64 ± 0.05 . TEX₈₆ reconstructed temperatures based on a soil calibration (Liu et al., 2013) are $-5 \pm 3^\circ\text{C}$, which is closer to the observed MAAT of 0.4°C. However, we observe no relationship between TEX₈₆ and BIT values for pond surface sediments, nor between TEX₈₆ and the abundance of archaeol; thus, the evidence for a strong impact of soil archaea or methanogen bias of the TEX₈₆ signal remains circumstantial. There is a negative correlation between TEX₈₆ and salinity observed in these pond surface sediments ($r^2 = 0.53$, $p = 0.009$), suggesting that halophiles and other archaea could have contributed high ratio of GDGT-1 dominance over the other isoGDGTs than a pure culture Thaumarchaeota (Fig. 3 and Table S2). However, this would have led to lower than expected temperatures (not higher as we observed), and it remains unclear if halophiles can synthesize such compounds (see Turich and Freeman (2011) and references therein).

650

651 5. Conclusion

652

We examined the distribution of archaeal isoGDGTs and DGDs in surface sediments and catchment soils of 11 ponds with different salinity located in Inner Mongolia, NE China to explore their sources. Both lipid- and DNA-based methods showed there are methanogens in these ponds, confirmed by the occurrence of GDGT-0, archaeol (C₂₀/C₂₀ DGD), hydroxyarchaeol and the *mcrA* gene. While the occurrence of extended archaeol (C₂₀/C₂₅ DGD) is presumed to have various biological sources, i.e., the Halobacteriales, non-halophilic methanogens and halophilic methanogens. The contribution of

1
2 661 Halobacteriales has been confirmed in these saline ponds based on the
3
4 662 occurrence of the archaeal diethers, and that could explain the correlation
5
6 663 between salinity, *mcrA* gene abundance and archaeol $\delta^{13}\text{C}$ values (-19‰ to
7
8 664 -30‰). The applicability of isoGDGT-based proxies such as TEX_{86} and ACE
9
10 665 has been tested in this study; however, due to the mixed sources of archaeal
11
12 666 tetraethers and diethers, and the rough determination of ACE index, these
13
14 667 proxies are evidently not applicable to these ponds and should be applied with
15
16 668 care to similar aquatic settings. Further, the comparative analysis of intact polar
17
18 669 lipids which are reflective of living community and the genetic data of the
19
20 670 archaeal community should be more helpful to unravel the autochthonous and
21
22 671 allochthonous sources.
23

24 672

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1 Multiple environmental and ecological controls on archaeal ether lipid
2 distributions in saline ponds
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Abstract

The distribution of archaeal ether lipids is thought to be governed by salinity in hypersaline and marine environments due to differences in their biological sources. However, little is known about their source and relationship with salinity in lacustrine environments. To explore this, we analyzed the distribution of specific archaeal lipids (isoprenoid GDGTs and diethers) of 11 ponds along a salinity gradient (0.8 ‰ to 279 ‰) in Inner Mongolia, NE China. Functional gene *mcrA* and compound-specific stable carbon isotope data were used to explore the biological source(s) of archaeal diether and tetraether lipids. Both lipid- and DNA-based methods showed methanogens to be abundant in ponds, which is consistent with the dominance of GDGT-0 relative to other isoprenoid GDGTs as well as the occurrence of archaeol (C_{20}/C_{20} DGD), hydroxyarchaeol and the *mcrA* gene. However, the occurrence of the extended archaeol (C_{20}/C_{25} DGD) also suggests the presence of Halobacteriales, non-halophilic methanogens and halophilic methanogens. This means that the biological sources of archaeal diethers and tetraethers are complicated. We subsequently evaluated the applicability of TEX₈₆ temperature and ACE salinity proxies in these settings; however, due to the mixed sources of archaeal tetraethers and diethers, these proxies are not applicable in these ponds and it appears that salinity is not the dominant control on archaeal ether lipid distributions in these saline ponds.

Keywords: isoGDGTs; archaeal diethers; ACE; TEX₈₆; *mcrA* gene; saline ponds

1. Introduction

Dialkyl glycerol diether (DGD) and glycerol dialkyl glycerol tetraether lipids (GDGTs), membrane lipid constituents of many archaea and certain bacteria, are increasingly used to explore biogeochemistry and paleoclimate in aquatic and terrestrial environments (Schouten et al., 2013). Isoprenoid GDGTs (isoGDGTs), comprising acyclic or ring-containing isoprenoidal biphytanyl carbon chains (see Fig.1), are synthesized by a wide range of Archaea, including Euryarchaeota, Crenarchaeota and Thaumarchaeota (Schouten et al., 2007; Pearson and Ingalls, 2013; Schouten et al., 2013).

GDGT-0, comprising two acyclic biphytanes, is a common isoGDGT with a large variety of biological sources. It occurs in all major groups of cultivated Archaea and likely is also produced by many of the uncultivated Archaea [see Schouten et al. (2013) for a review]. In lacustrine settings, GDGT-0 is often predominantly derived from methanogens and ammonia oxidizing Thaumarchaeota (Blaga et al., 2009; Powers et al., 2010; Sinninghe Damsté et al., 2012a). In addition, uncultured crenarchaeotal groups (group 1.2 or Bathyarchaeota (group 1.3)) and marine benthic group C (MBG-C) might also be a dominant source of GDGT-0 in the lake water column and sediments (Sinninghe Damsté et al., 2009; Buckles et al., 2013). Crenarchaeol, containing four cyclopentane rings in addition to one cyclohexane ring, is believed to be unique to Thaumarchaeota (Sinninghe Damsté et al., 2002; Schouten et al., 2013), although this has been debated for marine environments (Lincoln et al., 2014; Schouten et al., 2014). IsoGDGTs containing 1 to 3 cyclopentane moieties (isoGDGTs 1-3) are non-specific and are commonly found in a wide range of Archaea, including Thaumarchaeota (Wuchter et al., 2006), methanogenic Euryarchaeota (Gattinger et al., 2002; Zhang et al., 2011), anaerobic methane-oxidizing archaea (Pancost et al., 2001) and extremophiles

74 (Lattuati et al., 1998; Ellen et al., 2008).

75 Among the isoGDGT-based proxies used in lakes are the TEX₈₆ (TetraEther
76 indeX of 86 carbons) palaeothermometer and the Archaeol to Caldarchaeol
77 Ecometric (ACE) salinity index. TEX₈₆ was originally developed in marine
78 environments (Schouten et al., 2002); it is based upon the relative distribution
79 of isoGDGTs containing cyclopentane moieties, synthesized by aquatic
80 Thaumarchaeota, and is significantly correlated with sea surface temperature
81 (Schouten et al., 2002; Kim et al., 2008; 2010). Subsequent studies
82 demonstrated that TEX₈₆ in lacustrine sediments is also correlated with lake
83 surface water temperatures (Powers et al., 2004; Blaga et al., 2009; Powers et
84 al., 2010). However, in lakes, the contribution of isoGDGTs derived from soils
85 in the surrounding catchment and/or production by methanogenic archaea
86 within the lake can influence the distribution of sedimentary GDGTs, yielding
87 unreliable TEX₈₆-inferred temperatures (Sinninghe Damsté et al., 2009; Bechtel
88 et al., 2010; Sinninghe Damsté et al., 2012a). Before the TEX₈₆
89 paleothermometer can be applied confidently in certain lacustrine systems,
90 therefore, it is important to constrain the origins of the associated GDGTs. It is
91 likely that application of TEX₈₆ is inappropriate in some saline lakes, but this
92 has not been extensively explored.

93 The ACE salinity index was originally developed for and applied to marine
94 environments and is based upon the relative abundance of archaeol relative to
95 GDGT-0 (Turich and Freeman, 2011). The index is based on the hypothesis that
96 Halobacteriales appear to exclusively/predominantly produce DGDs as core
97 lipids (Teixidor et al., 1993; Kates, 1996; Kamekura and Kates, 1999; Dawson
98 et al., 2012), predominantly C₂₀/C₂₀ DGDs (archaeol) and C₂₀/C₂₅ DGDs
99 (extended archaeol) (Xu et al., 2001; Hezayen et al., 2002; Dawson et al., 2012).
100 In hypersaline environments the shift in archaeal community from marine
101 Euryarchaeota and Thaumarchaeota to halophilic Euryarchaeota (Jiang et al.,

1 2009), causes isoGDGTs to disappear, while archaeol becomes abundant
2
3 103 (Turich and Freeman, 2011). The ACE index has been applied to some lakes
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5 104 (Wang et al., 2013). However, a prerequisite for the application of the ACE
6
7 105 index is to constrain the source of archaeol and GDGT-0. For example,
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9 106 GDGT-0 can be synthesized by Thermoplasmatales (Turich and Freeman, 2011),
10
11 107 ANME Archaea and methanogens (Koga et al., 1998; Pancost et al., 2000a;
12
13 108 2005; Naeher et al., 2014a). In addition, archaeol could be produced by
14
15 109 Methanosarcinales, Methanopyrales, Methanococcales and Thermococcales
16
17 110 other than Halobacteriales (Villanueva et al., 2014), which may bias the ACE
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19 111 proxy. Due to the complexity of the archaeal community in lake systems, the
20
21 112 ACE index was suggested not to be applicable to tropical ponds (Huguet et al.,
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23 113 2015), Tibetan saline lakes (Günther et al., 2014) and a Chinese hypersaline
24
25 114 lake (Li et al., 2016).

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28 115 For these reasons, it is useful to distinguish the biological sources of archaeal
29
30 116 diethers and tetraethers in any given setting. Methanogens are slow growers and
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32 117 sensitive to oxygen, difficult to isolate or culture in laboratory conditions, but
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34 118 the methyl coenzyme M reductase (*mcrA*) gene sequences can be used for the
35
36 119 identification of methanogens in a wide range of environments (Luton et al.,
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38 120 2002; Steinberg and Regan, 2008; Ma et al., 2012). Another promising way to
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40 121 examine archaeal ecology is via compound-specific stable carbon isotope
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42 122 analysis ($\delta^{13}\text{C}$) of their lipid biomarkers (Summons et al., 1998; Blumenberg et
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44 123 al., 2004; Naeher et al., 2014a). $\delta^{13}\text{C}$ values of microbial lipids produced by
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46 124 methanogens can be strongly depleted if lipid biosynthesis is linked to the
47
48 125 production of isotopically light methane; however, ^{13}C -depletion does not occur
49
50 126 in many cases (Pancost and Sinninghe Damsté, 2003).

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52
53 127 Previously, we investigated the distribution of branched GDGTs that are
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55 128 derived from bacteria in 11 ponds from Inner Mongolia, NE China,
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57 129 representing a salinity gradient from 0.8 ‰ to 279 ‰, and the surrounding soils
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(Li et al., 2017). In this study, we combine biomarker distributions, functional gene abundance and compound-specific stable carbon isotopic compositions to explore the biological source(s) of archaeal diethers and tetraethers lipids, as well as to evaluate the applicability of archaeal ether lipid-based proxies in these saline aquatic settings.

135

136 2. Materials and methods

137

138 2.1. Sampling

139

140 Eleven ponds with a broad salinity range (from freshwater ponds with a
141 salinity of 0.8 ‰ to hypersaline ponds with a salinity of 279 ‰) have been
142 investigated previously, and the sampling information is provided in Li et al.
143 (2017) (Fig. 2 and Table S1). The pH of pond water was measured with a
144 portable pH meter (Table S1), and the salinity is calculated as the sum of the
145 measured ions in the unit of g L⁻¹ (Table S1), as reported in Li et al. (2017). In
146 general, these small (1.5–7.0 km² surface area) and shallow (~0.5 m water
147 depth) ponds are located in New Barag Left Banner of Inner Mongolia in the
148 Northeast China, and all developed in the mid to late Holocene (Zheng et al.,
149 1992; 2002). Local Meteorological Station records indicate that the mean
150 annual air temperature (MAAT) is 0.4 °C, while the mean annual minimum and
151 maximum temperatures are –5.1 and 6.7 °C, respectively; the mean annual
152 precipitation was about 284 mm during the period from 1971 to 2013
153 (<http://cdc.nmic.cn/home.do>). This area is characterized by a semi-arid desert
154 steppe climate, with grassland land use and the vegetation dominated by C3
155 plants, mainly *Leymus chinensis*, *Bromus inermis*, *Elytrigia repens*. The typical
156 soils of the study site are chernozem, castanozem and meadow soil.

157 Surface sediments (n=11; 0–2 cm) from the deepest point in each pond and

158 soil samples (n=11; 0–2 cm) were collected during a field campaign in August
159 2013. Water samples were collected above the surface sediments and sealed in
160 50 mL sterile centrifuge tubes. All samples for lipid analysis were rapidly
161 frozen and immediately transported to the laboratory and stored at –20 °C until
162 further analysis. DNA isolation was performed for only seven out of eleven
163 ponds, due to failure to store the other four samples at low temperature. Surface
164 sediment samples for DNA analysis were collected in 50 mL sterile centrifuge
165 tubes and immediately kept on dry-ice, then transported to the lab and stored at
166 –80 °C for future use.

167

168 *2.2. Total organic carbon analysis, lipid extraction and isoGDGT analysis*

169

170 The method for TOC content determination was reported in Li et al. (2016).
171 In general, it was measured by subtracting the inorganic carbon content from
172 the total elemental carbon content, determined by using a Carlo Erba EA1108
173 Elemental Analyzer and modified Coulomat 702 analyser at the University of
174 Bristol. All TOC measurements were performed in duplicate.

175 Lipid extraction was determined according to the procedures in Li et al.
176 (2017). Briefly, surface sediment and soil samples were freeze dried and ground
177 to powder by using a mortar and pestle, and ca. 10 g of grounded sediment and
178 soil samples were extracted in pre-cleaned cellulose extraction thimbles via
179 Soxhlet apparatus for 24 h with dichloromethane/methanol (2:1, v/v) (Naafs
180 and Pancost, 2014). An aliquot (50 %) of the total lipid extract (TLE) was
181 separated over activated alumina column into an apolar and a polar fraction
182 using Hexane/DCM (9:1, v/v) and DCM/MeOH (1:2, v/v), respectively.

183 The polar fraction containing GDGTs was dissolved in hexane/isopropanol
184 (99:1, v/v) and filtered through 0.45 µm PTFE filters and dried under N₂ prior
185 to analysis by high performance liquid chromatography with triple quadruple

186 mass spectrometry (HPLC-MS²) using an Agilent 1200 series, with
187 auto-injection and ChemStation manager software. Samples were re-dissolved
188 in hexane/isopropanol (99:1,v/v) and an internal synthetic C₄₆ standard (Huguet
189 et al., 2006) added for quantification. The separation of isoGDGTs was
190 achieved with a Thermo Finnigan silica column (150 mm × 2.1 mm, 1.9 μm).
191 The elution gradient follows the procedure of Yang et al. (2014) with some
192 modifications. It was first eluted isocratically (5 min) with 80% A and 20% B,
193 where A = hexane and B = hexane/isopropanol (9:1, v/v), followed by changing
194 the ratio to 72% A and 28% B from 5 to 45 min and then to 70% A and 30% B
195 for 1 min, followed by column washing with 100% B for 5 min and then 80% A
196 and 20% B to equilibrate the column. The flow rate was 0.2 ml/min. The
197 detection of GDGTs was achieved by using single ion monitoring (SIM) mode
198 of [M+ H]⁺, targeting *m/z* 744 for the C₄₆ internal standard, *m/z* 653 and 1302,
199 1300, 1298, 1296, 1292, 1050, 1048, 1046, 1036, 1034, 1032, 1022, 1020 and
200 1018 for archaeol, isoGDGTs and brGDGTs. The final quantification was
201 semi-quantitative as we assume the ionization efficiency and the relative
202 response factor between GDGTs and the C₄₆ internal standard is 1:1. However,
203 due to the lack of an archaeol standard, the difference in response factor
204 between archaeol and GDGT-0 is not available. Hence the ratio of archaeol/
205 GDGT-0 here represents a of ratio of instrumental response rather than of the
206 absolute abundance and could thus affect the reported ACE values. The
207 concentration of GDGTs was then normalized to TOC as determined in Li et al.
208 (2017).

209

210 2.3. Dialkyl glycerol diether (DGD) and compound-specific stable carbon 211 isotope analysis

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213 The other aliquot (50 %) of the TLE was separated over an aminopropyl NH₂

1 214 bond elute column (Biotage) into neutral, free fatty acid and phospholipid
2 215 fractions by elution of DCM/isopropanol (2:1, v/v), 2% acetic acid in ethyl
3 216 ether and MeOH, respectively (Zheng et al., 2014). The neutral fraction was
4 217 further separated using activated alumina column by elution of hexane/DCM
5 218 (9:1, v/v) and DCM/MeOH (1:1, v/v) into the apolar and polar fraction,
6 219 respectively. The polar fraction was silylated with N, *O*-Bis(trimethylsilyl)
7 220 trifluoroacetamide (BSTFA) and Pyridine (Sigma-Aldrich) at 70 °C for 1 h and
8 221 dissolved in hexane prior to analysis by gas chromatography-mass spectrometry
9 222 (GC-MS) and high temperature gas chromatography-mass spectrometry
10 223 (HTGC-MS) (Kaur et al., 2015).

11 224 The polar fraction containing DGDs was analyzed by GC-MS using a
12 225 ThermoQuest Finnigan Trace GC and MS instrument equipped with a
13 226 non-polar silica CP Sil5-CB column (50 m × 0.32 mm × 0.12 µm) using the
14 227 following temperature program, 70 °C to 130 °C at 20 °C min⁻¹, ramp to 300 °C
15 228 at 4 °C min⁻¹, and held at 300 °C for 25 min. The ionization potential was 70 eV,
16 229 and full scan spectra were obtained with the scanning range *m/z* 50-650. The
17 230 DGDs were reanalyzed by HTGC-MS using a PerkinElmer Turbomass Gold
18 231 MS and fitted with DB1-HT column (15 m × 0.32 mm × 0.12 µm) using the
19 232 following temperature program, 50 °C isotherm for 2 min, ramp to 350 °C at
20 233 10 °C min⁻¹ and isotherm for 10 min. Semi-quantification was achieved by
21 234 adding 5α-androstane and 2-hexadecanol as internal standards.

22 235 Compound specific δ¹³C values of archaeol were determined using gas
23 236 chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS)
24 237 with an IsoPrime 100 IRMS coupled with an Agilent 7693 GC. The fused silica
25 238 capillary CP-Sil-5 column (60 m × 0.32 mm × 0.10 µm) was used with the
26 239 same GC temperature program as the GC-MS. The δ¹³C values are reported
27 240 relative to the Vienna Pee Dee Belemnite (VPDB) standard, and the analytical
28 241 error was determined using co-injected standards and was ± 0.5 ‰. The

instrument stability was monitored by regular analysis of a fatty acid methyl ester standard mixture with a long-term precision of ± 0.3 %.

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245 2.4. DNA extraction, PCR and *mcrA* gene analysis

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247 Genomic DNA was extracted from 0.5 g dry samples with FastDNA[®] Spin
248 Kit for soil (MP, USA). Primer sets *mls* and *mcrA*-rev (Steinberg and Regan,
249 2009) were used for *mcrA* gene quantitative analysis. Quantification of *mcrA*
250 gene was performed using the Bio-Rad real-time system CFX96 with TAKARA
251 SYBR Premix Ex Taq (Tli RNaseH Plus, DRR420A). The primer set was tested
252 both with positive controls (plasmid with *mcrA* insert) and negative controls
253 (plasmid without *mcrA* insert) to avoid false positive detection and increase
254 positive detection. The standard copy number was achieved from the plasmid
255 containing *mcrA* gene through the clone library construction method according
256 to pMD18-T simple clone system (TAKARA, Dalian, China). A series of
257 ten-fold dilution was used to obtain a standard curve with efficiency close to
258 100% (95-105%). Real time PCR was conducted in a 20 μ L mixture containing
259 10 μ L of 2 $\times$ SYBR Green I premixture (DRR420A, TAKARA), 0.5 μ L of 10
260 μ M each primer, about 2 μ L of template DNA (100 ng) and 7 μ L PCR-grade
261 distilled water. The real time PCR was run for 40 cycles with the following
262 conditions: initial denaturing at 95 °C for 1 s, annealing at 55 °C for 30 s and
263 extension at 72 °C for 30 s, and fluorescent capture at 80 °C for 20 s with the
264 initial denaturation at 95 °C for 30 s.

265

266 2.5. GDGT-based proxy calculations

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268 Roman and Arabic numerals refer to the isoGDGT structures indicated in Fig.
269 1: 0, 1, 2, 3 indicate isoGDGTs containing zero, one, two or three cyclopentane

moieties, respectively; Cren and Cren' refer to crenarchaeol and its regioisomer. TEX₈₆ is calculated following Schouten et al. (2002) and reflects the degree of cyclization of particular isoGDGTs:

$$\text{TEX}_{86} = \frac{(\text{GDGT} - 2 + \text{GDGT} - 3 + \text{Cren}')}{(\text{GDGT} - 1 + \text{GDGT} - 2 + \text{GDGT} - 3 + \text{Cren}')} \quad (1)$$

The ACE index is based upon the relative abundance of archaeol relative to GDGT-0 (Turich and Freeman, 2011):

$$\text{ACE} = \frac{\text{archaeol}}{(\text{archaeol} + \text{GDGT} - 0)} \quad (2)$$

The R_{i/b} (Xie et al., 2012) represents the relative abundance of total isoGDGTs to total brGDGTs:

$$\text{R}_{i/b} = \sum \text{isoGDGTs} / \sum \text{brGDGTs} \quad (3)$$

The branched versus isoprenoid tetraether (BIT) index is calculated on the basis of Hopmans et al. (2004), reflecting the relative abundance of brGDGTs vs. crenarchaeol.

$$\text{BIT} = \frac{(\text{Ia} + \text{IIa} + \text{IIIa} + \text{IIa}' + \text{IIIa}')}{(\text{Ia} + \text{IIa} + \text{IIIa} + \text{IIa}' + \text{IIIa}' + \text{Cren})} \quad (4)$$

2.6. Statistical analysis

Canonical correspondence analysis (CCA) was performed using the software Canoco for Windows version 4.5. The fractional abundance of isoGDGTs, DGDs and the GDGT-based proxies as response variables and water chemistry parameters as explanatory variables were transferred into the Canoco software. Detrended correspondence analysis (DCA) was first used to measure the lengths of variances; however, all of these lengths were < 3, instead the CCA analysis based on a linear ordination method was selected to process the dataset. The variable significance was assessed using a Monte-Carlo significance permutation test. The significant difference between datasets was determined by a Pearson's coefficient (*p*-value) < 0.05 in the independent sample *t*-test by

297 using the SPSS software.

298

299 **3. Results**

300

301 *3.1. Measured pond water parameters and TOC contents*

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303 The pH of the eleven ponds varied from 7.7 to 10, whereas salinity varied
304 from 0.8 ‰ to 279 ‰ (Table S1). The lowest pH value and highest salinity was
305 observed in IM7, whereas other ponds had a similar pH values but lower
306 salinity. The pond water pH and salinity are linearly correlated ($r^2 = 0.5$, $p =$
307 0.02) and samples with high pH tend to have lower salinity. The TOC content
308 of surface sediments varies from 0.2 to 2.7 wt.% and that of soils ranges from
309 0.1 to 5.3 wt.%; the TOC contents of surface sediments increase with the
310 salinity ($r^2 = 0.66$, $p = 0.005$).

311

312 *3.2. Distribution of isoGDGTs from pond surface sediments and soils*

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314 As illustrated in Table S2 and Fig. 3, the isoGDGT distribution of both
315 surface sediments and soils is dominated by GDGT-0. The average fractional
316 abundance of GDGT-0 is 80% in surface sediments and 60% in soils (Fig. 3).
317 The second most abundant isoGDGT is crenarchaeol, with an average
318 proportion of 10 and 30% for surface sediments and soils, respectively. The
319 relative abundance of isoGDGTs-1 to -3 as well as the crenarchaeol
320 regioisomer is generally higher in soils than in surface sediments. However,
321 there is almost no relationship between isoGDGT abundances and pond water
322 salinity; only the fractional abundance of GDGT-1 is slightly correlated with
323 salinity ($r^2 = 0.32$, $p = 0.09$).

324 The isoGDGT-based proxies for both surface sediments and soils are

presented in Table S2. TEX₈₆ values for soils are 0.64 ± 0.05 , and the values of surface sediments are slightly lower with 0.57 ± 0.15 (Table S2). The relative abundance of isoGDGTs to brGDGTs ($R_{i/b}$) in surface sediments ranges from 0.6 to 9.1, with an average of 2.7, and it ranges from 0.2 to 1.9 with an average of 0.8 in soils (Table S2). The ratio of GDGT-0 to crenarchaeol (GDGT-0/Cren) ranges from 3 to 101 in surface sediments and from 0.5 to 40 in soils, and the ratio of crenarchaeol to crenarchaeol regioisomer (Cren/Cren') ranges from 8 to 17 in surface sediments and 11 to 22 in soils. The ACE is higher ($t = 4.188$; $p < 0.001$, shown by the paired sample t -test) in surface sediments than in soils, ranging from 0.14 to 0.97 in surface sediments and from 0.04 to 0.93 in soils (Table S2).

The TOC-normalized concentrations of isoGDGTs are highly variable, ranging from 0.1 to 8 $\mu\text{g/g}$ TOC for surface sediments and 0.05 to 44 $\mu\text{g/g}$ TOC for soils (Table S2). The concentration of isoGDGTs is generally higher in soils compared to the corresponding surface sediments, but with some exceptions (IM1, IM8 and IM10).

341

3.3. Distribution of DGDs and compound specific $\delta^{13}\text{C}$ values of archaeol

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The concentration of DGDs is shown in Fig. 4 and Table S3. The most abundant DGD in surface sediments and soils is archaeol ($\text{C}_{20}/\text{C}_{20}$ DGD, bio-*O*-phytanylglyceroldiether, see Fig. 1 for structure). However, a variety of other isoprenoidal DGDs, including hydroxyarchaeol and extended archaeol ($\text{C}_{20}/\text{C}_{25}$ DGD) are present in many surface sediment samples (Fig. 4 and Table S3). Archaeol is present in all surface sediments but not detected in four soil samples. Its concentrations are variable in both sediments (9–5800 $\mu\text{g/g}$ TOC), and soils (1–430 $\mu\text{g/g}$ TOC, Table S3). Due to the lower abundance of archaeol in soils, we only measured its stable carbon isotopic composition ($\delta^{13}\text{C}$) in pond

1 353 surface sediments. It ranges from -33 ‰ to -19 ‰, with the lowest value in IM2
2 354 and the highest value in IM6. In pond samples IM1, IM9 and IM10 the
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4 355 abundance of archaeol was too low to determine its $\delta^{13}\text{C}$ value.
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7 356 Extended archaeol, $\text{C}_{20}/\text{C}_{25}$ dialkyl glycerol diether (DGD) (Fig.1), has a
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9 357 similar structure as archaeol ($\text{C}_{20}/\text{C}_{20}$ DGD) but possesses phytanyl (C_{20}) and
10 358 sesterpanyl (C_{25}) isoprenoid chains instead of double phytanyl chains (Tindall
11 359 et al., 1984; Romano et al., 2007). This compound was found in Halobacteriales,
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13 360 as well as *Methanomassiliicoccus luminyensis* (the solo cultured representative
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15 361 of the seventh methanogenic order) and the halophilic methanogen
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17 362 *Methanonatronarchaeia* (Becker et al., 2016; Bale et al., 2019). In these ponds,
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19 363 the $\text{C}_{20}/\text{C}_{25}$ DGD is less abundant in surface sediments compared to archaeol
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21 364 (Fig. 4 and Table S3). It occurs in seven out of eleven surface sediment samples
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23 365 and its concentration ranges from 20–550 $\mu\text{g/g}$ TOC. However, it is present in
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25 366 only one soil sample (IMS6), with a concentration of 65 $\mu\text{g/g}$ TOC (Table S3).
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28 367 Hydroxyarchaeol has a structure similar to that of archaeol but with an
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30 368 additional hydroxyl group on the third carbon of the phytanyl carbon skeleton
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32 369 linked to either the second (*sn*-2-hydroxyarchaeol, Fig.1) or the third
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34 370 (*sn*-3-hydroxyarchaeol, Fig.1) glycerol carbon (Hinrichs et al., 2000a; Pancost
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36 371 et al., 2001). Hydroxyarchaeol is present in seven out of eleven surface
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38 372 sediment samples, but absent in all soil samples (Table S3). The concentration
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40 373 of *sn*-2-hydroxyarchaeol varies from 0.3 to 13 $\mu\text{g/g}$ TOC, and the concentration
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42 374 of *sn*-3-hydroxyarchaeol ranges from 0.6 to 25 $\mu\text{g/g}$ TOC in surface sediments
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44 375 (Table S3), but neither shows a correlation with salinity. As with extended
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46 376 archaeol and hydroxyarchaeol, the carbon isotopic composition could not be
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48 377 determined due to low concentrations.
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51 378 The statistical analysis indicates that none of the fractional abundances of
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53 379 isoGDGTs, isoGDGTs-based proxies or the distribution of DGDs has a
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55 380 significant correlation with pH. However, TEX_{86} and the ratio of
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GDGT-2/GDGT-3 are correlated with pond salinity (correlated with $r^2 = 0.53$, $p = 0.009$ and inversely correlated with $r^2 = 0.5$, $p = 0.014$, respectively). To test this further, we performed RDA to measure the impact of solute species, including Na^+ , K^+ , Mg^{2+} , Ca^{2+} , Cl^- , SO_4^{2-} , HCO_3^- and CO_3^{2-} concentrations, on the distribution of isoGDGTs and DGDs in pond surface sediments (Fig. 5). In the first RDA dataset, the analysis confirms that there is no significant correlation between water chemistry and the distribution of isoGDGTs and DGDs, and only HCO_3^- seems to affect the distribution of these archaeal lipids with the explanation of 45.2% ($p = 0.046$).

3.4. Distribution of *mcrA* gene in pond surface sediments

The *mcrA* was developed to target the gene sequence by determining methanogen gene copy numbers through the use of qPCR. Seven pond surface sediments were analyzed for the methanogen-specific marker *mcrA* gene. The functional molecular marker of methanogenic archaea could only be detected in four samples (IM3, IM5, IM6, and IM 8) (Fig. 4). The archaeal 16S rRNA gene copies number from these seven pond surface sediments are listed in Table S3, the archaeal 16S rRNA gene from IM8 is higher than the other three ponds. While ponds IM3, IM6, and IM8 all contain similar abundances of the *mcrA* gene, with an average of 65 copy numbers per ng DNA, the relative abundance of *mcrA* gene to the archaeal 16S rRNA gene in IM5 is higher than the other three ponds (Fig. 4).

4. Discussion

4.1. Provenance of archaeal lipids

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409 4.1.1. *isoGDGTs*

410 In pond surface sediments and surrounding soils, GDGT-0 is the most
411 abundant component among *isoGDGTs*, consistent with previous studies of
412 lacustrine sediments (Blaga et al., 2009; Tierney et al., 2012; Wang et al., 2012;
413 Naeher et al., 2014b; Li et al., 2016). However, in some soil samples (e.g., IMS
414 7) crenarchaeol was more abundant, as previously observed in other soils
415 (Sinninghe Damsté et al., 2009; Wang et al., 2012; Naeher et al., 2014b; Hu et
416 al., 2016). Similar distribution of *isoGDGTs* between surface sediments and
417 surrounding soils indicating that the main source of organic matter is soil input
418 as these are shallow ponds.

419 GDGT-0 is fairly non-specific and a commonly found lipid in a wide range
420 of archaea (Schouten et al., 2013); in lacustrine environments, the source of
421 GDGT-0 can be highly variable and it can be produced in different layers of the
422 water column and sediment. For example, GDGT-0 can originate from
423 Thaumarchaeota living in the surface water or from Crenarchaeota Group 1.2
424 and MBG-C that are resident in the anoxic lower water column as seen in Lake
425 Challa of equatorial East Africa (Sinninghe Damsté et al., 2009). Furthermore,
426 new evidence has indicated that the uncultured crenarchaeotal groups 1.2 and
427 Bathyarchaeota could also produce GDGT-0 in the anoxic lake settings
428 (Buckles et al., 2013). However, more recently, the *mcrA* gene that related to
429 methane metabolism has been detected in Bathyarchaeota based on the genomic
430 evidence (Evans et al., 2015; Zhou et al., 2018). In Lake Lucerne,
431 Thaumarchaeota are the dominant source of GDGT-0 (Blaga et al., 2011).
432 Methanogenic archaea can also be an important source of GDGT-0 within the
433 water column and sediment (Blaga et al., 2009; Powers et al., 2010; Naeher et
434 al., 2014b). The influence of methanogenic archaea can be explored by the ratio
435 of GDGT-0/crenarchaeol proposed by Blaga et al. (2009) as the methanogens
436 produce GDGT-0 but no crenarchaeol. Consequently, high ratios in these

1 437 surface sediments (3~102), especially high ratio occurred in IM10, IM3 and
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3 438 IM5 (Table S2), could indicate a strong methanogenic contribution.
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5 439 Seasonal fluctuation of these shallow ponds such as the water level and salinity
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7 440 could affect the microbial community composition and the distribution of
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9 441 GDGTs, however in the present study we only sampled in summer time and the
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11 442 core lipids analysis only which is not suffice to unravel the time when these
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13 443 lipids were produced. So the seasonal affect and intact polar lipid analysis
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15 444 should be considered in the future to examine the relationship between the
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17 445 temperature and/or salinity. However, archaea in soils also produce
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19 446 isoGDGTs (Weijers et al., 2006), and substantial input of soil organic matter
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21 447 into these ponds could alter the observed isoGDGT distributions that are
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23 448 produced *in situ* within the ponds. A strong contribution of soil-derived
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25 449 isoGDGTs is consistent with the high BIT indices of these surface sediments (>
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27 450 0.7). The higher abundance of GDGT-0 in surface sediments compared to soils
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29 451 – as well as higher GDGT-0 to crenarchaeol ratios – suggests that GDGT-0 is
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31 452 not solely derived from surrounding soils, and *in situ* production of GDGT-0
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33 453 occurs in these ponds (likely by methanogens), consistent with previous
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35 454 findings from different type of lakes (Blaga et al., 2009; Naeher et al., 2014b;
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37 455 Hu et al., 2016).

41 456 This conclusion is supported by the presence of the *mcrA* gene in four out
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43 457 of seven ponds (Fig. 4). However, the concentration of GDGT-0 for IM7 is
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45 458 higher than that in IM3 and IM6, but the IM7 sediment contained no detectable
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47 459 *mcrA*, indicating that high concentrations of GDGT-0 do not necessarily
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49 460 correspond to high *mcrA* gene concentrations (Fig. 4). The high concentration
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51 461 of GDGT-0 in IM7 is also intriguing, given the fact that this is the most saline
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53 462 pond and Halobacteriales are not thought to produce GDGT-0 (Schouten et al.,
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55 463 2013), or there is a possibility that GDGTs could produce during a low salinity
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57 464 season. This is consistent with the presence of other archaeal lipids, such as
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archaeol and hydroxyarchaeol that are phylogenetically widespread (Koga et al., 1993; Sprott et al., 1993). Based on this, we interpret the source of GDGT-0 to be primarily methanogens in this setting, although the contribution from Thaumarchaeota and other Euryarchaeota or other phyla of archaea cannot be excluded. However, GDGT-0 and GDGT-1 have been detected in halophilic methanogens, *Methanonatronarchaeia* (Sorokin et al., 2018), and it could also be a specific biological source of isoGDGTs in these ponds. Further, the carbon specific analysis of biphytanes derived from crenarchaeol and other GDGTs could shed light on the origin of GDGTs, which will be consider in the future study.

4.1.2. DGDs

Archaeol is the most common and ubiquitous compound among the archaeal diethers (De Rosa and Gambacorta, 1988), and is produced by Halobacteriales, Thermophiles, Thaumarchaeota and methanogens (Teixidor et al., 1993; Sprott et al., 1997; Koga et al., 1998; Elling et al., 2017; Bale et al., 2019). It has been detected in a range of environments, including marine subsurface sediments (Lipp et al., 2008; Oba et al., 2015), cold seeps (Pancost et al., 2001; Rossel et al., 2011), peats (Pancost et al., 2011), soils (Lim et al., 2012), and hypersaline environments (Birgel et al., 2014; Li et al., 2016). In this study, relatively high amounts of archaeol are present in all pond surface sediment samples (Fig.4 and Table S3), whereas in soil samples the concentration of archaeol is low or below the detection limit (Table S3). This indicates that archaeol is mainly produced within these ponds; the contribution of Halobacteriales to archaeol may be higher in the more saline ponds, whereas methanogens may show a dominant contribution in the freshwater ponds. However, the contribution of halophilic methanogens cannot be excluded.

The occurrence of other lipid biomarkers may also help identify the

1 493 biological source(s) of archaeol. For example, hydroxyarchaeol including both
2 494 the *sn*-2-and *sn*-3 isomers, occurs in seven out of eleven surface sediment
3 495 samples and one soil sample (IMS6) (Table S3). A high abundance of archaeol
4 496 and hydroxyarchaeol is usually related to the occurrence of methanogens and
5 497 anaerobic methanotrophs in environmental samples, including marine
6 498 sediments (Blumenberg et al., 2004; Elvert et al., 2005; Oba et al., 2006; Rossel
7 499 et al., 2011), lake sediments (Bechtel and Schubert, 2009; Naeher et al., 2014a),
8 500 peats (Pancost et al., 2011; Zheng et al., 2014). There is some evidence that
9 501 Halobacteriales (Upasani et al., 1994), Thermoacidophiles (Sprott et al., 1997)
10 502 and halo(alkali)philic methanogens isolated from hypersaline lakes (Bale et al.,
11 503 2019) synthesize hydroxyarchaeol. Hydroxyarchaeol is also associated with
12 504 anaerobic methanotrophic archaea (ANMEs), predominantly ANME-2 and/or
13 505 ANME-3 (Hinrichs et al., 2000b; Blumenberg et al., 2004; Rossel et al., 2011),
14 506 but less so ANME-1 (Aquilina et al., 2010). However, *sn*-2-hydroxyarchaeol is
15 507 found mostly in *Methanosarcinales* and *Methanococcales* (Koga et al., 1998),
16 508 whereas the *sn*-3-hydroxyarchaeol is found predominantly in *Methanosaeta*
17 509 *concilii* (Ferrante et al., 1988) and in most strains of *Methanococcales* and
18 510 *Methanosarcinales* (Koga and Nakano, 2008). Also, *sn*-3-hydroxyarchaeol is
19 511 less common and abundant than *sn*-2-hydroxyarchaeol in environmental
20 512 samples (Elvert et al., 2005; Niemann et al., 2005).

21 513 There is a significant positive correlation between the abundance of
22 514 *sn*-2-hydroxyarchaeol and *sn*-3-hydroxyarchaeol ($r^2 = 0.77$, $p = 0.009$) in pond
23 515 surface sediments, suggesting that these two diether isomers are from the same
24 516 biological source. However, there is no correlation between the concentrations
25 517 of archaeol and the two hydroxyarchaeol isomers. The occurrence of archaeol
26 518 and the absence of hydroxyarchaeol in soils, as well as the observation that the
27 519 archaeol concentrations are much higher than those of *sn*-2-hydroxyarchaeol
28 520 and *sn*-3-hydroxyarchaeol, suggests that archaeol has an additional source in

1 521 these settings. Intriguingly, the concentrations of archaeol (C_{20}/C_{20} DGD) and
2 522 extended archaeol (C_{20}/C_{25} DGD) are strongly correlated ($r^2 = 0.98$, $p < 0.001$,
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4 523 $n = 7$).

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6 524 Carbon isotopic compositions can help elucidate the carbon substrates of the
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8 525 archaeol source organisms (Pancost and Sinninghe Damsté, 2003). We could
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10 526 only determine $\delta^{13}C$ values for archaeol in eight pond surface sediments
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12 527 because the concentrations of archaeol from other three samples are too low to
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14 528 do carbon isotopic analysis. The values range from -33‰ to -19‰ (Table S3),
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16 529 falling out of the highly ^{13}C -depleted range commonly associated with archaeal
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18 530 methanotrophy ($\sim -60\text{‰}$ to -130‰ ; see Zhuang et al. (2016) for reference).
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20 531 They are, however, similar or slightly depleted relative to those (avg. -12‰)
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22 532 from other hypersaline environments (Birgel et al., 2014).
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26 533 Although the abundances of archaeol and its isotopic composition from
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28 534 pond surface sediments preclude an allochthonous source and an AOM source,
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30 535 it is less clear whether it derives from methanogens or Halobacteriales, or
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32 536 halophilic methanogens. The presence of *mcrA* gene, as discussed above,
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34 537 clearly indicates that methanogens are present in at least some ponds and likely
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36 538 contribute to some of the archaeol observed, but the isotopic compositions are
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38 539 ambiguous. ^{13}C -depletion in archaeal lipids has been invoked as evidence for
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40 540 methanogenesis, but there are diverse controls on their isotopic composition
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42 541 and ^{13}C -depletion is not expected in all cases (Summons et al., 1998). For
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44 542 example, the carbon isotopic composition of inferred methanogen lipids has
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46 543 been shown to be low in some ancient and modern settings (Freeman et al.,
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48 544 1990; 1994), but this is rarely observed in modern environments, including
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50 545 some thought to be characterized by methanogens (Hayes et al., 1987; Schouten
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52 546 et al., 2001; Naeher et al., 2014a). Acetoclastic and hydrogenotrophic
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54 547 methanogenesis are the two main methanogenic pathways in lake environments
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56 548 (Liu and Whitman, 2008). Indeed, the mixture of two methanogenic pathways
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can coexist in lake settings (Chan et al., 2005; Conrad, 2005), and both of them could be the source of archaeol. However, the dominant methanogenic pathway in the cold lake environment is usually the acetoclastic representatives with the contribution of hydrogenotrophic methanogenesis (f_{H_2}) < 10% (Zepp Falz et al., 1999; Nozhevnikova et al., 2007). The hydrogenotrophic methanogens decrease in abundance as the salinity increase in high altitude lake sediments on the Tibetan Plateau, while the trend for acetoclastic methanogens is opposite (Liu et al., 2016). Although the archaeal community structure of these ponds is not available, hydrogenotrophic methanogenesis has a larger kinetic isotope effect than acetotrophic methanogenesis, which results in a more negative $\delta^{13}C$ (Conrad, 2005). Where archaeol of these ponds has high $\delta^{13}C$ values, this has been mainly attributed to acetoclastic methanogens (Pancost et al., 2000b; Pancost and Sinninghe Damsté, 2003; Valentine et al., 2004; Govert and Conrad, 2009; Naeher et al., 2014a). Moreover, archaeol $\delta^{13}C$ values are positively correlated to salinity ($r^2 = 0.47$, $p = 0.009$, Fig. 6a), with low salinity ponds having lower values. Therefore, the biological source of archaeol appears to be a mixture of ^{13}C -depleted acetoclastic methanogens, Halobacteriales and halophilic methanogens, varying in dominance between low and high salinity ponds, respectively. This interpretation is consistent with the presence of extended archaeol. The presence of both hydroxyarchaeol isomers, and diverse GDGTs also suggests a diverse archaeal lipid producing community.

4.2. Relationship between ether lipids and salinity in these ponds

The ACE index was initially proposed to reconstruct salinity in marine environments by Turich and Freeman (2011), and then applied successfully in inland lakes along the salinity gradient on the Qinghai-Tibetan Plateau (Wang et al., 2013). However, the methanogen-inferred contribution to archaeol and

GDGT-0, as well as the possible contribution of GDGT-0 from halophilic archaea could affect the application of ACE index in tropical saline ponds (Huguet et al., 2015) and saline lakes (Günther et al., 2014; Li et al., 2016), leading to a complicated relationship between ACE and salinity.

In our study, the ACE values of soils (mean value 0.19) are consistently lower than the surface sediments (mean value 0.68), consistent with a higher salinity in the ponds compared to soils. Moreover, although there is a weak non-linear correlation between salinity and the ACE index of surface sediments ($r^2 = 0.38$, $p = 0.048$, Fig. 6b), ACE values are high at elevated salinities. For example, ACE indices are all higher (> 0.6) in ponds with salinity > 50 ‰ (Fig. 6b). However, these values are lower than those observed in marine environments (with a salinity of ~ 35 ‰) (Turich and Freeman, 2011; Huguet et al., 2015) but higher than those in Chaka Salt Lake surface sediments (salinity ~ 320 ‰ with an average ACE of 0.41) (Li et al., 2016). Therefore, the ACE index seems to record some characteristics of these ponds, but it remains unclear how those relationships differ from or align with different types of aquatic environments. However, for comparing ACE indices from different studies the standard is necessary to correct the response factor between archaeol and GDGT-0. We also determined the relationship between DGDs (C_{20}/C_{20} DGD, C_{20}/C_{25} DGD) and salinity. There is no correlation between DGD concentration and salinity in these ponds (Fig. 6c&d). For example, both the highest concentration of C_{20}/C_{20} DGD and C_{20}/C_{25} DGD was found in pond IM1 with a low salinity of 4.2 ‰ (Fig. 4 and Table S3), whereas pond IM7 (highest salinity of 280‰) contained a relatively low concentration of archaeal DGDs (Fig. 4 and Table S3). Also, there is no correlation between salinity and the ratio of archaeol/extended archaeol, indicating that there are various factors control the abundance of archaeal DGDs.

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605 4.4. Reconstructed LST based on TEX_{86}

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607 The application of isoGDGTs in lacustrine environments as a temperature
608 proxy was first investigated by Powers et al. (2004), leading to TEX_{86}
609 calibration for large lakes (Powers et al., 2005; Tierney et al., 2010). However,
610 TEX_{86} is often not applicable in lacustrine environments due to the potential
611 contribution from other sources such as methanotrophs or non-aquatic
612 Thaumarchaeota (Blaga et al., 2009; Naeher et al., 2014b). We would expect
613 that to be the case here, given potential contributions of methanogens and
614 halophiles and suppression of Thaumarchaeotal growth at elevated salinity.
615 However, in these ponds, there is no correlation between the ring index of
616 isoGDGTs and salinity indicating that the Thaumarchaeota in these saline
617 ponds do not change their ring composition in response to salinity.
618 Allochthonous contribution would also bias TEX_{86} values (Sinninghe Damsté
619 et al., 2012b); nonetheless, we sought to explore the performance of TEX_{86} in
620 these ponds and assess under what conditions it appears to lose fidelity. To do
621 this, we converted isoGDGT distributions into TEX_{86} -pond temperatures using
622 several lake calibrations (Powers et al., 2010; Tierney et al., 2010; Castañeda
623 and Schouten, 2011).

624 TEX_{86} values for surface sediments show some variability (0.57 ± 0.15 ;
625 Table S2), given the range of environmental conditions, and yield reconstructed
626 temperatures around 15°C. This is significantly higher than the observed
627 MAAT of 0.4°C and the summer air temperature of 6.7°C. It is possible that
628 this is due to allochthonous inputs, especially since TEX_{86} values for soils are
629 also high with an average value of 0.64 ± 0.05 . TEX_{86} reconstructed
630 temperatures based on a soil calibration (Liu et al., 2013) are $-5 \pm 3^\circ\text{C}$,
631 which is closer to the observed MAAT of 0.4°C. However, we observe no
632 relationship between TEX_{86} and BIT values for pond surface sediments, nor

between TEX₈₆ and the abundance of archaeol; thus, the evidence for a strong impact of soil archaea or methanogen bias of the TEX₈₆ signal remains circumstantial. There is a negative correlation between TEX₈₆ and salinity observed in these pond surface sediments ($r^2 = 0.53$, $p = 0.009$), suggesting that halophiles and other archaea could have contributed high ratio of GDGT-1 dominance over the other isoGDGTs than a pure culture Thaumarchaeota (Fig. 3 and Table S2). However, this would have led to lower than expected temperatures (not higher as we observed), and it remains unclear if halophiles can synthesize such compounds (see Turich and Freeman (2011) and references therein).

5. Conclusion

We examined the distribution of archaeal isoGDGTs and DGDs in surface sediments and catchment soils of 11 ponds with different salinity located in Inner Mongolia, NE China to explore their sources. Both lipid- and DNA-based methods showed there are methanogens in these ponds, confirmed by the occurrence of GDGT-0, archaeol (C₂₀/C₂₀ DGD), hydroxyarchaeol and the *mcrA* gene. While the occurrence of extended archaeol (C₂₀/C₂₅ DGD) is presumed to have various biological sources, i.e., the Halobacteriales, non-halophilic methanogens and halophilic methanogens. The contribution of Halobacteriales has been confirmed in these saline ponds based on the occurrence of the archaeal diethers, and that could explain the correlation between salinity, *mcrA* gene abundance and archaeol $\delta^{13}\text{C}$ values (−19‰ to −30‰). The applicability of isoGDGT-based proxies such as TEX₈₆ and ACE has been tested in this study; however, due to the mixed sources of archaeal tetraethers and diethers, and the rough determination of ACE index, these proxies are evidently not applicable to these ponds and should be applied with

1 661 care to similar aquatic settings. Further, the comparative analysis of intact polar
2 662 lipids which are reflective of living community and the genetic data of the
3
4 663 archaeal community should be more helpful to unravel the autochthonous and
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6 664 allochthonous sources.
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1 Figure Captions

2 **Fig. 1.** Chemical structures of isoprenoid GDGTs, archaeal diethers and branched
3 GDGTs.

4 **Fig. 2.** Map of 11 ponds located in New Barag Left Banner of Inner Mongolia, NE
5 China, with the sampling sites indicated.

6 **Fig. 3.** Fractional abundance of individual isoGDGTs in 11 pond surface sediments
7 and soils.

8 **Fig. 4.** Concentrations of C₂₀/C₂₀ DGD (μg/g TOC), C₂₀/C₂₅ DGD (μg/g TOC) and
9 the ratio of *mcrA* gene and archaeal 16S rRNA gene copies number in pond surface
10 sediments with different salinity. (n.a., samples are not available; n.d., *mcrA* gene are
11 not detectable in our samples.)

12 **Fig. 5.** The redundancy analysis showing relationships between water chemical
13 parameters and (a) the distribution of isoGDGTs and DGDs and (b) GDGT-based
14 indices.

15 **Fig. 6.** Cross plots of salinity and the δ¹³C values of archaeol (a; from the 8 pond
16 surface sediments where it could be determined), ACE indices (b), and the
17 concentrations (μg/g TOC) of C₂₀/C₂₀ DGD (c), C₂₀/C₂₅ DGD (d),
18 *sn*-2-hydroxyarchaeol (e), and *sn*-3-hydroxyarchaeol (f).

19 Figure 1

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Multiple environmental and ecological controls on archaeal ether lipid distributions in saline ponds

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Supplemental tables

Table S1. Information for 11 ponds located in New Barag Left Banner of Inner Mongolian (IM for Inner Mongolia; n.d., not determined).

ID	Location	Altitude (m)	Depth (m)	Area (km ²)	pH	Salinity (%)	Na ⁺ (mg/l)	K ⁺ (mg/l)	Mg ²⁺ (mg/l)	Ca ²⁺ (mg/l)	Cl ⁻ (mg/l)	SO ₄ ²⁻ (mg/l)	HCO ₃ ⁻ (mg/l)	CO ₃ ²⁻ (mg/l)
IM1	48°15.114' N; 118°25.102' E	685	0.2	2	9.3	0.42	1400	30	16	0	1200	800	620	140
IM2	48°14.557' N; 118°26.564' E	682	0.2	n.d.	10	1.22	4400	30	15	0	2340	1360	1700	2350
IM3	48°18.785' N; 118°32.166' E	658	0.3	1.8	9.7	13.3	43000	160	70	0	46200	24200	5700	14000
IM4	48°18.850' N; 118°32.872' E	671	0.2	n.d.	9.8	0.5	1600	10	25	0	1200	800	760	500
IM5	48°14.922' N; 118°27.864' E	675	0.2	2	8.6	0.08	90	0	40	2	270	n.d.	330	40
IM6	48°53.214' N; 118°05.653' E	545	0.3	3.1	9.1	5.74	18000	30	1500	90	26000	11300	250	290
IM7	48°16.734' N; 117°39.334' E	565	0.2	n.d.	7.7	27.87	66000	306	12000	340	100000	98600	1130	n.d.
IM8	48°23.130' N; 118°42.539' E	657	0.5	7	8.8	10.43	27600	90	670	20	30000	44900	500	240
IM9	48°22.700' N; 118°49.277' E	678	0.2	1.6	9.4	0.32	900	26	30	n.d.	640	220	1000	350
IM10	48°12.591' N; 118°25.237' E	683	0.2	1.2	9.3	0.44	1460	25	50	n.d.	1120	630	780	320
IM11	48°10.722' N; 118°26.041' E	665	0.5	3.8	9.3	6.23	19000	70	20	n.d.	24000	14800	2400	1980

Table S2 Fractional abundance of isoGDGTs and isoGDGT-based proxies for surface sediments and soils of 11 ponds in Inner Mongolia

(Total Con., total concentration of isoGDGTs, isoGDGT-based proxies are referred in section 2.5).

ID	GDGT-0	GDGT-1	GDGT-2	GDGT-3	Cren	Cren'	Total Con. ($\mu\text{g/g}$ TOC)	TOC(%)	TEX ₈₆	BIT	Ri/b	Cald/Cren	Cren/Cren'	GDGT2/3	ACE
IM1	0.82	0.02	0.02	0.01	0.12	0.01	0.15	0.24	0.68	0.88	1.0	7	16	1.4	0.90
IMS1	0.87	0.02	0.02	0.01	0.08	0.00	0.05	2.04	0.68	0.84	1.9	11	18	2.2	0.07
IM2	0.74	0.03	0.03	0.02	0.17	0.02	0.1	0.94	0.68	0.82	0.9	4	10	1.6	0.87
IMS2	0.96	0.00	0.00	0.00	0.02	0.00	1	0.24	0.68	0.95	1.7	40	11	2.0	0.10
IM3	0.95	0.02	0.01	0.00	0.02	0.00	0.3	2.68	0.30	0.94	3.5	60	10	4.4	0.81
IMS3	0.64	0.05	0.04	0.02	0.24	0.01	1	0.67	0.62	0.93	0.3	3	22	1.6	0.05
IM4	0.90	0.01	0.01	0.01	0.06	0.00	3	0.20	0.64	0.95	0.7	14	13	2.0	0.59
IMS4	0.91	0.01	0.01	0.00	0.06	0.00	5	0.14	0.54	0.94	0.9	16	13	2.2	0.39
IM5	0.95	0.01	0.00	0.00	0.03	0.00	5	0.49	0.49	0.97	0.8	32	15	2.1	0.49
IMS5	0.73	0.03	0.03	0.02	0.19	0.01	44	0.37	0.63	0.96	0.2	4	22	1.8	0.04
IM6	0.63	0.08	0.07	0.02	0.19	0.01	0.4	1.33	0.58	0.79	1.2	3	14	2.9	0.97
IMS6	0.53	0.07	0.07	0.02	0.30	0.02	1	1.32	0.63	0.81	0.7	2	19	2.9	0.93
IM7	0.85	0.07	0.02	0.00	0.05	0.01	0.9	2.25	0.30	0.66	9.1	17	8	3.8	0.83
IMS7	0.26	0.06	0.07	0.03	0.54	0.03	3	0.24	0.69	0.72	0.7	0	16	2.6	0.12
IM8	0.77	0.03	0.03	0.02	0.14	0.01	8	0.55	0.66	0.87	0.8	6	12	1.4	0.73
IMS8	0.28	0.10	0.07	0.07	0.45	0.03	3	0.75	0.63	0.88	0.2	1	17	1.1	0.22
IM9	0.68	0.04	0.04	0.02	0.20	0.02	2	0.38	0.70	0.88	0.6	3	10	1.9	0.14
IMS9	0.35	0.08	0.10	0.03	0.41	0.03	3	0.54	0.64	0.78	0.6	1	15	3.8	0.07
IM10	0.99	0.00	0.00	0.00	0.01	0.00	2	0.77	0.60	0.92	8.1	101	17	1.4	0.25

IMS10	0.57	0.07	0.07	0.03	0.25	0.02	0.7	5.39	0.63	0.90	0.4	2	15	2.1	0.04
IM11	0.88	0.01	0.01	0.01	0.08	0.01	0.6	1.17	0.69	0.78	3.1	11	11	1.8	0.89
IMS11	0.31	0.06	0.07	0.02	0.49	0.04	3	0.74	0.71	0.62	1.1	1	12	3.0	0.10

Table S3 Abundance of C₂₀/C₂₀ DGD, C₂₀/C₂₅ DGD, *sn*-2- and *sn*-3-hydroxyarchaeol (µg/g TOC), δ¹³C values of archaeol, and PCR analysis of general archaea sequences (Copies/g) in pond surface sediments, and the abundance of C₂₀/C₂₀ DGD and C₂₀/C₂₅ DGD (µg/g TOC) in soils from ponds in Inner Mongolia (n.a., samples are not available).

ID	C ₂₀ /C ₂₀	C ₂₀ /C ₂₅	<i>sn</i> -2- hydroxyarchaeol	<i>sn</i> -3- hydroxyarchaeol	δ ¹³ C C ₂₀ /C ₂₀	Archaeal sequence	ID	C ₂₀ /C ₂₀	C ₂₀ /C ₂₅
IM1	5800	550	-	-	-	1.82E+09	IMS1	-	-
IM2	340	30	-	-	-33.0	1.98E+08	IMS2	-	-
IM3	550	100	1	8	-22.0	2.10E+09	IMS3	-	-
IM4	35	-	-	-	-28.9	n.a.	IMS4	19	-
IM5	110	-	13	25	-29.9	4.77E+09	IMS5	20	-
IM6	230	20	0.9	5	-19.5	2.71E+09	IMS6	430	65
IM7	295	30	0.3	0.7	-20.0	8.05E+07	IMS7	-	-
IM8	380	50	0.9	1.2	-20.7	1.14E+10	IMS8	6	-
IM9	9	-	7	5	-	n.a.	IMS9	1	-
IM10	9	-	-	-	-	n.a.	IMS10	0.8	-
IM11	10	80	0.8	0.6	-22.6	n.a.	IMS11	2	-