

1 **Effects of temperature and pH on archaeal membrane lipid distributions in freshwater**
2 **wetlands**

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13
14 **Abstract**

15 Freshwater wetlands harbour diverse archaeal communities and associated membrane
16 lipid assemblages, but the effect of environmental factors (e.g. pH and temperature) on
17 the distribution of these lipids is relatively poorly constrained. Here we explore the effects
18 of temperature and pH on archaeal core-lipid and intact polar lipid (IPL) derived core lipid
19 distributions in a range of wetlands. We focus not only on the commonly studied
20 isoprenoidal glycerol dialkyl glycerol tetraethers (isoGDGTs), but also widen our analyses
21 to include more recently identified but relatively widespread archaeal lipids such as
22 isoGDGT isomers, methylated isoGDGTs (Me-GDGTs), and butanetriol and pentanetriol
23 tetraethers (BDGTs and PDGTs). Based on multivariate analysis and a globally distributed
24 set of wetlands, we find that the degree of isoGDGT cyclisation does increase along with

25 temperature and pH in wetlands; however and unlike in some other settings, this
26 relationship is obscured in simple scatterplots due to the incorporation of isoGDGTs from
27 highly diverse archaeal sources with multiple ring-temperature or ring-pH relationships.
28 We further show that the relative abundance of early eluting to later eluting isoGDGT
29 isomers increases with pH, representing a previously unknown and seemingly
30 widespread archaeal membrane homeostasis mechanism or taxonomic signal. The
31 distribution and abundance of crenarchaeol, a marker for Thaumarchaeota,
32 demonstrates that in wetlands these Archaea, likely involved in ammonia oxidation, are
33 restricted primarily to the generally dryer, soil/sediment surface and typically are more
34 abundant in circumneutral pH settings. We identify Me-GDGTs and Me-isoGMGTs
35 (homologs of isoGDGTs and isoGMGTs, but with additional methylation on the biphytanyl
36 chain) as being ubiquitous in wetlands, but variation in their abundance and distribution
37 suggests changing source communities and/or membrane adaptation. The high relative
38 abundance of BDGTs and PDGTs in the perennially anoxic part of the peat profile
39 (catotelm) as well as their elevated abundance in a circumneutral pH wetland is
40 consistent with an important input from their only known culture source, the
41 methanogenic Methanomassiliicoccales. Our results underline the diversity of archaeal
42 membrane lipids preserved in wetlands and provide a baseline for the use of archaeal
43 lipid distributions in wetlands as tracers of recent or ancient climate and biogeochemistry.

44

45 **Keywords:** temperature; pH; GDGTs; BDGTs; Me-GDGTs; crenarchaeol; isomers;
46 Archaea; wetlands; biogeochemistry

47 Highlights

- 48 • IsoGDGT cyclisation is linked to pH/temperature, but controls are complex.

- 49 • The relative abundance of early/late-eluting isoGDGT isomers changes with pH.
- 50 • Early-eluting isoGDGT isomers can dominate (~70%) in near-neutral pH
- 51 wetlands.
- 52 • BDGT producers, possibly methanogens, are likely selected for by near-neutral
- 53 pH.
- 54 • Me-GDGTs distributions vary, reflecting changing sources/membrane
- 55 adaptation.

56

57 1 Introduction

58 Wetland sediments are unique terrestrial archives that can provide insights into climatic
59 and environmental change on land on both recent and geological timescales (Barber,
60 1993; Pancost et al., 2007; Huguet et al., 2010; Coffinet et al., 2015, 2018; Zheng et al.,
61 2015; Naafs et al., 2018b; Inglis et al., 2019). They are also key components of the global
62 carbon cycle, being the largest natural source of CH₄ to the atmosphere, a greenhouse
63 gas with 25 times the warming potential of CO₂ on a centennial time-scale (Tian et al.,
64 2015). In response to rising global temperatures, wetland CH₄ emissions are projected to
65 increase by 33-60% by 2100 (Collins et al., 2013; Wania et al., 2013; Dean et al., 2018),
66 acting as a positive feedback to anthropogenic climate change. Such methane emissions
67 are ultimately driven by diverse archaeal assemblages with key roles in the processing of
68 organic matter, notably mediating methanogenesis and the anaerobic oxidation of
69 methane (AOM) (Cadillo-Quiroz et al., 2006; Zhu et al., 2012; Andersen et al., 2013;
70 Bridgham et al., 2013; Segarra et al., 2015; Valenzuela et al., 2017).

71 Wetland environments preserve diverse archaeal lipid assemblages (Pancost and
72 Sinninghe Damsté, 2003; Pancost et al., 2003; Weijers et al., 2004; Zheng et al., 2011;
73 Naafs et al., 2018b, 2019; Yang et al., 2018) that have the potential to inform studies of
74 archaeal-mediated carbon cycle-climate dynamics both in modern and ancient settings,
75 and/or to be used as palaeoclimatic markers. In recent years an increasingly diverse suite

76 of archaeal core tetraether structures have been identified in environmental samples
77 (including peat) (Liu et al., 2012, 2016; Naafs et al., 2018a) and cultures (Bauersachs et
78 al., 2015; Becker et al., 2016), increasing the potential of lipid-focused chemotaxonomic
79 and/or functional microbial studies and opening up novel avenues for proxy development.
80 With a few notable exceptions (Weijers et al., 2004; Naafs et al., 2018b, 2018a; Yang et
81 al., 2018), many of these compounds remain poorly characterised in wetland
82 environments. Despite this, and unlike in other environments such as the open ocean
83 (Schouten et al., 2002, 2013), the main environmental and ecological drivers of archaeal
84 membrane lipid composition in wetlands - particularly with regards to core lipid types such
85 as isoGDGT isomers and Me-GDGTs - are relatively poorly constrained. This contributes
86 to an overall incomplete understanding of archaeal ecology and carbon cycling in
87 wetlands, particularly in tropical regions, and the complex relationships of such microbial
88 communities with climate and environmental change. In addition, it limits the
89 interpretation of potentially informative lipid signatures in ancient sediments and other
90 mesophilic settings.

91 The aims of this study are to examine the composition of archaeal lipids in three
92 different types of modern wetlands, and to explore the ecological and environmental
93 factors that drive differences in their distribution. We focused not only on the more widely
94 studied isoGDGTs (De Rosa and Gambacorta, 1988; Schouten et al., 2013) and their
95 chromatographically distinct earlier eluting isomers (Becker et al., 2013; Hopmans et al.,
96 2016; Liu et al., 2016) but also examined the broader archaeal tetraether lipid distribution
97 (Fig. S1 for structures) in our three main study sites. This includes i) butane-/pentane-
98 dibiphytanyl glycerol tetraethers (B-/PDGTs) that have butanetriol or pentanetriol
99 backbones instead of one of the more common glycerol moieties (Zhu et al., 2014;

100 Becker et al., 2016), ii) methyl-GDGTs (Me-GDGTs) that incorporate up to three
101 additional methyl groups on their biphytanyl chain (Knappy et al., 2015), and iii) Me-
102 GMGTs, which incorporate an extra methyl group on the biphytanyl chain as well as the
103 covalent cross-link found in regular isoGMGTs (Knappy et al., 2014). GMGTs are a lipid
104 class that were recently found to be abundant in some peats (Naafs et al., 2018a). We
105 characterised both core lipids and the acid-hydrolysed core lipid derivatives of intact polar
106 lipids (IPL-derived core lipids): IPLs are commonly used as markers for in situ, live
107 microbial cells in the environment due to their relatively rapid degradation following cell
108 lysis (White et al., 1979; Harvey et al., 1986; Lipp et al., 2008), although it has been
109 shown that they can also be preserved over longer time-scales in some settings
110 (Bauersachs et al., 2010; Logemann et al., 2011; Lengger et al., 2013, 2014; Xie et al.,
111 2013). We focused in detail on three wetland sites, coming from Sebangau (Indonesia),
112 the Florida Everglades (USA), and Tor Royal, Dartmoor (UK). These three sites constitute
113 distinct wetland types with differing physicochemical and environmental characteristics.
114 In addition, two of these sites are tropical wetland regions (i.e. the Florida Everglades and
115 Sebangau, Indonesia), a poorly studied ecosystem type in terms of lipid geochemistry.
116 As well as these three sites, we examine the composition of certain archaeal lipids
117 (isoGDGT-0-4, their isomers and crenarchaeol) in our local sites as well as a globally
118 distributed set of wetlands (Naafs et al., 2017), allowing for the identification and
119 illustration of global patterns in archaeal lipid distributions. Collectively, this study provides
120 insights into the environmental controls on archaeal lipid membrane regulation in
121 mesophilic settings such as wetlands, and provides context for future studies utilising
122 archaeal lipids to elucidate biogeochemical processes in modern and ancient wetlands.

123

124 **2 Materials and Methods**

125 2.1 Sites and Sampling

126 We primarily focused on three wetland sites. These were: Sebangau (Indonesia),
127 Everglades (USA), and Tor Royal (UK). Site details are summarised in Table 1 (with
128 additional details in Table S1). For each site, one core was analysed, though we recognise
129 that peatlands are spatially heterogenous environments and therefore each core can only
130 be considered partially representative of a particular wetland site.

131 Table 1: Geographical location and major physicochemical parameters of the three
132 primary wetland sites.

Wetland	Country	Latitude	Longitude	Pore water pH	Mean Annual Air Temperature (°C)	Reference
Sebangau	Indonesia	02° 19' 16.96" S	113° 53' 54.29" E	3.2	26.2	Könönen et al., 2015; 2016
Tor Royal	United Kingdom	50° 32' 8.44" N	3° 58' 15.51" W	4.8	8.1	Collected for this study
Everglades	United States	26° 30' 18.00" N	80° 15' 52.00" W	6.8	23	Collected for this study

133

134 *Sebangau National Park, Indonesia*

135 The Sebangau peat swamp forest covers an area of around 5000 km² that forms the
136 catchment of the Sebangau River around 200 km north of the Java Sea in south-central
137 Borneo, Indonesia (Page et al., 1999, 2004). Peat accumulation in this region began
138 around 26,000 cal. yr BP (Page et al., 2004). Whilst some logging occurred in the 1990s,
139 a portion of undrained pristine swamp forest peat remains with a mixed tropical forest
140 vegetation assemblage (Page et al., 2004; Sundari et al., 2012). Yearly average
141 temperatures in the region are 26.2 °C, and precipitation is strongly influenced by El
142 Niño/La Niña oscillations, averaging 2540 ± 596 mm per year (Sundari et al., 2012).

143 Rainfall occurs throughout the year, although there is a more pronounced wet-season
144 between November and April (Page et al., 2004).

145 A peat core measuring 1 m length was taken from a hollow in a section of
146 undrained swamp forest in March 2015 (02° 19' 16.96" S, 113° 53' 54.29" E) (as
147 detailed in Könönen et al., 2015, 2016 and at an elevation of around 18 m above sea-
148 level. The median water table depth at this site is 10.3 cm below the surface (Könönen
149 et al., 2015, 2016). The peat swamp forest is ombrotrophic and highly acidic with a
150 pH of 3.2 ± 0.3 recorded at time of sampling.

151

152 *Florida Everglades, USA*

153 The Florida Everglades covers around 6000 km² and is a predominantly freshwater
154 subtropical wetland in south Florida that has experienced peat accumulation for
155 approximately 4,000 years (Wright and Comas, 2016). Whilst the freshwater Florida
156 Everglades was originally a predominantly oligotrophic system, significant agricultural
157 run-off from the adjacent Everglades Agricultural Area during the 20th and 21st century
158 has increased nutrient levels in many areas, in particular towards the northern extent of
159 the wetland (Bae et al., 2015). Most rainfall occurs during the wet season (mid-May to
160 October), with an annual average of approximately 1400 mm (Wang et al., 2007). Mean
161 yearly temperature at a nearby weather station (Belle GL) is 23°C (Abtew et al., 2011).
162 In both oligotrophic and eutrophic areas of the Everglades, anoxic conditions tend to exist
163 at or near (~ 20 mm) the sediment surface, whilst SO₄²⁻ reduction and methanogenesis
164 are particularly enriched under eutrophic conditions (King et al., 1990; Castro et al.,
165 2004). Sulphate-rich water intrusion, primarily originating from agriculture, has been
166 shown to occur throughout the sampling area (Wang et al., 2007).

167 Sampling was conducted in May 2018 at the very end of the dry season, at a site
168 in the north-east of Water Conservation Area One (WCA-1) within the Loxahatchee
169 National Wildlife Refuge (26° 30' 18.00" N, 80° 15' 52.00" W), at an elevation of around 5
170 m above sea-level. The sampling area is dominated by Loxahatchee peat formed
171 predominantly from water lily (*Nymphaea odorata*) remains (Wright and Comas, 2016). A
172 2 m peat/sediment core was collected with a Russian corer, and core-sections were
173 immediately transported to a - 20 °C freezer at Florida Atlantic University (USA) before
174 shipment on ice to the University of Bristol, UK, where they were sub-sampled and freeze-
175 dried prior to lipid extraction. The water table was 36 cm above the peat surface at the
176 time of sampling, remaining above the sediment surface all year-round at this site. The
177 pH at the time of sampling was measured as near neutral at 6.8.

178

179 *Tor Royal, UK*

180 Tor Royal is a small domed mire situated at an altitude of 390 m within Dartmoor National
181 Park, South-West UK. It is a designated Site of Special Scientific Interest due to its
182 relatively pristine nature, which encourages the growth of many species of Sphagnum,
183 ericaceous shrubs, sedges and grasses (Amesbury et al., 2008). Peat accumulation has
184 occurred over the last ~6,000 years, with a maximum depth of 6.2 m (Charman et al.,
185 1999). Based on a distinct change in the appearance and texture of the peat, the average
186 water table depth was designated to be at ~ 30 cm below the surface. The average
187 temperature in Princetown, 2 km to the north, is 8.1 °C (Burt and Holden, 2010). Rainfall
188 is relatively consistent throughout the year, with an annual average precipitation of
189 2058 mm (Burt and Holden, 2010).

190 A core measuring 1 m length was collected in April 2018 with a Russian corer
191 from the centre of the dome (50° 32' 8.44" N, 3° 58' 15.51" W), at an altitude of around
192 391 m. The core was immediately sub-sampled in the laboratory, before freeze-drying
193 prior to analysis. Pore-water pH at time of sampling was measured as 4.8.

194

195 2.2 Lipid extraction

196 For the three new peat cores from the Indonesia, USA, and UK around 1.0 g of freeze-
197 dried and homogenized peat was extracted using a modified Bligh-Dyer protocol (Bligh
198 and Dyer, 1959). An aqueous phosphate buffer (pH = 7.2) was prepared through the
199 addition of KOH pellets to a 0.5 M aqueous KH_2PO_4 solution. A monophasic mixture was
200 subsequently made up containing methanol (MeOH), dichloromethane (DCM) and
201 phosphate buffer (PB) in the ratio of 2:1:0.8 (MeOH:DCM:PB v:v). Subsequently, 16 ml
202 of this extraction mixture was added to the freeze-dried sediment. The 16 ml g^{-1} mixture
203 used is higher than that in used in many similar studies employing Bligh-Dyer extraction
204 protocols in peat (e.g. 5 ml g^{-1} in Peterse et al., (2011) and 8 ml g^{-1} in Huguet et al.,
205 (2010)), since it has recently been demonstrated that higher solvent:sediment ratios
206 maximise extraction efficiency of prokaryotic lipids, particularly in organic rich matrices
207 such as wetland sediments or peat (see supplementary material of Chaves-Torres and
208 Pancost, (2016). The solvent-sediment mixture was capped, ultrasonicated for 15
209 minutes, centrifuged at 3000 rpm for 12 minutes, and the supernatant was collected. This
210 was repeated a total of 4 times and the supernatants were combined. The combined
211 supernatants were adjusted to a final solvent ratio of 1:1:0.9 (MeOH:DCM:PB v:v) and
212 the mixture was centrifuged at 2500 rpm for 10 minutes to separate the aqueous (MeOH
213 and PB) and lower organic phase (DCM). This was repeated a total of 4 times, and the

214 organic phases were combined before being dried by rotary evaporation to yield the total
215 lipid extract (TLE).

216

217 2.3 Processing of total lipid extract (TLE) for high performance liquid chromatography 218 – mass spectrometry (HPLC-MS)

219 A glass column was packed with 1.5 g silica gel and pre-conditioned with Hex:EtAC
220 (1:1 v/v). An aliquot of the TLE was loaded onto the column with a small amount of
221 Hex:EtAC (1:1 v/v). Following the method of Lengger et al., (2013), core lipids (CLs)
222 were eluted through with 8 ml of Hex:EtAC (1:2) and IPLs were eluted with 10 ml
223 MeOH. Both fractions were dried under a gentle flow of N₂. In order to convert IPLs
224 into their core lipid derivatives, the IPL fraction was heated with 5 ml of 5% methanolic
225 HCl for 3 hours at 70 °C, cleaving polar head-groups and forming IPL-derived CLs.
226 After allowing the solution to cool, 5 ml of double distilled water was added, and pH
227 was adjusted to 4-5 using 1 M methanolic KOH. 5 ml of dichloromethane (DCM) was
228 added, vortexed for 10 seconds, and the DCM phase was liquid-liquid extracted and
229 collected in a separate vial. This was repeated 3 more times and the DCM extracts
230 containing the IPL-derived CLs were combined before drying under a gentle stream
231 of N₂. Both IPL-derived CL and CL fractions were then re-dissolved in
232 hexane:isopropanol (Hex:IPA 99:1, v/v) and filtered using 0.45 µm PTFE filters
233 (Thermo Fisher Scientific, Rockwood, TN, USA) before analysis.

234

235 2.4 HPLC-MS

236 CL and IPL-derived CL fractions were analysed separately. They were dissolved in 100
237 µl Hex:IPA (99:1 v/v) and 15 µl of this was injected and analysed by high performance

238 liquid chromatography / atmospheric pressure chemical ionisation – mass spectrometry
239 (HPLC/APCI-MS) using a ThermoFisher Scientific Accela Quantum Access triple
240 quadrupole mass spectrometer. As detailed by Hopmans et al., (2016), analyte
241 separation was achieved in normal phase using two ultra-high performance liquid
242 chromatography silica columns (1.7 μm , 2.1 x 150 mm). The column flow rate was 0.2
243 ml min⁻¹ and compounds were eluted isocratically with eluent A (hexane:IPA 9:1 v/v) and
244 eluent B (hexane): starting with 18% eluent A for 25 minutes, followed by a 55 minute
245 gradient to 100% eluent A for 14 minutes, before decreasing to 18% in 5 minutes where
246 it was held for a further 10 minutes. Selective ion monitoring (SIM) mode was used to
247 improve sensitivity and reproducibility, targeting the protonated [M+H]⁺ adducts of
248 tetraether lipids at the following masses and at a scan time of 0.234 scans/s: m/z 1018,
249 1020, 1022, 1032, 1034, 1036, 1046, 1048, 1050, 1162, 1190, 1218, 1236, 1240, 1242,
250 1244, 1246, 1290, 1292, 1294, 1296, 1298, 1300, 1302, 1310, 1312, 1314, 1316,
251 1318, 1328, 1330.

252

253 2.5 Additional analysis of samples from global peat database

254 In addition to data from the three newly collected peat cores, we analysed the archaeal
255 core lipid distribution in a globally distributed set of peatlands (Naafs et al., 2017). Regular
256 isoGDGT data from the global peat database was previously published (Naafs et al.,
257 2018b) while the distribution of the isoGDGT isomers included here is novel. The global
258 peat database is made up of 470 samples from 96 globally distributed peatlands,
259 spanning a temperature range of - 8 to 27 °C and a pH range of between 3 - 8.
260 Temperature data was generated for all sites via a bioclimatic model, PeatStash (Kaplan
261 et al., 2003; Gallego-Sala and Prentice, 2013), whilst pH data is available for 52 of the 96

262 sites (Table S4). For full analytical details, please see Naafs et al., (2017). In short, the
263 majority of samples were extracted using microwave extraction with 20 ml of
264 dichloromethane:methanol (DCM:MeOH 9:1, v/v). The total lipid extract was re-dissolved
265 in hexane:isopropanol (99:1, v/v), filtered using 0.45 μm PTFE filters and subsequently
266 analysed via HPLC-APCI-MS, with the same conditions described in section 2.4 (though
267 this time using m/z 1302, 1300, 1298, 1296, 1294, 1292, 1050, 1048, 1046, 1036, 1034,
268 1032, 1022, 1020, 1018, 744, and 653).

269

270 **2.6 Statistical analysis**

271 Hierarchical cluster analysis (HCA) was performed in R (RStudio v.
272 1.1.453; <http://cran.r-project.org/>) to examine the associations between different relative
273 abundances of archaeal lipids identified in the three wetland sites and to explore the
274 relationship between wetland environment and archaeal lipid composition. The `hclust()`
275 function was used with a Euclidean distance metric (Jackson et al., 2009; Elling et al.,
276 2017). In order to aid visualisation, when possible, samples were rearranged in depth
277 order, providing this resulted in no fundamental change in grouping. Principal component
278 analysis was also performed in R, using the same dataset as for HCA, to further examine
279 variation and the relative weights of different lipid variables in driving clustering between
280 our three sites and depths. Prior to PCA analysis, data was rescaled so that the mean =
281 0 and standard deviation was = 1. Canonical correspondence analysis (CCA) was also
282 performed on a global dataset of specific archaeal lipid distributions. CCA is an ordination
283 technique based on the chi-squared metric, widely applied to explore the ecological
284 relationships between multiple 'species' variables (i.e. lipid relative abundances) and
285 environmental variables (in this case pH and mean annual air temperature) (Braak and

286 Verdonshot, 1995; Jiang et al., 2014; Gong et al., 2015; Borcard et al., 2018). Following
287 Borcard et al., (2018), we did not include compounds which were of very low relative
288 abundance and absent in most sites (Cren' and isoGDGT-5), due to the potential 'many-
289 zeros' skewing effect. Although some variation in pH and lipid abundance and
290 distributions are expected with depth (Naafs et al., 2017), MAAT and pH were not
291 resolved with depth in this dataset. Therefore, the site composition of lipids were
292 averaged across several depths to perform CCA. As these are not weighted averages,
293 they do not account for possible changes in absolute lipid concentrations with depth.

294 When required, a Shapiro-Wilk test was used to test for normality, with an alpha level
295 of 0.05. Following this, unpaired t-tests and non-parametric Mann-Whitney significance
296 tests were used for normally distributed and non-normally distributed data, respectively,
297 when comparing differences in lipid biomarker composition between different sites and
298 depths, with a cut-off value of $P < 0.05$.

299

300 3 Results

301 3.1 Occurrence and depth variation of CL and IPL archaeal lipids within three primary 302 wetland sites

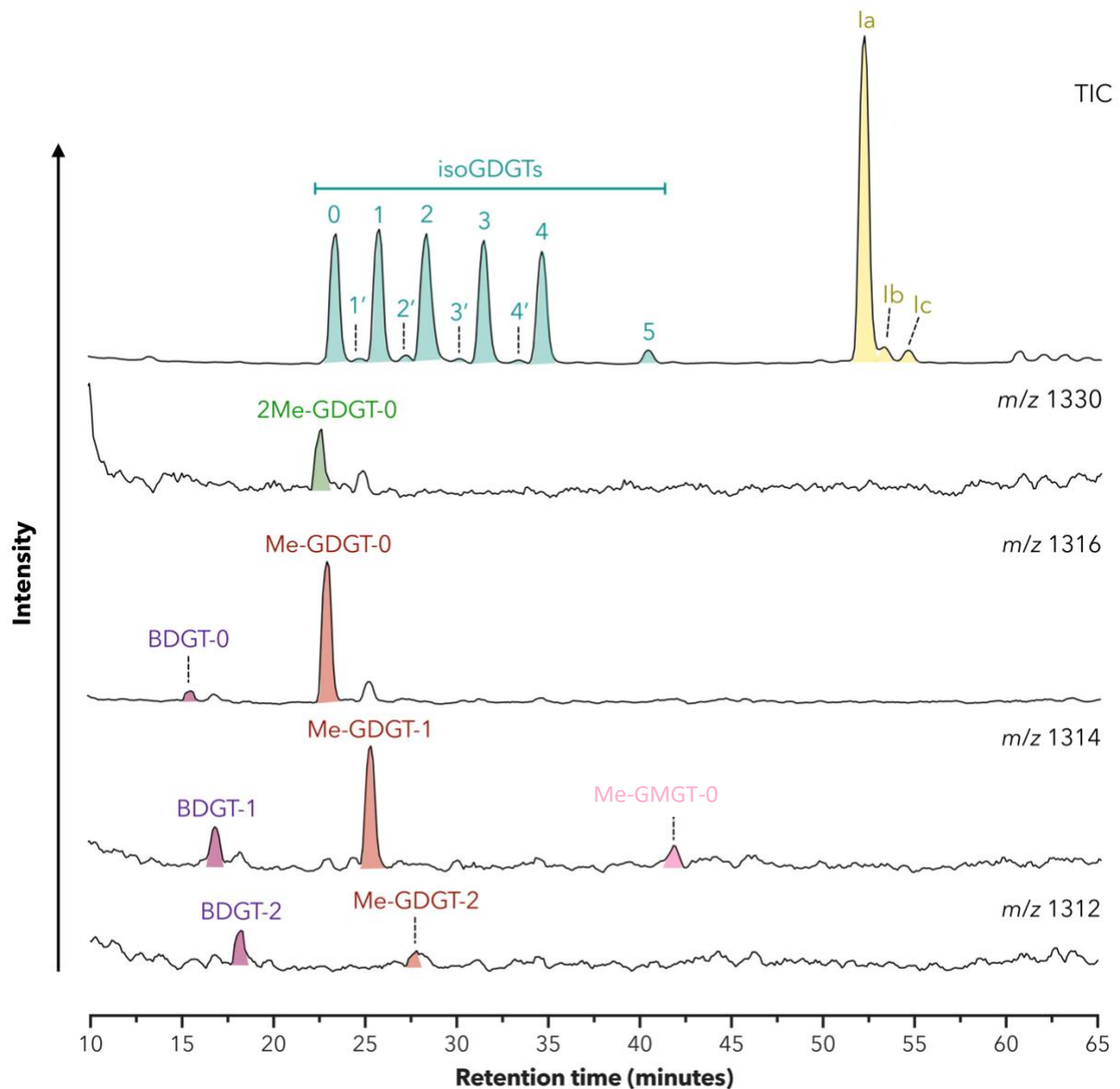
303 The relative abundance of individual compounds and classes varied both between and
304 within the three sites. Whilst we characterised both IPL-derived and core lipids, the depth
305 profiles of both lipid groups were similar, except where noted, and are therefore largely
306 referred to collectively.

307 Lipids detected at our sites include characteristic archaeal membrane lipids such
308 as the isoGDGTs, in particular isoGDGT-0, which despite varying in relative abundance
309 among sites was generally the dominant archaeal lipid (average of 54% of total archaeal

310 lipids in both CL and IPL-derived pools in all sites). IsoGDGTs 1-4 were present at all sites
311 but were much less abundant than isoGDGT-0 in the Everglades and Tor Royal. In
312 Sebangau they were typically of a similar relative abundance as isoGDGT-0 (Fig. 1). Due
313 to the consistently low abundance of crenarchaeol (which can contribute to m/z 1294),
314 we did not correct the abundance of isoGDGT-4 for the abundance of crenarchaeol in
315 our samples. IsoGDGT-5, identified recently for the first time in mesophilic settings,
316 including in other samples from the Sebangau wetland (Naafs et al., 2018b), was also
317 present above detection limits here. It was absent in the Everglades and Tor Royal.

318 Based on their mass and relative retention time, nearly all samples contained
319 earlier eluting isomers of isoGDGTs 1-4 (denoted isoGDGT-1'-4', Fig. 1), similar to that
320 seen in other environmental samples (Pitcher et al., 2009; Becker et al., 2013; Hopmans
321 et al., 2016; Liu et al., 2018; Sinninghe Damsté et al., 2018) and cultures (Sinninghe
322 Damsté et al., 2018; Bale et al., 2019). Based on this data alone, the exact structural
323 configuration of the isoGDGT isomers cannot be identified, although they could represent
324 isomers with different ring stereochemistry (Becker et al., 2013; Sinninghe Damsté et al.,
325 2018; Bale et al., 2019), or regioisomers with parallel or antiparallel glycerol
326 arrangements (Becker et al., 2013; Liu et al., 2018, 2019).

327 In addition to these relatively common isoGDGTs and their isomers, several
328 recently identified archaeal ether lipids were detected in our samples, with structures
329 inferred from their relative elution time, characteristic [M+H]⁺ ion, and comparison to
330 previous studies. These included BDGTs with up to three cyclopentane rings (Zhu et al.,
331 2014; Meador et al., 2015; Becker et al., 2016), Me-GDGTs with up to two rings (Knappy
332 et al., 2012, 2015; Zhu et al., 2014) and Me-GMGTs with up to two rings (Knappy, 2010;
333 Yang et al., 2018) (Fig. 1).



335

336 **Figure 1.** HPLC-APCI-MS total ion chromatogram (TIC) and selected ion chromatograms of the
337 tropical Sebangau wetland in Indonesia (37.5 cm depth), showing elution order of key archaeal
338 compounds discussed in the text. isoGDGT isomers, which elute just before their respective
339 cyclic isoGDGT, are distinguished from regular isoGDGTs by the presence of a prime symbol
340 ('). Me-GDGTs typically elute ~ 0.25 minutes prior to their isoGDGT homolog, whilst 2Me-
341 GDGTs elute ~0.5 minutes prior to their respective isoGDGT. Compounds la-c represent
342 bacterial branched brGDGTs (Sinninghe Damsté et al., 2000) which are not the focus of this
343 study.
344

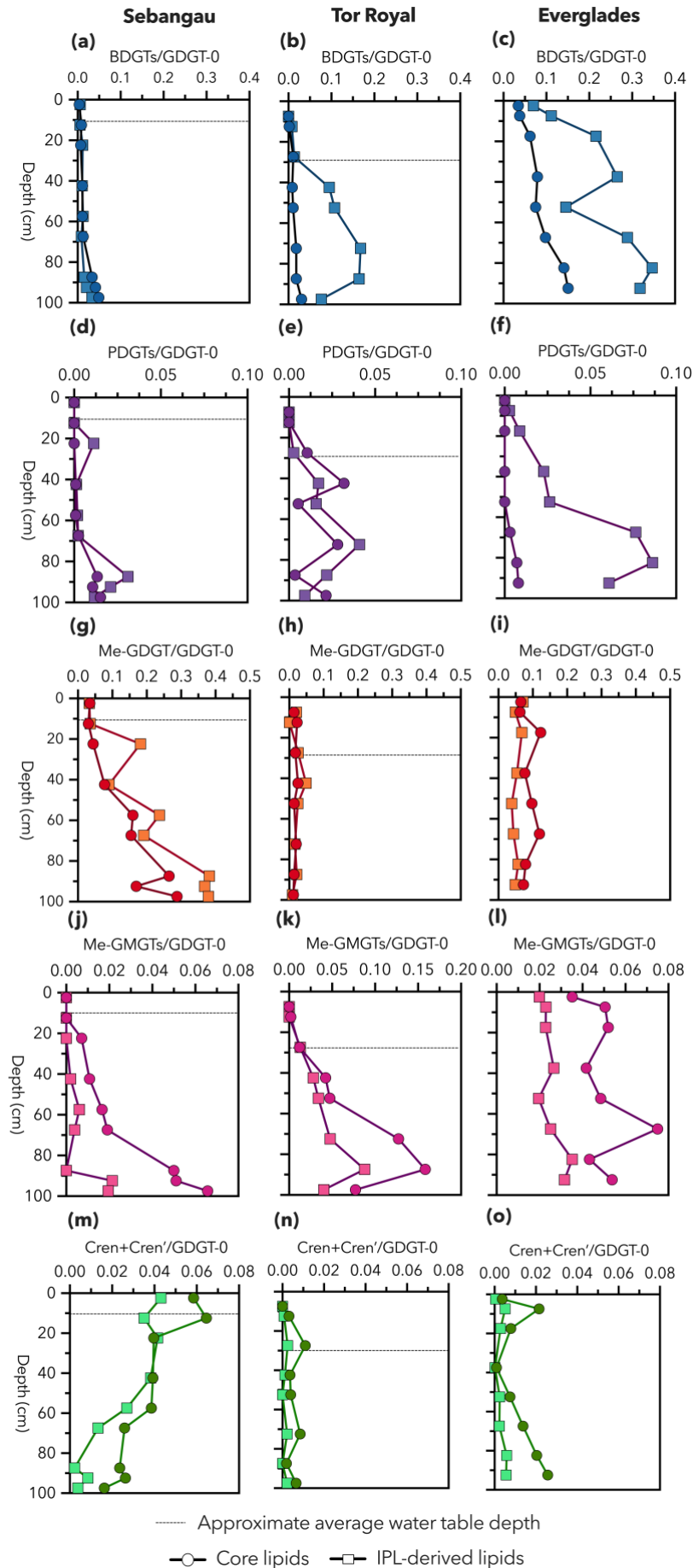
345

We visualise and analyse the compositional differences between different sites and

346

depths in detail via hierarchical cluster analysis below, but briefly summarise the depth

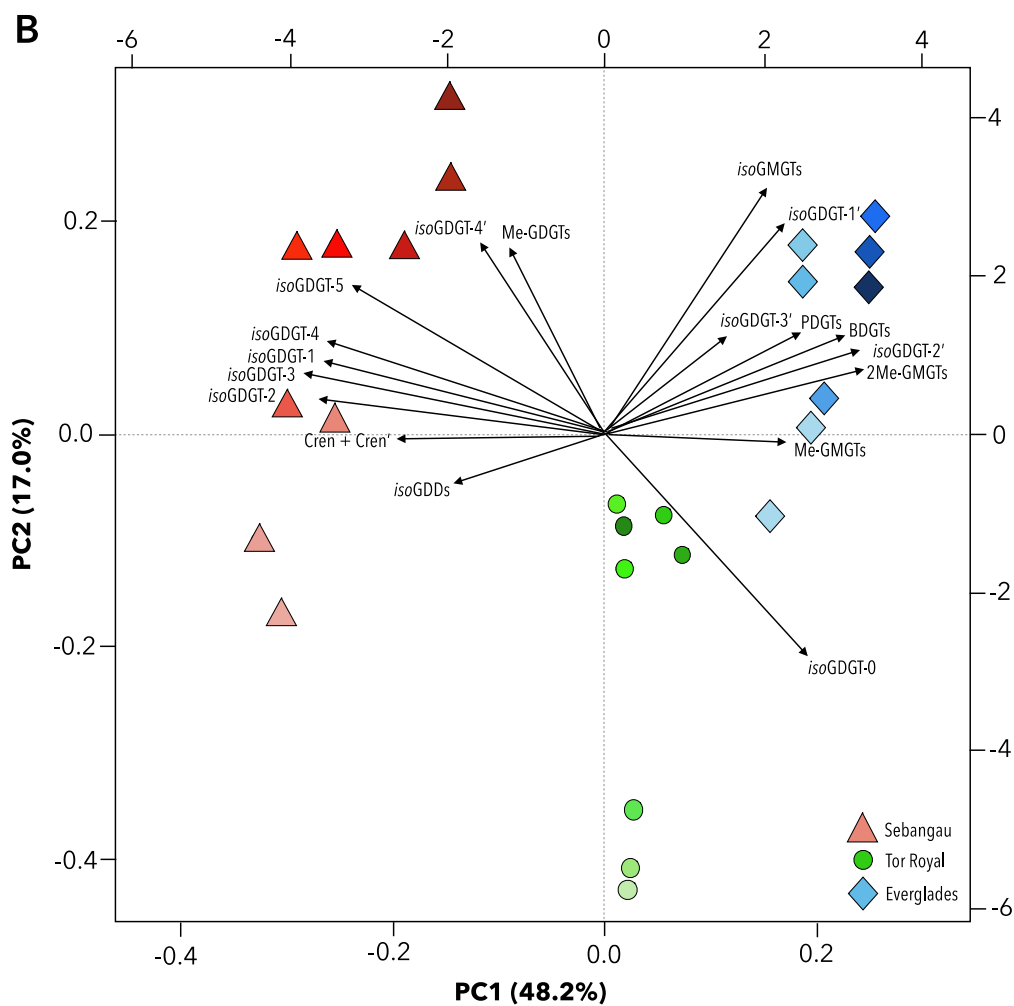
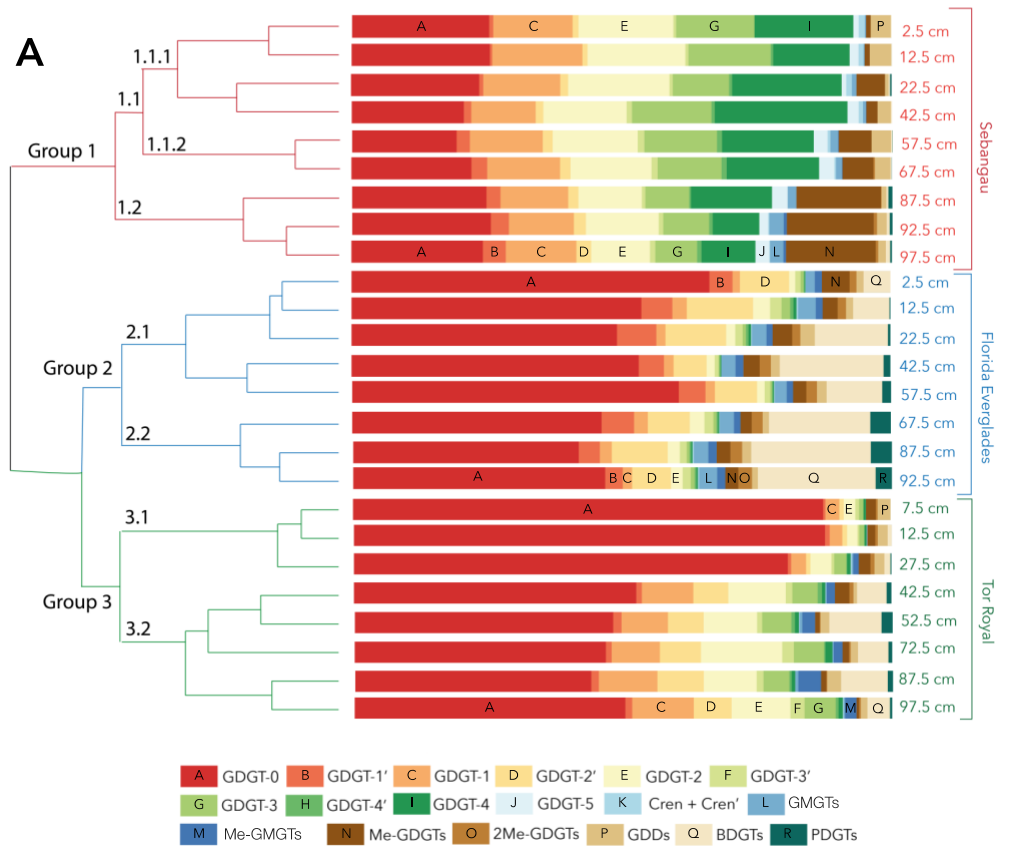
347 behaviour of key compounds here. BDGT abundances relative to those of isoGDGT-0
348 increased in the catotelm (the deeper, permanently waterlogged and anoxic part of the
349 peat column) of Sebangau and Tor Royal, and at depth in the Everglades, which is likely
350 anoxic throughout (King et al., 1990; Castro et al., 2004). IPL-derived BDGTs made up a
351 higher relative abundance of the total IPL-derived archaeal lipids than in the core lipids
352 and increased at depth particularly in this fraction (Fig. 2a-c). PDGTs exhibit similar
353 increases in abundance relative to the BDGTs with depth in all sites (Fig. 2d-f). The
354 relative abundance of Me-GDGTs also increased with depth in Sebangau, whilst staying
355 relatively stable in Tor Royal and the Everglades. In contrast, Me-GMGTs increased
356 substantially in relative abundance below the acrotelm-catotelm boundary in Sebangau
357 and Tor Royal, with only a minor increase with depth in the likely permanently anoxic
358 Everglades core (Fig. 2j-i). Crenarchaeol and its isomer (Cren'; Fig. 2m-o), which were
359 present in very low abundances in all sites, decreased with depth progressively in both
360 IPL-derived and CL fractions of Sebangau. In both Tor Royal and the Everglades, the
361 relative abundances of Crenarchaeol and Cren' showed no clear downcore trend,
362 although concentrations were just above the detection limit in these peats, potentially
363 obscuring subtle downcore trends.



365 **Figure 2.** *Depth profile of key archaeal compounds, relative to isoGDGT-0, in the three*
366 *principal wetland sites. A dotted line denotes the approximate position of the acrotelm-*
367 *catotelm boundary, whilst the core lipid and IPL-derived lipid pools are shown by circles and*
368 *squares respectively. Note the average water table depth in the Everglades site is above the*
369 *peat surface.*
370

371 3.2 Variation in relative abundance of archaeal lipids between sample sites and depths

372 We performed hierarchical cluster analysis (HCA) on both the IPL-derived (Fig. 3) and CL
373 fractions (Fig. S2). Both IPL-derived and CL fractions showed near-identical partitioning
374 in HCA. We performed this alongside a principal component analysis on the same dataset
375 (Fig 3B) which we address below. For both the HCA and PCA, we predominantly focused
376 on IPL-derived lipids below; even when accounting for long term preservation of certain
377 IPLs (Lengger et al., 2013; Chaves-Torres and Pancost, 2016), these more likely reflect
378 the actual distribution of live biomass than their core lipid counterparts (Harvey et al.,
379 1986; Lipp and Hinrichs, 2009; Buckles et al., 2013).



381 **Figure 3.** Panel A shows a hierarchical cluster analysis (HCA) dendrogram of the IPL-derived
382 archaeal lipid fractions in our three wetland sites, with relative proportion bar-plots
383 illustrating the relative proportion of different archaeal lipid groups. An analogous figure for
384 CLs can be found in the supplements (Fig. S2). Where possible, in the upper and lowermost
385 samples of each site, bars are labelled with a letter corresponding to the key. Panel B shows a
386 principal component analysis biplot for the same set of archaeal lipid relative abundances in
387 Sebangau (Indonesia), Tor Royal (UK), and Everglades (USA). Points represent different depths
388 within each site, with darkening shades of sample points corresponding to progressively
389 deeper samples within a site (e.g. light red represents shallow samples from Sebangau, whilst
390 darker red corresponds to deeper samples from the same site). See Table S3 for loadings.
391

392 In the IPL-derived lipids, the data clustered into three groups, exclusively defined
393 by wetland site: Sebangau (group 1), Florida Everglades (group 2) and Tor Royal (group
394 3). The CLs partitioned in the same way with only one exception: the sample from 2.5 cm
395 in the Everglades clustered within the same group as samples from Tor Royal, most likely
396 driven by the high relative abundance of isoGDGT-0 in this particular sample (Fig. S2),
397 which was similar to that of the shallow samples in Tor Royal.

398 Samples from Sebangau (group 1) have a characteristic distribution in which
399 isoGDGTs-1-4 are of a comparable abundance to isoGDGT-0 ($24\% \pm 2\%$), with
400 isoGDGT-4 particularly enriched in Sebangau relative to the other groups. In comparison,
401 both the Florida Everglades (group 2) and Tor Royal (group 3) are characterised by higher
402 isoGDGT-0 proportions ($52\% \pm 8\%$ and $61\% \pm 20\%$ average respectively). Sebangau
403 also has the lowest relative abundances of isoGDGT-1' to -4' isomers. In order to illustrate
404 these differences in the relative abundance of the early and late eluting isoGDGT isomers
405 between different sites, we calculated the following ratio for each sample. We chose to
406 exclude the isoGDGT-4 isomers from the calculation in order to enable comparison of the
407 ratio with other wetland sites in which isoGDGT-4 and its isomer are not present above
408 detection limits:

409

410
$$isoGDGT_{Isomer\ Index} = \frac{\sum_{1'}^{3'} isoGDGT}{\sum_{1'}^{3'} isoGDGT + \sum_1^3 isoGDGT}$$

411

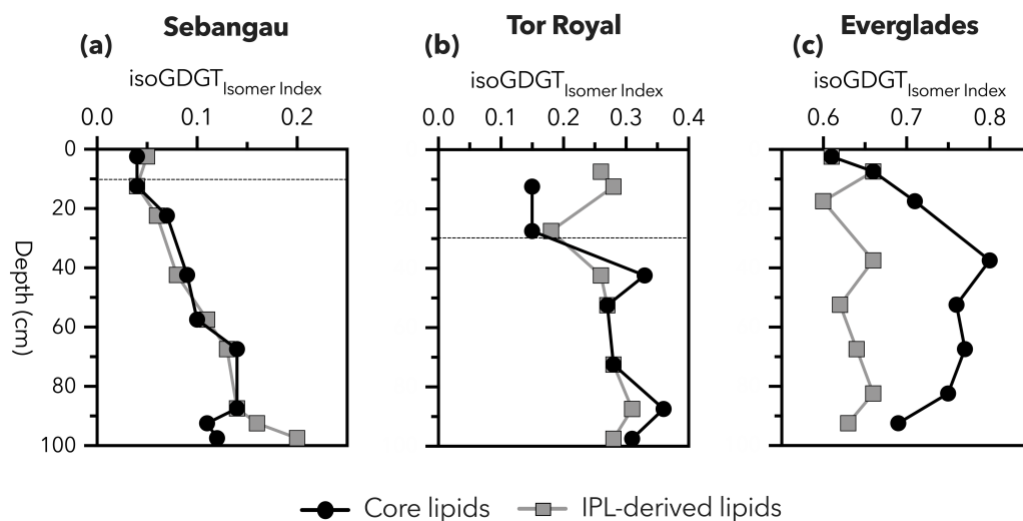
412 The averaged $isoGDGT_{Isomer\ Index}$ ratio for core lipids in Sebangau was 0.09 ± 0.04 .

413 Tor Royal and the Everglades had ratios of 0.26 ± 0.08 and 0.72 ± 0.06 , respectively.

414 This ratio is plotted against depth for each site in Figure 4. Both core lipids and IPL-derived

415 lipids had similar depth profiles, varying downcore and with a higher ratio deeper in the

416 core, particularly for CLs in the Everglades.



417

418 **Figure 4.** Depth profile of $isoGDGT_{Isomer\ Index}$ in the 3 primary wetland study sites. A dotted
419 line denotes the approximate position of the acrotelm-catotelm boundary, whilst the core
420 lipid and IPL-derived lipid pools are shown by circles and squares respectively. Note the
421 average water table depth in the Everglades site is above the peat surface.
422

423 Both the Everglades and Tor Royal contain elevated proportions of BDGTs compared to

424 Sebangau: making up $15 \% \pm 7$ of total lipids in the Everglades and $4 \% \pm 4$ of total

425 lipids in Tor Royal. The Florida Everglades are also characterised by relatively abundant

426 PDGTs, particularly at depth ($\sim 2 \% \pm 2$), as opposed to < 1.0 % in Tor Royal and

427 Sebangau).

428 The samples did further cluster by depth within our HCA analysis, but only within
429 their particular geographic locality, suggesting that depth (reflecting redox conditions) is
430 a secondary control on the archaeal lipid composition; that is to say that samples from
431 the same depth in different wetlands do not have systematically similar compositions. For
432 example, in the IPL-derived lipids of Sebangau (Fig. 3a), samples split into three distinct
433 depth groups, from between 2.5 cm – 42.5 cm (group 1.1.1), 57.5 cm – 67.5 cm (group
434 1.1.2), and 77.5 cm – 97.5 cm (group 1.2). These three groups were primarily
435 characterised by increasing relative abundances of Me-GDGTs, making up 2 % ± 2 % of
436 total archaeal lipids in shallow group 1.1.1, 6 % ± 0.5% in intermediate group 1.1.2, and
437 16 % ± 0.5% in deep group 1.2.

438 Despite anoxic conditions existing throughout the sediment profile of the
439 Everglades, archaeal lipid compositions also show clear vertical zonation: samples from
440 the top 52.5 cm (group 2.1) clustered separately from those from the bottom 25 cm (i.e.
441 67.5 cm – 92.5 cm; group 2.2). Shallow group 2.1 was predominantly characterised by
442 lower relative abundances of BDGTs (11 % ± 6 %) and PDGTs (0.7%) than in the deep
443 group 2.2: 19 % ± 3 % ($p = 0.0364$) and 3 % ± 0.5% ($p = 0.0008$), respectively.

444 Tor Royal also showed clear depth-zonation; group 3.1 contains only samples from
445 within the oxic acrotelm, whilst group 3.2 is made up uniquely of samples from the anoxic
446 catotelm. This is mainly driven by differences in the relative abundance of isoGDGT-0:
447 group 3.1 (2.5 cm – 27.5 cm) is characterised by a significantly higher average relative
448 isoGDGT-0 abundance of 86 % ± 4 %, whilst those in catotelm group 3.2 (42.5 cm – 97.5
449 cm) have average isoGDGT-0 abundance of 47 ± 3 % ($p = 0.0357$). In addition, acrotelm
450 group 3.1 has a very low relative abundance of Me-isoGMGTs and BDGTs (<1%), with

451 both compound classes increasing significantly in catotelm group 3.2 ($2\% \pm 1\%$ and 7%
452 $\pm 2\%$ respectively).

453 However, it must be noted that the effect of changes in redox state on the total
454 archaeal lipid assemblage is likely underestimated in this HCA, as we treat each individual
455 isoGDGT as an individual variable rather than grouping archaeal isoGDGTs into one
456 class. Grouping them into one class removes the effect of temperature and pH on
457 isoGDGT distribution. Indeed, when individual isoGDGTs are assimilated into one
458 compound class and HCA variable, the strength of clustering between the different
459 wetland localities is slightly weakened with deeper samples from Sebangau and Tor Royal
460 instead occupying the same cluster. This suggests that – at least in terms of the relative
461 proportion of different compound classes – depth (potentially redox state) also exerts a
462 strong influence on archaeal lipid assemblage, alongside pH and temperature.

463 As above, we further ran a principal component analysis (PCA) on the same data
464 as for the HCA, in order to further elucidate differences in archaeal lipids between our
465 three primary sites, and to gain a more quantitative understanding of the relative weight
466 of lipid variables in determining clustering (Fig. 3B and Table S3 for loadings on PC1 and
467 PC2). When considering the relative abundance of IPL-derived lipids, the first two
468 principal components explained 65.2% of the total variance (Fig. 3B). Consistent with
469 HCA, the samples were split into clusters that generally corresponded to individual
470 wetland sites. Also largely consistent with the results from HCA, depth generally affected
471 clustering, but only within sites rather than systematically across all sites, with shallower
472 and deeper samples generally clustering separately within their site-clusters. isoGDGT-
473 1-5 and Me-GDGTs were negatively associated with PC1, representing lipids that were
474 more abundant in the acidic, tropical Sebangau site. Positive scores on PC1 therefore

475 likely reflect an increase in pH. isoGDGT-1'-4', isoGMGTs, BDGTs, PDGTs and 2Me-
476 GDGTs were positively associated with PC2, representing lipids that were particularly
477 abundant in the tropical, higher pH Everglades site. isoGDGT-0 was strongly negatively
478 associated with PC2, and was particularly abundant in shallower Tor Royal samples.
479 Higher scores on PC2 could possibly reflect increasing temperature or depth, although
480 more data, particularly in colder regions, would be required to explore this further.

481

482 3.3 Globally-resolved analysis of isoGDGT distributions via Canonical Correspondance

483 Analysis

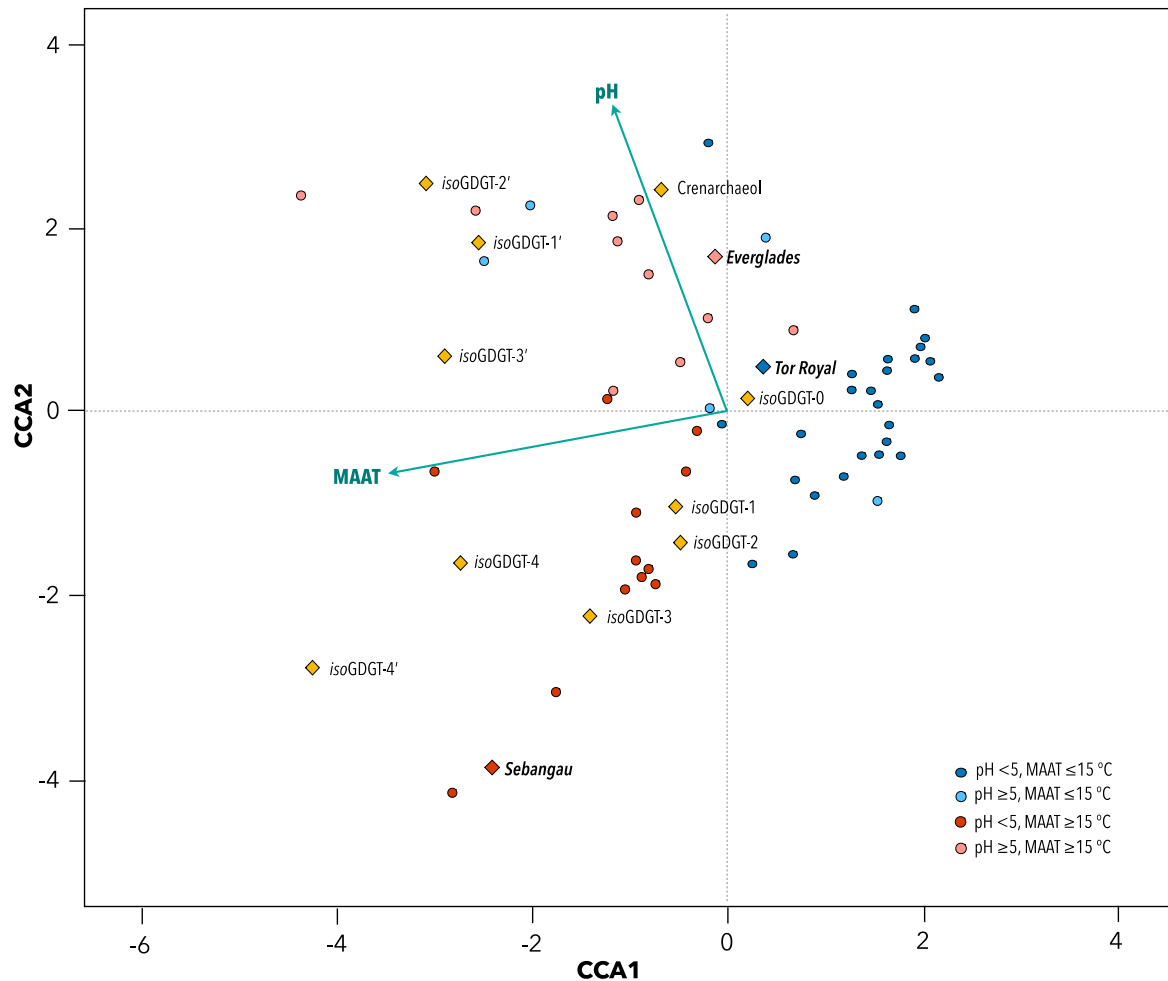
484

485 We chose to use the widespread and widely-studied isoGDGTs to place our three
486 primary study sites within a global context, and to further deconvolve the relationships
487 between pH, temperature and the distribution of archaeal membrane lipids in peat. To do
488 this we conducted a canonical correspondence analysis (CCA) algorithm (Braak and
489 Verdonschot, 1995) using the vegan 2.3-1 package in R, following Borcard et al., (2018)
490 (Fig. 5) (Oksanen et al., 2017). We performed this analysis on sites from the global
491 database of wetland core lipid distributions generated by Naafs et al., (2017, 2018b) for
492 which both pH and temperature measurements were available (Table S6), as required for
493 CCA, as well as our three additional study sites (Fig. 5) (n = 55). Low Variance Inflation
494 Factors (VIFs) of <2 for both pH and temperature demonstrate an absence of collinearity,
495 as required for CCA (Borcard et al., 2018). We used ANOVA (999 permutations) (using
496 the anova.cca function) to test (a) the overall significance of the CCA model, (b) the
497 significance of each environmental variable and (c) the significance of the CCA1 and
498 CCA2 axes. All were found to be significant ($p = < 0.005$). In order to aid visualisation of

499 our CCA biplot (Fig. 5), we sub-divided the data into four wetland 'end-member'
500 categories, broadly representing different wetland temperature and pH regimes. It must
501 be stressed that these categories were chosen to aid illustration in our CCA analysis,
502 rather than reflecting real-world thresholds. We note that other compounds which we
503 discuss in later sections (e.g. BDGTs and Me-GDGTs) likely vary widely in response to
504 changing environmental conditions and may therefore represent key differences between
505 wetlands, as suggested by our three primary study sites. Therefore, although not included
506 here, future studies should build on these findings and focus on exploring their variability
507 on a similar global scale.

508 The clustering of sites belonging to each wetland type category in our CCA
509 analysis illustrates the effect of the chosen environmental parameters on archaeal lipid
510 composition on a global scale. The three in depth study sites – Sebangau, Tor Royal and
511 the Everglades – broadly cluster with other sites in the global database (Fig. 5) also
512 assigned to their temperature/pH category, suggesting that – at least in terms of the
513 relative abundance of the compounds in this analysis – they can be considered
514 representative for their particular wetland type. In this CCA (Fig. 5), the position of each
515 lipid corresponds to its ecological optimum in relation to the respective temperature and
516 pH gradients (shown by arrowed lines). This means that possible relationships between
517 species variables (i.e. lipids) and environmental variables (i.e. temperature and pH), often
518 not visible in 2-dimensional property:property space, can be elucidated (Pearson et al.,
519 2008). To summarise what is shown in Figure 7: the relative abundance of isoGDGT-0
520 was most closely associated with the lower MAAT cluster containing Tor Royal, whilst
521 cyclic isoGDGTs, in particular isoGDGT-3 and -4, were most closely associated with
522 acidic and high MAAT wetlands, including Sebangau. These results from the global

523 database replicate trends observed in our primary three sites. Interestingly, isoGDGT-1'-
524 3' isomers showed opposing behaviour to their later eluting isoGDGT isomers, being most
525 closely associated with higher pH wetlands, such as the Florida Everglades (where early-
526 eluting isomers make up ~ 70% of all isoGDGTs 1-3). Crenarchaeol was most closely
527 associated with more alkaline wetlands.



528

529 **Figure 5.** Canonical correspondence analysis biplot showing study sites (averaged composition
530 across core) and a subset of peat database samples from Naafs et al., (2017) (See Table S6).
531 This subset corresponds to localities in the database for which both temperature and pH
532 metadata are available (a necessary condition of CCA). In this CCA, the position of each lipid
533 corresponds to its ecological optimum in relation to the respective temperature and pH
534 gradients (shown by arrowed lines). Sites have been placed in categories (see key) in order to
535 illustrate the impact of different temperature regimes and physicochemical conditions (pH) on
536 sample composition. These categories represent arbitrary boundaries in mean annual air
537 temperature and pH, and aim to aid interpretation rather than representing real-world
538 threshold conditions.

539

540 In the following sections we couple findings from this CCA with insights from our
541 three main sites to explore the relationships of these archaeal lipids with temperature, pH
542 and depth. This lipid-focused study provides constraints on the present-day global
543 distribution of Archaea in wetland sediments and provides insights into how archaeal
544 communities moderate their lipid biochemistry in response to their external environment.

545

546 4 Discussion

547 The relatively low taxonomic specificity of most archaeal lipids (de Rosa et al., 1986;
548 Schouten et al., 2013; Bauersachs et al., 2015; Elling et al., 2017) makes it challenging
549 to directly assign specific sources to compounds in environmental samples. This is made
550 more challenging by the high diversity of archaeal communities in wetlands (Cadillo-
551 Quiroz et al., 2008; Narrowe et al., 2017) and the likelihood of a significant input from
552 uncultured phyla for which lipid compositions are not known. For example, the uncultured
553 phylum Bathyarchaeota (formerly Miscellaneous Crenarchaeotal Group) can in some
554 cases be the most abundant archaeal taxa in wetlands (Narrowe et al., 2017; Bai et al.,
555 2018), but their membrane composition is unknown and so their contribution cannot be
556 easily assessed.

557 Several decades of culture and incubation experiments (De Rosa et al., 1980; Uda
558 et al., 2001; Wuchter et al., 2004; Schouten et al., 2007; Boyd et al., 2011) together with
559 data from a range of environments (Schouten et al., 2002; Pearson et al., 2008; Yang et
560 al., 2016; Naafs et al., 2018b) and biophysical and computational molecular dynamic
561 studies (De Rosa et al., 1994; Chong et al., 2005, 2012; Shinoda et al., 2005; Chuginov
562 et al., 2014; Caforio and Driessen, 2017; Huguet et al., 2017), predominantly focusing

563 on the degree of cyclisation of isoGDGTs, have shown that Archaea modify their
564 membrane compositions in order to maintain fluidity in response to changes in
565 extracellular pH and temperature. Differences between the composition of archaeal lipids
566 in different wetlands are therefore the product of both differences in archaeal community
567 and that community's adaptation to extracellular conditions, integrating both
568 accumulating in situ community production but also input from the time of deposition at a
569 given depth interval.

570 It is important to stress that we focus on relationships with pH and temperature,
571 but other variables that we have not measured (e.g., electron donor flux and energy
572 availability) can also control archaeal lipid distributions (Elling et al., 2014, 2015; Qin et
573 al., 2015; Hurley et al., 2016; Evans et al., 2018; Zhou et al., 2020). Additionally, pH in
574 particular is a 'master-variable', regulating other important geochemical characteristics
575 in wetlands which we do not directly measure such as nutrient speciation and
576 concentration. However, pH has been shown to be the main determining variable for soil
577 archaeal community composition and diversity, even between tropical and temperate
578 biomes (Tripathi et al., 2015), although climatic factors including temperature are
579 important additional determinants of microbial community composition (Delgado-
580 Baquerizo et al., 2018).

581 Our three main study sites each have a unique lipid fingerprint, with clear
582 variations between sites but also across redox boundaries (Fig. 2 - Fig. 3). This provides
583 possible insights into both the ecology of various lipid source-organisms, and/or their
584 membrane lipid adaptation to the measured environmental conditions. In the following
585 sections, we begin by discussing the isoGDGTs, their isomers and crenarchaeol,
586 contextualising our observations from our three main sites with a database of global

587 wetlands generated by Naafs et al., (2017) to examine the parallels between our local
588 observations and those of wetlands globally. Finally, we discuss the distributions of Me-
589 GDGTs and Me-GMGTs, BDGTs and PDGTs in our three primary sites.

590

591 4.1 IsoGDGTs, isoGDGT isomers and Crenarchaeol

592 Regular isoGDGTs and crenarchaeol are the most widely studied archaeal lipids, nearly
593 ubiquitous and the most abundant archaeal membrane lipids in most environments
594 (Schouten et al., 2013). Previous work has shown that the degree of cyclisation of
595 isoGDGTs is influenced by temperature and pH (De Rosa et al., 1980; Schouten et al.,
596 2002; Pearson et al., 2008; Sinninghe Damsté et al., 2012a; Qin et al., 2015; Yang et al.,
597 2016). This appears to be supported by the data from our three study sites with higher
598 proportions of isoGDGTs-1-4 at the tropical Sebangau peatland. However, in a previous
599 analysis of the global dataset of peatlands, no clear relationship between ring index (or
600 TEX₈₆) and temperature was found (Naafs et al., 2018b) (see below). Crenarchaeol
601 abundances in wetlands appear to be governed by pH but also aridity via its influence on
602 redox conditions (Zheng et al., 2015, 2018; Naafs et al., 2019), consistent with the largely
603 aerobic ammonia oxidising ecology of its Thaumarchaeota source (Pester et al., 2011).
604 There is little known about the controls on the abundance and distribution of isoGDGT
605 isomers in wetlands.

606

607 4.1.1. Distinct differences in isoGDGT distribution in global wetlands in response to 608 temperature and pH

609 The distribution of isoGDGTs is highly variable between different sites and climatic
610 and physicochemical regimes. The tropical and acidic site Sebangau is characterised by

611 a distinct distribution in which the abundances of isoGDGTs-1 to -4 are similar to
612 isoGDGT-0 (Fig. 1 and 3a). Other acidic and high temperature wetland sites share this
613 relative enrichment in the abundance of isoGDGT-1 to -4 as demonstrated by their
614 behaviour in our CCA analysis (Fig. 5). This distribution pattern is similar to that of
615 isoGDGT distributions found in acidic and high temperature terrestrial hot springs
616 (Pearson et al., 2008), with the slight dominance of isoGDGT-4 amongst the cyclic
617 isoGDGTs also reminiscent of the distribution in (hyper)thermophilic Archaea (Uda et al.,
618 2001; Sinninghe Damsté et al., 2012b). This observation is consistent with archaeal
619 membrane adaptation in acidic and high temperature environments: that is, the presence
620 of cyclopentane rings on the isoGDGT core lipid increases membrane impermeability to
621 protons, whilst also limiting the rotational freedom of the chain helping to maintain
622 appropriate membrane fluidity and stability (Dannenmuller et al., 2000; Gabriel and Lee
623 Gau Chong, 2000; Caforio and Driessen, 2017). These findings are consistent with the
624 recent identification of isoGDGT-5 – a lipid previously thought to be restricted to Archaea
625 inhabiting extremophilic environments – in acidic and tropical wetlands with a pH of < 5.1
626 and a mean annual air temperature > 19.5 °C (Naafs et al., 2018b).

627 Whilst our multivariate data show a clear link between cyclic isoGDGTs and
628 acidic/higher temperature wetlands, recent work on the same dataset of globally
629 distributed wetlands as used in this study showed no clear correlation with either pH or
630 temperature with TEX₈₆ or the Ring Index (Naafs et al., 2018b), two established molecular
631 ratios which reflect the degree of cyclisation of isoGDGTs. This has also been observed
632 for hot-spring environments, where neither index directly correlates with pH or
633 temperature (Pearson et al., 2004), though a link between cyclic isoGDGTs and low pH
634 or high temperatures is shown when multivariate methods, which take into account the

635 effect of both temperature and pH, are applied (Pearson et al., 2008). The lack of a clear
636 relationship is likely compounded in wetlands by their relatively high archaeal diversity,
637 encompassing inputs from several cultured and uncultured phyla (Pazinato et al., 2010;
638 Narrowe et al., 2017) with differing ring-temperature (and pH) relationships. This is in
639 contrast to open ocean environments, where planktonic Thaumarchaeota are purported
640 to be the dominant source (Weijers et al., 2011; Elling et al., 2015; Besseling et al., 2018)
641 and a clear relationship with temperature exists (Schouten et al., 2002). In addition,
642 wetlands are highly heterogenous systems, with sharp gradients in geochemical
643 parameters occurring over small spatial scales that can dramatically alter archaeal
644 communities (Narrowe et al., 2017). This is particularly important as several studies in
645 recent years have demonstrated that environmental parameters other than temperature
646 and pH can also affect isoGDGT cyclisation in Archaea (Elling et al., 2014, 2015; Qin et
647 al., 2015; Hurley et al., 2016; Evans et al., 2018), likely further clouding the ring-
648 temperature or ring-pH relationships. Nonetheless, our multivariate analysis
649 demonstrates that the isoGDGT distribution, and especially ring number, is responsive to
650 changes in temperature and pH on a global scale in terrestrial mesophilic settings,
651 consistent with previous work showing that isoGDGTs in soils correlate with temperature
652 in local-scale altitudinal transects (Yang et al., 2016).

653

654 **4.1.2 isoGDGT isomers respond to pH in global wetlands**

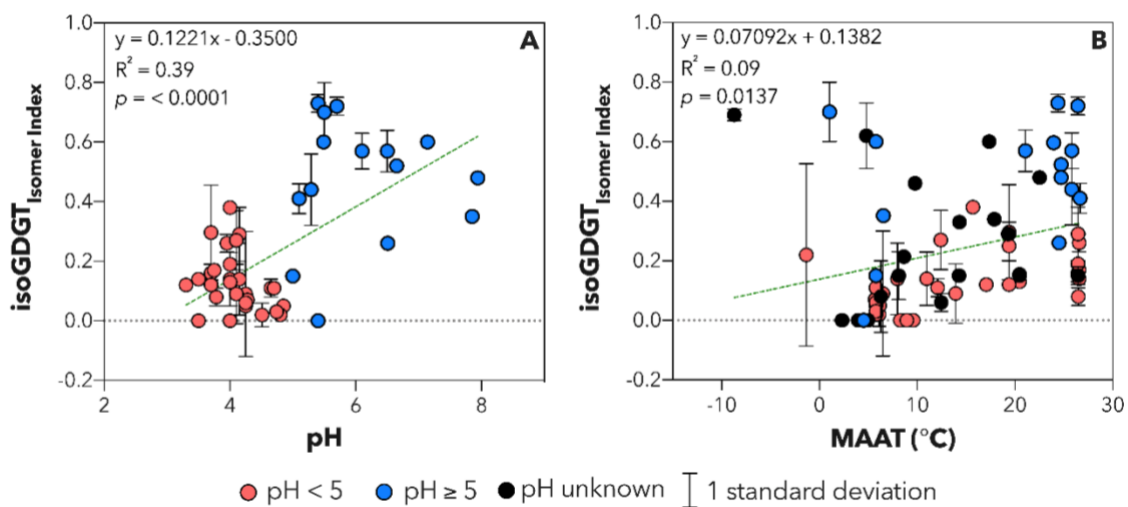
655 Analytical developments in the last two decades have revealed the existence of several
656 isoGDGT isomers in environmental samples and culture (Sinninghe Damsté et al., 2002;
657 Sinninghe Damsté et al., 2012; Becker et al., 2013; Liu et al., 2018). Theoretically,
658 isomerism in isoGDGT core lipid structures can arise in several ways, and are discussed

659 in detail in Becker et al., (2013). Possible differences include (a) regioisomeric differences
660 in the configuration of the two glycerol units, which can be parallel or anti-parallel to each
661 other (Grather and Arigoni, 1995; Becker et al., 2013; Liu et al., 2018, 2019); (b)
662 structural differences in the position of the ring(s) on the biphytane moieties (Becker et
663 al., 2013) and; (c) differences in cyclopentane ring stereochemistry (Becker et al., 2013;
664 Sinninghe Damsté et al., 2018; Bale et al., 2019). Based on HPLC-MS data alone, it is
665 not possible to detect the configuration of the isoGDGT isomers present in the wetlands
666 in this study.

667 Both core lipids and IPL-derived lipids generally show relatively minor increases in
668 the isoGDGT_{Isomer Index} ratio with depth in all three sites (except in the CLs in the perennially
669 anoxic Everglades; Fig. 4), possibly representing changing archaeal communities with
670 depth or adaptation to changing growth conditions. Clear differences in the relative
671 abundance of these isomers do exist between different sites: the higher pH Everglades
672 study site is characterised by a dominance of early eluting isoGDGT isomers compared
673 to the later eluting isoGDGTs and a high isoGDGT_{Isomer Index}, in contrast to the low pH
674 Sebangau site in which they are almost absent, and the moderate pH Tor Royal where
675 they occur in low abundances (Fig. 3a). CCA suggests similar relationships exist globally
676 (Fig. 5). Scatterplots (Fig. 6) demonstrate that the isoGDGT_{Isomer Index} is moderately and
677 significantly correlated with pH, although, based on these, no clear relationship with
678 temperature exists.

679 Whilst the precise taxonomic or physiological significance of these isomers
680 remains unknown, the correlation with pH is also consistent with a previous study
681 analysing isoGDGT distributions in soils immediately adjacent to two terrestrial hot
682 springs, in which the early eluting isomers of GDGT-1 and GDGT-2 were present in larger

683 proportions in the more alkali hot-spring soils (Pitcher et al., 2009). Moreover, a recent
684 culture study on the Thaumarchaeota *Ca. Nitrosotenuis uzonensis* also showed increases
685 in the relative proportion of early eluting isomers with increasing temperature (Bale et al.,
686 2019). Whilst there is no such clear relationship with temperature within our datasets, the
687 results of this previous study do demonstrate that changes in isoGDGT_{Isomer Index} could
688 occur a physiological advantage to certain Archaea under evolving growth conditions,
689 as may be the case with changing pH in wetlands.



690

691 **Figure 6.** *isoGDGT_{Isomer Index} plotted against pH (A) and temperature (B). See S6 for data.*

692

693 However, at present, it is not possible to tell whether our observations correspond to an
694 archaeal membrane homeostasis mechanism, or rather whether they are a taxonomic
695 signal caused by broad changes in archaeal community in different environments.
696 Previous work argued that changes in the relative abundance of Thaumarchaeota versus
697 AOM-related archaeal communities, could possibly drive differences in regioisomerism in
698 marine settings (Liu et al., 2019). This specific change in archaeal community likely does
699 not underly our observations, since crenarchaeol (see Section 4.1.3) is consistently found
700 in very low abundance in wetlands, but the influence of other community changes cannot
701 be excluded.

702 Despite the need for detailed further study, our findings in globally distributed wetlands
703 indicate that this change in isomerisation with pH represents a poorly understood yet
704 potentially widespread taxonomic or physiological signal, with potential
705 palaeoenvironmental or geobiological utility.

706

707 **4.1.3 Distinct habitat preferences of Thaumarchaeota in wetlands are revealed by the** 708 **abundance and distribution of crenarchaeol**

709 Crenarchaeol, a structurally unique isoGDGT produced by Thaumarchaeota
710 (Sinninghe Damsté et al., 2002; Elling et al., 2017), is only present at very low
711 abundances in all three of our primary study sites, and in most sites in the global peat
712 database (Table S5 and S6), suggesting that these Archaea are only minor constituents
713 of the archaeal communities in such environments (Fig. 2m-o, and Fig. 3a). This is
714 consistent with genomic evidence from wetlands that generally indicate a low abundance
715 or absence of Thaumarchaeota OTUs in wetlands (Lv et al., 2014; Narrowe et al., 2017;
716 Bai et al., 2018).

717 The depth profile of crenarchaeol and its isomer at all sites, but particularly
718 Sebangau, is consistent with other reports of lipids from Thaumarchaeota in wetlands
719 (Yang et al., 2018). Crenarchaeol is most abundant at the surface because the
720 periodically oxygenated peat surface is most likely to harbour aerobic Thaumarchaeota
721 (Stieglmeier et al., 2014), and its abundance decreases with depth (Fig. 3m).
722 Furthermore, crenarchaeol is most closely associated with more circumneutral pH
723 wetlands in our CCA analysis (Fig. 5), though not in all of them, suggesting that while pH
724 may in part regulate the abundance of Thaumarchaeota, other factors also clearly play
725 an important role. These observations are in line with the understanding that most

726 Thaumarchaeota are neutrophilic, aerobic ammonia-oxidisers, predominantly linked in
727 mesophilic terrestrial environments to dry, well-aerated soils of circumneutral pH rather
728 than waterlogged, anoxic and often acidic peat soil or wetland sediment (Stieglmeier et
729 al., 2014; Zheng et al., 2015). Therefore, it can be said that in general, Thaumarchaeota
730 are not important contributors to the wider cyclic-GDGT pool in most wetlands, and could
731 be used as a viable tracer for drying of ancient wetlands (Zheng et al., 2015).

732 In the following section, we concentrate on the distributions of Me-GDGTs, Me-
733 GMGTs, BDGTs and PDGTs in our three primary sites.

734

735 4.2 Methylated-GDGTs (Me-GDGTs) and Me-GMGTs are widespread in wetlands

736 Me-GDGTs are higher mass homologs of regular isoGDGTs, containing up to
737 three additional methylations on the biphytanyl chain and identified in culture with up to 6
738 cyclopentane rings (Knappy et al., 2015). Similarly, Me-isoGMGTs are higher mass
739 methylated homologs of the isoGMGTs (Knappy et al., 2015), a class of monoalkyl
740 tetraethers with a covalent cross link between the biphytanyl chains. Regular isoGMGTs
741 were recently identified as being abundant in peat, especially in tropical sites (Naafs et
742 al., 2018a). They have previously been identified in cultures of several methanogenic
743 Euryarchaeota: (hyper)thermophiles *Methanothermobacter thermautotrophicus* (Knappy
744 et al., 2015; Yoshinaga et al., 2015), *Methanobacter marbugensis*, *Methanosaeta*
745 *thermophila* (Bauersachs et al., 2015), and mesophiles *Methanobrevibacter smithii* and
746 *Methanosphaera stadtmanae* (Bauersachs et al., 2015). They were also detected in the
747 core lipids of the heterotrophic Euryarchaeota *Thermococcus kodakarensis* (Meador et
748 al., 2014), and in two Crenarchaeota species: *Sulfolobus acidocaldarius* and
749 *Pyrobaculum* sp. AQ1.S2 (Knappy et al., 2015). Our identification of Me-isoGDGTs and

750 Me-GMGTs in all three of our wetland sites is only the second time these compounds
751 have been identified in wetlands (Yang et al., 2018). Although they do not systematically
752 share a depth trend across the three sites, both compound classes increase relative to
753 isoGDGT-0 below the oxic-anoxic acrotelm-catotelm boundary in Sebangau and are
754 relatively stable with depth throughout the completely anoxic Everglades sediment profile
755 (Fig. 2g-l). Whilst there is a putative biosynthetic link between the Me-GDGT and
756 isoGMGT chain adaptations (Knappy, 2010; Knappy et al., 2014), 2Me-GDGTs and Me-
757 GMGTs show different behaviour than Me-GDGTs in PCA (Fig. 3B), suggesting
758 potentially different controls on their production. However, their depth trends in our
759 wetland sediments, which harbour large and diverse communities of anaerobic
760 Euryarchaeota (Cadillo-Quiroz et al., 2008; Pazinato et al., 2010; Bräuer et al., 2011;
761 Narowe et al., 2017), is broadly consistent with culture evidence that supports a
762 predominant source amongst the Euryarchaeota, possibly predominantly methanogens
763 (Knappy et al., 2015) inhabiting anaerobic niches in wetlands. This is further supported
764 by their absence above detection limits in an oxygenated mineral soil (Yang et al., 2018),
765 and their relatively high abundance in thermogenic compost soils, eutrophic lake
766 sediments and Messinian marls (Knappy et al., 2014). Me-GDGTs have additionally been
767 detected in anoxic estuarine sediments (Zhu et al., 2014), deep sub-surface marine
768 sediments from the Peru Margin (Zhu et al., 2014), and marine hydrothermal vent
769 sediments (Reeves et al., 2014).

770 The precise controls on Me-isoGDGT production are thus far unclear. Me-
771 isoGDGT-0 production was shown to increase relative to isoGDGT-0 when detergents
772 were added to *M. marbugensis* cultures (Grather et al., 2007), and when *M.*
773 *thermautotrophicus* was grown outside of its normal growth temperature (at 45 °C rather

774 than 70 °C; Knappy et al., 2015). These findings collectively suggest that additional
775 isoprenoid chain methylation could be a stress-response mechanism in certain Archaea.
776 Whilst the effect of additional methylation on the isoGDGT membrane structure is not well
777 understood, it has been suggested that chain methylation would cause a twisting of the
778 isoGDGT backbone, modifying membrane packing tightness in a similar fashion to the
779 addition of methyl groups at lower temperatures and higher alkalinities in brGDGT
780 producing Bacteria (Knappy et al., 2015). We do not see evidence for this relationship in
781 our wetland sites: the highest proportion of Me-GDGTs relative to isoGDGT-0 occurs in
782 Sebangau, our most acidic and highest temperature site. Thus, this could be a taxonomic
783 rather than physiological signal, linked perhaps to the putative enrichment of thermo-
784 and/or acidophilic Archaea at this site. However, as observed with the isoGDGTs, it is
785 possible that the level of cyclisation of Me-GDGTs also responds to temperature and pH.
786 Their relative distribution in our sites does indeed suggest a possible environmental
787 response in line with archaeal membrane regulation or changes in source community,
788 with no cyclic homologs present in our circumneutral Everglades site, and the highest
789 relative abundance of cyclic Me-GDGTs ring index in Sebangau, our highest temperature
790 and most acidic site (Table S2). More work is required to confirm this possible
791 temperature-pH dependence.

792

793 **4.3 BDGT and PDGT producers are anaerobes with a possible habitat preference for** 794 **circumneutral pH wetlands**

795 BDGTs and PDGTs are isoprenoid-based archaeal lipids that were first identified as
796 orphan lipids in estuarine and subseafloor sediments (Zhu et al., 2014). Both sets of
797 compounds were subsequently identified in a culture of the only isolate of the newly

798 identified seventh order of methanogens, *Methanomassiliicoccus luminyensis*. Screening
799 of 25 other cultured Archaea, including members of the Euryarchaeota, Crenarchaeota
800 and Thaumarchaeota, failed to detect BDGTs or PDGTs, suggesting that these
801 compounds could indeed be specific biomarkers for the Methanomassiliicoccales
802 (Becker et al., 2016). This could be a potentially unique trait alongside their distinctive H₂
803 dependent methylotrophic metabolism and energy conservation mechanisms (Becker et
804 al., 2016; Kröninger et al., 2016; Kallistova et al., 2017). However, the BDGTs have also
805 been linked to the uncultured Bathyarchaeota in estuarine sediments, based on the depth
806 correlation of IPL-BDGTs and the Bathyarchaeota 16s gene (Meador et al., 2015).
807 Moreover, more recent $\delta_{13}\text{C}$ characterisation of BDGTs suggested that they might have
808 multiple archaeal sources in marine environments (Coffinet et al., 2020). This could
809 include a mixture of autotrophic, potentially methanogenic, and heterotrophic Archaea
810 (Coffinet et al., 2020).

811 Our work is the first identification of PDGTs in wetlands and only the second of
812 BDGTs, which were recently characterised in peat from Southern China (Yang et al.,
813 2018). As is observed for the BDGTs in the Southern Chinese peat, in our peat cores
814 both compound classes become more abundant at depth, being absent or only present
815 in negligible amounts within the partially oxygenated acrotelms of Sebangau and Tor
816 Royal (Fig. 2a-f). This suggests that both BDGTs and PDGTs have a predominantly
817 anaerobic source in wetland environments. Both BDGTs and PDGTs are significantly less
818 abundant in acidic peatlands, and have the highest relative abundance in the Everglades,
819 the most minerotrophic, circumneutral pH site, with intermediate relative abundances
820 found in Tor Royal. This suggests that BDGT and PDGT producers are selected for in
821 more neutral pH environments. Consistent with these results, the optimum growth of the

822 only cultured isolate of the seventh order of methanogens that produces BDGTs and
823 PDGTs, *Methanomassiliicoccus luminyensis*, is at pH 7. In addition,
824 *Methanomassiliicoccus* were recently identified as important members of the
825 methanogen community in the Florida Everglades (Bae et al., 2015) as well as other
826 wetland types (Söllinger et al., 2016), but particularly in minerotrophic wetland soils (Yang
827 et al., 2017). This is consistent with an important input into the BDGT pool from members
828 of the *Methanomassiliicoccus* in our Everglades site. Interestingly, the higher relative
829 abundance of BDGTs in the IPL-derived fraction than in the core lipid fraction is mirrored
830 by the findings of Coffinet et al., (2020), which the authors explain could be due to their
831 preferential preservation as a result of the steric hindrance of the glycosidic bond by the
832 additional methyl group, limiting the action of extra-cellular enzymes. This could lead to
833 longer term preservation of IPL-BDGTs in sediments relative to other archaeal IPL types.

834 Whilst further work is required to validate the potential of BDGTs and PDGTs as
835 markers for *Methanomassiliicoccus* in wetlands, our results are consistent with
836 substantial input from this order. If confirmed, this finding would add to the growing body
837 of evidence that suggests *Methanomassiliicoccus* play a key but previously overlooked
838 role in the global carbon cycle, particularly in minerotrophic wetlands. It also suggests
839 that PDGTs and BDGTs could be useful to trace the contribution of this order to wetland
840 biogeochemistry.

841

842 5 Conclusions

843 We determined the relative abundances of diverse archaeal lipid types in three wetland
844 study sites, and further contextualised these, where possible, with a re-analysis of a global
845 database of archaeal lipids in wetlands. The latter broadly confirms that findings based

846 on our three in-depth study sites are representative but further global analysis is
847 necessary. We demonstrate using multivariate methods that the degree of archaeal
848 isoGDGT cyclisation does in fact vary in response to temperature and acidity in wetlands,
849 consistent with archaeal membrane homeostasis. This contrasts with findings that focus
850 on the 2-dimensional relationships of common indices such as TEX86 or Ring Index with
851 pH or temperature in global wetlands. Intriguingly, we find that the ratio of isoGDGT
852 isomers (IsoGDGT_{isomer Index}) is globally correlated to pH, likely signalling a distinct,
853 widespread and poorly understood adaptation or taxonomic signal which demands
854 further investigation. Crenarchaeol, indicative of Thaumarchaeota, is only present in small
855 proportions in almost all wetlands, and is more closely linked with mesophilic,
856 minerotrophic sites. We also focus on four main newly identified archaeal core lipid groups
857 present in our three principal wetland study sites, the Me-GDGTs, Me-GMGTs, BDGTs
858 and PDGTs, which we identify as being abundant in wetlands. In most cases these
859 compounds become more abundant at depth which suggests that they are produced
860 predominantly by anaerobes. Furthermore, the BDGTs and PDGTs have depth profiles
861 and appear to be more abundant in circumneutral wetlands, consistent with their putative
862 source, the *Methanomassiliicoccales*, highlighting the potentially key role of this newly
863 identified seventh order of methanogens in global carbon cycling. These findings provide
864 a critical context for lipid-based investigations of Archaea in modern wetland
865 environments, as well as in reconstructions of archaeal biogeochemistry and their
866 environmental/climatic context in ancient wetland sediments.

867

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888

889 **Competing Interests**

890 The authors declare that they have no competing interests.

891

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