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[Methanotroph-derived bacteriohopanepolyol \(BHP\) signatures as a function of temperature related growth, survival, cell death and preservation in the geological record.](#)

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1 **Methanotroph-derived bacteriohopanepolyol (BHP) signatures as a**
2 **function of temperature related growth, survival, cell death and**
3 **preservation in the geological record**

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23 Interpretation of bacteriohopanepolyol (BHP) biomarkers tracing microbiological
24 processes in modern and ancient sediments relies on understanding environmental
25 controls of production and preservation. BHPs from methanotrophs (35-aminoBHPs)
26 were studied in methane-amended aerobic river-sediment incubations at different
27 temperatures. It was found that: 1. With increasing temperature (4-40°C) a tenfold
28 increase in aminopentol (associated with *Crenothrix* and *Methylobacter* spp. growth)
29 occurred with only marginal increases in aminotriol and aminotetrol; 2. A further increase
30 in temperature (50°C) saw selection for the thermophile *Methylocaldum* and mixtures of
31 aminopentol and C-3 methylated aminopentol, again, with no increase in aminotriol and
32 aminotetrol. 3. At 30°C more aminopentol and an aminopentol isomer and unsaturated
33 aminopentol were produced after methanotroph growth and the onset of substrate
34 starvation/oxygen depletion; 4. At 50°C, aminopentol and C-3 methylated aminopentol,
35 only accumulated during growth but were clearly resistant to remineralisation despite cell
36 death. These results have profound implications for the interpretation of aminoBHP
37 distributions and abundances in modern and past environments. For instance, a
38 temperature regulation of aminopentol production but not aminotetrol or aminotriol is
39 consistent with and, corroborative of, observed aminopentol sensitivity to climate
40 warming recorded in a stratigraphic sequence deposited during the Paleocene-Eocene
41 thermal maximum (PETM).

42

43 **Introduction**

44 Bacteriohopanepolyols (BHPs, see supplementary information and Fig. S1) are
45 widely produced by bacteria and are generally implicated in protection from
46 environmental stress (Poralla et al., 1984; Kannenberg and Poralla, 1999; Welander and
47 Summons, 2012; Kulkarni et al., 2013). Methanotrophs have their own distinctive BHP
48 structures collectively known as the 35-aminoBHPs with an amine functionality at the

49 terminal C-35 position and variable numbers of additional hydroxyl groups (see
50 supplementary information). Increasing temperatures lead to increases in aminoBHP
51 concentration and/or variation (Jahnke et al., 1999), as does growth stage (e.g.
52 Wellander and Summons, 2012), however, understanding environmental controls of
53 aminoBHP production goes beyond simply understanding their ecophysiology.
54 AminoBHPs are apparently produced by all methanotrophs unlike other markers e.g. 4,4-
55 dimethyl sterols (Rohmer et al., 1984; Jahnke et al., 1999; Cvejic et al., 2000; Talbot et
56 al., 2001; van Winden et al., 2012; Banta et al., 2015). Such diagenetic markers can
57 trace methanotrophs in past and present global carbon and climate cycles (e.g. Talbot
58 et al., 2014; Schefuß et al., 2016).

59 Based on the geological principle that the present is the key to the past, this
60 current study aimed to provide an environmental and whole population perspective on
61 aminoBHP synthesis relatable to the geological record. Our focus on the aminoBHPs
62 and aminopentol, in particular, was based on the knowledge that these biomarkers are
63 present both in modern environments and likely preserved for millions of years (Talbot
64 et al., 2014). To this end, we have used a microcosm based approach in contrast to
65 previous studies which have focused on cultures. Specifically we have analysed
66 methanotroph sediment enrichments from the River Tyne estuary, UK, which hosts a
67 range of indigenous methanotrophic species that responded to changing environmental
68 controls (Sherry et al., 2016). Here we report temperature induced effects on
69 aminoBHP production during and after methanotroph growth at temperatures
70 realistically encountered in the Tyne and that selected for thermophiles, also identified
71 in the Tyne. The study hypothesised that environmental stresses such as temperature
72 and starvation (see supplementary information) either singly or in combination regulate
73 individual aminoBHP production profiles. The results of aminoBHP analysis of pure
74 cultures of 3 marine strains of *Methylobacter* spp. are included for comparison.

75

76 **Results and Discussion**

77 *The influence of temperature and methanotroph community on AminoBHP synthesis*

78 Sherry et al., (2016) previously demonstrated, by analysis of pMMO genes as a function
79 of imposed temperatures (4°C to 50°C), the enrichment of psychrotolerant and
80 mesophilic *Methylobacter* and *Methylomonas* spp. and a thermophilic *Methylocaldum*
81 sp. all of which are Type I methanotrophs. Follow-up analysis of 16S rRNA genes
82 generated by next generation sequencing of amplicon libraries from the same
83 microcosms but reported here for the first time (see supplementary information and Table
84 S2) confirmed this Type I dominance and temperature related succession. However, an
85 expanded inventory of Type I genera were identified, specifically; a psychrotolerant
86 *Crenothrix* sp. based on its enhanced enrichment at 4°C. *Crenothrix* spp. have been
87 previously identified in low temperature environments, for instance, cold methane seeps
88 in West Siberian river flood plains (Oshkin et al. 2014). Conversely, Type II
89 methanotrophs (*Alphaproteobacterial*, *Rhizobiales*) were absent in the CH₄ amended
90 incubations (Table S2). Type II *Beijerinckiaceae* were also absent and Verrucomicrobial
91 methanotrophs *Methylacidimicrobium* and *Methylacidiphilum*, were at very low relative
92 abundances.

93 With respect to temperature induced shifts in aminoBHP compositions, aminotriol
94 (III), by far the most abundant aminoBHP detected, was only enriched in 4-30°C
95 incubations (maximally at 21 and 30°C; Fig. 1a). The dominant methanotrophs
96 previously identified at these temperatures (Sherry et al., 2016) were from the genus
97 *Methylobacter* suggesting that such species produce significant quantities of aminotriol
98 (III) not just in pure cultures (as also observed for the closely related *Methylobacter* strain
99 BB5.1, Fig. S2c) but within a more physically, chemically and biologically complex
100 sediment setting. However, additional contributions from other microorganisms within the

101 sediment cannot be ruled out as aminotriol has a diverse range of biological sources
102 (Neunlist and Rohmer, 1985a,b, Talbot et al., 2001, 2008, 2016a; van Winden et al.,
103 2012). Aminotetrol (II) was also significantly enriched at 4-30°C albeit at lower absolute
104 concentrations (Fig. 1b) again consistent with the dominance of *Methylobacter* spp.
105 identified by Sherry et al. (2016). Little is known about BHPs in *Crenothrix*, however, a
106 metagenome (NCBI accession number PRJNA336651 for *Crenothrix polyspora* described
107 by Stoecker et al. (2006) and physically enriched from a German waterworks contained
108 two contiguous sequences identified as squalene synthetase (*ERG9*, an enzyme that
109 synthesises squalene from farnesyl diphosphate) and squalene-hopene cyclase (*sqhC*,
110 an enzyme that catalyzes the cyclization of squalene into hopene). At 40°C aminotriol
111 and aminotetrol were not enriched (Fig. 1a,b) suggesting that the different *Methylobacter*
112 species selected for (Sherry et al., 2016) produced a low abundance of this compound.
113 Likewise the thermophilic genus *Methylocaldum* at 50°C (Sherry et al. 2016 and Table
114 S2) did not produce aminotriol and aminotetrol suggesting their production is restricted
115 to lower temperatures.

116 In contrast, a temperature-dependent and, critically, linear (R^2 0.92) increase in
117 aminopentol (I) was observed between 4 and 40°C (Fig 1c). Even considering
118 differences in growth yields for Type I methanotrophs, it seems likely that this aminoBHP
119 is a physiological response to temperature expressed either by individual organisms or
120 as a part of community succession.

121 Despite a temperature dependent physiological role for aminopentol (I) there is a
122 threshold above which additional BHPs are required. The aminopentol isomer (I'; Fig.
123 1c) detected at 40°C has been described previously (van Winden et al., 2012) and found
124 in environmental settings where aminopentol is abundant relative to other BHPs (Talbot
125 et al., 2014; Wagner et al., 2014; Spencer-Jones et al., 2015). Additionally at 40°C, which
126 was dominated by a *Methylobacter* sp. not observed at other temperatures, the
127 unsaturated aminopentol (Δ I; Fig. 1d) was present in substantial quantities even though

128 only trace levels of this compound were observed in our *Methylobacter* cultures (Fig.
129 S2). It remains to be seen if unsaturated aminopentol (Δ I) is only produced by some or
130 all *Methylobacter* in substantial quantities close to their maximum temperature tolerance
131 and otherwise trace levels render it undetectable.

132 At 50°C the total amount of aminopentol-like BHPs remained broadly the same
133 but with lower levels of aminopentol (I). This temperature was dominated by
134 *Methylocaldum* (Sherry et al., 2016; Table S2) and unsaturated 3-methylaminopentol
135 (Δ IV) and particularly 3-methylaminopentol were produced (Fig. 1c,d) in substantial
136 amounts suggesting their high temperature requirement. In corroboration, the *hpnR* gene
137 (Welandar and Summons, 2012) involved in C-3 methylation of BHPs was detected (see
138 supplementary information and Fig. S3). Interestingly, a pure culture of the closely
139 related *M. szegediense* OR2 also exhibited a greater abundance of the methylated
140 homologue (IV; Cvejic et al., 2000). This pattern indicates that relative abundances
141 observed in pure culture reflect sediment signatures from methanotrophs enriched in
142 pseudo-natural settings. 3-Methylaminopentol (IV) in one replicate incubated at 40°C
143 was likely also produced by *Methylocaldum* spp. identified as a faint band in the *pmoA*
144 DGGE profiles (Sherry et al., 2016). Cultures of *Methylobacter* spp. (Fig. S2) also
145 produced C-3 methylated compounds although only at low levels. It remains to be seen
146 whether such cultures produce larger quantities of methylated compounds at their upper
147 temperature limits. The occurrence of low levels of the C-3 methylated aminopentol (IV,
148 identified in two replicates) at 60°C (Fig. 1d) with no significant methane oxidation was
149 intriguing but consistent with the identification of the *hpnR* gene at this temperature (Fig.
150 S3). A low abundance of *Methylocaldum* spp. (Table S2) implies slow/stationary growth
151 with continuing synthesis of 3-methylaminopentol in agreement with Summons et al.,
152 1994; and Welandar and Summons, 2012.

153 Overall, it appears that aminoBHPs reflect methanotroph populations and their
154 activity at a given temperature. For example, a *sphagnum* peat, known to host
155 methanotroph symbionts was incubated at 5-25°C with methane oxidation rates highest
156 around 20°C (van Winden et al., 2011). Here aminopentol was only identified between
157 15 and 25°C with the most significant increase between 20 and 25°C (van Winden,
158 2011). Aminotetrol at concentrations five times that of aminopentol showed a similar
159 temperature response but was not correlated with methanotroph activity (van Winden,
160 2011). Conversely, in cultures of the psychrotolerant methanotroph CEL 1932 (related
161 to *Methylomonas* sp.) between 10 and 35°C, a decline in aminopentol concentration was
162 observed across the entire temperature range with no change in aminotetrol
163 concentrations (Jahnke et al., 1999). The reasons for these striking differences are
164 unclear but possibly CEL 1932 was unable to grow optimally across the full temperature
165 range whereas sediment enrichments allowed for community successional adaptation.

166

167 *Microbial processes and methanotroph growth/survival in sediment enrichments as a*
168 *function of time*

169 Additional experiments in the form of sacrificial time series were performed at 30 and
170 50°C (Fig. 2). Temporal profiles of methane removal in the early phase of incubations
171 were consistent with the experiments described by Sherry et al. (2016) with rapid
172 removal of all added methane suggesting comparable methanotroph growth. After
173 methane removal headspace gas compositions diverged markedly with 50°C incubations
174 showing rapid and extensive re-emergence of methane (Fig. 2b) suggesting oxygen
175 depletion and onset of methanogenesis by day 11 (cf. Gray et al. 2002). Oxygen
176 depletion also occurred at 30°C microcosms but the evidential methane re-emergence
177 was slower and less extensive. Regardless of temperature this oxygen depletion cannot
178 be ascribed wholly to consumption by aerobic methanotrophs since their metabolism
179 requires an oxygen to methane molar ratio of 2:1 compared to the actual headspace ratio

180 at time zero of 4:1. Consequently, it can be deduced that surplus oxygen was partly
181 consumed by the oxidation of indigenous organic matter (OM) present in the
182 microcosms. Indigenous OM also likely fuelled subsequent production of methane.
183 Disregarding the fate of the biomass enriched during methane oxidation (discussed in
184 detail below) a reason for the earlier and more extensive methane production in both the
185 amended and unamended incubations at 50°C compared to 30°C may be an increased
186 bio-availability of OM at higher temperatures (Parkes et al. 2014). Specifically, lysis of
187 indigenous mesophilic cells present in the sediment termed 'necromass' likely
188 augmented by thermal activation of sediment macromolecular OM at higher
189 temperatures (Parkes et al. 2014). Enhanced methanogenesis at high temperatures (50-
190 70°C) without carbon amendment has been observed for Tyne sediments (Bell, 2016).

191 Regardless of the contribution of thermally activated OM to methane under
192 anaerobic conditions, this indigenous OM cannot explain the higher methane yields in
193 the methane amended enrichments relative to the unamended controls since both
194 treatments comprised the same sediment material. An obvious explanation is the
195 decomposition of methanotroph cell biomass generated during the methane
196 consumption phase. Certainly, the temporal pattern of *pmoA* amplicon intensities (as a
197 proxy for methanotroph biomass growth and degradation) indicated an accumulation of
198 *pmoA* up to day 11 and its decline thereafter. This rise and fall of *pmoA* was contrary to
199 the pattern observed in the 30°C experiments which indicated a steady accumulation of
200 *pmoA* template up to day 11 after which intensities were broadly maintained. It has long
201 been recognised (Tanner and Wallace 1925; Imšenecki and Solnzeva 1945) that
202 thermophiles (in this case most likely thermophilic *Methylocaldum* sp., Sherry et al. 2016)
203 die-off more quickly than their mesophilic counterparts after substrate exhaustion even
204 when grown at their optimum temperature.

205 Rapid die-off and subsequent biomass degradation might, therefore, account for
206 the methane observed after day 11 in the high temperature amended experiments,

207 however, a simple calculation based on literature reported growth yields for Type I
208 methanotrophs e.g. *Methylomagnum ishizawai* (340 mg_(dry mass) mole_(CH₄)⁻¹; Khalifa et al.
209 2015) indicated that this re-cycled biomass would have been inconsequential. This
210 methane, is however, explicable in the context of sediment 'priming effects' (Bianchi
211 2011) which refers to the empirically observed enhanced remineralization of less
212 bioavailable organic matter on the addition of bioavailable substances, a phenomenon
213 widely recognised in soil science, the mechanism of which is not fully understood
214 (Bianchi, 2011). Soil priming experiments have shown CO₂ evolution from indigenous
215 OM markedly increased after plant residue addition (Blagodatskaya and Kuzyakov
216 2008). The priming of a marine sediment with algal biomass increased levels of
217 background remineralization under anoxic conditions (van Nugteren et al., 2009).

218

219 *The effect of mesophilic methanotroph growth, survival and death on aminoBHPs*

220 Aminotriol production in 30°C time series experiments was broadly consistent
221 with analogous 30°C shorter-term experiments, namely, a moderate enrichment (Fig. 3a,
222 Table S1b), However, enrichment actually occurred in the 'early stationary phase' 7 to
223 15 days after methane consumption (fig. 2). One interpretation is a synthesis of additional
224 aminotriol putatively by *Crenothrix* and *Methylobacter* spp. adapting to substrate
225 depletion. However, production from aerobic heterotrophs (Talbot et al., 2008)
226 demonstrably active in these incubations cannot be excluded.

227 In contrast, aminotetrol and to a greater extent aminopentol, were produced in
228 progressively greater amounts during incubation throughout both methane consumption
229 and stationary growth phases (Fig. 3b,c) supportive of a physiological response to
230 increasing temperatures, the onset of starvation or, both for aminopentol synthesis. This
231 interpretation of the data was tentatively supported, albeit using a non-quantitative
232 endpoint PCR approach, by the apparent accumulation of *pmoA* up to day 11 (see
233 supplementary information and Fig. S4). After this *pmoA* was, apparently, broadly

234 maintained providing evidence of prolonged cell survival. In support of multiple drivers of
235 aminoBHP production, the aminopentol isomer only detected at 40°C in the shorter
236 incubations was detected in the longer lower temperature experiments (Fig. 3c) after a
237 7 day lag phase and substrate exhaustion (fig 2). An even longer lag coincident with
238 oxygen depletion was required before the detection and accumulation of the unsaturated
239 aminopentol and 3-methylaminopentol was detected in one replicate at day 20 (data in
240 Osborne, 2016).

241 These environmental effects have not been reported previously but are certainly
242 consistent with culture studies suggesting BHPs play an important role in maintaining
243 cell homeostasis under environmental stress and stationary phase. For
244 *Rhodopseudomonas palustris* TIE-1, the deletion of the squalene-hopene cyclase
245 (*sqhC*) gene required for the biosynthesis of hopanoids (Wendt et al., 1997a,b), resulted
246 in increased sensitivity to pH extremes particularly in the stationary phase (Welander et
247 al., 2009). In *Streptomyces coelicolor* A3(2) hopanoids were not produced in liquid
248 culture but were on solid medium when sporulating; a response hypothesised to protect
249 spores by decreasing cell membrane water permeability (Poralla et al., 2000). BHP
250 production by the cyanobacterium *Nostoc punctiforme* in response to N and P limitation
251 found higher levels of BHPs (Doughty et al., 2009). Phosphorous limitation had the
252 greatest effect after 3 weeks of starvation. Hopanoid levels 34 times that of vegetative
253 cells were found in the outer membrane of cells. Intriguingly these cells were
254 differentiated into thick walled akinete survival structures (Doughty et al., 2009).

255

256 *The effect of thermophilic growth, survival and death on aminoBHP synthesis*

257 Temperature limits for aminotriol and aminotetrol production were confirmed and
258 extended in the 50°C time series experiments (Fig 4 a, b). These experiments also
259 confirmed the high temperature enrichment of a consistent mixture of aminopentol-like
260 BHPs namely, aminopentol, 3-methylaminopentol and unsaturated 3-methylaminopentol

261 during growth (Fig. 4c). At 4 days, comparable to the short-term experiments, absolute
262 quantities of these aminoBHPs were almost identical suggesting their composition is
263 highly regulated under specific conditions. However, in contrast to lower temperatures
264 there were no significant increases after methane removal (Fig. 4c). This cessation of
265 BHP production is likely the result of cell death as inferred by the coincident 'priming' of
266 methane production from the breakdown of biomass (Fig 2). This interpretation of
267 quantitative data was tentatively, albeit non-quantitatively, supported by an endpoint
268 PCR of *pmoA* which indicated an apparent decline of after methane consumption (Fig.
269 S4b).

270 High temperature growth-associated production, but not stationary phase
271 production of 3-methylaminopentol putatively by *Methylocaldum* are interesting because
272 previously, and apparently contradictorily, it has been suggested that 3-methylhopanoid
273 production may be related to growth stage. Summons et al. (1994) identified 3-
274 methylhopanoids in the late stationary phase of growth and more recently, Welander and
275 Summons (2012) demonstrated in *Methylococcus capsulatus* Bath grown at 37°C, its
276 potential physiological role in the maintenance of intracytoplasmic membranes (ICM) and
277 late stationary phase survival as cysts. This apparent contradiction may be resolved
278 when the interplay of multiple environmental stresses are considered. For instance, it is
279 likely that temperature and substrate availability in combination determine the
280 physiological response of methanotrophs. Whereas starvation and the onset of anoxia
281 at 50°C led to rapid cell death, starvation at lower temperatures might have led to survival
282 via aminoBHP synthesis. It has been previously proposed that the maintenance of ICM
283 may aid survival under low oxygen (Welander and Summons, 2012).

284 A final interesting point about high temperatures and the apparent death of
285 methanotrophs by substrate exhaustion is that aminoBHPs appear to have survived
286 intact (Fig. 4) despite biomass degradation, oxygen depletion and ultimately

287 methanogenesis. This persistence attests to their recalcitrance in sediments and survival
288 in the geological record relative to more labile compounds.

289

290 *Implications for hopanoid distributions in the environment and geological records*

291 AminoBHPs are found in soils, wetlands, lakes, river, estuarine and marine
292 sediments across different climate regions (Talbot et al. 2016a and references therein).
293 Aminotetrol, aminopentol and C-3 methylated aminoBHPs in particular, are of interest in
294 distinguishing Type I and II methanotrophs and, critically, are used to identify sites of
295 intense aerobic methane oxidation and the dispersal of materials from such locations.
296 For instance, high aminopentol concentrations measured in the Congo and Amazon
297 deep-sea fans have been interpreted as originating from the continent reflecting the
298 persistent delivery over geological timescales of terrestrial organic carbon to these
299 sediments (Talbot et al., 2014; Wagner et al., 2014; Spencer-Jones et al., 2016; Schefuß
300 et al., 2016).

301 Critically, it is clear that a fundamental understanding of environmental controls
302 on aminoBHP production is needed to fully interpret palaeoenvironmental records of
303 these biomarkers (Talbot et al. 2016a). For instance, the identification of temperature
304 regulated production of aminopentol can be used to re-interpret aminoBHP patterns in
305 the Cobham lignite. The Cobham lignite is a terrestrial lacustrine/mire sedimentary
306 sequence in southern England which spans the Palaeocene–Eocene Thermal Maximum
307 (PETM). The PETM is the most extreme warming event in the last 55 million years where
308 central-western European mean annual air temperature averages rose to 23-26°C (Inglis
309 et al. 2017) relative to current means for the UK of 8-11°C. Isotopically depleted
310 hopanoids measured in the Cobham lignite (Pancost et al. 2007) have previously
311 suggested an increase in the methanotroph population at the onset of the PETM in this
312 local, driven by changes to a warmer, wetter and methane rich environment. Talbot et
313 al. 2016a, subsequently reported a correspondence between negative ¹³C carbon

314 excursions indicative of high methane and abundances of aminopentol but not
315 aminotetrol (as a proportion of total biohopanoids) and reported generally higher levels
316 of aminopentol in lignite deposited during the PETM. Talbot et al. 2016a suggested that
317 these BHP patterns recorded environmental change with a potential shift from the
318 dominance of Type II (indicated by the presence of aminotetrol and absence of
319 aminopentol) to Type I methanotrophs (additional presence of aminopentol) during
320 periods of intense methane cycling. A caveat given by Talbot et al., however, was the
321 recent laboratory finding of Sherry et al. 2016, that changes in methanotrophic
322 community composition are not induced by differences in methane concentration. This
323 contradiction can be resolved with the conclusion that, regardless of the intensification
324 of methane oxidation during the PETM, the abundance of aminopentol relative to other
325 aminoBHPs was principally regulated by a response to temperature. The wider
326 implications of these results is that interpretation of aminoBHP relative abundances in
327 modern environments and in the geological record should only be made in the context of
328 measured temperatures or temperature proxies.

329 Studies of aminoBHP production as a function of substrate availability, redox and
330 growth phase (Figs. 3 and 4) further emphasises the need for a wider understanding of
331 depositional conditions and processes. For instance, aminopentol production at 30°C
332 after methane removal suggests that its detection does not, necessarily, represent
333 periods of persistent methane oxidation but instead highly variable methane conditions,
334 such as typically encountered at seafloor methane seeps (Valentine, 2011). Highly
335 variable methane flux trends may be a common feature of many methanogenic
336 environments due to periodic changes in hydrology and atmospheric pressure changing
337 redox conditions or gas flow. For instance, in a UK landfill site biogenic methane was
338 found to be absent in the ground gas for 70% of the time and methane flux correlated
339 closely with atmospheric pressure (Teasdale *et al.* 2014).

340 At high temperatures, 3-methyl aminoBHP production only occurred during active
341 methane oxidation (Fig. 4) rather than in the late stationary phase as identified by
342 Welander and Summons (2012) which underlies the importance of a paleo-
343 environmental context. C-3 methylated hopanes and other geohopanoids are regularly
344 reported in ancient settings (e.g. Collister, 1992; Farrimond et al., 2004; Birgel and
345 Peckmann, 2008; Talbot et al., 2016a) but reports of 3-methyl aminoBHPs are rare.
346 Intriguingly they include: a neo-volcanic, eutrophic, shallow saline lake sediment (Talbot
347 et al., 2003; Talbot and Farrimond 2007), a geothermal microbial mat (Zhang et al., 2007)
348 and a geothermal silica sinter deposit (Gibson et al., 2008) which suggest a high
349 temperature control on their production as identified in our laboratory experiments.
350 However, potentially they are also produced at lower temperatures in response to
351 starvation and oxygen depletion as described by Welander and Summons (2012) and
352 here observed for one microcosm replicate after long-term incubation. 3-Methyl
353 aminotriol has been observed in some soils, primarily from temperate settings (Cooke,
354 2010; Talbot et al. 2016b; Zhu et al., 2011) indicating their occasional production under
355 mesophilic conditions.

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362

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