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

**Introduction**

Bacteriohopanepolyols (BHPs, see supplementary information and Fig. S1) are widely produced by bacteria and are generally implicated in protection from environmental stress (Poralla et al., 1984; Kannenberg and Poralla, 1999; Welander and Summons, 2012; Kulkarni et al., 2013). Methanotrophs have their own distinctive BHP structures collectively known as the 35-aminoBHPs with an amine functionality at the
terminal C-35 position and variable numbers of additional hydroxyl groups (see supplementary information). Increasing temperatures lead to increases in aminoBHP concentration and/or variation (Jahnke et al., 1999), as does growth stage (e.g. Wellander and Summons, 2012), however, understanding environmental controls of aminoBHP production goes beyond simply understanding their ecophysiology. AminoBHPs are apparently produced by all methanotrophs unlike other markers e.g. 4,4-dimethyl sterols (Rohmer et al., 1984; Jahnke et al., 1999; Cvejic et al., 2000; Talbot et al., 2001; van Winden et al., 2012; Banta et al., 2015). Such diagenetic markers can trace methanotrophs in past and present global carbon and climate cycles (e.g. Talbot et al., 2014; Schefuß et al., 2016).

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

The influence of temperature and methanotroph community on AminoBHP synthesis

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

With respect to temperature induced shifts in aminoBHP compositions, aminotriol (III), by far the most abundant aminoBHP detected, was only enriched in 4-30°C incubations (maximally at 21 and 30°C; Fig. 1a). The dominant methanotrophs previously identified at these temperatures (Sherry et al., 2016) were from the genus *Methylobacter* suggesting that such species produce significant quantities of aminotriol (III) not just in pure cultures (as also observed for the closely related *Methylobacter* strain BB5.1, Fig. S2c) but within a more physically, chemically and biologically complex sediment setting. However, additional contributions from other microorganisms within the
sediment cannot be ruled out as aminotriol has a diverse range of biological sources (Neunlist and Rohmer, 1985a, b, Talbot et al., 2001, 2008, 2016a; van Winden et al., 2012). Aminotetrol (II) was also significantly enriched at 4-30°C albeit at lower absolute concentrations (Fig. 1b) again consistent with the dominance of *Methylobacter* spp. identified by Sherry et al. (2016). Little is known about BHPs in *Crenothrix*, however, a metagenome (NCBI accession number PRJNA336651 for *Crenothrix polyspora* described by Stoecker et al. (2006) and physically enriched from a German waterworks contained two contiguous sequences identified as squalene synthetase (*ERG9*, an enzyme that synthesises squalene from farnesyl diphosphate) and squalene-hopene cyclase (*sqhC*, an enzyme that catalyzes the cyclization of squalene into hopene). At 40°C aminotriol and aminotetrol were not enriched (Fig. 1a, b) suggesting that the different *Methylobacter* species selected for (Sherry et al., 2016) produced a low abundance of this compound. Likewise the thermophilic genus *Methylocaldum* at 50°C (Sherry et al. 2016 and Table S2) did not produce aminotriol and aminotetrol suggesting their production is restricted to lower temperatures.

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

Despite a temperature dependent physiological role for aminopentol (I) there is a threshold above which additional BHPs are required. The aminopentol isomer (I'; Fig. 1c) detected at 40°C has been described previously (van Winden et al., 2012) and found in environmental settings where aminopentol is abundant relative to other BHPs (Talbot et al., 2014; Wagner et al., 2014; Spencer-Jones et al., 2015). Additionally at 40°C, which was dominated by a *Methylobacter* sp. not observed at other temperatures, the unsaturated aminopentol ($\Delta$I; Fig. 1d) was present in substantial quantities even though...
only trace levels of this compound were observed in our Methylobacter cultures (Fig. S2). It remains to be seen if unsaturated aminopentol ($\Delta I$) is only produced by some or all Methylobacter in substantial quantities close to their maximum temperature tolerance and otherwise trace levels render it undetectable.

At 50°C the total amount of aminopentol-like BHPs remained broadly the same but with lower levels of aminopentol (I). This temperature was dominated by Methylocaldum (Sherry et al., 2016; Table S2) and unsaturated 3-methylaminopentol ($\Delta IV$) and particularly 3-methylaminopentol were produced (Fig. 1c,d) in substantial amounts suggesting their high temperature requirement. In corroboration, the hpnR gene (Welander and Summons, 2012) involved in C-3 methylation of BHPs was detected (see supplementary information and Fig. S3). Interestingly, a pure culture of the closely related M. szegediense OR2 also exhibited a greater abundance of the methylated homologue (IV; Cvejic et al., 2000). This pattern indicates that relative abundances observed in pure culture reflect sediment signatures from methanotrophs enriched in pseudo-natural settings. 3-Methylaminopentol (IV) in one replicate incubated at 40°C was likely also produced by Methylocaldum spp. identified as a faint band in the pmoA DGGE profiles (Sherry et al., 2016). Cultures of Methylobacter spp. (Fig. S2) also produced C-3 methylated compounds although only at low levels. It remains to be seen whether such cultures produce larger quantities of methylated compounds at their upper temperature limits. The occurrence of low levels of the C-3 methylated aminopentol (IV, identified in two replicates) at 60°C (Fig. 1d) with no significant methane oxidation was intriguing but consistent with the identification of the hpnR gene at this temperature (Fig. S3). A low abundance of Methylocaldum spp. (Table S2) implies slow/stationary growth with continuing synthesis of 3-methylaminopentol in agreement with Summons et al., 1994; and Welander and Summons, 2012.
Overall, it appears that aminoBHPs reflect methanotroph populations and their activity at a given temperature. For example, a *sphagnum* peat, known to host methanotroph symbionts was incubated at 5-25°C with methane oxidation rates highest around 20°C (van Winden et al., 2011). Here aminopentol was only identified between 15 and 25°C with the most significant increase between 20 and 25°C (van Winden, 2011). Aminotetrol at concentrations five times that of aminopentol showed a similar temperature response but was not correlated with methanotroph activity (van Winden, 2011). Conversely, in cultures of the psychrotolerant methanotroph CEL 1932 (related to *Methylomonas* sp.) between 10 and 35°C, a decline in aminopentol concentration was observed across the entire temperature range with no change in aminotetrol concentrations (Jahnke et al., 1999). The reasons for these striking differences are unclear but possibly CEL 1932 was unable to grow optimally across the full temperature range whereas sediment enrichments allowed for community successional adaptation.

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

Additional experiments in the form of sacrificial time series were performed at 30 and 50°C (Fig. 2). Temporal profiles of methane removal in the early phase of incubations were consistent with the experiments described by Sherry et al. (2016) with rapid removal of all added methane suggesting comparable methanotroph growth. After methane removal headspace gas compositions diverged markedly with 50°C incubations showing rapid and extensive re-emergence of methane (Fig. 2b) suggesting oxygen depletion and onset of methanogenesis by day 11 (cf. Gray et al. 2002). Oxygen depletion also occurred at 30°C microcosms but the evidential methane re-emergence was slower and less extensive. Regardless of temperature this oxygen depletion cannot be ascribed wholly to consumption by aerobic methanotrophs since their metabolism requires an oxygen to methane molar ratio of 2:1 compared to the actual headspace ratio.
at time zero of 4:1. Consequently, it can be deduced that surplus oxygen was partly consumed by the oxidation of indigenous organic matter (OM) present in the microcosms. Indigenous OM also likely fuelled subsequent production of methane. Disregarding the fate of the biomass enriched during methane oxidation (discussed in detail below) a reason for the earlier and more extensive methane production in both the amended and unamended incubations at 50°C compared to 30°C may be an increased bio-availability of OM at higher temperatures (Parkes et al. 2014). Specifically, lysis of indigenous mesophilic cells present in the sediment termed ‘necromass’ likely augmented by thermal activation of sediment macromolecular OM at higher temperatures (Parkes et al. 2014). Enhanced methanogenesis at high temperatures (50-70°C) without carbon amendment has been observed for Tyne sediments (Bell, 2016).

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

Rapid die-off and subsequent biomass degradation might, therefore, account for the methane observed after day 11 in the high temperature amended experiments,
however, a simple calculation based on literature reported growth yields for Type I
methanotrophs e.g. *Methylomagnum ishizawai* (340 mg\text{dry mass} \text{mole}^{-1} \text{CH}_4; Khalifa et al.
2015) indicated that this re-cycled biomass would have been inconsequential. This
methane, is however, explicable in the context of sediment ‘priming effects’ (Bianchi
2011) which refers to the empirically observed enhanced remineralization of less
bioavailable organic matter on the addition of bioavailable substances, a phenomenon
widely recognised in soil science, the mechanism of which is not fully understood
(Bianchi, 2011). Soil priming experiments have shown CO\text sub{2} evolution from indigenous
OM markedly increased after plant residue addition (Blagodatskaya and Kuzyakov
2008). The priming of a marine sediment with algal biomass increased levels of
background remineralization under anoxic conditions (van Nugteren et al., 2009).

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

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

In contrast, aminotetrol and to a greater extent aminopentol, were produced in
progressively greater amounts during incubation throughout both methane consumption
and stationary growth phases (Fig. 3b,c) supportive of a physiological response to
increasing temperatures, the onset of starvation or, both for aminopentol synthesis. This
interpretation of the data was tentatively supported, albeit using a non-quantitative
endpoint PCR approach, by the apparent accumulation of *pmoA* up to day 11 (see
supplementary information and Fig. S4). After this *pmoA* was, apparently, broadly
maintained providing evidence of prolonged cell survival. In support of multiple drivers of aminoBHP production, the aminopentol isomer only detected at 40°C in the shorter incubations was detected in the longer lower temperature experiments (Fig. 3c) after a 7 day lag phase and substrate exhaustion (fig 2). An even longer lag coincident with oxygen depletion was required before the detection and accumulation of the unsaturated aminopentol and 3-methylaminopentol was detected in one replicate at day 20 (data in Osborne, 2016).

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

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

Temperature limits for aminotriol and aminotetrol production were confirmed and extended in the 50°C time series experiments (Fig 4 a, b). These experiments also confirmed the high temperature enrichment of a consistent mixture of aminopentol–like BHPs namely, aminopentol, 3-methylaminopentol and unsaturated 3-methylaminopentol
during growth (Fig. 4c). At 4 days, comparable to the short-term experiments, absolute quantities of these aminoBHPs were almost identical suggesting their composition is highly regulated under specific conditions. However, in contrast to lower temperatures there were no significant increases after methane removal (Fig. 4c). This cessation of BHP production is likely the result of cell death as inferred by the coincident ‘priming’ of methane production from the breakdown of biomass (Fig 2). This interpretation of quantitative data was tentatively, albeit non-quantitatively, supported by an endpoint PCR of pmoA which indicated an apparent decline of after methane consumption (Fig. S4b).

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

A final interesting point about high temperatures and the apparent death of methanotrophs by substrate exhaustion is that aminoBHPs appear to have survived intact (Fig. 4) despite biomass degradation, oxygen depletion and ultimately
methanogenesis. This persistence attests to their recalcitrance in sediments and survival in the geological record relative to more labile compounds.

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

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

Critically, it is clear that a fundamental understanding of environmental controls on aminoBHP production is needed to fully interpret palaeoenvironmental records of these biomarkers (Talbot et al. 2016a). For instance, the identification of temperature regulated production of aminopentol can be used to re-interpret aminoBHP patterns in the Cobham lignite. The Cobham lignite is a terrestrial lacustrine/mire sedimentary sequence in southern England which spans the Palaeocene–Eocene Thermal Maximum (PETM). The PETM is the most extreme warming event in the last 55 million years where central-western European mean annual air temperature averages rose to 23-26°C (Inglis et al. 2017) relative to current means for the UK of 8-11°C. Isotopically depleted hopanoids measured in the Cobham lignite (Pancost et al. 2007) have previously suggested an increase in the methanotroph population at the onset of the PETM in this local, driven by changes to a warmer, wetter and methane rich environment. Talbot et al. 2016a, subsequently reported a correspondence between negative $^{13}$C carbon
excursions indicative of high methane and abundances of aminopentol but not aminotetrol (as a proportion of total biohopanoids) and reported generally higher levels of aminopentol in lignite deposited during the PETM. Talbot et al. 2016a suggested that these BHP patterns recorded environmental change with a potential shift from the dominance of Type II (indicated by the presence of aminotetrol and absence of aminopentol) to Type I methanotrophs (additional presence of aminopentol) during periods of intense methane cycling. A caveat given by Talbot et al., however, was the recent laboratory finding of Sherry et al. 2016, that changes in methanotrophic community composition are not induced by differences in methane concentration. This contradiction can be resolved with the conclusion that, regardless of the intensification of methane oxidation during the PETM, the abundance of aminopentol relative to other aminoBHPs was principally regulated by a response to temperature. The wider implications of these results is that interpretation of aminoBHP relative abundances in modern environments and in the geological record should only made in the context of measured temperatures or temperature proxies.

Studies of aminoBHP production as a function of substrate availability, redox and growth phase (Figs. 3 and 4) further emphasises the need for a wider understanding of depositional conditions and processes. For instance, aminopentol production at 30°C after methane removal suggests that its detection does not, necessarily, represent periods of persistent methane oxidation but instead highly variable methane conditions, such as typically encountered at seafloor methane seeps (Valentine, 2011). Highly variable methane flux trends may be a common feature of many methanogenic environments due to periodic changes in hydrology and atmospheric pressure changing redox conditions or gas flow. For instance, in a UK landfill site biogenic methane was found to be absent in the ground gas for 70% of the time and methane flux correlated closely with atmospheric pressure (Teasdale et al. 2014).
At high temperatures, 3-methyl aminoBHP production only occurred during active methane oxidation (Fig. 4) rather than in the late stationary phase as identified by Welander and Summons (2012) which underlies the importance of a paleo-environmental context. C-3 methylated hopanes and other geohopanoids are regularly reported in ancient settings (e.g. Collister, 1992; Farrimond et al., 2004; Birgel and Peckmann, 2008; Talbot et al., 2016a) but reports of 3-methyl aminoBHPs are rare. Intriguingly they include: a neo-volcanic, eutrophic, shallow saline lake sediment (Talbot et al., 2003; Talbot and Farrimond 2007), a geothermal microbial mat (Zhang et al., 2007) and a geothermal silica sinter deposit (Gibson et al., 2008) which suggest a high temperature control on their production as identified in our laboratory experiments. However, potentially they are also produced at lower temperatures in response to starvation and oxygen depletion as described by Welander and Summons (2012) and here observed for one microcosm replicate after long-term incubation. 3-Methyl aminotriol has been observed in some soils, primarily from temperate settings (Cooke, 2010; Talbot et al. 2016b; Zhu et al., 2011) indicating their occasional production under mesophilic conditions.

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