Revisiting the precursors of the most abundant natural products on Earth: a look back at 30+ years of bacteriohopanepolyol (BHP) research and ahead to new frontiers

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Abstract

In this review we look back on 30+ years of bacteriohopanepolyol (BHP) research within the field of organic geochemistry. BHPs are ubiquitous, intact polar lipids in modern environments. They have been found in lacustrine, marine, riverine, and soil and peat environments, and they are noteworthy lipids in biological symbiont studies. BHPs are the precursors of hopanoids, which are the most abundant fossil lipids found in the geological record. BHPs are synthesized by members of various bacterial taxa, and their distributions are often used to help to identify bacterial communities, in studies of both modern and past environments. However, less than 10% of known bacterial species are genetically capable of synthesizing BHPs, and many BHPs are not specific to particular bacterial sources. Nonetheless, a range of BHPs with specific side chain configurations and/or A-ring modifications have proven very useful for tracing bacterial metabolism and for identifying ecological niches in various environments (e.g., aerobic methanotrophy, possibly nitrite-dependent intra-aerobic methanotrophy, and anaerobic ammonium oxidation) or for tracing environmental processes (e.g., soil input into aquatic settings). Moreover, BHPs (with previously unknown terminal groups and side chain configurations) are continuously being discovered, thanks to recent methodological and instrumental advances. These highlight the advent of a new era of BHP lipidomics which awaits full exploitation in organic geochemistry. Here, we provide a summary of the state-of-the-art of BHP knowledge, analytical frontiers, and suggest directions for future research.

1. Background

Over the past 30+ years, considerable research within the field of organic geochemistry has been dedicated to the complex lipids known as bacteriohopanepolyols (BHPs; Fig. 1). BHPs are biosynthetic products produced exclusively by prokaryotes (e.g., Ourisson et al.,

1979; Ourisson and Rohmer, 1992; Rohmer et al., 1984), although by no means do all prokaryotes produce BHPs (e.g. Farrimond et al., 1998; Pearson et al., 2007; Pearson and Rusch, 2009; Rohmer et al., 1984; Talbot et al., 2008b). BHPs are embedded in the outer and inner membranes of gram-negative bacteria (e.g., Wu et al., 2015a) as well as in the cytoplasmic membrane of gram-positive bacteria, in part acylated to lipopolysaccharides (Komaniecka et al., 2014; Silipo et al., 2014). They are thought to control cell membrane permeability and rigidity (e.g. Kannenberg and Poralla, 1999; Welander et al., 2009; Wu et al. 2015a). Work by Sáenz et al. (2012a; 2015) has shown that BHPs likely play a role in membrane lipid ordering of bacteria (and possibly lipid raft formation; Sáenz, 2010), much like sterols in eukaryotes. BHPs have also been shown to be localized in microdomains accumulating in akinete cells and near cell junctions in the cyanobacterium *Nostoc punctiforme*, which potentially aids cell curvature (Doughty et al., 2014). Functionally, BHPs increase the antibiotic, pH, temperature, and detergent resistance of bacterial cells (Welander et al., 2009; Schmerk et al., 2011; Doughty et al., 2011; Sáenz, 2010), and may foster cell survival in the late stationary phase (Welander and Summons, 2012).

BHPs are synthesized via the cyclization of squalene to form the characteristic pentacyclic triterpenoid ring system (Fig. 1A). Squalene cyclization to 17β(*H*),21β(*H*)-hop-22(29)-ene (diploptene) is performed by squalene-hopene cyclase (SHC, encoded by the *shc* gene) in a complex one-step enzymatic reaction (Ochs et al., 1992; Perzl et al., 1997; 1998; Wendt et al., 1997; Tippelt et al., 1998; Siedenburg and Jendrossek, 2011) that can be performed independently of molecular oxygen (Ourisson and Rohmer, 1982). The addition of an adenine by the radical SAM protein HpnH, results in the intermediate to all extended polyfunctionalized BHPs: 30-(5'-adenosyl)hopane (adenosylhopane; Fig. 1C) (Bradley et al., 2010). The phosphorylase HpnG then removes the nitrogenous base (adenine) to form ribosylhopane (Liu et al., 2014; Bodlenner et al., 2015). The ribose moiety can be transformed

into an acyclic form (the ether bond is broken to form a carbonyl moiety non-enzymatically; Bradley et al., 2010), which is reduced by an unknown protein to form bacteriohopane-32,33,34,35-tetrol (BHT; Fig. 1C). Various proteins further alter either the side chain to form a variety of amino-BHPs (HpnO) and composite (i.e., containing a sugar head group) BHPs (HpnI, HpnK, HpnJ), or methylate the A-ring (HpnP, HpnR) (Welander et al., 2010; Welander and Summons, 2012; Schmerk et al., 2015; Sohlenkamp and Geiger, 2016).

Modifications to the ring system of BHPs include methylations (Fig. 1A) at the carbon positions of C-2 (e.g., Talbot et al., 2008b and references therein), C-3 (e.g., Cvejic et al., 2000a), both C-2 and C-3 (e.g., Sinninghe Damsté et al., 2017), C-31 (e.g., Simonin et al., 1994), and C-12 (e.g., Costantino et al., 2000). Double bonds have been reported in the BHP ring system at C-6 (Δ^6), C-11 (Δ^{11}), or both ($\Delta^{6,11}$) (Fig. 1A; e.g. Talbot et al., 2007b and references therein) and in the BHP side chain (van Winden et al., 2012). With the exception of the C_{30} hopanoids diploptene and diplopterol, BHPs have modified extended side chains derived from ribose (e.g., Duvold and Rohmer, 1999) that typically contain 4, 5 or 6 functional groups (e.g., Rohmer et al., 1984; Rohmer et al., 1993) termed tetra-, penta-, and hexa-functionalized BHPs, respectively. These may retain the cyclic ether ring system of the ribose with a complex moiety; usually this is either an (amino)sugar, or a nucleoside such as adenosine in adenosylhopane (Fig. 1C). The unmodified ring system with a linear side chain is usually the dominant BHP in cultures and environmental samples (overviews of BHP sources were included in Talbot and Farrimond, 2007 and in Talbot et al., 2008b). However, BHPs with novel side chains (e.g., Kool et al., 2014; Rush et al., 2016; Hopmans et al., 2021) or new isomers (e.g., Kusch et al., 2018; Schwartz-Narbonne et al., 2020) are continually being described and these lead to adjustments of our understanding of the structural diversity of BHPs encountered in the environment and pave the way to the possibility of BHP lipidomics studies of environmental samples.

BHPs are also the precursors of geohopanoids, which are the related defunctionalized products (e.g., hopanes, hopanols and hopanoic acids) that occur ubiquitously in ancient sediments, kerogens, coals and oils, as well as in recent sediments. Diagenesis of BHPs leads not only to partial or total loss of the side chain, but also to changes in the stereochemistry of the core hopane skeleton. Over time, the "biological" 17β,21β(*H*) configuration isomerizes to the thermally more stable "geological" 17α,21β(*H*) and 17β,21α(*H*) configurations (e.g., Mackenzie et al., 1981). This process may be accelerated in acidic environments, such as in peats (e.g., Inglis et al., 2018) and there are some notable biological exceptions. For example, BHT with the 17α,21β(*H*) configuration was found in Holocene sediments from an Antarctic lake (Talbot et al., 2008a), and species of the genus *Frankia* have been shown to synthesize BHT with the 17α,21β(*H*)-configuration (Rosa-Putra et al., 2001). Likewise, some acetic acid bacteria, including *Komagataeibacter xylinus*, synthesize BHT with a 22*S* configuration, which is more typically considered to result from diagenesis (Peisler and Rohmer, 1992). Regardless, geohopanoids are often used as geochemical markers (proxies) of sample maturity. For example, the 17β,21β(*H*) to 17α,21β(*H*) ratio, along with the ratio of "geological" 22*S* to the "biological" 22*R* configuration is often used to determine thermal maturity (Ourisson and Albrecht, 1992; Peters et al., 2005). Reports of geohopanoids include those in rocks dating back to the Archaean (e.g., Eigenbrode, 2008), though the authenticity of biomarkers in Archaean samples has been questioned (French et al., 2015). Nonetheless, intact BHPs have been detected in mudstone samples as old as ca. 50 Ma (Kilwa area outcrops, Tanzania; van Dongen et al., 2006) and possibly the 55 Ma old Cobham Lignite (Talbot et al., 2016a). Anhydro-BHT has been detected in samples from the Upper Jurassic (Gorodische outcrop, Russia; Bednarczyk et al., 2005), although this represents an abiotic etherization/degradation product, rather than a biosynthetic compound (Schaeffer et al., 2008; Eickhoff et al., 2014). Given their high preservation potential and widespread occurrence, the global abundance of

121 geohopanoids has been estimated at 1 Pg C (1×10^9 tons) and they were termed "*the most*
122 *abundant natural products on Earth*", 30 years ago, by Ourisson and Albrecht (1992). Since
123 then BHP research has been reinvigorated thanks partly to analytical improvements in mass
124 spectrometry and the application of genomics tools.

125 The diverse nature of BHP lipid structures (Fig. 1) and their high preservation potential
126 have led to the use of particular BHPs as biomarkers of unique bacterial source organisms
127 (Cvejic et al., 2000b; Kool et al., 2014; Rush et al., 2014; van Winden et al., 2012), of
128 environmental conditions (Ricci et al., 2014; Welander and Summons, 2012), and for invoking
129 the origin of bacterial organic matter (Zhu et al., 2011). Here, we review the state-of-the-art of
130 BHP analytical methodologies and the application of BHP lipidomics to the modern (and by
131 inference, geological) sedimentary records. We highlight possible future avenues of focus for
132 these important lipids. Our focus is the environmental distributions and proxy potential of
133 BHPs, rather than on their physiological role in bacterial membranes. For in-depth overviews
134 of the biological roles of BHPs, readers are referred to the reviews by Belin et al. (2018) and
135 Newman et al. (2016).

136 In Section 2, we provide an overview of the methodological approaches so far taken for
137 the analysis of BHPs; these not only lie at the heart of our discipline, but also showcase the
138 tremendous improvements that have been made recently. We review the sensitivity of BHPs to
139 different extraction methods, how their detection using gas and liquid chromatography-mass
140 spectrometry has evolved/improved and has paved the way for compound-specific BHP
141 analysis, and which analytical obstacles we still face (i.e., quantification) during routine
142 analysis. In Section 3, we review the structural and environmental diversity of BHPs,
143 highlighting BHPs that have proven to be either reliable or promising proxies for specific
144 bacterial metabolisms, such as aerobic methanotrophy, nitrite-dependent intra-aerobic

methanotrophy, anaerobic ammonium oxidation, or which carry environmental information (e.g., of terrestrial origin). In Section 4, the frontiers of BHP lipidomics are examined, and we outline the possibly untapped proxy potential of BHPs. This outlook includes a call for further investigations of BHP remodeling as a stress response (e.g., going beyond classic culturing approaches), and draws attention to the critical need for further methodological developments (specifically those diversifying the compound-specific isotope toolbox), the extension of multi-omics to BHP studies to include a broader implementation of untargeted lipidomics, as well as a larger-scale exploitation of the information afforded by genomics tools.

2. Analytical considerations

2.1 Extraction methods

To date, a suite of different extraction methods has been used to extract BHPs. These include different versions of modified Bligh & Dyer protocols (e.g., Talbot et al. 2007a; Sáenz et al., 2011a), microwave and ultrasound (Berndmeyer et al., 2014), as well as Soxhlet (e.g., Wakeham et al., 2007, 2012; Kusch et al., 2021b,c). However, it is important to note that earlier tests revealed that extraction efficiencies may differ for different BHPs and that certain BHPs, such as bacteriohopane-31,32,33,34-tetrol cyclitol ether (BHT-CE; a composite tetra-functionalized structure with an aminosugar group at C-35; Fig. 1C), may evade routine solvent extraction due to complexation in the membrane (e.g., Herrmann et al., 1996).

One of the most common Bligh & Dyer-based methods for BHP extraction is based on ultrasonication (and shaking) of samples in methanol (MeOH)/chloroform/water (2:1:0.8, *v:v:v*); typically three times. Separation of the resulting combined total lipid extract from the aqueous phase is subsequently achieved using separatory funnels (Saenz et al., 2011a), or through centrifugation (Talbot et al., 2007a). Further modifications of this method have been made at times, for instance, substitution of chloroform with dichloromethane (DCM) (e.g.,

Sáenz et al., 2011a), or use of a MeOH/DCM/buffer (e.g., Berndmeyer et al., 2014; Rush et al., 2016). Buffers used have included those based on ammonium acetate (Rush et al., 2016), phosphate (Berndmeyer et al., 2014), or trichloroacetic acid (TCA; Matys et al., 2017). In case of the latter, the TCA needed to be quickly removed since it can lead to BHP degradation (e.g., Matys et al., 2017).

Soxhlet-based extractions have usually been performed using DCM/methanol (2:1, *v:v*). Nonetheless, this approach has been chosen simply as the most feasible way of extracting suspended particulate matter (SPM) filters from water column depth profiles, which otherwise disintegrate during ultrasonication (e.g., Wakeham et al., 2007, 2012; Kusch et al., 2021b,c). Although the effects of Soxhlet extraction have yet to be compared directly to those of other extraction methods, no obvious bias (recovery, diversity) was observed for either intact polar lipids (Schubotz et al., 2009), or BHPs (e.g., Kusch et al., 2021b,c).

Few comparative studies have been published; but it is reasonable to assume that reporting laboratories have protocols in place for testing the efficiency of the different extraction methods used (e.g., Osborne, 2016). Berndmeyer et al. (2014) tested the extraction efficiencies of microwave (DCM/MeOH 3:1, *v:v*), ultrasound (DCM/MeOH 3:1, *v:v*) and Bligh & Dyer (MeOH/DCM/phosphate buffer 2:1:0.8, *v:v:v*; samples were shaken, rather than sonicated) methods, using two sediment samples from the Baltic Sea. These authors did not find substantial differences in total BHP yields for these methods: the quantities of eight BHP analytes extracted were the same within the standard deviations of replicate analyses. However, they reported superior extraction efficiencies for some of the low abundance BHPs using the Bligh & Dyer method, including for 35-aminobacteriohopane-32,33,34-triol (aminotriol; Fig. 1C), 35-aminobacteriohopane-31,32,33,34-tetrol (aminotetrol; Fig. 1C), 35-aminobacteriohopane-30,31,32,33,34-pentol (aminopentol; Fig. 1C), and BHT-CE. Osborne (2016) tested various modifications of the Bligh & Dyer protocol, i.e., solvent substitutions

(chloroform vs. DCM and water vs. phosphate buffer), while keeping the physical extraction parameters identical. Most extraction protocols yielded no significant differences: only the method employing a mix of MeOH/DCM/phosphate buffer (2:1:0.8, *v:v:v*) resulted in significantly lower total BHP yields. In the case of BHT-CE, Osborne (2016) found a superior extraction efficiency using a MeOH/chloroform/water (2:1:0.8, *v:v:v*) solvent mixture. No differences were observed when ultrasonication times were varied (Osborne, 2016). Recently, a comparison of the Bligh & Dyer method with harsher extraction conditions (i.e. methanolysis, acid hydrolysis, and direct acetylation) showed that these four extraction methods yielded similar amounts of bacteriohopanetetrols from *Komagataeibacter xylinus* cells (Schaeffer et al., 2021). However, methanolysis may have partially converted a portion of BHT to anhydro-BHT. Surprisingly, methanolysis and direct acetylation recovered higher yields of composite BHPs than the Bligh & Dyer method, because >20% of the composite pool was left in the aqueous phase when the latter method was used. Clearly, there is variability between extraction methods, which should be considered and tested when deciding how to proceed with specific sample types and analysis needs. Further extraction efficiency tests might include the use of detergents and mechanical disruption, such as freeze-thaw cycles, which seem to improve the extraction of intact polar lipids from cells (Evans et al., 2022). Potential inter-laboratory biases should be assessed in the future (Section 4.2.1).

2.2 BHP analysis using GC-MS

The first reports of BHPs in natural environments were based on analyses using gas chromatography-mass spectrometry (GC-MS; Rohmer et al., 1980). Until recently, GC instrumentation was only used to detect a limited number of sufficiently volatile polyfunctionalized hopanoids and simple intact polyfunctionalized BHP side chains, e.g., BHT. The methylated and/or unsaturated homologs of these lipids (Fig. 1) can also be identified by

GC-MS (e.g., Sessions et al., 2013). Traditionally, GC-MS identification and quantification of BHPs in organic extracts was based on a treatment of extracts with periodic acid (H_5IO_6) followed by sodium borohydride ($NaBH_4$) or super hydride ($LiEt_3BH$) reduction, also known as the Rohmer reaction (Rohmer et al., 1984). This reaction converts polyols into C_{30}–C_{32} primary alcohols, which are subsequently acetylated using acetic acid and pyridine. Analyses of these derivatized hopanols provide information about the number of functional groups present in the original intact molecules (Rohmer et al., 1984). Using this chemical cleavage method, Farrimond et al. (2000) demonstrated for the first time that BHT was not necessarily the primary BHP in sediments and revealed the presence of a greater diversity of compounds, including tetra-, penta-, and hexafunctionalized analogs and their methylated homologs.

The analysis of hopanoids using GC-MS certainly represented a step forward in understanding the distributions of BHPs in environmental samples. However, by removing all but one functional group from the side chain using the Rohmer reaction, much of the source, environment, or process-specific information of the BHPs was lost. The Rohmer method is also laborious, and BHPs with a cyclized side chain (e.g. nucleoside BHPs and composite BHPs; Fig. 1C) are not detected, with the consequence that BHP abundance is underestimated. To elucidate fully the complex arrays of BHPs in samples, it proved necessary to develop alternative methodologies. In 2013, Sessions et al. published a high temperature (HT)-GC-MS method that achieved elution and separation of more complex acetylated intact BHPs on two different GC stationary phases (BHT, bacteriohopane-31,32,33,34,35-pentol [BHpentol], and aminotriol on DB-5HT; C-2 methylated BHPs on DB-XLB). Though HT-GC shows promise for BHP analysis, as it has in other areas of organic geochemistry, the vast majority of work analyzing intact BHPs since has been performed using high performance liquid chromatography-MS (HPLC-MS).

2.3 BHP analysis using HPLC-MS

Following initial analytical developments using GC-MS (Section 2.2), HPLC-MS analysis of acetylated BHPs has been the most commonly used method throughout recent decades. In the 1990s, a normal phase HPLC gradient was developed for analysis of underivatized BHPs in the ethanologenic bacterium *Zymomonas mobilis* (Moreau et al., 1995). Ionization was achieved via negative ion chlorine addition atmospheric pressure chemical ionization (APCI). The first HPLC-MS analysis of a sedimentary BHP distribution (using a surface sediment of a small eutrophic lake in the UK; Fox et al., 1998) was carried out using this method. However, only two BHPs were identified: BHT and BHT-CE. Further developments revealed that the chlorine adducts formed with this method proved difficult to fragment under atmospheric pressure chemical ionization (APCI) conditions and at best only yielded information on the number of BHP functional groups, but not the ring system. This meant that it was not possible to identify unknown BHPs using this method (Talbot et al., 2001). Additionally, the use of normal phase chromatography proved unsuitable for BHPs containing 1 or more amine functionalities, particularly when these were located at C-35 (e.g., amino-BHPs; Fig. 1C). Therefore, alternative separation protocols were developed using derivatized BHPs.

Schulenberg-Schell et al. (1989) developed a reversed phase HPLC method for analysis of BHPs after acetylation, which was modified by Talbot et al. (2001) to reveal the BHP profiles of a group of methanotrophic bacteria. Advances followed with the application of ion-trap mass spectrometry, which allowed for a more precise control of the fragmentation of the precursor ions. Most environmental and culture studies of BHPs since 2003 have used a version of this reversed-phase chromatographic method, with either a ternary or binary solvent system and a linear gradient from MeOH/water (90:10) to MeOH/propan-2-ol/ water (59:40:1) on a C_{18} column, followed by detection via positive ion APCI (Blumenberg et al., 2007; Kusch et

al., 2019, 2021b,c; Saenz et al., 2011a,b; Talbot et al., 2003a,b). Subsequent investigations of a wider range of hopanoid-producing bacterial cultures (Talbot et al., 2003b,c; 2007a,b; 2008b) led to improved understanding of the APCI fragmentation pathways of BHPs. This allowed the identification of both known and related unknown BHPs in sedimentary systems (e.g., Rush et al., 2016). Based on the above reversed-phase chromatographic method, Kusch et al. (2018) developed an isocratic HPLC method that enabled the detection of a range of new BHP isomers, including five isomers of BHT, in marine sediment samples. Due to the baseline separation of the most common isomers, this method was also employed as a first step for isolation of BHPs for stable carbon isotope analysis (Hemingway et al., 2018).

Nevertheless, the analysis of derivatized BHPs by HPLC-MS has disadvantages. The relative acetylation efficiencies of individual BHPs vary, and assumptions must be made for BHPs for which there is a lack of authentic standards. Additionally, BHPs with 'masked' acetylation sites, e.g., BHT-CE on its terminal sugar, often produce acetylomers, the concentrations of which need to be summed for quantification. With the development of improved ultra-high performance liquid chromatography-mass spectrometry (UPLC-MS) instruments and advanced LC column chemistries, it has been possible to introduce improved methods for analyzing non-derivatized BHPs. Based on a Waters™ lipid application note (Issac et al., 2011), non-derivatized BHPs were successfully identified in bacterial isolates and purified culture material, using a UPLC-tandem MS system. However, a follow-up study applying this method revealed discrepancies with known BHP producers. For example, Malott et al. (2014) did not report BHPs known to be synthesized by *Burkholderia* spp. (i.e., BHT and unsaturated BHT-CE; Cvejic et al., 2000b). Furthermore, Wu et al. (2015b) reported a reduction in ionization efficiencies of non-acetylated BHPs compared to their acetylated counterparts. More recently, analytical efforts dedicated to the analysis of underivatized BHPs have succeeded (Talbot et al., 2016c; Hopmans et al., 2021) and the advent of high-resolution

accurate mass (HRAM) mass spectrometry, such as orbitrap, has enabled the detection of a range of BHPs with previously unknown moieties. Talbot et al. (2016c) used an ultra-inert Excel C_{18} column and modified the established solvent gradient described above to transition from MeOH/water/formic acid (90:10:0.1) to propan-2-ol/MeOH/water (59:40:1), within a total run time of 9 minutes. Detection was achieved in positive ion atmospheric pressure ionization mode on a triple quadrupole MS system. The authors demonstrated detection of some commonly occurring BHPs including BHT, aminotriol, and adenosylhopane (Talbot et al., 2016c). Hopmans et al. (2021) demonstrated BHP separation on a C_{18} BEH column using a solvent gradient from MeOH/water/formic acid/aqueous ammonia (85:15:0.12:0.04) to MeOH/propan-2-ol/formic acid/aqueous ammonia (50:50:0.12:0.04) over a much longer run time (i.e., 80 minutes). Using a quadrupole-orbitrap HRAM system and positive ion heated electrospray ionization (HESI), these authors demonstrated the detection of approximately 130 underivatized BHPs within a single environmental sample (Fig. 2) - substantially expanding the range of known and previously undescribed BHPs. The sample, a soil from a terrestrial methane seep in Italy, contained a large suite of BHPs with ethenolamine moieties (Fig. 1C), as well as novel nucleoside BHPs structurally related to adenosylhopane (Hopmans et al., 2021). Application of this method to a range of environmental samples will probably extend the BHP lipidomics window substantially.

2.4 BHP isotope analysis

Due to the diversity of carbon assimilation pathways used by the various bacteria that synthesize BHPs, the ^{13}C isotopic composition of these lipids can vary greatly, depending on the source organism. Thus, compound-specific isotope ratio values of BHPs can be useful when determining source organisms in environmental settings. Currently, the only means of determining the $\delta^{13}C$ values of BHPs is by GC-isotope ratio mass spectrometry (GC-irMS). In

320 the future BHPs may be isolated at sufficient purity (e.g., using preparative HPLC or GC) to
321 be analyzed using (nano)EA-irMS or spooling-wire irMS (analogous to a method for GDGTs;
322 Pearson et al., 2016).

323 Recently, two different HT-GC-irMS methods have been developed for $\delta^{13}C$ analysis
324 of intact BHPs, building upon the HT-GC method of Sessions et al. (2013). Hemingway et al.
325 (2018) first isolated BHP fractions via UPLC using the method described by Kusch et al.
326 (2018), which allows baseline separation of a range of different BHPs and their isomers, such
327 as BHT and BHT isomer, but not all structurally different BHPs. Subsequently, the individual
328 fractions were analyzed using HT-GC-irMS and BHPs that co-eluted during LC analysis (e.g.,
329 BHT isomer and 2β-methyl-bacteriohopane-32,33,34,35-tetrol (2Me-BHT)), were separated
330 via the HT-GC method (using a Zebron ZB-5HT stationary phase heated to 350°C). While the
331 method of Hemingway et al. (2018) circumvents problems with GC co-elution of BHP isomers,
332 Lengger et al. (2019) achieved BHP separation via HTGC alone using a slower temperature
333 ramp rate (7°C/min rather than 10°C/min). Both, the work of Hemingway et al. (2018) and
334 Lengger et al. (2019) revealed strong ^{13}C-depletion of the so-called BHT-x isomer (Rush et al.,
335 2014; Schwartz-Narbonne et al, 2020), providing evidence for an anaerobic ammonium
336 oxidizing (anammox) source of this BHP in marine sediments (Section 3.3). We anticipate that
337 further insights into the source organisms of diverse (known and so far unknown) BHPs will
338 be gained from applying the above methods and by continuing efforts to further develop BHP
339 isotope techniques (Section 4.2.2).
340

341 **2.5 BHP quantification**

342 One of the primary pitfalls in BHP analysis is the absence of authentic standards. This
343 paucity limits any ability to accurately quantify BHPs with different moieties. Until authentic
344 standards are available, any BHP quantification remains semi-quantitative since response

345 factors between BHPs, including those with different moieties/head groups, and commercially
346 available surrogate standards, such as 5α-pregnane-3β,20β-diol (analyzed as the acetylated
347 pregnane diacetate) cannot be determined and may vary substantially. Using isolated BHT,
348 aminotriol, adenosylhopane, bacteriohopane-31,32,33,34-tetrol glucosamine (BHT
349 glucosamine; Fig. 1C), and BHT-CE, Cooke et al. (2008a) determined that the HPLC-MS
350 signal response of BHT was 8x higher than pregnane diacetate and that of aminotriol,
351 adenosylhopane, BHT glucosamine, and BHT-CE 12x higher than pregnane diacetate when
352 examined on a LCQ™ ion trap mass spectrometer. However, few comparable data on
353 ionization efficiencies exist for other instruments and/or laboratories. Wu et al. (2015b) isolated
354 BHT and 2Me-BHT (Fig. 1C), as well as diplopterol and 2Me-diplopterol, from
355 *Rhodopseudomonas palustris* TIE-1 biomass. These authors tested response factor differences
356 for both GC and LC-based analyses and found substantial differences between hopanoid lipids
357 and surrogate standard materials (androsterone and pregnane acetate) as well as between
358 instruments. Using GC-MS and GC-flame ionization detection (FID), diplopterol and 2Me-
359 diplopterol had roughly an order of magnitude higher signal response than BHT and 2Me-BHT,
360 whereas the opposite was observed on a UPLC-time of flight (TOF) MS system. In this case,
361 the response detected for BHT and 2Me-BHT was ca. 1.2x and 1.3x higher, respectively, than
362 the signal detected for pregnane actetate (Wu et al., 2015b). This differs from the observations
363 of Cooke et al. (2008) by up to an order of magnitude and further emphasizes the need for
364 authentic standards for calibrations. Wu et al. (2015b), also synthesized tetra-deuterated
365 diplopterol for use as an internal standard and demonstrated that it was superior for
366 quantification of diploterol in environmental samples, which often suffers from ion suppression
367 effects due to co-elution of other compounds. To our knowledge, no extended hopanoid lipids
368 (BHPs) have thus far been synthesized successfully, which may make the isolation-from-
369 culture approach to obtaining external authentic BHP standards more feasible. Nonetheless,

culturing bacteria and isolating/purifying BHPs is tedious work that goes beyond the capabilities of most laboratories interested in (and equipped for) BHP analysis. In most cases, such a culturing approach will also only yield a limited number of individual BHPs that can be used as reference materials, depending on the BHP diversity produced by the cultured bacterial species. Likewise, mass production of even a handful of authentic standards at large scale is likely beyond the resources of many organic geochemistry laboratories. Although some laboratories have used purified standards (e.g., van Winden et al., 2012; Matys et al., 2019b; Schwartz-Narbonne et al., 2020), these materials are not available universally. Until this conundrum is solved, BHP quantification will remain semi-quantitative, and BHP abundances (absolute and relative) will either be reported by comparsion with responses of surrogate standards without response factor corrections (e.g., Kusch et al., 2019; 2021b,c) or by integrated chromatographic peak areas only (e.g., Rush et al., 2019; van Kemenade et al., 2022). While these approaches probably ensure that data obtained in the same laboratory and with the same instrument are comparable, they strongly limit the comparability of data sets generated in different laboratories, and likely introduce biases when reporting BHP proxy values such as R_{soil} or normalizing ratios between BHPs with different moieties, e.g., 2Me-BHT/[2Me-BHT+BHT] (Matys et al., 2019b; Kusch et al., 2022).

3. Structural and environmental BHP diversity – a brief summary

Advances in analytical methods have revealed large structural and stereochemical diversity in BHPs. This can be exploited by organic geochemists (i.e., by BHP lipidomics approaches) to elucidate the origin of organic matter, to study community assemblages and system functionalities, and to investigate the structures of present and past ecosystems. For example, where the source organism of a BHP is known, BHPs have been used as biomarkers to trace specific bacteria and their biogeochemical processes in the environment. However, our

views of BHP-producing bacteria have changed quite dramatically in recent years, aided by improved analytical methodology on the one hand and by the expansion of (meta)genomics on the other hand – just as lipidomics and (meta)genomics have aided molecular organic biogeochemical research overall (Steen et al., 2020).

Unlike in the biological production of sterols, BHP biosynthesis does not require oxygen (Ourisson and Rohmer, 1982). However, despite the observation of BHP synthesis by facultative anaerobes including *Rhodomicrobium vannielii* (Neunlist et al., 1985), *Rhodopseudomonas* spp. (Neunlist and Rohmer, 1988), *Rhodospirillum rubrum* (Llopiz et al., 1992), and the fermentative *Zymomonas mobilis* (e.g., Renoux and Rohmer, 1985), early studies indicated that BHPs were not produced by any obligate anaerobes (Rohmer et al., 1984; Farrimond et al., 1998). This led to the assumption that all BHP source organisms were aerobic. Nevertheless, BHPs were soon detected in sediments from modern and ancient anoxic systems (e.g., Elvert et al., 2000; Pancost et al., 2000; Thiel et al., 2003), and this opened speculation that anaerobic BHP source(s) exist. Sinninghe Damsté et al. (2004; 2005) found BHT production in enrichment cultures of strictly anaerobic ammonium oxidizing bacteria (anammox), and Blumenberg et al. (2007; 2009a) reported that some species of dissimilatory sulfate reducers (sulfate reducing bacteria [SRB]) of the genus *Desulfovibrio* make significant but varying amounts of both BHT and aminotriol, and minor traces of aminotetrol in some cases. Moreover, the presence of *shc* gene sequences in, e.g., *Geobacter* spp. and *Magnetospirillum* spp. indicated that further anaerobes are capable of BHP biosynthesis (Fisher et al., 2005; Härtner et al., 2005); although it should be noted that gene presence does not equate to lipid production. Nonetheless, BHP production by *Geobacter sulfurreducens* and *G. metallireducens* was later confirmed by Eickhoff et al. (2013a). Although only few (as mentioned above) anaerobic BHP producers are known in culture, recent studies from marine

oxygen-minimum-zone (OMZ) settings indicate that BHP synthesis in anoxic and even sulfidic waters seems to more prevalent than previously thought (Kusch et al., 2021b,c; 2022).

It is also possible to extract information about environments in which BHPs are synthesized without knowing the exact organism(s) responsible for their synthesis. In the case of nucleoside BHPs (Fig. 1), observed primarily in soils (Section 3.4), these orphan biomarkers have been used to estimate terrestrial organic matter input into marine systems. Below, we outline specific cases of the application of BHPs to environmental settings and highlight future work that might be considered to try to improve BHP applications in environmental studies.

3.1 Methanotrophy

3.1.1 Aerobic methanotrophy (amino-BHPs, MC-BHPs, and C-3 methylated BHPs)

Bacteria performing aerobic methane oxidation (AMO) have been shown to synthesize a range of unique amino-BHPs and methylcarbamate-BHPs (MC-BHPs; Rush et al., 2016) as well as their corresponding unsaturated and/or C-3 methylated homologs (e.g., van Winden et al., 2012; Osborne et al., 2017) (Fig. 1). In culture, gammaproteobacterial Type I methane oxidizing bacteria (MOB) produce aminopentol in high abundances, as well as minor amounts of aminotetrol and the more ubiquitous aminotriol (Fig. 4A). In contrast, alphaproteobacterial Type II MOB primarily synthesize aminotetrol and aminotriol (Rohmer and Ourisson, 1984; Jahnke et al., 1999; Cvejic et al., 2000a; Talbot et al., 2001; Zhu et al., 2011; van Winden et al., 2012; Rush et al., 2016), although there are notable exceptions (Fig. 4A). For example, *Methylomicrobium* sp. only synthesizes aminotetrol and aminotriol and *Methylomarinum* sp., *Methylomarinovum* sp., and *Methylovulum* sp. produce uncharacteristically low aminopentol abundances (Rush et al., 2016). Despite these exceptions, overall the amino-BHP distributions of bacterial cultures (Fig. 4A) provide strong support for their use as AMO proxies. Yet, a puzzling observation is that many samples from marine environments (water column

444 suspended particulate matter (SPM), sediments, carbonates; Fig. 4C, summarized in Table 1 of
445 Rush et al., 2016) do not contain aminopentol - although Type I MOB are typically isolated
446 from marine/aquatic settings (Sieburth et al., 1987; Lindstrom, 1988; Hirayama et al., 2014;
447 Takeuchi et al., 2014; Tavormina et al., 2015), whereas aminotetrol is much more abundant
448 and aminotriol is ubiquitous. High relative abundances of aminopentol have been reported in
449 pelagic water column SPM along strong redox gradients such as in the Black Sea (Wakeham
450 et al., 2007; Blumenberg et al., 2007; Kusch et al., 2022) or the Baltic Sea (Berndmeyer et al.,
451 2013). However, overall, aminopentol is much more frequently found in terrestrial settings
452 including soils, peat bogs, wetlands, and river and lake sediments (Table 1 of Rush et al., 2016)
453 and, consequently, in SPM and sediments deposited off major rivers such as the Amazon
454 (Wagner et al., 2014), Congo (Spencer-Jones et al., 2015), Yenisei (de Jonge et al., 2016), and
455 Yangtze (Zhu et al., 2010). This observation has led to the suggestion that aminopentol in
456 marine sediments - especially in near-shore settings - may more often be a proxy for terrestrial
457 wetland AMO rather than for marine AMO (Talbot et al., 2014; Spencer-Jones et al., 2015;
458 Rush et al., 2016). For example, variations in the concentrations of aminopentol found in a 2.5
459 Ma sediment record off the Congo Fan was interpreted to be caused by shifts in methanotrophy
460 and wetland cover of the Congo River catchment area (Schefuß et al., 2016; Spencer-Jones et
461 al., 2017). Nonetheless, the absence of aminopentol in some Type I MOB cultures implies that
462 its absence in methane-influenced marine samples (Rush et al., 2016) does not a priori imply
463 the absence of Type I MOB in environmental samples. Yet, traces of aminopentol and
464 aminotetrol are also produced by SRB of the *Desulfovibrio* genus, typically in very low
465 abundances (1-3 orders of magnitude lower) relative to aminotriol (Blumenberg et al., 2006;
466 2009a; 2012). Aminopentol has also recently been detected in the biomass of cultured nitrite-
467 oxidizing *Nitrospira defluvii* and *Nitrobacter vulgaris* (Elling et al., 2022), various
468 thermophilic *Alicyclobacillus*, *Brevibacillus*, *Geobacillus*, *Meiothermus*, and *Thermus* strains

isolated from a terrestrial hot spring (Kolouchová et al., 2021), and in Antarctic microbial mats for which 16S rRNA gene sequencing did not reveal the presence of MOB (Evans et al., 2022). Thus, detection of aminopentol in marine samples may neither be taken as unequivocal evidence for the presence of Type I MOB.

MC-BHPs, which are structurally similar to amino-BHPs but contain a methylcarbamate moiety at C-35 instead of an amine moiety (Fig. 1C), have been identified in Type I cultures by Rush et al. (2016). These authors found the respective analogs of the tetra-, penta-, and hexa-functionalized amino-BHPs, i.e., 35-methlcarbamate-amino-bacteriohopane-32,33,34-triol (MC-triol), 35-methlcarbamate-amino-bacteriohopane-31,32,33,34-tetrol (MC-tetrol), and 35-methlcarbamate-amino-bacteriohopane-30,31,32,33,34-pentol (MC-pentol), produced by *Methylobacter* sp., *Methylomicrobium* sp., *Methylomarinum* sp., and *Methylomarinovum* sp. Retrospective data mining of previous analyses of cultured MOB biomass revealed that Type I *Methylococcus capsulatus* does not synthesize MC-BHPs. MC-BHPs were also detected in a range of marine methane-influenced samples that did not contain aminopentol. MC-triol was present in high abundance (>50%) in nearly all investigated samples, whereas MC-tetrol and MC-pentol abundances ranged from 0-37% and 0-19%, respectively (Rush et al., 2016). This distribution potentially makes MC-BHPs more useful markers of AMO in marine settings than amino-BHPs. However, few studies have since reported MC-BHPs in environmental samples and at least MC-triol has been identified in culture in nitrite-oxidizing *Nitrococcus mobilis* and *Nitrobacter vulgaris*, albeit in low relative abundances, under certain growth conditions (Elling et al., 2022). Accordingly, the proxy potential of MC-BHPs still needs to be confirmed further.

Methylation at the C-3 position of the A-ring, especially in amino-BHPs, has long been associated with AMO, specifically Type I MOB (e.g., Neunlist and Rohmer, 1985a; Zundel and Rohmer, 1985; Cvejic et al., 2000a; Talbot et al., 2001, 2003a). However, genetic evidence

494 revealed that the *hpnR* gene coding for C-3 methylation is found in various non-methanotrophic
495 bacteria, including acetic acid bacteria and Actinobacteria (Streptomyces), and that not all
496 methanotrophs possess *hpnR* (Welander and Summons, 2012). Likewise, 3β-methyl-
497 bacteriohopane-32,33,34,35-tetrol (3Me-BHT; Fig. 1C) was recently shown in the anaerobic
498 phototrophic purple nonsulfur bacterium *Rhodopila globiformis* (Mayer et al., 2021). More
499 conclusive evidence that C-3 methylated BHPs in specific environments have a methanotrophic
500 source should reside in the stable carbon isotope compositions of 3Me-BHPs. The assimilation
501 of strongly ^{13}C-depleted methane by methanotrophs should be mirrored by a substantial ^{13}C-
502 depletion of 3Me-BHPs (Jahnke et al., 1999). So far, δ^{13}C values for intact C-3 methylated
503 BHPs (or amino-BHPs) have not been obtained, but analyses of related hopanoid lipids in
504 ecosystems influenced by methane have revealed compounds with substantially ^{13}C-depleted
505 values. For example, Talbot et al. (2014) derived C_{30} hopanols from aminopentol via the
506 Rohmer reaction and found their δ^{13}C values to be around -40‰, supporting the contention that
507 aminopentol in a 1.2 Ma sediment record off the Congo River was synthesized by MOB. The
508 utilization of methane as a carbon substrate during lipid synthesis is also mirrored in the
509 strongly depleted δ^{13}C values of hop-17(21)-ene and C_{29}-ββ hopanes in lignite from the
510 Paleocene-Eocene Thermal Maximum (−31 to −76‰; Pancost et al., 2007; Inglis et al., 2020).
511 Diploptene δ^{13}C values in laminated Holocene sediments from Saanich Inlet ranged from
512 −26‰ to −40‰ and Elvert et al. (2001) interpreted the ca. 14‰ shift from heavier to lighter
513 δ^{13}C values at ca. 6000 ^{14}C yrs BP as reflecting a shift in the microbial community from
514 nitrifying bacteria and cyanobacteria towards MOB, resulting from intensified bottom water
515 anoxia. Nonetheless, it should be noted that the fractionation against ^{13}C depends both on the
516 metabolic pathway used by MOB to assimilate carbon (the ribulose monophosphate pathway
517 in Type I vs. the serine pathway in Type II, for instance) and on ambient methane
518 concentrations (Jahnke et al., 1999). For example, Type II MOB-derived hopanoids (pooled

519 hop-17(21)-ene and 2Me-hop-17(21)-ene, bishomohopanol) in *Sphagnum* sp. symbionts do not
520 seem to incorporate strongly ^{13}C-depleted signatures (δ^{13}C values range from −31 to −38‰;
521 van Winden et al., 2010), although fractionation against ^{13}C seems to increase at higher
522 temperatures, reflected in δ^{13}C values of −34‰ at 5°C vs. −41‰ at 25°C (van Winden et al.,
523 2020). The less pronounced ^{13}C-depletion of hopanoids produced by Type II MOB can be
524 explained by the assimilation of CO_2 in addition to CH_4 in the serine pathway. Furthermore,
525 only moderate hopanoid ^{13}C-depletion (δ^{13}C from −17 to −32‰) was observed by Inglis et al.
526 (2019) for C_{31} αβ-hopane in a total of 102 recent global peatland samples, consistent with
527 earlier results from modern peats (e.g., Pancost et al., 2003) and C_{31}-ββ hopanes in lignite from
528 the Paleocene-Eocene Thermal Maximum where co-occurring C_{29}-ββ hopanes were strongly
529 ^{13}C-depleted (Pancost et al., 2007; Inglis et al., 2020). Similar values were found for hop-
530 22(29)-ene, C_{30} hopene(s), C_{27}-α hopane, C_{29}-βα hopane, C_{29}-ββ hopane and C_{30}-ββ hopane in
531 smaller sample subsets (Inglis et al., 2019), suggesting the addition of hopanes from non-
532 methanotrophic source organisms. Future work should aim to better constrain the carbon
533 isotopic fractionation of aerobic methanotrophs.
534
535 **3.1.2 Nitrite-dependent intra-aerobic methanotrophy**
536 The unusual methanotroph *Ca.* Methylomirabilis oxyfera that performs nitrite-
537 dependent intra-aerobic methanotrophy (n-damo) under anoxic conditions (Ettwig et al., 2010)
538 has been shown to be the source of a range of previously unknown hopanoids. Rather than the
539 MOB-characteristic amino-BHPs mentioned above, *Ca.* M. oxyfera synthesizes BHT,
540 BHpentol, and bacteriohopane-30,31,32,33,34,35-hexol (BHhexol; Fig. 1C) as well as their C-
541 3 methylated homologs (Kool et al., 2014). While BHT, 3Me-BHT, and BHpentol are rather
542 common BHPs, hexa-functionalized BHhexol and its C-3 methylated homolog are the
543 dominant BHPs in this organism. Only one other bacterium, thermophilic *Alicyclobacillus*

acidoterrestris (Řezanka et al., 2011), and a marine *Petrosia* sponge, or more likely its bacterial symbiont (Shatz et al., 2000), have so far been shown to produce BHhexol and *Ca.* M. oxyfera is the only known source of 3Me-BHhexol (Kool et al., 2014). This BHP distribution potentially carries taxonomic information and, thus, may have proxy potential. Furthermore, *Ca.* M. oxyfera characteristically biosynthesizes novel demethylated hopanoids, including 22,29,30-trisnorhopan-21-ol, 3Me-22,29,30-trisnorhopan-21-one, and 3Me-22,29,30-trisnorhopan-21-ol (Smit et al., 2019). A putative demethylase was identified in the genome, supporting an intentional metabolic origin of these hopanoids rather than abiotic demethylation (Smit et al., 2019). Thus far, BHhexol, 3Me-BHhexol (analyzed as hopanols) and the demethylated hopanoids have been used to trace *Ca*. M. oxyfera in peatland environments (Fig. 5) (Kool et al., 2014; Smit et al., 2019).

The novel BHPs produced by *Ca*. M. oxyfera may prove useful for tracing n-damo in environmental samples, and the specific environmental niche occupied by n-damo should also aid deciphering the source of BHhexol. *A. acidoterrestris* has high optimum growth temperature (42-53°C), thrives at low pH (2.5-5.8), and primarily lives aerobically (Deinhard et al., 1987). This places *A. acidoterrestris* in an ecological niche that is significantly different from that of *Ca.* M. oxyfera. Depending on the environmental setting, contributions from *A. acidoterrestris* can probably be excluded. For example, BHhexol and 3β-methyl-bacteriohopane-30,31,32,33,34,35-hexol (3Me-BHhexol; Fig. 1C) in anoxic settings, such as stratified oceanic water columns, may therefore be indicative of n-damo, at least until any potential additional source organism(s) of these BHPs are identified. Indeed, BHhexol was found in the hypoxic and anoxic water column in Vancouver Island fjords, as well as the sulfidic Black Sea water column (Kusch et al., 2021b; 2022). The latter is somewhat puzzling if n-damo bacteria are indeed the primary source of BHhexol in the ocean since NC10 bacteria may not tolerate sulfide; direct culture evidence is thus far missing, but NC10 gene sequences

were not detected in the sulfidic Golfo Dulce water column (Padilla et al., 2016). However, the distribution of NC10 bacteria (or *Ca.* M. oxyfera specifically) in the ocean is poorly constrained. Regardless, detection of BHhexol and/or 3Me-BHhexol, in concert with the novel demethylated trisnorhopanols and trisnorhopanones mentioned above, may provide strong evidence for the presence of n-damo/*Ca.* M. oxyfera in environmental samples. Their proxy potential should be elucidated in future studies combining BHP/hopanoid analysis with metagenomic surveys.

3.2 N_2-fixation and nitrification (C-2 methylated BHPs)

Some (but not all) cyanobacteria synthesize C-2 methylated BHPs (2Me-BHPs), many of which were initially shown to be diazotrophs, especially terrestrial strains (Talbot et al., 2008b and references therein). These 2Me-BHPs are preserved in the geological record, i.e., in some ancient sediments and oils, as 2Me-hopanes (e.g., Farrimond et al., 2003). Thus, 2Me-hopanes (and the 2Me-hopane index: C_{30} 2Me-hopanes/[C_{30} 2Me-hopanes+C_{30} desmethlyhopanes]) have been used as biomarkers to infer the presence of molecular atmospheric oxygen in ancient rocks such as from the Proterozoic or Archean (Brocks et al.,1999; Summons et al., 1999) as well as periods of intensified marine diazotrophy during global ocean anoxic events (OAEs; e.g., Kuypers et al., 2004; Sepúlveda et al., 2009). This interpretation was challenged when the anoxygenic phototrophic purple non-sulfur bacterium *R. palustris* was shown to synthesize 2Me-BHT under oxygen-depleted growth conditions (Rashby et al., 2007). Subsequent work by Welander et al. (2010) revealed the gene necessary for C-2 methylation of the A ring (*hpnP*) and that it is present not only in some groups of cyanobacteria, but also in the alphaproteobacteria (including non-photosynthetic organisms). Furthermore, at least one species of Acidobacteria and three species of Actinobacteria possess the *hpnP* gene (Naafs et al., 2021). A recent genbank meta-analysis by Naafs et al. (2021)

revealed that roughly equal proportions of cyanobacteria and alphaproteobacteria that possess the *shc* gene also possess the *hpnP* gene, and the relative abundance of cultured strains that indeed produce 2Me-BHPs is also similar in both phyla/classes (Fig. 6A). However, among all cyanobacteria that possess the *nifH* gene (encoding nitrogenase), approximately 27% also possess the *hpnP* gene (5% of all available genomes). In the case of marine cyanobacteria, however, only 2% of those possessing *nifH* also possess the *hpnP* gene, i.e., a single genome/species (Elling et al., 2020; Fig. 6B). Cyanobacteria possessing both genes are slightly more common in various terrestrial environments, yet the capacity for N_2 fixation is rather low overall (Elling et al., 2020 provide details). Thus, the presence of 2Me-BHPs and 2Me-hopanes alone can no longer be considered indicative of phototrophy (either oxygenic or anoxygenic), nor N_2 fixation/diazotrophy (Doughty et al., 2009; Sáenz et al., 2012b), especially in modern settings and even including distinct ecological niches such as saline microbial/cyanobacterial mats (Blumenberg et al., 2013). Moreover, Ricci et al. (2015) proposed that the capacity to produce 2Me hopanoids has in fact originated in the alphaproteobacteria and that cyanobacteria obtained the *hpnP* gene via lateral gene transfer later in Earth history. Accordingly, a growing body of evidence now suggests limited use of 2Me-BHPs as biomarkers for cyanobacteria, although metagenomics can still reveal cyanobacteria as the sole source of 2Me-BHPs in specific ecological niches/settings, e.g., Antarctic freshwater microbial mats (Matys et al., 2019b).

Rather recently, 2Me-BHPs have been associated with alphaproteobacterial nitrite oxidizing bacteria (NOB) specifically (Kharbush et al., 2018; Elling et al., 2020, 2021). Elling et al. (2020) showed that 2Me-BHP synthesis in *Nitrobacter vulgaris* is induced by cobalamin (vitamin B12), which could be supplied by ammonia oxidizing archaea in the upper ocean. Rather than a proliferation of N_2-fixation during times of ocean stratification and anoxia (e.g., during OAEs), Elling et al. (2020; 2021) interpret high 2Me-hopane abundances in the

sedimentary record as resulting from intensified nitrification in response to high nutrient inputs. This interpretation may be supported by the fact that many marine cyanobacteria neither produce 2Me-BHPs (e.g., Sáenz et al., 2012b; Bauersachs et al., 2017; Naafs et al., 2021) nor contain *shc* (or other related) genes, whereas NOB (not limited to alphaproteobacteria) have been shown to account for the majority of marine *shc* sequences in oxygen-depleted settings (e.g., Kharbush et al., 2016) and various Nitrobacter species carry both the *shc* and *hpnP* genes, including marine *Nitrobacter* Nb-311A (Elling et al., 2020). Yet, Elling et al. (2022) only found trace amounts of 2Me-hopanoids in most other culturing conditions of *N. vulgaris* and 2Me-hopanoids were also notably absent in other marine NOB cultures (i.e., *Nitrospira marina*, *Nitrospina gracilis*, *Nitrococcus mobilis*; Elling et al., 2022), limiting any link between 2Me-hopanes and intensified nitrification in the ocean. In addition to nitrite-oxidizing *Nitrobacter* Nb-311A, Naafs et al. (2021) identified *hpnP* gene sequences only in one other marine alphaproteobacterial species (methylotrophic *Methylobacterium salsuginis*) known to synthesize 2Me-BHPs and posited that 2Me-hopanes may in fact indicate intensified denitrification during OAEs. Importantly, Elling et al., (2022) found that novel nitrogen-containing BHPs similar to those reported in a terrestrial methane seep by Hopmans et al., (2021) seem to be synthesized by NOB, which could be used in the future to confirm a nitrifier origin of the 2Me-BHPs in the sedimentary record. Additional constraints will also come from advanced BHP lipidomics studies that produce an inventory of the distributions of 2Me-BHPs in the environment and identify relevant ecological niches. Reports of 2Me-BHPs in modern marine systems have most commonly included only 2Me-BHT (e.g., Blumenberg et al., 2010; Zhu et al., 2011), but more recent studies have revealed greater 2Me-BHP diversity in marine samples, likely due to the use of HRAM MS technology (e.g., Kusch et al., 2019; 2021b; 2022). These studies show that common 2Me-BHPs in marine SPM and sediments also include 2β-methyl-bacteriohopane-31,32,33,34-tetrol pentose (2Me-BHT pentose) and 2β-methyl-35-

644 amino-bacteriohopane-32,33,34-triol (2Me-aminotriol; Fig. 1C), as well as C-2 methylated
645 nucleoside BHPs produced in situ in marine oxygen minimum zones (OMZs); these are all
646 theoretical precursors of 2Me-hopanes. In fact, Kusch et al. (2021b; 2022) identified the highest
647 abundances of 2Me-BHT in the deepest, anoxic and/or sulfidic SPM samples in stratified
648 Vancouver Island fjords, as well as in the Black Sea, where Wakeham et al. (2007) had also
649 identified 2Me-BHT only in SPM from the anoxic zone. These observations highlight the
650 benefits of increased analytical sensitivity for capturing 2Me-BHP production in the
651 environment and place yet another constraint on the use of 2Me-BHPs and 2Me-hopanes in the
652 geological record.
653
654 **3.3 Anammox (BHT isomers)**
655 An isomer of BHT (previously termed BHT-II) had been shown to accumulate under
656 suboxic and/or anoxic marine conditions (Sáenz et al., 2011b; Kharbush et al., 2013).
657 Subsequently, Rush et al. (2014) demonstrated that this isomer was produced in high
658 abundances by the marine anammox genus *Ca.* Scalindua profunda and deposited in high
659 concentrations in sediments underlying OMZ settings known to harbor anammox bacteria
660 (Golfo Dulce, Costa Rica). Yet, small amounts of BHT-II had also been reported in oxic marine
661 sediments (Sáenz et al., 2011b; Matys et al., 2017; Kusch et al., 2018, 2019), implying that the
662 presence of BHT-II in environmental samples alone is not sufficient to invoke an anammox
663 origin and leading to the suggestion that a threshold BHT-II ratio (BHT/[BHT+BHT-II]) may
664 need to be defined before anammox contributions in the environment can be invoked (Kusch
665 et al., 2018). Nonetheless, supporting evidence came from stable carbon isotope analysis of
666 BHT-II in Mediterranean sapropels (Hemingway et al., 2018; Elling et al., 2021) and of BHT-
667 II in Arabian Sea sediments (Lengger et al., 2019). In these studies, BHT-II $\delta^{13}C$ values were
668 strongly ^{13}C-depleted (by 14 to up to 26‰ relative to BHT and by 18 to up to 29‰ relative to

669 TOC; Fig 7), in agreement with the kinetic isotope effect associated with the reductive acetyl-
670 CoA pathway used by anammox organisms (observed for different ladderane lipids and hop-
671 17(21)-ene; Schouten et al., 2004).

672 Bacterial cultures and marine sediments have indeed been shown to contain more than
673 one BHT isomer (up to 5; Peiseler and Rohmer, 1992; Rosa-Putra et al., 2001; Talbot et al.,
674 2003c; van Winden et al., 2012; Kusch et al., 2018). The recent work of Schwartz-Narbonne
675 et al. (2020) revealed that there are in fact two late-eluting isomers of BHT which co-elute
676 when the HPLC method of Talbot et al. (2001) – and subsequent modifications thereof (Section
677 2.3) – is used to analyze acetylated BHPs. The marine *Ca.* Scalindua genus was shown uniquely
678 to produce one of them (termed BHT-*x*). The second late-eluting isomer, with known
679 stereochemistry elucidated by nuclear magnetic resonance (NMR) spectroscopy
680 (17β,21β(H),22R,32R,33R,34R; BHT-34*R*, Peiseler and Rohmer, 1992), is synthesized by
681 other bacteria known to produce late-eluting BHT isomers (i.e., *Frankia* spp, *Acetobacter*
682 *pasteurianus*, *Komagataeibacter xylinus*, *Methylocella* spp., and *Ca.* Brocadia spp.). Schwartz-
683 Narbonne et al. (2020) separated the two isomers using GC or the HPLC method of Hopmans
684 et al. (2021) for underivatized BHPs. Although the commonly used reverse phase HPLC
685 method used to analyze acetylated BHPs does not allow separation of BHT-x and BHT-34*R*,
686 the total 'BHT isomer', (i.e., the combined BHT-x + BHT-II (BHT-34*R*) inventory), in samples
687 deposited under oxygen-limited conditions in the ocean, seems to indeed comprise primarily
688 BHT-x and thus primarily derives from anammox bacteria with only small contributions from
689 BHT-34*R* (Rush et al., 2019; Zindorf et al., 2020; Kusch et al., 2021b; 2022). The relative
690 abundance of BHT isomer is substantially elevated in these samples and BHT isomer
691 abundances in SPM sharply trace the ecological niche of anammox bacteria in the water column
692 (e.g., Kusch et al., 2022). This may, however, may not be the case in lakes (e.g., Matys et al.,
693 2019a). Care now needs to be taken not to confuse the two BHT isomers, i.e., reports of BHT-

694 II in oxygen-limited settings in earlier (Sáenz et al., 2011b; Matys et al., 2017; Kusch et al.,
695 2018) and more recent publications (Elling et al., 2021) indeed refer to BHT-x, rather than the
696 non-specific term used by these authors (i.e., BHT-II). BHT-x concentrations and ratios are
697 already being applied in multiproxy approaches to demonstrate intensified loss processes in the
698 marine nitrogen cycle during periods of anoxic perturbations, such as the deposition of
699 Mediterranean sapropels during the Cenozoic (Rush et al., 2019; Elling et al., 2021) and the
700 expansion of the Gulf of Alaska OMZ during the last Glacial (Zindorf et al., 2020). These
701 studies demonstrate that BHT-x may be a powerful proxy to study the past N cycle and ocean
702 deoxygenation, particularly when other proxies (e.g., trace metals) do not accurately record
703 paleo redox conditions (Zindorf et al., 2020).
704
705 **3.4 Soil input (nucleoside BHPs)**
706 The first nucleoside BHP to be identified, adenosylhopane, was isolated from a purple
707 non-sulfur bacterium and structurally elucidated using NMR spectroscopy (Neunlist and
708 Rohmer, 1985b). The synthesis of adenosylhopane has since been shown to be a crucial
709 intermediate step in the side chain elongation of all extended C_{35} BHPs (Bradley et al., 2010).
710 As such it would be expected that all hopanoid-producing bacteria produce adenosylhopane;
711 however, it has only been detected in a limited number of earlier culture studies via NMR
712 spectroscopy (Neunlist and Rohmer, 1985b; Seeman et al., 1999; Bravo et al., 2001) or ion-
713 trap HPLC-MS (Talbot et al., 2007a, 2008b). Instead, adenosylhopane and its C-2 methylated
714 homolog were observed to be abundant in soils and mostly absent in open marine environments
715 (Seeman et al., 1999; Bravo et al., 2001; Cooke et al., 2008a; Xu et al., 2009; Rethemeyer et
716 al., 2010). This led to adenosylhopane and the related nucleoside BHPs with (at the time)
717 unknown side chain, being termed “soil-marker BHPs” (Zhu et al., 2011) and these nucleoside

718 BHPs were also proposed as tracers for fluvially transported terrestrial (soil) organic matter in
719 aquatic sediments (Talbot and Farrimond, 2007; Cooke et al., 2008b).

720 Cooke et al. (2009) carried out a small pilot study investigating the BHP compositions
721 of surface sediments off the Great Russian Arctic Rivers. These authors found increasing
722 relative and total concentrations of adenosylhopane towards the east and related the
723 compositional changes to enhanced permafrost preservation and longer summer thaw periods
724 (Cooke et al., 2009). In a study from the Western Canadian Arctic, Taylor and Harvey (2011)
725 also found significant amounts of nucleoside BHPs in river and shelf transect sediments,
726 particularly from areas draining peatlands, which are known to have complex and abundant
727 BHP distributions (van Winden et al., 2012; Höfle et al., 2015). Subsequently, BHP analysis
728 of a comprehensive set of surface sediments from the Yangtze River-Estuary-East China Sea
729 (ECS) by Zhu et al. (2011) led to the suggestion of a new BHP-based soil OC input proxy. Soil
730 samples from the catchment revealed the greatest level of BHP structural diversity (an average
731 of 21 compounds), whereas this number decreased rapidly along the export transect to only
732 four in the ECS. Moreover, absolute and relative nucleoside BHP abundances declined whereas
733 the absolute and relative abundances of BHT overall increased offshore. This observation led
734 to the definition of the R_{soil} index as

735

736 $$R_{soil} = [\text{soil-marker BHPs}]/[\text{BHT+soil-marker BHPs}]$$

737

738 where, all nucleoside BHPs and their methylated components were summed and related
739 to BHT, which is frequently the major, and in many cases only, BHP observed in open marine
740 settings (e.g., Zhu et al., 2011). However, BHT cannot be considered a true marine end member
741 as it is also found in soils and lacustrine settings (e.g., Talbot et al., 2003a; Talbot and
742 Farrimond, 2007; Cooke, 2010; Kim et al., 2011). Follow-up work was aimed at testing the

743 R_{soil} proxy (Fig. 8A) through comparison with other organic proxies used to trace allochthonous
744 soil input, such as the branched isoprenoid tetraether index (BIT) and/or bulk TOC stable
745 carbon isotopes. Results from Bothnian Bay sediments revealed strong correlations between
746 R_{soil} and BIT and bulk $\delta^{13}C$, but only a rather sporadic occurrence of methylated nucleoside
747 BHPs (Doğrul Selver et al., 2012). The methylated nucleoside BHPs were thus excluded in a
748 modified R'_{soil} index, recommended by these authors for (sub)Arctic settings. Further
749 applications of the R'_{soil} index included the Siberian shelf region off the Great Russian Arctic
750 Rivers (Bischoff et al., 2016; de Jonge et al., 2016). These studies demonstrated good
751 agreement of the spatial trends described by the nucleoside BHP-based soil-input proxy (Fig.
752 8A) and BIT and bulk carbon $\delta^{13}C$.

753 Several caveats need to be considered when using the R_{soil} index:

754 1) Heterogeneity in soils. For example, peats tend to contain relatively less
755 adenosylhopane than mineral soils (e.g., Taylor and Harvey, 2010; van Winden et al., 2012;
756 Höfle et al., 2015), and many soils contain abundant BHT (e.g., Cooke et al., 2008b; Xu et al.,
757 2009; Wagner et al., 2014; Höfle et al., 2015; Spencer-Jones et al., 2015). The abundance of
758 nucleoside BHPs has been shown to vary with soil pH, temperature, and precipitation (e.g.,
759 Kim et al., 2011; Höfle et al., 2015; Talbot et al., 2016b; Rush et al., 2021). However, trends
760 are inconsistent across environments; soil pH, the most commonly documented parameter, has
761 been shown to negatively (Kim et al., 2011) or to positively (Höfle et al., 2015) correlate with
762 nucleoside BHP abundances, or to show no relationship at all with the latter (Spencer-Jones et
763 al., 2015). The observed heterogeneity complicates direct comparison of absolute R_{soil} values
764 between sites, and may also explain very low R_{soil} values (<0.1) in settings where other organic
765 and bulk proxies indicate high terrestrial OC contributions, such as surface sediments off the
766 Mississippi River (Kusch et al., 2019).

767 2) Selective degradation. Early observations in Congo fan sediments (e.g., Cooke et al.,
768 2008a; Handley et al., 2010) revealed strong diagenetic degradation of adenosylhopane (to
769 anhydro-BHT), which disappears at ca. 90 mbsf (~900 ka BP), whereas co-occurring BHPs,
770 such as aminopentol and composite BHPs, are still present at >100 mbsf (Handley et al., 2010).
771 The likely preferential degradation of nucleoside BHPs when compared to the better preserved
772 BHT will consequently bias R_{soil}-based reconstructions of soil OC input through time, where
773 paleo-reconstructions will show artificial trends of decreasing soil input downcore. The
774 environmental settings in which the paleo-record is deposited should also be considered:
775 nucleoside BHPs have been reported to degrade rapidly under acidic peat conditions (Talbot et
776 al., 2016b). Thus, the preservation potential of BHPs, including composite BHPs, needs to be
777 investigated in more detail.
778 3) In situ production in the ocean. Nucleoside BHPs were also recently shown to be
779 produced along the redoxclines in marine OMZs (Kusch et al., 2021c). The most pronounced
780 in situ production was observed for adenosylhopane and N1-methyl-inosylhopane (Fig. 1C) as
781 well as their C-2 methylated homologs. However, in this case the corresponding R_{soil} index
782 values were very low (Fig. 8B), which will likely limit potential biases. Nonetheless, little is
783 known about in situ BHP production in marine settings. The presence of nucleoside BHPs, as
784 well as BHT synthesis in sediments, may also cause additional changes in R_{soil} index values.
785 All of these factors show that whilst nucleoside BHPs and the R_{soil} index can provide
786 useful information, their presence must be considered in the context of the environmental
787 setting and the composition of the source materials. Given the importance of adenosylhopane
788 as the first BHP intermediate (Bradley et al., 2010) and its in situ production in OMZs (Kusch
789 et al., 2021c), it is currently a mystery why adenosylhopane is so abundant in soils when
790 compared to sediments. Also, why would substantially higher amounts of BHPs in soils remain
791 in an arrested biosynthetic state (i.e., adenosylhopane is the terminal product much more

792 frequently in soils in comparison to other environments)? A further conundrum is that if
793 adenosylhopane is the sole intermediate for side chain elongation, why does it so frequently
794 occur and cluster with the other nucleoside BHPs in soils? Hopmans et al., (2021) reported,
795 and tentatively identified, more than 18 individual nucleoside BHPs using the HRAM method
796 to analyze BHP abundances in an Italian soil (Fig. 2). This structural and isomeric diversity of
797 nucleoside BHPs had not been apparent previously and potentially may indicate other BHP
798 intermediates such as those based on N1-methylinosine instead of adenine. Since lots of BHP
799 producers occupy the rhizosphere (Belin et al., 2018), the function of nucleoside BHPs may be
800 related to this niche. Yet the significance and biosynthetic pathway(s) of other related
801 nucleoside BHPs with alternative terminal groups at the C-35 position (Fig. 1) are currently
802 unknown. If these other nucleoside BHPs are synthesized via different enzymes (and possibly
803 via further intermediates), they may play very different roles in cell and physiological
804 functioning than does adenosylhopane.
805
806 **4. Frontiers in BHP research**
807 **4.1 Exploiting the proxy potential of BHP adaptation and remodeling in response to**
808 **environmental stressors**
809 Lipid remodeling is a common organismic response to external environmental stressors
810 and is relatively well constrained for membrane monolayer and bilayer-forming lipids; as well
811 as membrane regulating lipids, such as sterols (e.g., reviews by Harayama and Riezmann, 2018;
812 Sohlenkamp and Geiger, 2016). Changes in membrane hopanoid composition have also been
813 observed in various studies (e.g., Joyeux et al., 2004; Neubauer et al., 2015; Doughty et al.,
814 2009, 2011; Kulkarni et al., 2013; Sáenz et al., 2015) and provide opportunities to be exploited
815 for new BHP proxies that, for example, allow temperature, pH, and other environmental
816 parameters to be traced; much like those based on other bacterial biomarkers, such as branched

GDGTs (for which an overview is provided by Schouten et al., 2013a). The physiological role of hopanoids/BHPs and general hopanoid gene expression has been investigated using gene knock-out experiments, where BHP production in wild type bacteria is compared to production in mutants (e.g., Welander et al., 2009; Doughty et al., 2011; Kulkarni et al., 2013; Welander and Summons, 2012; Neubauer et al., 2015; Sáenz et al., 2015). Such experiments primarily targeted the *shc* or *hpnP* genes to examine membrane fitness after loss of hopanoids or 2Me-hopanoids. Few studies have investigated more subtle BHP adaptation and remodeling in response to changing environmental/culturing conditions, and most have focused on total hopanoid abundances, or simple methylated BHPs/hopanoids, rather than identifying changes in the BHP lipidome. For example, increased C-2 methylation was observed in response to pH increase in chemoheterotrophically grown *R. palustris* TIE-1, whereas total BHP production was not affected (Welander et al., 2009). Doughty et al. (2009) observed an increase in total hopanoid production and a relative increase in 2Me-hopanoids during P-limited and light-limited growth of *N. punctiforme*, a response to cell differentiation into akinete cells. More recently, Chwastek et al. (2020) did not observe any significant changes in diplopterol or 2Me-diplotpterol abundances in *Methylobacterium extorquens* exposed to varying temperatures, salt, detergent, and methanol concentrations, although lipid remodeling was indeed observed for intact polar lipids (especially with temperature). In contrast, Brenac et al. (2019) found BHP remodeling in the fermenter *Zymomonas mobilis* when exposed to various levels of ethanol, i.e., sugar moieties (glucosamine, cyclitol ether) seemed to enhance ethanol resistance. Moreover, Cordova-Gonzalez et al. (2021) observed a general trend towards enhanced BHP production in the MOB *Methylotuvimicrobium alcaliphilum* when grown at decreasing salinities and increasing nitrate concentrations and, more specifically, roughly 2-3 times higher aminotriol abundances in response to low salinity or high NO_3^- and 2-3 times higher 3Me-

aminotriol (two isomers) in response to high salinity or high NO_3^-. To date, no analogous studies have investigated BHP adaptation in any of the anaerobic BHP-producing organisms, likely because anaerobic culturing techniques are more intricate and usually have slower growth rates compared to aerobic incubations. To our knowledge, only the effect of different electron donors (H_2 vs. Fe^{2+}) during anoxic photoautotrophic growth of *R. palustris* strain TIE-1 has been reported by Eickhoff et al. (2013b). These authors showed that growth on Fe^{2+} led to substantially increased production of C-2 methylated homologs of most hopanoids. Moreover, the overall BHP composition also changed, regardless of changes in methylation, indicating further lipid remodeling. TIE-1 grown on H_2 was characterized by much higher BHT and aminotriol abundances, whereas TIE-1 grown on Fe^{2+} had higher adenosylhopane and diplopterol abundances.

The focus on C-2 and C-3 methylated hopanoids in incubation studies has been motivated by the importance of methylated hopanes in the geological record. Much less is known about the adaptation of more complex BHPs (composite BHPs, amino-BHPs, nucleoside BHPs), including either modifications to their ring structure or to the (amino)sugar side chain. This lack of knowledge may in part be explained by the relatively low diversity of BHPs in many of those cultured model organisms (e.g., Welander et al., 2009; Liu et al., 2014; Kulkarni et al., 2015; Chwastek et al., 2020) which are easy to maintain and manipulate in the laboratory. In part, this lack of knowledge may also be due to previous analytical constraints (e.g., analysis via GC-MS in the past, use of less sensitive instrumentation formerly) or simply prioritization of the dominant BHPs. Given perpetual improvements in the specificity, sensitivity, and availability of analytical methods and structural diversity of BHPs that can now be detected (e.g., Hopmans et al., 2021), it may be worthwhile to re-examine the BHP distributions of extracts or biomass appropriately stored from previous studies (ideally at –20 °C or below) or repeat some of the incubation experiments to provide more comprehensive

866 studies of BHP lipidome remodeling in response to environmental factors. Nonetheless, given
867 that the vast majority of bacteria are not available in culture or even cultivable at all ('microbial
868 dark matter') (Rappé and Giovannoni, 2003; Epstein, 2013) and that in vitro culturing
869 conditions do not reflect in situ environmental conditions (use of nutrient-rich media, absence
870 of competing species, but also syntrophic networks, lack of interaction with abiotic matrices
871 etc.), important constraints will also come from testing BHP adaptation in situ or in vivo in
872 conjunction with metagenomics, e.g., along environmental gradients or micro- and mesocosm
873 experiments. Such studies will better reflect the complexities and interactions inherent to
874 natural systems and also allow assessment of species selection effects.

875 For example, microcosm incubations of River Tyne estuarine sediments dominated by
876 *Crenothrix* sp., *Methylobacter* sp. and *Methylocaldum* sp. (all Type I MOB) revealed
877 temperature-dependent changes in relative amino-BHP abundances (Fig. 9; Osborne et al.,
878 2017). All microcosms had highest abundances of aminotriol (>60%), but aminopentol
879 abundances increased with temperature from 2-5% at 4 °C and 21 °C to up to 22% at 40 °C
880 (possibly the optimum growth temperature of the organism responsible for aminopentol
881 synthesis). Further increases in temperature were associated with a decrease to 10% at 50 °C
882 and lack of aminopentol at 60 °C (Osborne et al., 2017). In each experiment, increasing
883 abundances of aminopentol occurred at the expense of aminotriol, whereas relative aminotetrol
884 abundances remained rather constant (9-12%) in all microcosms. These observations were
885 explained by temperature-driven selection for mesophilic *Crenothrix* sp. and *Methylobacter* sp.
886 at lower temperatures and thermophilic *Methylocaldum* sp. at higher temperatures (Sherry et
887 al., 2016; Osborne et al., 2017). If temperature has a direct effect on aminopentol production
888 in specific Type I MOB, or selection for certain Type I MOB, this mechanism could provide
889 an explanation for the previously mentioned absence of aminopentol in deep marine settings
890 (such as seep carbonates), which typically have low in situ water temperatures. Osborne (2016)

also incubated the Tyne sediment under varying CH_4 concentrations, pH values, and salinities (Fig. 9). These results indicate that CH_4 concentrations did not change the amino-BHP composition substantially, but changes in pH and salinity had effects similar to temperature. Salinity affected aminopentol abundances, which were highest at 15 g/L NaCl (24%) and lowest at 120 g/L NaCl (4%), whereas aminotetrol remained at 9-12% abundance irrespective of salinity (Osborne, 2016). In contrast, changes of pH led to a relatively linear response towards higher abundances of both aminopentol and aminotetrol with decreasing pH, i.e., aminopentol and aminotetrol accounted for as much as 18% and 23% at pH 4, respectively, and as low as 0% and 8% at pH 9, respectively (Osborne, 2016).

An example study using a combination of lipidomics and metagenomics to uncover BHP adaptation along environmental gradients comes from Antarctic ice-covered Lake Vanda. Matys et al. (2019b) obtained microbial mat samples across a small-scale (ca. 15 cm) irradiance gradient and showed that decreased photosynthetically active radiation upregulated 2Me-BHP production in cyanobacteria (specifically in the green-pigmented zone). In this case, all HpnP protein sequences obtained from different mat layers in the lake belonged to cyanobacteria (with two different HpnP copies in the green-pigmented zone), suggesting that 2Me-BHP upregulation is indeed a direct response to solar irradiance rather than selection for different species. In a pilot study of nucleoside BHP distributions across an Alaskan soil transect, Rush et al. (2021) found significant correlation between the abundance of nucleoside BHP (including those newly identified by Hopmans et al., (2021)) and environmental variables such as pH, temperature, and precipitation. It seems that the soil bacterial community uses these modifications in nucleoside BHP to adapt to environmental conditions. Further lipidomic and metagenomic work is required to determine the genes responsible for nucleoside BHP synthesis and subsequent structural modification, as well as how these BHPs function to regulate cell membrane stability under different climatic conditions.

916

917 **4.2 Future analytical needs and frontiers**

918 **4.2.1 Improving comparability between laboratories**

919 As outlined above (Section 2.4), quantification remains an obstacle in making BHP data
920 fully comparable between laboratories, even when data are reported in relative abundances
921 only. One step towards at least realizing (and to a certain extent, alleviating) the apparent
922 differences between laboratories is the organization of an inter-laboratory round robin study
923 that will compare analytical precision and reproducibility; similar to those achieved for GDGTs
924 and highly branched isoprenoids (e.g., Schouten et al. 2009; 2013b; Belt et al. 2014). Analytical
925 biases should be assessed in and compared between laboratories and instruments using
926 gravimetric mixtures of purified BHPs, as well as aliquots of the same environmental extracts
927 containing many known BHPs (which could be achieved by admixing various terrestrial and
928 marine samples). In the same way, biases arising from BHP extraction methods (Section 2.1)
929 can be tested by providing each laboratory with the same sediment or biomass material. The
930 environmental extract would afterwards be applied by the BHP community as a shared 'in-
931 house' standard mix, provided with consensus abundances (relative and absolute) from the
932 round robin. This would allow laboratories to normalize BHP abundances onto a common basis
933 and monitor instrument performance long-term, across data sets and time.

934

935 **4.2.2 Isotope analysis**

936 Following the relatively recent development of HTGC-irMS methods for the $\delta^{13}C$
937 analysis of intact BHPs (Hemingway et al., 2018; Lengger et al., 2019), these await exploitation
938 of their full potential. For instance, isotopic analysis of BHPs can then be used to trace methane
939 as a carbon source of BHP-producing bacteria, to confirm an anammox origin of BHT isomer
940 (including when BHT-x and BHT-II co-elute during HPLC analysis) in the water column and

941 sediments, or to evaluate kinetic fractionation factors during BHP synthesis under autotrophic
942 and heterotrophic growth conditions (as a means to infer the 'metabolic state' of BHPs).
943 The next analytical frontiers should include modifying these HTGC-irMS methods
944 (Hemingway et al., 2018; Lengger et al., 2019) to allow δ^2H analysis of intact BHPs; extending
945 what is thus far only available for hopanols (e.g., Li et al., 2009). The feasibility of analyzing
946 the ^2H isotopic composition of high polarity compounds via HTGC-irMS has been
947 demonstrated by Lengger et al. (2021) for GDGTs. Scrutinizing the substantial differences in
948 the hydrogen isotopic composition of precipitation (annual mean δ^2H varies roughly between
949 −20 to −200‰, depending on latitude) and seawater (δ^2H=0‰) (Fig. 10A), δ^2H analysis of
950 BHPs might, for instance, aid in distinguishing BHPs produced on land from those produced
951 in situ (in marine water columns or sediments). The latitudinal ^2H_2O gradients should be
952 conserved in BHPs synthesized by the same source organisms and/or via the same metabolism,
953 given that kinetic ^2H fractionation strongly depends on the carbon flux through the different
954 hydrogenases reducing $NADP^+$ to NADPH and growth water (Wijker et al., 2019).
955 Accordingly, BHP δ^2H analysis will aid in solving a long-standing question about the origin of
956 the majority of BHPs in the ocean/sediments (e.g., Pearson et al., 2009; Sáenz et al., 2011a).
957 Concurrent compound-specific ^{14}C analysis would strongly support this type of 'fingerprinting'
958 given the strong latitudinal $\Delta^{14}C$ gradient observed for sedimentary plant waxes (Fig. 10B),
959 which is mainly controlled by climatic factors (Eglinton et al., 2021; Kusch et al., 2021a).
960 Hydrogen and carbon isotope analysis will also aid in identifying the utilization of dissolved
961 methane (vs. dissolved inorganic carbon) by bacteria in the ocean and, once kinetic
962 fractionation factors are constrained, may hold clues regarding which type of methane (e.g.,
963 biogenic vs. thermogenic) is used (Fig. 10C) (e.g., Whiticar, 1999). In the case of amino-BHPs,
964 their dual ^{13}C and ^2H isotope compositions could also help to identify (and possibly to quantify)
965 relative proportions of aminotetrol synthesized by Type I and Type II MOB, respectively, in

966 that the assimilation of CO_2 in addition to CH_4 by Type II (serine pathway) could be revealed
967 (Jahnke et al., 1999). Likewise, potential contributions of aminotetrol and aminopentol from
968 SRB could be revealed in marine sediments, since these bacteria utilize a range of organic
969 substrates, such as amino acids, sugars, and long-chain alkanoic acids (Muyzer and Stams,
970 2008), which would not carry methane isotope imprints if derived from the surface ocean or
971 the continent.

972 Additional information on the terrestrial/soil nitrogen cycle may reside in the ^{15}N
973 isotope composition of the head group of nucleoside BHPs, pending method developments that
974 allow cleaving the nitrogen-containing nucleosides (e.g., adenine, inosine) for analysis. Such
975 data could reveal the nitrogen species used by/the metabolism of the hitherto unknown source
976 organisms (potentially aided by δ^2H analysis; Wijker et al., 2019), which in turn should help
977 constrain the bacterial producers (both in soils and in situ in marine OMZs). Given that many
978 BHPs in soils accumulate in the rhizosphere and are produced by N_2-fixing plant symbionts
979 (e.g., Ricci et al., 2014; Kulkarni et al., 2015; Belin et al., 2019; Tookmanian et al., 2021),
980 nucleoside BHPs may be synthesized by N_2-fixing bacteria, rather than by heterotrophic
981 bacteria. Fixation of atmospheric N_2 and nitrate/ammonia utilization should be directly
982 distinguishable in nucleoside $\delta^{15}N$ values, especially in settings where soil N is artificially ^{15}N-
983 enriched due to the use of fertilizers or bacterial denitrification (e.g., Hobbie and Ouimette,
984 2009; Denk et al., 2017). If the source organisms are indeed heterotrophic bacteria and the
985 metabolic routing is constrained/fractionation factors are established, potential climatic effects
986 on nucleoside BHP $\delta^{15}N$ values could be investigated, exploiting the natural soil $\delta^{15}N$ gradient
987 (increase with mean annual precipitation and decrease with mean annual temperature) observed
988 with latitude (e.g., Amundson et al., 2003).

989

990 **4.2.3 Pairing BHP lipidomics and other -omics**

991 Owing to the recent analytical advances in HPLC-MS methodologies and isotope
992 analysis outlined above, the field of organic geochemistry is entering a new era of BHP
993 lipidomics. This opportunity also comes with its own challenges. A longstanding complication
994 to identifying potential (BHP) biomarkers for bacteria and/or environmental processes in
995 complex systems is that this task depended on manual mass spectral interpretations and focus
996 was usually only on the dominant lipids. This inherently leaves a large proportion of the
997 lipidome hidden, which means that microorganisms that do not make up a significant part of
998 the microbiome and likely do not contribute large stocks to the total lipid pool, are easily
999 overlooked. Traditionally, this problem was circumvented through analysis of culture and
1000 enrichment material of microbes known to be important players in biogeochemical cycles. For
1001 example, the lipid biomarkers of anammox bacteria were identified using HPLC-MS analysis
1002 of enrichment cultures (Sinninghe Damsté et al., 2002) and only applied to environmental
1003 settings afterwards using targeted (e.g., MS/MS) methodology (Kuypers et al., 2003). An
1004 alternative approach is untargeted lipidomics, using high resolution mass spectrometry (Pluskal
1005 et al., 2010; 2020), and making use of advances in data processing (e.g., Steen et al., 2020).
1006 Such an untargeted lipidomics approach applied to Black Sea SPM revealed microbial
1007 networks and niche partitioning across the oxic and anoxic marine water column (Bale et al.,
1008 2021; Ding et al., 2021). This method has the advantage of allowing determination of the
1009 diversity of unknown lipids and their importance in specific environmental niches. Future
1010 applications of this method to environmental lipidomes will allow the identification of novel
1011 lipids, including BHPs, and their potential function and proficiency as biomarkers for important
1012 microbial processes within Earth's biogeochemical cycles. Further breakthroughs will come
1013 from pairing untargeted BHP lipidomics with other -omics. Pioneering genomics work in the
1014 last decade has tremendously improved our understanding of the diversity of BHP producers
1015 in the environment (metagenomics) as well as the physiological/functional role of BHPs in

bacterial membranes (gene knock-out experiments) (e.g., Welander et al., 2009; Bradley et al., 2010; Doughty et al., 2011; Kulkarni et al., 2013; Welander and Summons, 2012; Neubauer et al., 2015; Sáenz et al., 2015). Screening for hopanoid synthesis genes has now become a standard tool in many BHP studies for identification of putative BHP producers in environmental samples (e.g., Pearson et al., 2009; Kharbush et al., 2013; Matys et al., 2019a,b). Such combined -omics have revealed a much higher diversity of bacteria capable of synthesizing BHPs than would have ever been known from culturing work alone, yet BHP production by many of the bacterial species/genera identified in environmental samples can ultimately not be confirmed given the prevalent lack of cultured representatives. However, thus far, genomics-assisted studies have mostly targeted only 1-2 genes (e.g., *shc* and either *hpnP* or *hpnR*), whereas genes required to synthesize other side chains/head groups (e.g., *hpnO*, *hpnI*, *hpnK*, *hpnJ*) have received virtually no attention. Screening for these genes (as well as the synthesis genes for newly identified BHPs; Hopmans et al., 2021) may, for example, help unlock the untapped potential of composite BHPs, for which sources at present seem to be diverse (e.g., Renoux and Rohmer, 1985; Flesch and Rohmer, 1989; Talbot and Farrimond, 2007; Höfle et al., 2015). Yet, the distribution and diversity of composite BHPs has been shown to be highly variable, especially in settings such as microbial mats or geothermal systems, where they seem to reflect environmental conditions (e.g., Gibson, 2009; Gibson et al., 2014). Important insights will be gained about the controls on environmental BHP diversity when the full suite of known hopanoid synthesis genes is included in metagenomic surveys and directly linked to the diverse environmental BHP inventory.

5. Conclusions

Much work has been done since bio- and geohopanoids were first declared 30 years ago to be the “most abundant natural products on Earth”. The identification of specific bacteria

1041 responsible for the synthesis of unique BHPs including methane-oxidizing bacterial
1042 communities and anaerobic ammonium-oxidizing bacteria, as well as the suite of BHPs derived
1043 from soils, ground truths their applications as biomarkers. New BHPs with chemotaxonomic
1044 potential have also recently been identified, both in cultures and in the environment. The onset
1045 of molecular microbiology approaches to dig deeper into the biological mechanisms that
1046 underpin hopanoid structural transformation has contextualized BHP application to important
1047 organic geochemistry questions. With the advent of advanced multi-omics techniques and more
1048 sensitive mass spectrometric analyses, we are on the brink of an explosion in BHP research and
1049 leaps forward in understanding the synthesis and function of BHPs in the bacterial cell, their
1050 sources, distribution, and diversity in the environment, and improved organic
1051 geochemical/proxy applications.
1052
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Figures

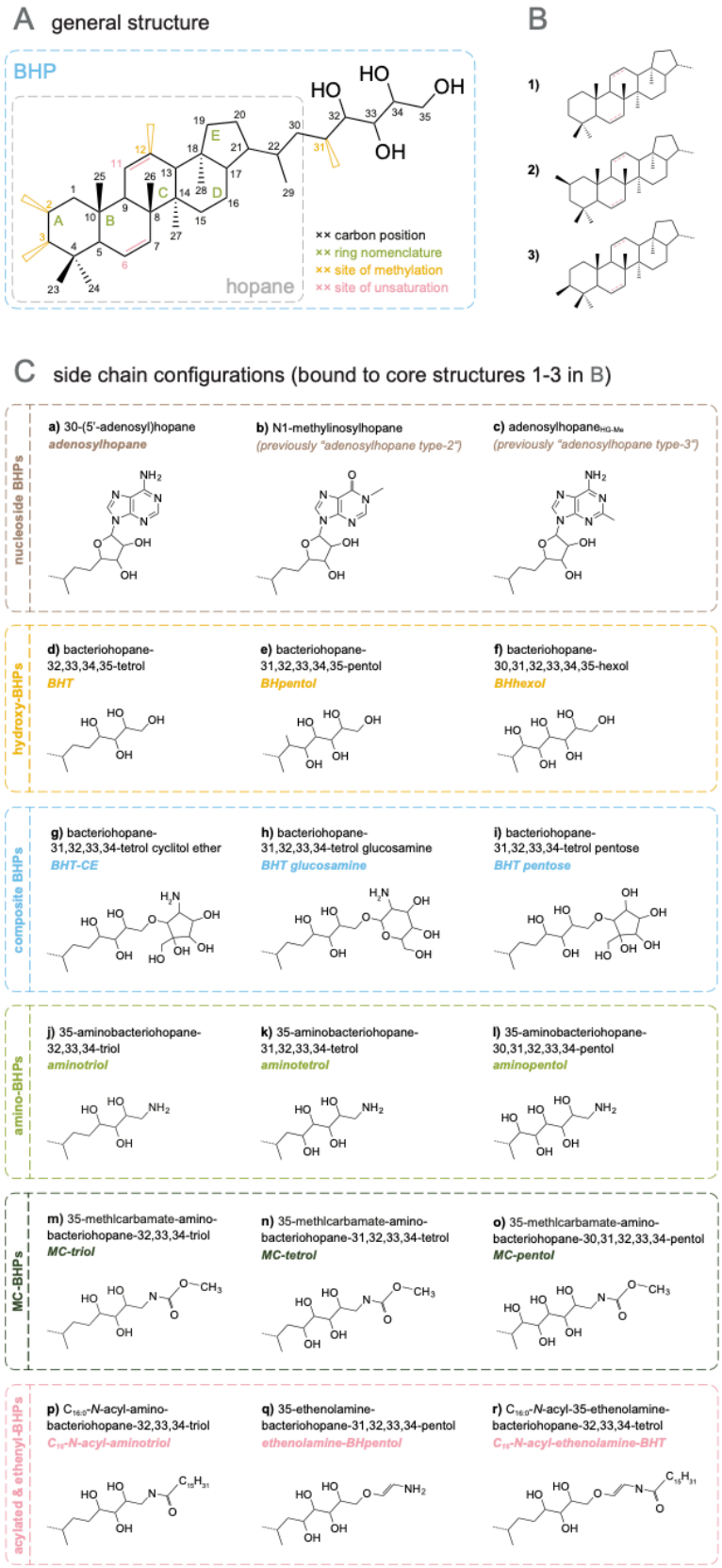

A general structure
BHP
hopane
×× carbon position
×× ring nomenclature
×× site of methylation
×× site of unsaturation
B
1)
2)
3)
C side chain configurations (bound to core structures 1-3 in B)
nucleoside BHPs
a) 30-(5'-adenosyl)hopane
adenosylhopane
b) N1-methylinosylhopane
(previously "adenosylhopane type-2")
c) adenosylhopane
(previously "adenosylhopane type-3")
hydroxy-BHPs
d) bacteriohopane-32,33,34,35-tetrol
BHT
e) bacteriohopane-31,32,33,34,35-pentol
BHpentol
f) bacteriohopane-30,31,32,33,34,35-hexol
BHhexol
composite BHPs
g) bacteriohopane-31,32,33,34-tetrol cyclitol ether
BHT-CE
h) bacteriohopane-31,32,33,34-tetrol glucosamine
BHT glucosamine
i) bacteriohopane-31,32,33,34-tetrol pentose
BHT pentose
amino-BHPs
j) 35-aminobacteriohopane-32,33,34-triol
aminotriol
k) 35-aminobacteriohopane-31,32,33,34-tetrol
aminotetrol
l) 35-aminobacteriohopane-30,31,32,33,34-pentol
aminopentol
MC-BHPs
m) 35-methlcarbamate-amino-bacteriohopane-32,33,34-triol
MC-triol
n) 35-methlcarbamate-amino-bacteriohopane-31,32,33,34-tetrol
MC-tetrol
o) 35-methlcarbamate-amino-bacteriohopane-30,31,32,33,34-pentol
MC-pentol
acylated & ethenyl-BHPs
p) C16:0-N-acyl-amino-bacteriohopane-32,33,34-triol
C16-N-acyl-aminotriol
q) 35-ethenolamine-bacteriohopane-31,32,33,34-pentol
ethenolamine-BHpentol
r) C16:0-N-acyl-35-ethenolamine-bacteriohopane-32,33,34-tetrol
C16-N-acyl-ethenolamine-BHT

Fig 1: A) Generalized structure of bacteriohopanepolyols including the hopane skeleton and the extended side chain (example shown: BHT). Other BHP side chain configurations for commonly occurring BHPs and/or those referred to in the text are also shown (B and C). The side chain classification is based on the structure of the moieties. Nucleoside BHPs (a-c) are characterized by adenosine or inosine head groups (these BHPs have previously also been termed 'soil-marker' BHPs and adenosyl-BHPs), hydroxy-BHPs (d-f) group simple polyols; composite BHPs (g-i) include those containing an (amino)sugar head group; amino-BHPs (j-l) have a simple amine group at C-35; MC-BHPs (m-o) contain a methylated carbamic acid ester at C-35; acylated and/or ethenyl-BHPs (p-r) include BHPs that are bound to an alkanoic acid moiety via an *N*-acyl or an ethenolamine group or have a simple ethenolamine group.

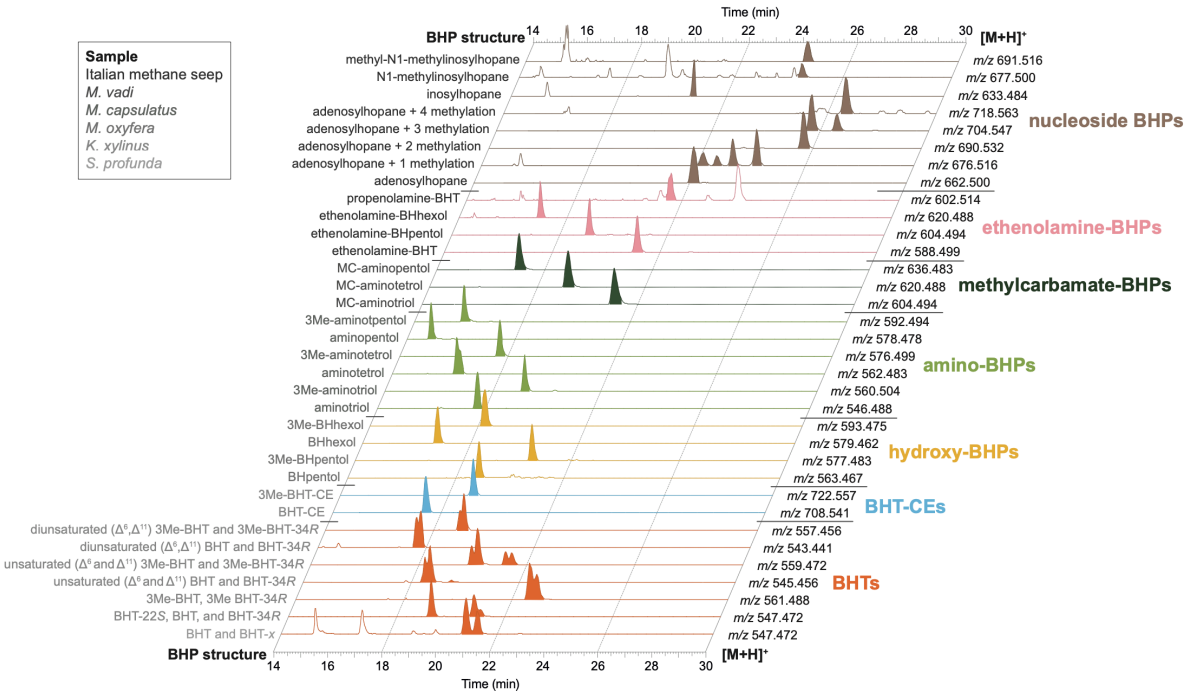


Fig 2: Example extracted ion chromatograms (EICs) of environmental and culture samples (Fuoco di Censo seep Sicily, Italy,, *Methylomarinum vadi* (strain IT-4), *Methylococcus capsulatus* (strain Bath), '*Ca*. M. oxyfera', *Komagataeibacter xylinus* strain R-2277, and '*Ca*.

Scalindua profunda') taken from Hopmans et al. (2021) and aligned to the retention time of BHT. We report the protonated ion ($[M+H]^+$). Note that peak heights are plotted as best fit and are not comparable between EICs. Further details of high resolution masses used to generate these chromatograms as well as reference to novel *N*-acylated BHPs, which elute between 30–45 minutes, can be found in Hopmans et al. (2021).

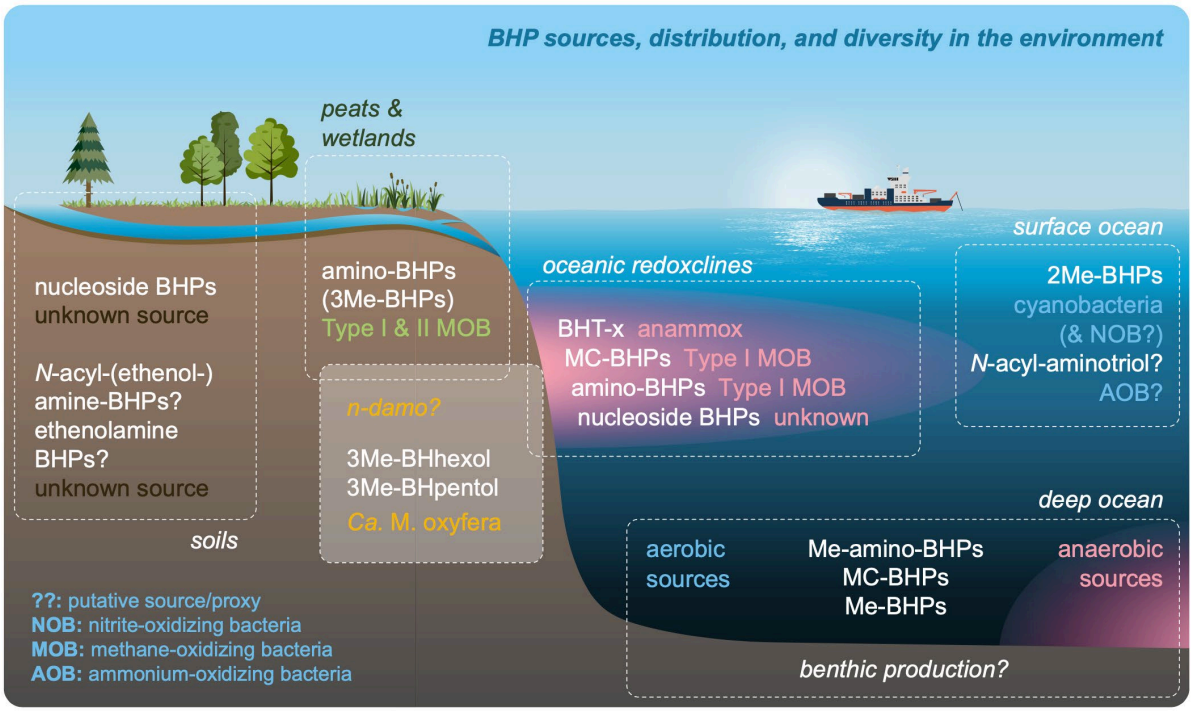


Fig 3: Schematic showing BHP distribution and diversity in the environment, including novel BHPs and their putative sources/origin such as *N*-acyl-amine-BHPs or (*N*-acyl-)ethenolamine-BHPs (for more detail, see Talbot et al., 2007a; Kusch et al., 2019; 2021b; Hopmans et al., 2021). See Fig. 1 for details on BHP structures and 'classification'.

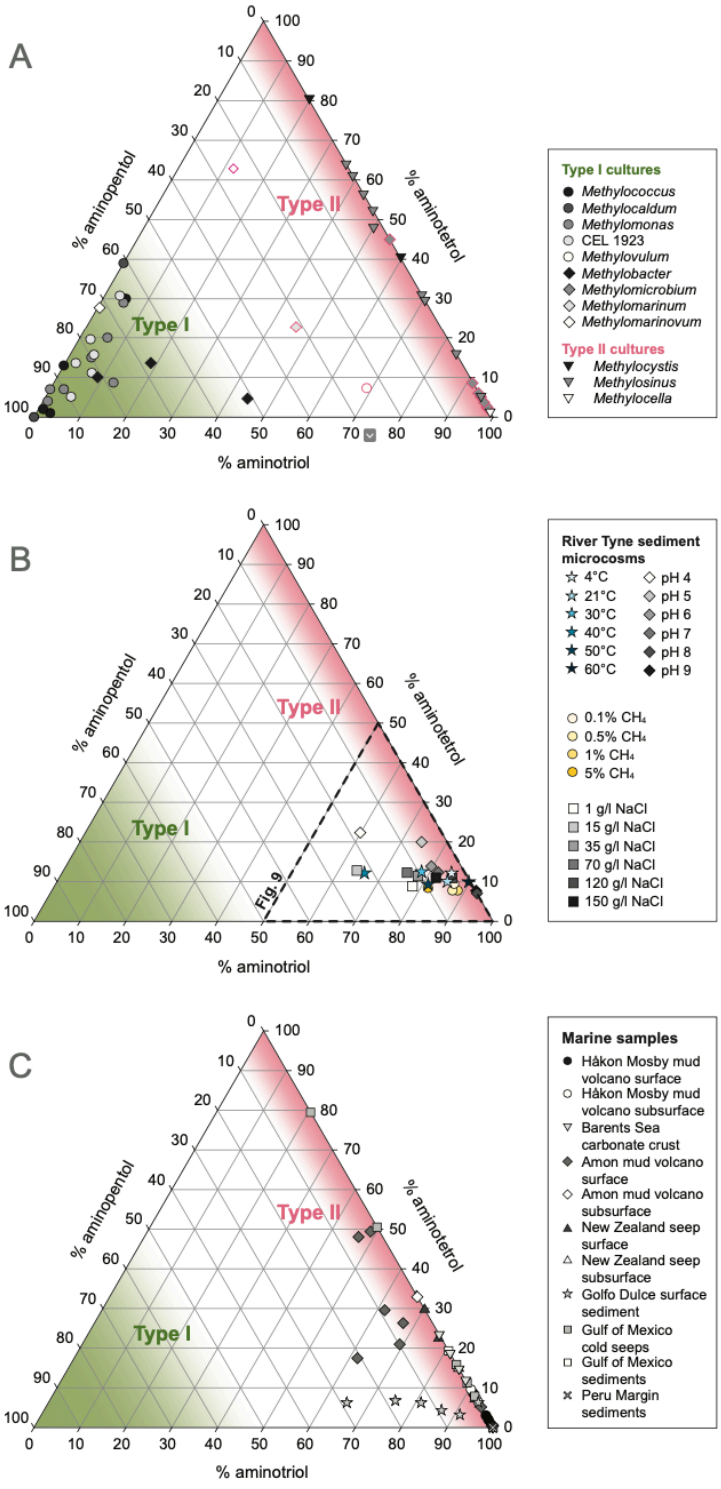


Fig 4: Amino-BHP (aminopentol, aminotetrol, and aminotriol) distributions in (A) MOB cultures (data from Rohmer and Ourisson, 1984; Jahnke et al., 1999; Talbot et al., 2001; Zhu et al., 2011; van Winden et al., 2012; Rush et al., 2016), (B) microcosm incubations of River Tyne sediment (data from Osborne, 2016; Osborne et al., 2017), and (C) marine environmental

samples from methane-influenced settings (data from Rush et al., 2016). Green and pink shaded areas indicate the presumed Type I and Type II MOB endmember ranges based on data in (A); outliers from these ranges are highlighted by pink contour lines. Dashed contour line in B) indicates the close-ups shown in Fig. 9.

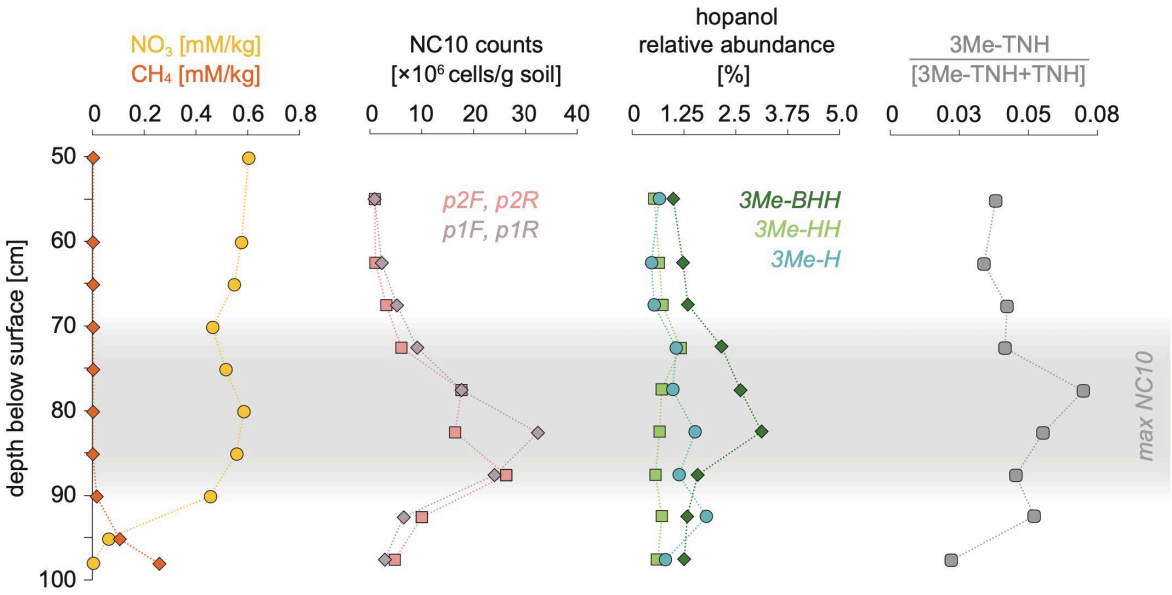
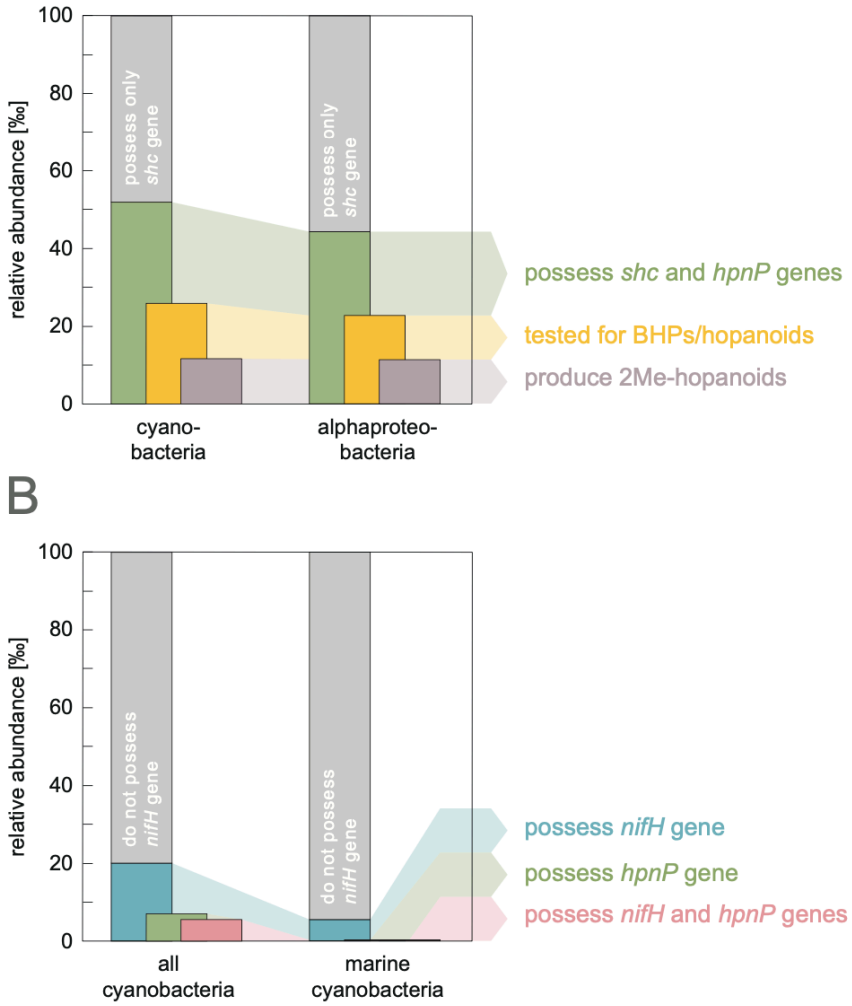


Fig 5: Nutrient, cell count, and hopanoid depth profiles of a peat core from the Brunssummerheide, SE Netherlands. NC10 cell numbers as assessed by qPCR (results shown for primer pairs p1F, p1R and p2F, p2R). 3Me-BHH: 3Me-bishomohopanol; 3Me-HH: 3Me-homohopanol; 3Me-H: 3Me-hopanol; 3Me-TNH/[3Me-TNH+TNH]: 3Me-22,29,30-trisnorhopan-21-ol/(3Me-22,29,30-trisnorhopan-21-ol+22,29,30-trisnorhopan-21-ol. Data from Zhu et al. (2012), Kool et al. (2014), and Smit et al. (2019).



1873

1874 Fig 6: A) Nested relative abundance of strains that possess the *hpnP* and *shc* genes, have been
1875 tested for BHP/hopanoid production, and produce 2Me-hopanoids among all cyanobacterial
1876 and alphaproteobacterial cultures possessing the *shc* gene. Data from Naafs et al. (2021). B) A)
1877 Nested relative abundance of cyanobacteria that possess the *nifH* gene, the *hpnP* gene, and both
1878 the *nifH* and *hpnP* genes. Data from Elling et al. (2020).

1879

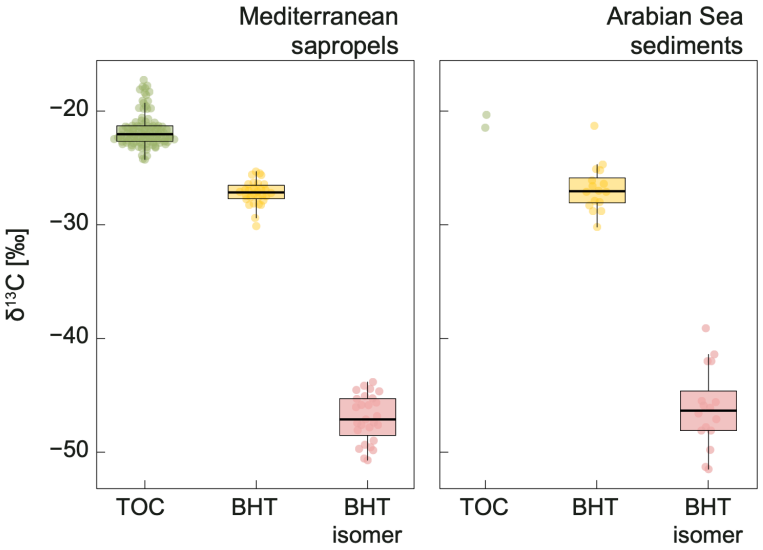


Fig 7: Stable carbon isotope distribution of total organic carbon, BHT, and BHT isomer in Mediterranean sapropels and Arabian Sea surface sediments. Data from Elling et al. (2021) and Lengger et al. (2019). Arabian Sea sediments include data obtained on unamended samples and data from oxic or suboxic isotope probing incubations (amendment of ^{13}C-labeled dissolved and/or particulate organic matter).

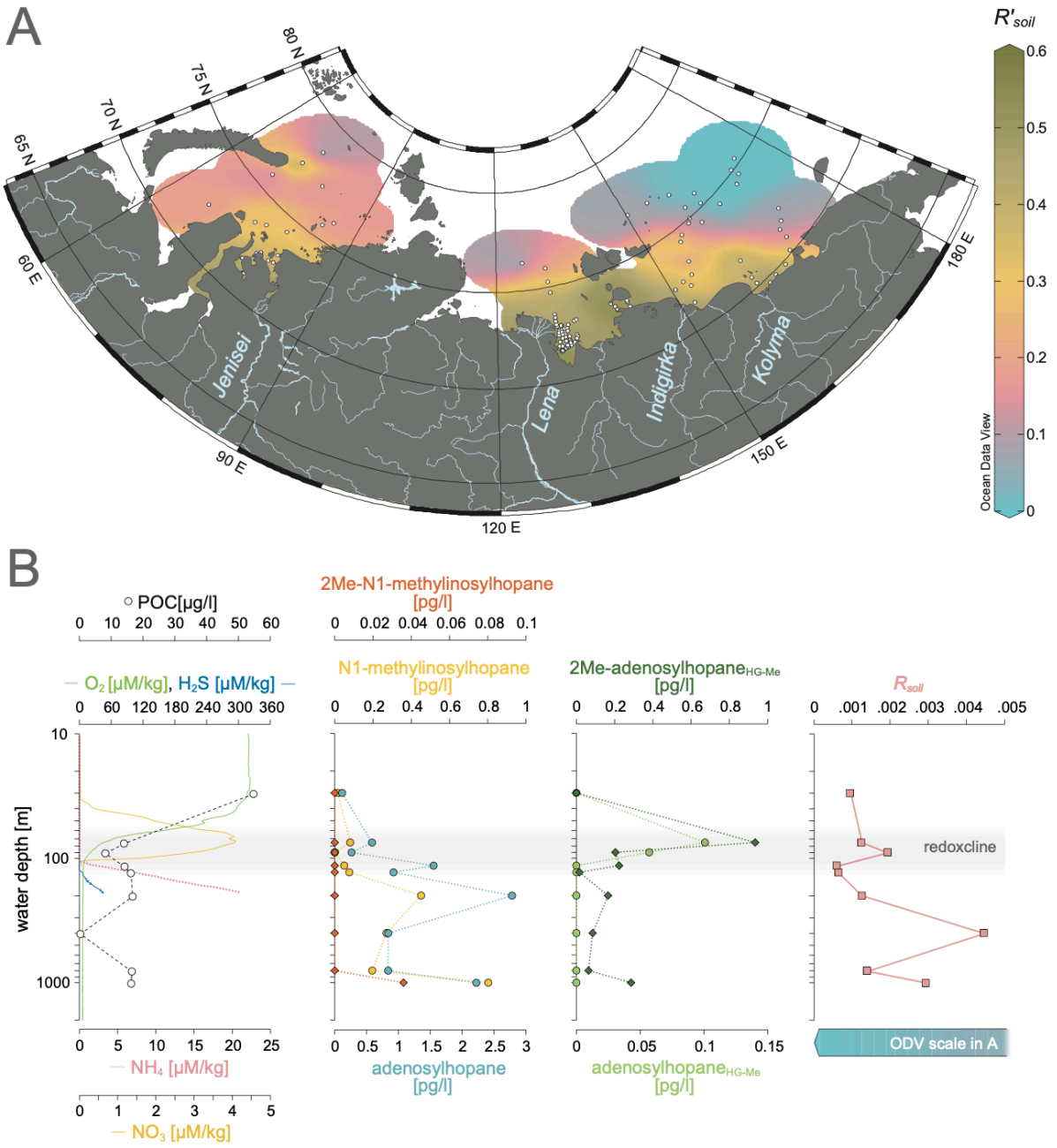


Fig 8: A) Spatial *R'soil* pattern in surface sediments off major Siberian rivers. Data from Bischoff et al. (2016) and De Jonge et al. (2016); B) nucleoside BHP depth profiles in the eastern central gyre of the Black Sea showing *in-situ* production at depth (data from Kusch et al., 2021c). Note that *Rsoil* and *R'soil* values are essentially identical within ±0.001 for this water column profile.

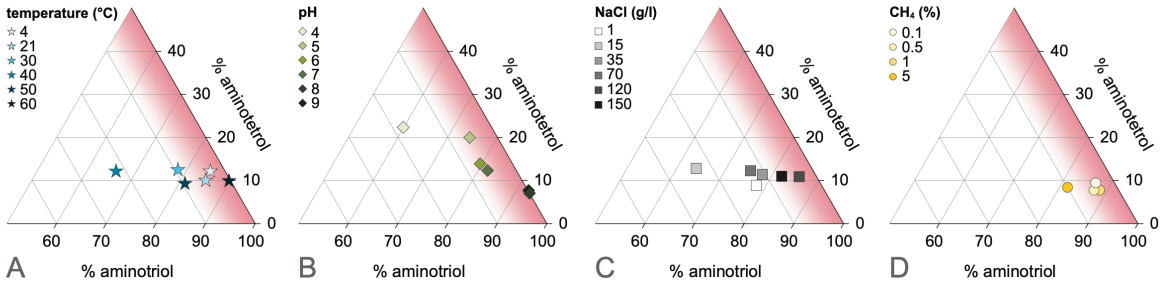


Fig 9: Close-up of amino-BHP (aminopentol, aminotetrol, and aminotriol) distributions in microcosm incubations of River Tyne sediment. Shown are the effects of changes in A) temperature, B) pH, C) salinity, and D) ambient methane concentrations. Data from Osborne (2016) and Osborne et al. (2017). See Fig. 4 for reference.

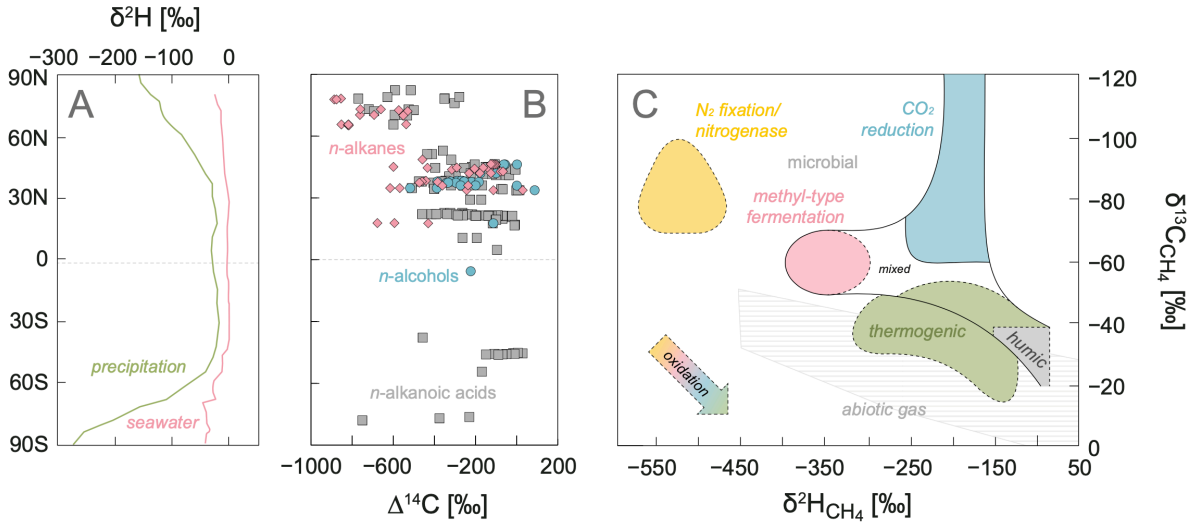


Fig 10: Natural isotope gradients to be exploited for BHP isotope analysis. A) Latitudinal precipitation and seawater δ^2H gradients. Precipitation δ^2H modeled using GNIP data (Speelman et al., 2010), seawater δ^2H modeled using GEOSECS data (Xu et al., 2012). Assuming similar kinetic fractionation effects, BHPs produced on land can be expected to have substantially lower δ^2H values; B) Latitudinal $\Delta^{14}C$ gradient of *n*-alkyl plant waxes in marine core-top sediments (figure re-drawn from Kusch et al., 2021a). BHP $\Delta^{14}C$ values can be expected to show the same trend if BHPs have a terrestrial origin; C) Stable carbon and

hydrogen isotopic composition of methane sources (endmember ranges from Whiticar, 1999; Etiope et al., 2013; Niemann and Whiticar, 2017; Luxem et al., 2020). Arrow depicts the generalized trajectory of methane ^{2}H and ^{13}C enrichment resulting from preferential uptake of lighter isotopes during bacterial oxidation. BHP $\delta^{2}H$ and $\delta^{13}C$ values can be expected to follow the opposite trend.