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Title

Environmental controls on bacteriohopanepolyol profiles of benthic microbial mats from Lake Fryxell, Antarctica

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Abstract

Bacteriohopanepolyols (BHPs) are pentacyclic triterpenoid lipids that contribute to the structural integrity and physiology of some bacteria. Because some BHPs originate from specific classes of bacteria, BHPs have potential as taxonomically and environmentally diagnostic biomarkers. For example, a stereoisomer of bacteriohopanetetrol (informally BHT II) has been associated with anaerobic ammonium oxidation (anammox) bacteria and suboxic to anoxic marine environments where anammox is active. As a result, the detection of BHT II in the sedimentary record and fluctuations in the relative abundance of BHT II may inform reconstructions of nitrogen cycling and ocean redox changes through the geological record. However, there are uncertainties concerning the sources of BHT II and whether or not BHT II is produced in abundance in non-marine environments, both of which are pertinent to interpretations of BHT II signatures in sediments. To address these questions, we investigate the BHP composition of benthic microbial mats from Lake Fryxell, Antarctica. Lake Fryxell is a perennially ice-covered lake with a sharp oxycline in a density stabilized water column. We describe the diversity and abundance of BHPs in benthic microbial mats across a transect from oxic to anoxic conditions. Generally, BHP abundances and diversity vary with the morphologies of microbial mats, which were previously shown to reflect local environmental conditions, such as irradiance and oxygen and sulfide concentrations. BHT II was identified in mats that exist within oxic to anoxic portions of the lake. However, anammox bacteria

have yet to be identified in Lake Fryxell. We examine our results in the context of BHPs as biomarkers in modern and ancient environments.

Keywords: Anammox, Bacteriohopanepolyol, Bacteriohopanetetrol isomer, Biomarker, McMurdo Dry Valleys

1. Introduction

Stable lipid biomarkers such as hopanes preserve evidence of ancient biological activity in the geological record (Brocks and Pearson, 2005; Peters et al., 2005). Hopane parent molecules, bacteriohopanepolyols (BHPs), are pentacyclic triterpenoid lipids that contribute to membrane structural integrity and physiology in some α -, β -, and γ -proteobacteria, cyanobacteria and planctomycetes. A large diversity of BHPs that mainly differ in the side chain structures, has been described (e.g. Talbot et al., 2007). Side chain structures may be specific to particular organisms or be formed in response to particular environmental conditions. For this reason, BHPs have great potential as taxonomically and environmentally diagnostic biomarkers. However, an incomplete understanding of BHP

diversity and source organisms in modern environments precludes the robust interpretation of hopanes in the rock record.

Analytical advances, such as the development of high performance liquid chromatography mass spectrometry (HPLC-MS) methods for the analysis of intact BHPs, (Talbot et al., 2001, 2003; Kusch et al., 2018) and compound-specific response factors (Wu et al., 2015) have enabled robust identification and semi-quantification of BHPs in complex samples from a range of modern environments. For example, it is now possible to separate, identify, and quantify composite BHPs and stereoisomers. Furthermore, the development of culture-independent techniques to predict the capacity for hopanoid biosynthesis through the identification of biosynthetic genes (e.g. Pearson and Rusch, 2009; Welander et al., 2010; Ricci et al., 2014, 2015) has greatly increased our understanding of hopanoid source organisms and limits the necessity of culture-dependent techniques (Rashby et al., 2007). However, the complete biosynthetic pathways for some BHPs are not yet fully understood. It is therefore important to characterize BHP diversity, possible source organisms, and associated environmental conditions in a wide range of previously unexplored environments to constrain geobiological interpretations of hopanes in the rock record.

To date, environmental studies of BHPs have focused predominantly on low- to mid-latitude marine, lacustrine (Castañeda and Schouten, 2011), and terrestrial (Cooke et al., 2008) environments. For example, a stereoisomer of bacteriohopanetetrol (BHT), informally denoted BHT II, has been identified in marine oxygen minimum zones (OMZs; e.g. Sáenz et al., 2011; Wakeham et al., 2012; Kharbush et al., 2013; Matys et al., 2017), sediments from an anoxic marine fjord-like enclosure (Rush et al., 2014), and in enrichment cultures of anaerobic ammonium-oxidizing (anammox) bacteria (Rush et al., 2014). Additionally, ^{13}C -depleted BHT II values relative to BHT values observed in Mediterranean Sea sediments appear to support the utility of BHT II as a proxy for anammox bacteria (Hemingway et al., 2018). However, other sources of BHT II may exist in environments that have yet to be studied. For example, isomers of BHT, possibly BHT II, are produced by purple non-sulfur bacterium *Rhodopseudomonas acidophila* (Neunlist et al., 1988), acetic acid bacteria (Peiseler and Rohmer, 1992; e.g. *Acetobacter* spp.), *Frankia* sp. (Rosa-Putra et al., 2001), and an aerobic Type II methanotrophic bacteria (*Methylocella palustris*; van Winden et al., 2012), complicating the utility of BHT II as a proxy for any particular organism or environmental condition.

The “BHT II ratio” ($\text{BHT II}/\text{BHT} + \text{BHT II}$) has been proposed as a proxy for past changes in the relative importance of anammox and fluctuations in nitrogen cycling in response to ocean redox changes through the geological record. The development of this biomarker has significant implications for tracing past changes in the marine nitrogen cycle, as anammox bacteria are a major sink for fixed nitrogen in marine environments (Dalsgaard et al., 2005). However, the full diversity of BHT II sources remains unclear. A biosynthetic pathway specific to BHT II has yet to be elucidated, precluding the investigation of the capacity for BHT II production through phylogenomic approaches. As a result, environmental screening for BHT II and possible source organisms is, currently, the best way to document its environmental range, identify any additional sources of BHT II, and to assess the robustness of BHT II as a proxy for anammox bacteria in suboxic to anoxic environments.

Few studies have described BHPs in high latitude regions and in particular, Antarctica, despite the high diversity of BHPs identified relative to low-latitude settings, including tetra- and penta-functionalized, methylated, non-methylated and unsaturated BHPs identified there (Talbot et al., 2008; Cooke et al., 2009; Matys et al., 2018). Here, we describe BHP diversity and abundance in benthic microbial mats from Lake Fryxell, Antarctica. The benthic microbial communities in Lake Fryxell are structured according to local environmental conditions, such as irradiance and oxygenation (Jungblut et al., 2016). Our results suggest that BHP profiles vary with mat morphotype and local environmental conditions, with the most substantial change in BHP diversity occurring across the oxycline. Of particular interest is the abundance of BHT II and shifts in penta-functionalized hopanoids across the oxic-anoxic transition. These results extend knowledge concerning the distribution and diversity of BHPs in polar regions and afford insights for interpreting the distribution of BHPs, including BHT II, in modern environments and sedimentary archives.

2. Materials and methods

2.1. Site Description

Lake Fryxell (77°36'S 162°6'E) is a closed-basin, perennially ice-covered lake in the McMurdo Dry Valleys, Antarctica (Fig. 1). The ice cover (approximately 4-5 m thick; Vincent, 1981) has contributed significantly to the development of the lake structure. The balance between ablation of the ice cover and inflowing melt water determines annual changes in lake level (Dugan et al., 2013). Ice ablates from the top of the ice cover and

freezes on the bottom; as a result, stable or falling lake levels are associated with increasing salinity as solutes are excluded from the ice cover. Conversely, excess inflowing melt water during summer months, while an ice-free moat forms around much of the lake margin, can lead to development of a freshwater lens beneath the ice cover. As a result, the lake level history at Lake Fryxell has led to a strong salinity gradient and density stratified water column (Lyons et al., 2005).

Physical and chemical variables of Lake Fryxell at the time of sampling (November 2012) were reported and are described in detail in Jungblut et al. (2016; Table 1). Measurements were made both in the water column and at the mat-water interface. Briefly, irradiance was determined using a Li 192 photosynthetically active radiation (PAR) sensor connected to a Li-Cor Li 1400 m in a waterproof housing. Simultaneous measurement of irradiance incident to the lake surface (Li 190 PAR sensor) enabled the calculation of percent surface irradiance. Conductivity-temperature-depth-oxygen (CTDO) profiles were obtained using a Richard Brancker “Concerto” CTD.

The ice cover limits the transport and diffusion of solutes and gasses into and out of Lake Fryxell. As a byproduct of photosynthesis and freeze-concentration of dissolved gasses entering the lake through melt water streams, oxygen is present at high concentrations (137-622 $\mu\text{mol/kg}$) in the upper water column. Net photosynthesis transitions to net respiration with declining irradiance with water depth. This transition enables the development of an oxycline between 9-10 m depth and complete anoxia (O_2 limit) below 10 m depth (Sumner et al., 2015). The oxycline also coincides with an increase in sulfide, dissolved reactive phosphorus (DRP), and ammonium (NH_4^+) concentrations into the anoxic zone, from low concentrations in overlying oxic waters. Nitrate (NO_3^-) remains at low concentrations at all depths described here (Jungblut et al., 2016; Table 1).

Benthic microbial mats colonize the Lake Fryxell floor. The microbial communities are structured according to local environmental conditions, such as oxygenation and irradiance (Jungblut et al., 2016). While geochemical gradients are steep vertically throughout the water column, the transition from an oxic to anoxic water column intersects the gently sloping lake floor over a relatively long horizontal distance (Fig. 2). Across the lake bottom, phototrophic communities are present at depths of up to 10.5 m (Wharton et al., 1983), with a change in dominant metabolism from oxygenic to anoxygenic photosynthesis occurring below the oxycline (9-10 m). Microbial mat morphologies change considerably across the oxycline: (i) cusped pinnacle mats are present in the upper hyperoxic zone from

approximately 8.8–9.4 m depth, (ii) ridge-pit mats are present immediately above the oxic-anoxic transition from approximately 8.8–9.7 m, and (iii) prostrate mats are present in the upper anoxic zone from approximately 9.6–10.3 m. Flocculent biomass is present below (10.1–11 m) and is defined by a non-cohesive accumulation of dark gray-brown organic material (Jungblut et al., 2016).

Benthic microbial mats typically consist of well-defined internal zones, formed as a result of local environmental conditions and microorganisms performing distinct metabolisms. For example, in Lake Vanda, another ice-covered lake in the McMurdo Dry Valleys, the benthic microbial mats contain characteristic pigmented zones that reflect acclimation of cyanobacteria to changing spectral characteristics through the synthesis of particular pigments (Hawes et al., 2013). The photosynthetic communities in Lake Fryxell may also produce transient microenvironments with O₂ concentrations of ~50 µmol O₂/L (Sumner et al., 2015). Similarly, it is possible that transient suboxic to anoxic environments may also exist within individual mats, especially during the dark winter months.

2.2. Sample collection

Sampling at Lake Fryxell was conducted in November 2012 (Table 2), as described by Jungblut et al. (2016). Biomass was collected by SCUBA divers, who operated through a single dive hole melted through the ice cover at 77°36.4'S, 163°09.1'E.

Due to the gradual slope of the lake bottom, the horizontal benthic transect distance (8–11 m depth) was approximately 50 m (Jungblut et al., 2016). Whole benthic microbial mats were sampled at six depths along the transect between 8.9 and 11 m depth (Fig. 2; Table S2). The samples were taken with a 38-mm-diameter corer. Replicate core samples (same technique and, similar mat morphology) were taken from all depths and were divided between multiple studies (i.e. Jungblut et al., 2016). As a result, replicates from 9.0, 9.7, 9.9, 10.6, and 11 m were available for this study (Table S2). Individual cores were placed into a 60-ml wide-mouthed bottle underwater, sealed, and returned to the surface. On-site processing consisted of draining excess water and freezing (-20° C). Upon return to New Zealand, samples were freeze-dried and ground to a fine powder.

Benthic microbial mats of ridge-pit morphology were collected from 9.4 and 9.5 m depth in order to investigate the spatial heterogeneity of BHP concentrations within individual mat structures. Individual ridge-pit structures from 9.4 and 9.5 m depth were subsampled immediately following collection and dissected using flame-sterilized blades and

forceps (Table 2). Mats from 9.4 m depth were subsampled according to pigmentation: brown-purple (top), green (middle), and beige (bottom) layers. These samples were composed primarily of active microbial mat and few inorganic constituents, as evidenced by high organic matter content. Due to limitations in material quantity, pigmented subsamples were not collected and analyzed in replicate. Ridge-pit mats from 9.5 m depth were dissected in triplicate according to mat features: photosynthetic surface (surface), within the ridges including their edges (middle), and pit base (base). These samples were composed of both active microbial mat and older, underlying organic material and inorganic constituents. Dissected subsamples were rinsed in sterile deionized water, transferred into sterile plastic tubes, and frozen at -20°C until further analysis. Samples were freeze-dried in New Zealand prior to transport for lipid extraction and analysis at MIT.

2.3. Lipid extraction and analysis

Microbial biomass was placed in combusted glass centrifuge vials, homogenized and spiked with 40 ng of 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine (C16 PAF) as a recovery standard. Total lipid extracts (TLEs) were obtained by extracting the samples using a modified Bligh and Dyer method (Bligh and Dyer, 1959) as described by Matys et al. (2017). The modified extraction procedure includes the use of dichloromethane (DCM) in place of chloroform and isolates supernatants from methanol/dichloromethane/phosphate buffer solution (2:1:0.8, v/v/v) and methanol/dichloromethane/trichloroacetic acid (TCA) buffer (2:1:0.8, v/v/v) extractions until the TCA was removed from the samples, after the liquid-liquid extraction step due to reports that TCA can contribute to the degradation of hopanoids. The TLEs were kept at -20 °C until analysis.

A fraction of each TLE was acetylated with pyridine/acetic anhydride (1:1, v/v) at 70 °C for 1 h and left at room temperature overnight. The acetylated TLEs were analyzed by high-performance liquid chromatography-atmospheric pressure chemical ionization – mass spectrometry (HPLC-APCI-MS) as described in Matys et al. (2017). The LC-MS system comprised a 1200 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an auto sampler and a binary pump linked to a Q-TOF 6520 mass spectrometer (Agilent Technologies) via an APCI interface operated in positive ion mode. A Poroshell 120 EC-C18 column (2.1 x 150 mm, 2.7 µm; Agilent Technologies) was chosen to provide fast and high-resolution separations of a wide range of small molecules at lower pressures. The column temperature was set at 30°C. The eluent flow remained constant at 0.19 ml/min. Eluent A

contained a mixture of methanol: H₂O 95:5 v/v and eluent B comprised pure isopropyl alcohol (IPA). The HPLC gradient was as follows: isocratic flow of 100% eluent A (0-2 mins), a linear gradient from 0-20% of eluent B (2-20 mins), isocratic flow of 20% B (20-30 mins), linear gradients from 20-30% of eluent B (30-40 mins) and from 30-0% B (40-45 mins), isocratic flow of 100% A for 5 minutes. The column was conditioned for 5 minutes of post-run time at 100% A. The APCI parameters were as follows: gas temperature 325 °C, vaporizer temperature 350 °C, drying gas (N₂) flow 6 l/min, nebulizer (N₂) flow 30 l/min, capillary voltage 1200 V, corona needle 4 µA, and fragmentor 150 V.

BHPs were identified on the basis of accurate mass measurements of their protonated molecular ions, fragmentation patterns in MS-MS mode, and by comparison of relative retention times (Talbot et al., 2003, 2007; Welander et al., 2012). Quantification was achieved through an internal standard of 3 α ,12 α -Dihydroxy-5 β -pregnan-20-one,3,12-diacetate (PD) and external standard calibration curves of PD and authentic BHT, 2-MeBHT, Diplopterol, 2-MeDiplopterol, and aminotetrol. PD was chosen as an internal standard because of its structural similarity to hopanoids and because it has a retention time that does not overlap with the range of BHPs of interest. Calibrations with authentic BHP standards were necessary to account for variations in ionization efficiencies of different BHPs (Wu et al., 2015). PD and authentic BHP standard calibrations were completed throughout the duration of the sample analyses to confirm reproducibility.

2.4. Loss on ignition

In order to create a comparable dataset with Sutherland and Hawes (2009), Mackey et al. (2017), and Matys et al. (2019) the same LOI method was employed. After lipid extraction, all samples were dried and weighed in small ceramic crucibles. The samples were then combusted at 450 °C for 4 hours. Upon cooling to room temperature, the samples were weighed, and weight loss on ignition was taken as the organic matter content of individual samples.

2.5 Genomic analyses

Previously published metagenomic and amplicon datasets were used to examine the genes involved in the production of hopanoids and in anaerobic ammonium oxidation (anammox) in benthic microbial communities in Lake Fryxell. The collection, preservation, and extraction of DNA for these samples are described in full in Junblut et al. (2016) and

Dillon et al. (2018 and 2019). The samples were from mats of ridge-pit morphology. Subsamples were collected from the tops of ridges (top), within the ridges including their edges (middle), and from the bottoms of pits (bottom) of mats from 9.3 m depth. Briefly, the samples were preserved immediately after collection and using an Xpedition Soil/Fecal DNA MiniPrep kit (Zymo Research, Irvine, CA) and stored on ice/frozen until returning to UC Davis where they were stored at -80° C until DNA was extracted using the Xpedition Soil/Fecal DNA MiniPrep kit following the manufacturer's instructions.

Metagenomic sequence files for top (accessions SAMN09937131, SAMN09937132, SAMN09937146, SAMN09937147, SAMN09937133, SAMN09937148, SAMN09937135, SAMN09937136, SAMN09937150, SAMN09937151, SAMN09937137, SAMN09937138, SAMN09937152, SAMN09937153, SAMN09937154, SAMN09937155, SAMN09937139, SAMN09937140, SAMN09937141, SAMN09937142, SAMN09937156, SAMN09937157, SAMN09937143, SAMN09937144, SAMN09937158, SAMN09937159), middle (SAMN09937100, SAMN09937101, SAMN09937115, SAMN09937116, SAMN09937102, SAMN09937103, SAMN09937117, SAMN09937118, SAMN09937104, SAMN09937119, SAMN09937107, SAMN09937108, SAMN09937122, SAMN09937123, SAMN09937109, SAMN09937124, SAMN09937111, SAMN09937112, SAMN09937126, SAMN09937127, SAMN09937113, SAMN09937114, SAMN09937128, SAMN09937129), and bottom (SAMN09937066, SAMN09937067, SAMN09937083, SAMN09937084, SAMN09937068, SAMN09937069, SAMN09937085, SAMN09937086, SAMN09937070, SAMN09937071, SAMN09937087, SAMN09937088, SAMN09937089, SAMN09937072, SAMN09937073, SAMN09937074, SAMN09937075, SAMN09937091, SAMN09937092, SAMN09937093, SAMN09937094, SAMN09937076, SAMN09937077, SAMN09937079, SAMN09937080, SAMN09937096, SAMN09937097) ridge-pit mat subsamples from 9.3 m depth in Lake Fryxell were concatenated to form the “top,” “middle,” and “bottom” samples respectively. Metagenomes were assembled using MEGAHIT (Li et al., 2015) using a minimum contig length of 500 bp and the paired end setting. Reads were mapped back to the assembly using Bowtie2 (Langmead and Salzberg, 2013). A depth file was created using `jgi_summarize_bam_contig_depths` in Anvio (Eren et al., 2015). Translated nucleotide sequences were annotated using prokka (Seemann, 2014). Sequences annotated as SHC, HpnP, HpnR, HpnO, and HDH and HZS were retrieved for further analysis. The proteins SHC, HpnP, HpnR, and HpnO were chosen due to their involvement in hopanoid biosynthesis. The proteins HDH and HZS were chosen to search for the potential capability

for anammox. NirK and NirS are also used in this pathway, but are also involved in denitrification and are therefore, not indicative of the anammox process.

We refer to the diversity of SHC, HpnP, HpnR, and HpnO sequences identified in this study as unique sequences. The term unique sequence indicates that the translated nucleotide sequences identified are SHC, HpnP, HpnR, and HpnO but have small differences in the nucleotide sequences as a result of differing evolutionary histories. If each bacterial species has a single copy of the gene, then the number of unique sequences would indicate the number of species with the gene. However, it is possible that some species may have multiple copies of one unique sequence in their genomes or that individuals from the same species have slightly different sequences. As a result, we do not correlate the number of unique sequences directly to the number of species with the genes for SHC, HpnP, and HpnR. Additionally, we do not assign taxonomic affiliations to the sequences. Although the translated nucleotide sequences were long enough to be annotated as specific proteins, the retrieved sequences were significantly shorter than full-length and thus we were not confident in completing these types of analyses.

3. Results

3.1. Total organic matter

Bacteriohopanepolyol (BHP) abundances were normalized to total organic matter (TOM), which was measured for each sample analyzed (Supplementary Table 2). Mat samples (whole and subsampled) from Lake Fryxell were composed of 2.8-25.2% TOM. Generally, TOM in whole mat samples decreased from an average of 20.5% at 9 m depth (SD= 5.5; n= 3) to 6.6% (SD= 1.3; n= 2) at 11 m depth. TOM in ridge-pit mat subsamples from 9.4 m depth was high in brown-purple (21%; n= 1) and green (20.8%; n=1) subsamples and was relatively low in the beige sample (12.5%; n= 1). In ridge-pit mat subsamples (surface, middle, and pit base) from 9.5 m, TOM was consistently low, with an average of 4.5% (SD= 1.1, n= 9). The variability of TOM measurements within mat replicates is likely due to the spatial variability of supplementary constituents (i.e. inorganic grains) that greatly influence sample weight and as a result, the relative abundance of TOM.

3.2. Bacteriohopanepolyols

Bacteriohopanepolyol abundance

BHP abundance was normalized to total organic matter (TOM) for each sample in order to compare BHP concentrations across all samples. TOM of each sample was estimated using the loss on ignition (LOI) technique. Total BHPs, the summed concentration of all BHPs identified, ranged from 21–89 $\mu\text{g BHP/g TOM}$ in whole mat samples (Table 2). Generally, total BHPs increased with depth into Lake Fryxell from an average of 24.7 $\mu\text{g/g}$ (SD= 2.7; n= 3) at 9 m depth to 74.4 $\mu\text{g/g}$ (SD= 20.1; n=2) at 11 m depth. Total BHPs in ridge-pit subsamples from 9.4 m depth, sampled according to pigmentation, decreased with depth into the mat structure, from 90.7 $\mu\text{g/g}$ in the top (brown-purple) mat subsample to 48 $\mu\text{g/g}$ in the middle (green) and bottom (beige) subsamples. Total BHPs in dissected ridge-pit mat samples from 9.5 m depth, sampled according to mat feature, also generally decreased with depth into the mat structure, from an average of 31.7 $\mu\text{g/g}$ (SD= 5.9; n= 3) in the surface to 14.8 $\mu\text{g/g}$ (SD= 2.8; n= 3) in the middle and 18.8 $\mu\text{g/g}$ (SD= 2.5; n= 3) in the pit base samples.

Bacteriohopanepolyol diversity

Bacteriohopanepolyol (BHP) diversity in whole benthic mat samples collected between 9.7 and 11 m depth varies with mat morphology (Fig. 3). Seven BHPs were identified in cusped pinnate mats, collected from 9–9.3 m depth in the upper hyperoxic zone. Bacteriohopanetetrol (BHT, **1a**; numbers refer to the structures in Fig. S1) and aminobacteriohopanetriol (aminotriol, **1d**) comprised an average of 59% (mean= 14.1 $\mu\text{g/g}$; SD= 4.9; n=4) and 32.7% (mean= 7.7 $\mu\text{g/g}$; SD= 4.8; n=4) of total BHPs respectively. Three minor BHPs were identified, including a later eluting BHT stereoisomer (BHT II, **1b**), 2-methylbacteriohopanetetrol (2-MeBHT, **2a**), and 3-methylbacteriohopanetetrol (3-MeBHT, **3a**). The BHP profile of one sample from 9 m depth included low relative abundance of two penta-functionalized BHPs, as seen in the deeper ridge-pit morphology. The highest diversity of BHPs (8) was identified in mats of the ridge-pit morphology at 9.7 m depth, at the oxic-anoxic transition. BHT was the most abundant compound and comprised 68.4% (mean= 36.7 $\mu\text{g/g}$; SD= 13.6; n= 3) of total BHPs. Minor BHPs identified included those BHPs identified in pinnate mats in addition to three penta-functionalized BHP including bacteriohopanepentol (BHpentol, **1c**) and a later eluting isomer (BHpentol II; tentatively 30R, 32R, 34R, 33S, 34R based on Zhao (1996)) and a C-2 methylated variant (2-MeBHpentol, **2c**). BHP diversity decreased below the oxycline (9.8 m), in prostrate and flocculent mat

morphologies, which lie within the anoxic zone. Six BHPs were identified in prostrate and flocculent mats: BHT, BHT II, 2-MeBHT, BHpentol, aminotriol, 2-MeBHpentol.

The ridge-pit mat subsampled according to pigmentation (9.4 m depth) exhibited the same BHP diversity as whole mat samples from 9.7 m depth, the closest whole mat sample of similar mat morphology (ridge-pit). BHPs identified include BHT, BHT II, 2-MeBHT, 3-MeBHT, BHpentol, BHpentol II, 2-MeBHpentol, and aminotriol (Fig. 4). 3-MeBHT and BHpentol II were not present in the top (brown-purple) mat sample, but were present in both the middle (green) and bottom (beige) mat samples, accounting for an increase in diversity with depth into the mat structure. BHT accounted for an average of 76.5% of the total BHPs (SD= 4.2%; n=3). BHT II was abundant (17.8% of the total BHPs) in the top (brown- purple) mat sample and decreased toward the base of the mat (mean= 5%; SD= 0.7; n=2). The relative abundance of 2-MeBHT also decreased with depth into the mat structure, from 5% of the total BHPs in the top (brown-purple) mat sample to 3.8% and 3.3% in the middle (green) and bottom (beige) mat samples, respectively. The relative abundances of 3-MeBHT, BHpentol, BHpentol II, and 2-MeBHpentol all increased with depth into the mat structure, accounting for 0.6% of the total BHPs in the top (brown-purple), 2.6% in the middle (green), and 5.4% in the bottom (beige) mat sample. Aminotriol was abundant (11.3% total BHPs) in the middle (green) mat sample and averaged 5.3% of the total BHPs (SD= 0.9%; n=2) in the top (brown-purple) and bottom (beige) mat samples.

The ridge-pit mat that was dissected on the basis of its morphology (9.5 m depth) contained 7 BHPs, including BHT, BHT II, 2-MeBHT, BHpentol, BHpentol II, and aminotriol (Fig. 5). Unlike whole ridge-pit mat samples from 9.7 m depth (Fig. 3), the closest the closest whole mat sample of similar mat morphology (ridge-pit), 3-MeBHT was not detected and 2-MeBHpentol was only identified in one middle mat sample. BHP diversity did not vary substantially between dissected ridge-pit subsamples (top, middle, and pit).

BHP biosynthesis protein abundance

Translated nucleotide sequences for the hopanoid biosynthesis protein SHC were found in all ridge-pit mat subsamples from 9.3 m, with 3 unique sequences in top, 2 in the middle, and 6 in the bottom sample. The translated nucleotide sequence of the B-12 binding radical S-adenosylmethionine (SAM) protein (HpnP) that catalyzes the C-2 methylation of hopanoids, was found in all samples with 2 in the top sample and 4 in each of the middle and bottom samples. Similarly, the translated nucleotide sequence for HpnR, the protein

responsible for hopanoid methylation at the C-3 position, was found in all samples with 1 unique sequence found in each of the top and middle samples and 4 in the bottom. The translated nucleotide sequence for HpnO, the protein responsible for aminoBHP production, was not found in any samples.

The translated nucleotide sequences for anammox proteins HDH and HZS were not found in any sample.

4. Discussion

4.1. Distribution of BHPs in whole mat samples across redox conditions

BHP profiles (abundance and diversity) of whole benthic microbial mat samples vary with mat morphology and local environmental conditions, which range from oxic (9-9.3 m samples) to suboxic/anoxic (9.7-11 m samples) and differ in sulfide, dissolved reactive phosphorus (DRP), and ammonium (NH_4^+) concentrations (Jungblut et al., 2016; Table S1), which increase with decreasing oxygen concentrations. As a result, BHP abundance and diversity were generally interpreted as markers of active microbial communities that reflect and respond to local environmental conditions.

However, unlike nearby Lake Vanda (Matys et al., 2019), organic matter preserved in benthic microbial mats may not only be produced in situ (by modern and ancient communities), but are also likely to contain some contribution of organic matter from the overlying water column. The Lake Fryxell water column is productive with bacterial concentrations ranging from $1.0\text{-}3.8 \times 10^8$ /L in the oxic portion of the water column (<9.5 m depth) and increase to 20×10^8 /L in anoxic bottom waters (>9.5 m depth; Laybourn-Parry et al., 1996). Coincidentally, BHP abundance increases with depth into Lake Fryxell, with the highest abundances of BHPs occurring around 10 m depth in the anoxic bottom waters. However, $\delta^{13}\text{C}$ compositions of benthic organic matter and particulate organic matter indicate that benthic productivity dominates in Lake Fryxell at the depths described here (Lawson et al., 2004). Still, contributions by particulate organic matter input were considered throughout this study.

Similar increases in BHP abundances and diversity with decreasing oxygenation have been noted in the marine water column through oxygen minimum zones (OMZ; Sáenz et al., 2011; Kharbush et al., 2016; Matys et al., 2017). While BHPs have traditionally been considered most abundant in aerobic bacteria, the results of this study support the current hypothesis that hopanoid producers may be more diverse and abundant in low-oxygen

environments than previously thought, and possibly even more abundant than in environments that are well-oxygenated. In fact, this may account for the high diversity of BHPs in benthic microbial mats from suboxic to anoxic portions of Lake Fryxell, as compared to nearby Lake Vanda (Matys et al., 2019) where benthic microbial mats remain in contact with well oxygenated water throughout the year. As a result, it is of considerable interest to further investigate the abundance, diversity, distribution, possible biological sources and physiological functions of hopanoids in suboxic/anoxic environments.

It should be noted that organic matter is preferentially preserved in hypoxic environments as a result of decreased rates of remineralization (Jessen et al., 2017), which could account for the increase in total BHP abundance in anoxic portions of Lake Fryxell. However, the concurrent increase in BHP diversity in anoxic portions of Lake Fryxell suggests that additional hopanoid sources also exist below 9.6 m depth.

4.2. C-2 methylated hopanoids

2-Methylbacteriohopanepolyols (2-MeBHPs) have been widely applied as biomarkers for cyanobacteria (e.g. Summons et al., 1999), although additional sources have been identified, including multiple α -proteobacteria (Welanders et al., 2010; Ricci et al., 2014, 2015).

Cyanobacteria were identified as the only source of 2-MeBHPs in Lake Vanda, based on the phylogeny of translated nucleotide sequences SHC and HpnP (Matys et al., 2019). 2-MeBHPs and HpnP translated nucleotide sequences are also present in Lake Fryxell (Table S3). However, due to the limitations of metagenomic data available, we are unable to unequivocally resolve the taxonomy of the HpnP sequences identified. However, we expect that cyanobacteria comprise at least one source of 2-MeBHP in Lake Fryxell based on our understanding of 2-MeBHP source organisms in nearby Lake Vanda.

The relatively low diversity and abundance of 2-MeBHP in Lake Fryxell as compared to Lake Vanda may be due to differences in cyanobacteria abundance. Approximately 3% of surface irradiance is transmitted through the ice cover in Lake Fryxell (Jungblut et al., 2016) as compared to 15-20% transmittance in Lake Vanda (Howard-Williams, 1998; Hawes and Schwarz, 2000) as a result of greater ice thickness and sediment content in Lake Fryxell, which creates a very different habitat for photosynthetic communities. However, cyanobacterial 16S rRNA gene diversity showed that the mats were dominated by filamentous oscillatorian cyanobacteria such as *Leptolyngbya* and *Phormidium* (Jungblut et

al., 2016), known hopanoid-producers (Summons et al., 1999; Talbot et al., 2008; Hamilton et al., 2017). For example, the surfaces of ridge-pit mats from 9.4 m depth contain high abundances (absolute and relative) of 2-MeBHP and are dominated by *Leptolyngbya*. The decrease in 2-MeBHP with depth into the mat follows changes in the relative abundance of cyanobacteria (Dillon, 2018).

Alphaproteobacteria are also potential contributors to 2-MeBHP inventories in Lake Fryxell. For instance, 2-MeBHPs are abundant in flocculent mat samples (10.6 and 11 m depth), which do not contain cyanobacteria as a result of irradiance limitation (Sumner et al., 2015; Jungblut et al., 2016). Alternatively, a water column source of 2-MeBHP cannot be ruled out. Cyanobacteria and α -proteobacteria are present but comprise a very low proportion of the bacterial phyla in the Lake Fryxell water column (Kwon et al., 2017).

4.3. C-3 methylated hopanoids

3-MeBHT was the only C-3 methylated hopanoid identified in Lake Fryxell. 3-MeBHT was present in at least one sample from all depths described (9.0-11.0 m depth), spanning oxic to anoxic environments. 3-MeBHPs have been used as a biomarker for aerobic methanotrophs and aerobic acetic acid bacteria (Zundel and Rohmer, 1985), although alternative sources have been identified (Welander and Summons, 2012), including diverse proteobacteria (α -, β -, and γ -proteobacteria), which are abundant throughout Lake Fryxell (Dillon, 2018). For example, *Frankia* and *Burkholderia* have been shown to produce C-3 methylated hopanoids, based on the taxonomic distribution of HpnR (Welander and Summons, 2012). According to Dillon (2018), the relative abundances of *Frankia* and *Burkholderia* increase with depth (from top, to middle, and bottom) into mats collected from 9.0 and 9.3 m depth in Lake Fryxell. We also identify an increase in the concentration of 3-MeBHT and the number of unique HpnR sequences identified with depth into mats from 9.4 m depth (BHPs) and 9.3 m depth (HpnR). This relationship suggests that 3-MeBHT may be produced at least in part by *Frankia* and *Burkholderia* in oxic portions of the lake. However, neither *Frankia* nor *Burkholderia* were identified in anoxic portions of Lake Fryxell (9.8 m depth; Dillon, 2018), where we detect 3-MeBHT. Unfortunately, metagenomic data is not available for the same samples. This suggests that other sources of 3-MeBHT exist in benthic microbial mats from anoxic portions of Lake Fryxell, that a pelagic source of 3-MeBHT exists, or that 3-MeBHT was produced and outlasted the DNA of known source organisms such as *Frankia* and *Burkholderia*.

4.4. Penta-functionalized hopanoids

Penta-functionalized BHPs (BHpentol, BHpentol II, and 2-MeBHpentol) vary significantly among mat morphologies with clear differences between mats in the oxic and anoxic portion of Lake Fryxell. BHpentol, BHpentol II, and 2-MeBHpentol are typically associated with cyanobacteria (Bisseret et al., 1985; Zhao et al., 1996) and as a result, should decrease with depth into Lake Fryxell as the abundance of cyanobacteria decreases (Dillon, 2018). Instead, significant shifts in the diversity of penta-functionalized hopanoids occur across the oxycline. BHpentol II is only present in oxic portions of Lake Fryxell. BHpentol and its C-2 methylated counterpart, 2-MeBHpentol, are most abundant in the suboxic to anoxic portion of the lake, with the greatest abundances occurring at 9.9 m depth. The relative abundances of BHpentol and 2-MeBHpentol also increase with depth into the subsampled mat structure from 9.4 m, to greatest concentrations where cyanobacteria and O₂ are lowest. Similar trends in penta-functionalized BHP have been noted in marine oxygen minimum zones (Sáenz et al., 2011), where BHpentol is most abundant below the oxic-anoxic transition. It is possible that penta-functionalized BHPs have a greater diversity of source organisms than previously reported (Sáenz et al., 2011), or are produced in response to environmental conditions (Garby et al., 2017), such as low light and oxygen. However, studies that further examine the physiological role of penta-functionalized BHP must be performed in order to test this hypothesis.

4.5. BHT stereoisomer (BHT II)

BHT II has been proposed as a biomarker for anammox bacteria (Rush et al., 2014) and the BHT II ratio (BHT II/total BHT), a proxy for suboxic to anoxic conditions such as those associated with marine oxygen minimum zones (OMZs; Sáenz et al., 2011) where anammox bacteria are abundant. In characterizing the relative contribution of BHT II in respect to the earlier eluting isomer (BHT), it is possible to compare a wide range of environments with variable productivity and preservation potential. We describe the abundance of BHT II and local environmental conditions in order to consider BHT II source organisms in Lake Fryxell. We also compare BHT II ratio values from Lake Fryxell and marine OMZ systems to investigate implications of terrestrial BHT II production on marine BHT II signatures.

BHT II was detected in all benthic microbial mat samples collected from Lake Fryxell. However, we were unable to identify the anammox proteins HDH and HZS in benthic mat samples from Lake Fryxell. We explore four hypotheses that explain this discrepancy: (i) the BHT II signature is produced in the overlying water column and transported to benthic microbial mats below, (ii) anammox bacteria are present in seasonally occurring microenvironments within the mats that were not sampled for metagenomic analysis, (iii) anammox bacteria were once present within the mats and the hopanoids they produced outlasted their DNA and (iv) BHT II is produced by other bacteria, in addition to anammox. Since the biosynthetic reactions leading to BHT II have yet to be elucidated, it is not possible to unambiguously state that anammox bacteria are a source of BHT II in Lake Fryxell or that other BHT II producers exist. Instead, we explore the distribution of BHT II in Lake Fryxell in order to elucidate the likelihood of these scenarios.

It is not possible to exclude seasonal anammox activity in the water column and subsequent deposition of BHT II to benthic microbial mats. Planctomycetes, the phylum to which anammox bacteria belong, have been identified in the Lake Fryxell water column (Vick-Majors et al., 2014). Based on the distribution of anammox in marine systems (e.g. Kuypers et al., 2003; Thamdrup et al., 2006) we hypothesize that the metabolism would most likely exist in Lake Fryxell at the upper anoxic zone (approximately 9.6–10.3 m) where DO is low and NH_4^+ and NO_2 (albeit at low concentrations) are present (Table S1). However, if anammox were present in the water column, the abundance of BHT II would likely be consistent at all depths below that depth due to the production and export of the same signature through the underlying water column to the benthic microbial mats below. However, BHT II is present in benthic microbial mats < 9.6 m depth (Fig. 3) and BHT II abundance varies in mats < 9.6 m. In marine OMZ systems, BHT II abundance decreases below the oxycline due to the recycling of sinking organic matter (Matys et al., 2017). However, this is not likely to occur over the relatively short vertical distances (approx. 1 m) described here. Furthermore, BHT II abundance (absolute and relative) differs with mat morphologies. As a result, we assume that BHT II is being produced within the microbial mats and reflects the contemporary and/or recent communities.

The greatest abundance of BHT II was detected in whole mat samples from the oxycline, where O_2 is <10 $\mu\text{mol/kg}$ and NH_4^+ is present (8 $\mu\text{g/L}$). This result agrees with previous studies that describe the highest relative abundances of BHT II at oxic/anoxic transitions in marine OMZs (e.g. Sáenz et al., 2011), where anammox is most abundant (e.g.

Kuypers et al., 2003; Thamdrup et al., 2006). BHT II is also present in benthic microbial mats underlying an oxic water column (9.0 and 9.3 m). Seasonally suboxic to anoxic microenvironments, suitable for the anammox process, may form within the mats. While an extensive study of microenvironments in benthic microbial mats of Lake Fryxell has not been completed, a previous study described pervasive oxygenation (Sumner et al., 2015) throughout the mat-water interface of Lake Fryxell in the summer months due to photosynthetic O₂ production by cyanobacteria within the mats. Dissolved oxygen concentrations are especially high at the mat surface (<5 mm depth), where BHT II is most abundant in ridge-pit mat subsamples from 9.4 m and 9.5 m depth, compared to subsurface subsamples. If suboxic microenvironments were seasonally present below the mat surface, anammox could persist, albeit limited by nitrogen (NO₂⁻ and NH₄⁺). As a result, BHT II would be detectable but in very low abundance (0.5-1.0 µg/g), as seen in whole mat samples from 9.0 and 9.3 m. These results suggest that anammox bacteria comprise at least one of the sources of BHT II in benthic microbial mats in Lake Fryxell.

However, based on the absence of HDH and HZS in the dataset, we cannot confirm the presence of the anammox metabolism (contemporary or ancient) within benthic microbial mats of Lake Fryxell. As a result, we consider alternative sources of BHT II. Isomers of BHT have also been reported in other non-marine environments. For example, an isomer of BHT was detected in a stratified mat from a hypersaline lake and tentative sources of α -Proteobacteria or acetic acid bacteria were reported (Blumenberg et al., 2013). Proteobacteria are abundant in Lake Fryxell (Dillon, 2018), however, the putative sources which include the purple non-sulfur bacterium *Rhodospseudomonas acidophila* (Neunlist et al., 1988) and acetic acid bacteria (Peiseler and Rohmer, 1992; e.g. *Acetobacter* spp.) have not been identified in the lake. An isomer of BHT has also been reported in *Frankia* sp. a terrestrial nitrogen-fixing plant symbiont (Rosa-Putra et al., 2001) and an aerobic Type II methanotrophic bacteria (*Methylocella palustris*; van Winden et al., 2012). *Frankia* sp. has been identified in Lake Fryxell (Dillon, 2018) at 9.0 and 9.3 m. However, the relative abundance of *Frankia* sp. increases with depth into the mats as compared to the relative abundance of BHT II, which decreases with depth into mats from 9.4 m. This suggests that even if *Frankia* sp. is one source of BHT II in Lake Fryxell, additional sources are likely.

If BHT II was produced by extant microbial communities, the BHT II signature would be present in greater abundance with depth into mat structures and would not likely be present at the mat surface. However, the relative abundance of BHT II is consistent in the

ridge-pit microbial mat from 9.5 m depth (subsamped according to mat feature) and decreases with depth into the ridge-pit microbial mat from 9.4 m depth (subsamped according to pigmentation). However, it is possible that anammox bacteria could have existed in the recent past and contributed to the BHT II inventory in Lake Fryxell.

At this point, we are unable to confirm the source of BHT II in Lake Fryxell. As a result, implications for the use of BHT II as a proxy for anammox bacteria are unclear. However, this study provides evidence of yet another non-marine environment where BHT II has been identified, with implications for the application of the BHT II ratio as a proxy for suboxic to anoxic marine environments, such as marine OMZs, where anammox are active. However, the BHT II ratio values measured in Lake Fryxell (<0.1) are substantially lower than BHT II ratio values measured in modern OMZ systems which often range from 0.6 to 0.8 (Fig. 6; Sáenz et al., 2011; Matys et al., 2017). If we assume that anammox bacteria are the source of BHT II in Lake Fryxell, the low BHT II ratio values detected suggest that anammox bacteria are less abundant in Lake Fryxell than in marine OMZ systems. This could be due to the fact that nitrate and nitrite, essential to the anammox process, are normally present in excess in marine OMZ systems (Canfield, 2006). Conversely, nitrate and nitrite are limited/low in Lake Fryxell. As has been suggested (Kusch et al., 2018) we propose that a certain threshold value of the BHT II ratio may be established with the generation of a larger BHT II dataset from diverse environments and the BHT II ratio may serve as a proxy for anoxia/anammox or suboxic to anoxic marine environments above that certain value.

It should be noted that the abundance of BHT II produced by any one organism may also vary with environmental conditions. BHPs may be regulated in response to particular environmental conditions, such as temperature (Kulkarni et al., 2013; Osborne et al., 2017), pH (Kulkarni et al., 2013; Garby et al., 2017), and nutrient concentrations (Doughty et al., 2009). It is possible that environmental conditions present in marine OMZ systems may prompt the production of high abundances of BHT II while conditions in Lake Fryxell do not. At this point, we are hesitant to speculate as to what conditions those may be as much is still unknown concerning environmental conditions in Lake Fryxell in the Antarctic winter.

5. Conclusions

We have observed a high abundance and diversity of bacteriohopanepolyols in benthic microbial mats from Lake Fryxell, an ice-covered lake in the McMurdo Dry Valleys, Antarctica. This study contributes to the dearth of BHP research in polar regions and presents

insights for interpreting the distribution of BHPs in modern environments and sedimentary archives. BHP diversity varies with benthic microbial mat morphology, with significant differences between those mat morphologies present in oxic and suboxic/anoxic environments. Of particular interest is the ubiquity of a stereoisomer of bacteriohopanetetrol (BHT), informally referred to as BHT II and shifts in penta-functionalized hopanoids across the oxic-anoxic transition. BHT II has been associated with anammox bacteria and suboxic to anoxic marine environments where anammox is active. However, based on the absence of anammox proteins HDH and HZS in the available metagenomic dataset, we cannot confirm the presence of the anammox metabolism within microbial mats from Lake Fryxell. Notably, the BHT II ratio (BHT II/total BHT) values measured in Lake Fryxell are substantially lower than BHT II ratio values measured in modern OMZ systems. We suggest that, with the generation of a larger BHT II database from diverse environments, the BHT II ratio (BHT II/total BHT) may be a more reliable proxy for anoxia/anammox. We also observe shifts in the diversity of penta-functionalized hopanoids occur across the oxycline, in disagreement with previous studies that associate penta-functionalized hopanoids with cyanobacteria. This finding suggests that penta-functionalized BHPs have a greater diversity of source organisms than previously thought or that penta-functionalized BHPs are produced in response to particular environmental conditions. However, studies that further examine the physiological role of penta-functionalized BHP must be performed in order to test this hypothesis.

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To be added.

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Figures

Figure 1. Map of Antarctica (A) and the McMurdo Dry Valleys, including Lake Fryxell (elevation: 18 m; B). All lakes are color coded blue. All glaciers are color coded grey. Adapted from Sumner et al., 2016.

Figure 2. Schematic of Lake Fryxell bathymetry (slope from Junblut et al., 2016) and microbial mat morphotypes along the sampling transect, including reference points (stars) where samples were taken. White stars indicate sites where whole mat samples were collected. Black stars indicate where whole and dissected mat samples were collected. Images are representative of the mats present at the depths identified. The distance between red markers in each image indicates 3 cm.

Figure 3. Dissolved oxygen (DO) profiles and corresponding mat morphologies (CP: cusped pinnacles; RP: ridge-pit; Pr: prostrate; F: flocculent) and bacteriohopanepolyols (BHPs; μg BHP/g total organic matter) in whole mat samples in Lake Fryxell, Antarctica. Error bars indicate ± 1 standard deviation calculated through replicate analysis. The number of replicates is defined in Table S2 and Section 2.2.

Figure 4. Relative abundance (%; B) of bacteriohopanepolyols (BHPs) and total BHPs ($\mu\text{g/g}$; C) in ridge-pit benthic microbial mats from 9.4 m depth in Lake Fryxell, Antarctica. The number of BHPs identified in each subsample are shown in parentheses following each subsample label. White arrows indicate pigmented zones sampled for analysis: Brown-purple (Br.-p.), Green (Gr.), and Beige (B.) in representative ridge-pit mat cross section (A). Scale bar signifies 10 mm.

Figure 5. Relative abundance (%; B) of bacteriohopanepolyols (BHP) and total BHPs ($\mu\text{g/g}$; C) in ridge-pit benthic microbial mats from 9.5 m depth in Lake Fryxell, Antarctica. The number of BHPs identified in each subsample are shown in parentheses following each

subsample label. White arrows indicate mat features sampled for analysis: photosynthetic upper surface (Surf.), dark pigmented pit base (Base), and beige middle (Mid.) in representative ridge-pit mat cross section (A). Scale bar signifies 10 mm.

Figure 6. Bacteriohopanetetrol (BHT) II ratio ($\text{BHT}/\text{BHT} + \text{BHT II}$) vs. dissolved oxygen (DO) concentration measured in marine oxygen minimum zones (Arabian Sea, Peru Margin, Cariaco Basin, and Northern Chile) and Lake Fryxell, Antarctica (adapted from Matys et al., 2017).

Supplementary Files

Figure S1. Bacteriohopanepolyols identified in this study. The illustrated stereochemistry for BHT II is tentative, based on Rosa-Putra (2001).

Table S1. Physical and chemical variables measured at the microbial mat-water interface along sampling transect in November 2012 (from Junblut et al., 2016).

Table S2. Bacteriohopanepolyols identified in benthic microbial mats from Lake Fryxell, Antarctica.

Table S3. Bacteriohopanepolyols and associated protein sequences identified in benthic microbial mats of ridge-pit morphology from 9.3 m depth in Lake Fryxell, Antarctica. + and - symbols denote the presence and absence of the protein, respectively.

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