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Citation: Matys, Emily D., Mackey, Tyler, Grettenberger, Christen, Mueller, Elliott, Sumner, Dawn Y. et al. 2019. "Bacteriohopanepolyols across environmental gradients in Lake Vanda, Antarctica." *Geobiology*, 17 (3).

As Published: <http://dx.doi.org/10.1111/gbi.12335>

Publisher: Wiley

Persistent URL: <https://hdl.handle.net/1721.1/140509>

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

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Title:

BACTERIOHOPANEPOLYOLS ACROSS ENVIRONMENTAL GRADIENTS IN
LAKE VANDA, ANTARCTICA

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Acknowledgements:

We acknowledge the logistic support of the US Antarctic Program and Antarctica New Zealand. Anne Jungblut and Dale Andersen provided substantial support as field team members. We also thank three anonymous reviewers for their constructive comments that helped improving this manuscript.

Funding:

This research was supported by the NASA Astrobiology Institute through an award (NNA13AA90A) to RES. Field data and sample collection for this study were supported by funds from the NASA Exobiology Program (NNX13AI60G and NNX08AO19G) and

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/GBI.12335](https://doi.org/10.1111/GBI.12335)

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the New Zealand Foundation for Research, Science and Technology (CO1X0306). EDM and RES also thank the MIT-NZ MISTI Global Seed Funds grant program for additional support.

Author Manuscript

Article Type: Original Article

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KEY WORDS:

2-Methylhopane, Bacteriohopanepolyol, Biomarker, Antarctica, Lake Vanda, McMurdo Dry Valleys

ABSTRACT

Bacteriohopanepolyols (BHPs) are bacterial membrane lipids that may be used as biological or environmental biomarkers. Previous studies have described the diversity, distribution, and abundance of BHPs in a variety of modern environments. However, the regulation of BHP production in polar settings is not well understood. Benthic microbial mats from ice-covered lakes of the McMurdo Dry Valleys, Antarctica provide an opportunity to investigate the sources, physiological roles, and preservation of BHPs in high latitude environments. Lake Vanda is one of the most stable lakes on Earth, with microbial communities occupying specific niches along environmental gradients. We describe the influence of mat morphology and local environmental conditions on the diversity and distribution of BHPs and their biological sources in benthic microbial mats from Lake Vanda. The abundance and diversity of C-2 methylated hopanoids (2-MeBHP) is of particular interest, given that their stable degradation products, 2-methylhopanes, are among the oldest and most prevalent taxonomically informative biomarkers preserved in sedimentary rocks. Furthermore, the interpretation of sedimentary 2-methylhopanes is of great interest to the geobiology community. We identify cyanobacteria as the sole source of 2-MeBHP in benthic microbial mats from Lake Vanda and assess the hypothesis that 2-MeBHP are regulated in response to a particular environmental variable, namely solar irradiance.

1. INTRODUCTION

Bacteriohopanepolyols (BHPs) comprise a structurally diverse group of pentacyclic triterpenoids and a biomarker class that is widely used for environmental, paleoceanographic, and paleo-reconstruction studies (i.e. Talbot & Farrimond, 2007; Wakeham et al., 2012; Blumenberg et al., 2013). BHPs have long been considered bacterial sterol surrogates (Rohmer, Bouvier, & Ourisson, 1979; Ourisson, Rohmer, & Poralla, 1987), assisting in

1 membrane organization (Sáenz et al., 2015) and fluidity (Sáenz, Sezgin, Schwille, & Simons,
2 2012; Wu et al., 2015). However, the exact biological function of particular BHPs remains
3 uncertain. While bacteriohopanetetrol (BHT) is produced by diverse bacteria and is
4 ubiquitous in modern environments (e.g. freshwater, marine, and soil), other structural
5 variations are associated with particular taxonomic or environmental sources. For example,
6 amino-BHPs are prevalent in methanotrophs (Blumenberg et al., 2006; Rush et al., 2016), a
7 stereoisomer of BHT (BHT II) has been associated with anammox bacteria and suboxic-
8 anoxic environments (Sáenz, Wakeham, Eglinton, & Summons, 2011; Rush et al., 2014;
9 Matys et al., 2017), and hopanetriol and anhydrobacteriohopanetetrol have been
10 proposed as indicators of oxidizing and reducing environments, respectively (Bradley,
11 Pearson, Sáenz, & Marx, 2010). Identifying and understanding taxonomic sources and
12 environmental conditions that prompt the production of particular hopanoids in diverse
13 modern environments continues to be a geobiologically-significant research topic.

14
15 2-Methylhopanoids (2-MeBHPs) are of particular interest due to their preservation potential
16 and limited number of known biological sources. Through diagenesis, functionalized
17 hopanoids may lose their differentiating features. However, hydrocarbon backbone
18 methylations at the C-2 (2-MeBHP) or C-3 (3-MeBHP) position may be preserved. In fact, 2-
19 methylhopanes, the stable degradation products of 2-MeBHP, are present in sediments dating
20 back to 1.64 Ga (Brocks et al., 2005). The interpretation of sedimentary 2-methylhopanes is
21 of great interest to the geobiology community. Originally proposed as biomarkers for
22 cyanobacterial oxygenic photosynthesis (Summons, Jahnke, Hope, & Logan, 1999), some
23 alphaproteobacteria have since been shown to produce 2-MeBHPs in abundance (Rashby,
24 Sessions, Summons, & Newman, 2007; Welander, Coleman, Sessions, Summons, &
25 Newman, 2010; Ricci, Michel, & Newman, 2015). In fact, genomic data suggest that the
26 capacity for C-2 methylation may have arisen in the alphaproteobacteria (Ricci, Michel, &
27 Newman, 2015). 2-MeBHP have more recently been investigated as markers of particular
28 ecological niches (Ricci, Morton, Kulkarni, Summers, & Newman, 2017) and environmental
29 conditions such as nutrient limitation (Doughty, Hunter, Summons, & Newman, 2009), pH
30 and osmotic tension (Poralla, Härtner, & Kannenberg, 1984; Welander et al., 2009; Kulkarni,
31 Wu, & Newman, 2013; Kulkarni et al., 2015; Garby et al. 2017), and desiccation (Poralla,
32 Muth, & Härtner, 2000). However, few studies have examined the regulation of 2-MeBHP as
33 a response to prevailing environmental conditions in natural systems.

1 Benthic microbial ecosystems are abundant in ice-covered lakes of Antarctica's McMurdo
2 Dry Valleys (e.g. Wharton, McKay, Clow, & Andersen, 1993; Wharton, 1994; Paerl &
3 Priscu, 1998; Priscu et al., 1998, 2005; Quesada, Fernández-Valiente, Hawes, & Howard-
4 Williams, 2008) and provide a rare opportunity to examine hopanoid source organisms and
5 the BHPs they produce in response to environmental conditions. Due to the oligotrophic
6 nature of the water column (Vincent & Vincent, 1982) and limited influx of organic matter
7 (McKnight, Aiken, Andrews, Bowles, & Harnish, 2013) into Lake Vanda, the organic
8 content of benthic microbial mats is a result of in situ growth rather than sedimentary
9 processes. Furthermore, Lake Vanda is one of the most physically stable lakes on Earth
10 (Vincent, 1981). Microbial communities exist at specific positions across persistent
11 environmental gradients in response to local physical and chemical conditions, including low
12 light levels (Hawes, Sumner, Andersen, & Mackey, 2011; Hawes, Sumner, Andersen,
13 Jungblut, & Mackey, 2013; Zhang et al., 2015).

14
15 Lakes of the McMurdo Dry Valleys can be characterized as extreme shade environments due
16 to perennial ice cover and winter darkness. Shade-adapted benthic microbial mats dominated
17 by cyanobacteria are responsible for the majority of primary production in Lake Vanda
18 (Hawes & Schwarz, 2001; Quesada, Fernández-Valiente, Hawes, & Howard-Williams, 2008;
19 Zhang et al., 2015). Microbial communities are organized within pinnacles and prostrate mats
20 (Hawes & Schwarz, 2001; Hawes, Sumner, Andersen, Jungblut, & Mackey, 2013; Sumner et
21 al., 2016), contributing to the development of and exploiting steep environmental gradients.
22 For example, the mats are finely laminated and contain characteristic pigmented zones (from
23 orange to green and purple) that reflect acclimation to changing spectral characteristics with
24 depth into the mats through the synthesis of particular pigments (from carotenoids to
25 phycocyanin and phycoerythrin; Hawes, Sumner, Andersen, Jungblut, & Mackey, 2013).

26
27 BHPs may also be regulated in response to environmental conditions, as described above.
28 This is the first comprehensive study of BHPs in Antarctica. We describe the abundance and
29 diversity of BHPs in benthic microbial mats from Lake Vanda, identify BHP source
30 organisms, and consider environmental conditions which may prompt the production of
31 particular BHPs in this environment. The analyzed samples span stable environmental
32 gradients, from 9 m to 27 m depth and through microbial mat structures. We specifically
33 focus on the abundance and diversity of 2-MeBHP across environmental gradients such as

irradiance, and consider hopanoid C-2 methylation as an adaptation to membrane stress in polar environments.

2. METHODS

2.1 Study Site

Lake Vanda (77.52° S, 161.67° E) is a perennially ice-covered lake in Wright Valley, one of the McMurdo Dry Valleys in southern Victoria Land, Antarctica (Fig. 1). The ice cover (approx. 4 m thick in January 2011) minimizes wind mixing, which, in addition to temperature and strong salinity gradients, results in a stratified water column with two convecting cells in the upper part of the lake (Howard-Williams, Schwarz, Hawes, & Priscu, 1998). The upper convection cell (UCC; ca. 4-23 m) is well mixed with relatively consistent conductivity (~900 $\mu\text{S}/\text{cm}$), dissolved oxygen (~16 mg/L), temperature (~4.5 °C), and pH (8-8.3; Supplementary Table 1). The upper and lower (28-45 m) convection cells are separated by a pycnocline (23-28 m), where conductivity increases rapidly with depth. The lower convection cell (LCC) is well mixed with relatively consistent conductivity (~1550 $\mu\text{S}/\text{cm}$), dissolved oxygen (~19 mg/L), temperature (~6.5 °C), and pH (8.3-8.5).

The ice cover also affects the quantity and spectral quality of light available to benthic photosynthetic communities. Lake Vanda has the thinnest ice cover (3.5-4 m; Sumner et al., 2016) and highest transmittance ($K_{\text{ice}} = 0.6 \text{ m}^{-1}$) of any McMurdo Dry Valley lake (Howard-Williams, Schwarz, Hawes, & Priscu, 1998). Approximately 15-20% of incident photosynthetically active radiation (PAR) reaches the water column (Howard-Williams, Schwarz, Hawes, & Priscu, 1998; Hawes & Schwarz, 2001). Light is scattered within the ice, creating a diffuse light environment below. The water column is characterized by extreme clarity due to low suspended solids ($<0.1 \text{ g m}^{-3}$) and few terrestrial dissolved organic carbon sources (Howard-Williams, Schwarz, Hawes, & Priscu, 1998).

Lake Vanda is also one of the most oligotrophic lakes known due to the low supply and efficient scavenging of phosphorus (Vincent & Vincent, 1982). Consequently, communities of phytoplankton in the convection cells are extremely sparse, but are acclimated to low, seasonal PAR and persistent low temperature (Vincent & Vincent, 1982). The paucity of phytoplankton results in benthic microbial mats playing a major role in carbon and nutrient cycling and being responsible for the majority of primary production in Lake Vanda (Hawes & Schwarz, 2001; Quesada, Fernández-Valiente, Hawes, & Howard-Williams, 2008).

1
2 Benthic microbial mats extend to at least 50 m depth in Lake Vanda and are volumetrically
3 dominated by filamentous cyanobacteria including taxa within the *Leptolyngbya*,
4 *Phormidium*, and *Tychonema* genera with lesser representation by *Pseudanabaena* and
5 *Synechococcus* sp. (Hawes, Sumner, Andersen, Jungblut, & Mackey, 2013; Zhang et al.,
6 2015; Sumner et al., 2016). The mats have diverse topographies, including prostrate forms
7 and pinnacles that are from <1 mm to 30 cm tall (Love, Simmons, Parker, Wharton, &
8 Seaburg, 1983; Wharton, 1994; Sumner et al., 2016). They are internally laminated with
9 organic-rich hyaline layers alternating with thin (~0.4 mm) mud-rich laminae that form
10 annually (Hawes, Moorhead, Sutherland, Schmeling, & Schwarz, 2001; Hawes, Sumner,
11 Andersen, Jungblut, & Mackey, 2013; Sumner et al., 2016). The outermost mat layers are
12 dominated by *Leptolyngbya* (Hawes, Sumner, Andersen, Jungblut, & Mackey, 2013; Sumner
13 et al., 2016). The laminae are brown, and their geometry mimics the outer morphology of the
14 pinnacle. The interior of large pinnacles is comprised of thicker green or purple pigmented
15 hyaline layers (0.5-3.0 mm) between mud laminae. These subsurface photosynthetic zones
16 have significantly greater populations of *Phormidium* and other *Oscillatoriales* sp. than the
17 surface brown layers (Hawes, Sumner, Andersen, Jungblut, & Mackey, 2013; Sumner et al.,
18 2016). The geometry of these laminae, their response to pulse amplitude modulated (PAM)
19 photometry, and light penetration measurements all suggest active net photosynthetic carbon
20 fixation within the large pinnacles by cyanobacteria (Sumner et al., 2016), to ~5 mm depth.

21
22 The cyanobacterial composition of sample types is consistent across individual pinnacles,
23 including ones of different sizes (Hawes et al., 2013; Sumner et al., 2016). As a result, we
24 assume throughout this study that different mats of similar mat morphology and in close
25 proximity to one another are composed of similar microbial populations. Due to sampling
26 constraints, hopanoid and metagenomic analyses of the samples described are assumed to
27 reflect the hopanoid diversity and source organisms throughout the sampled areas.

28
29 A flood in summer of 2001-2002 deposited a thick layer of mud in Lake Vanda, and that
30 horizon can be used to constrain the age of pinnacles (Hawes, Sumner, Andersen, Jungblut,
31 & Mackey, 2013; Sumner et al., 2016). Therefore, the age of pinnacles can be determined by
32 counting their subsurface laminae. However, subsurface photosynthesis also contributes
33 significantly to the biomass of large pinnacles and formation of organics that span the time

range from the annual capture of mud at the pinnacle surface to the time of collection (Sumner et al., 2016).

2.2 Sample Collection

SCUBA divers sampled benthic microbial mats during austral summer (December 2013; Supplementary Table 2). The divers entered the lake through an access hole melted in the ice cover at 77° 31.6' S, 161° 36.6' E. Sample sets were collected to interrogate the production and preservation of BHPs in Lake Vanda benthic microbial mats. The analysis of whole prostrate mats and individual pinnacle samples provide an overview of BHPs present in this environment. In order to further interrogate the sources of BHPs throughout these mats, we dissected a large pinnacle to allow for the analysis of individual mat laminae and collected a set of samples along a light transect to characterize the effect of shading on BHP production. Core samples were collected in order to test the preservation potential of BHPs in Lake Vanda.

2.2.1. Whole prostrate mat samples

Whole prostrate mat samples were collected in duplicate at each sampled depth from the UCC (11-19 m) and pycnocline (23-27 m). Prostrate mat between pinnacles was generally flat-lying but contained centimeter-scale mounds and lows with relief of a few millimeters (Hawes, Sumner, Andersen, Jungblut, & Mackey, 2013). The samples consist of the upper cohesive layers of prostrate microbial mats, which were cut from the surrounding mat and delaminated from underlying organic material and sediment. All layers grew after the 2001 flood. Samples were originally collected in sterile Falcon tubes, and were transferred to combusted mason jars with foil between glass and rubber seal approximately 1 hour after sample collection. Jars were placed in a freezer approximately 2 hours after sampling.

2.2.2. Individual pinnacles

Individual pinnacles (small, medium, and large) were collected from within the UCC (19 m depth). Small sized pinnacles were defined as containing two or more hyaline-sediment laminae while medium sized pinnacles were defined as being larger than small pinnacles but lacking internal green and purple pigmented areas (Sumner et al., 2016). Thus, both small and medium sized pinnacles had low populations of Phormidium and other Oscillatoriales. All of these pinnacles grew after 2001 flood (Sumner et al., 2016).

One large (~20 mm tall) pinnacle was dissected in the field. Subsamples collected include surface, green, purple, and interior mat components. The “surface” subsample consisted of the outer brown surface mat. The “green” subsample consisted of green-pigmented areas in the pinnacle subsurface. The “purple” subsample consisted of pink-purple pigmented areas underlying green-pigmented areas in the pinnacle subsurface. The “interior” subsample was the central part of the pinnacle. The surface, green, and purple subsamples all grew between 2001 and 2013, whereas the interior sample grew before the 2001 flood (Sumner et al., 2016). Pinnacles and subsamples were transferred directly into combusted glass vials using a metal spatula. The vials were placed in the freezer within 2 hours of sampling.

2.2.3. Shading transect

At 9 m depth, microbial mats grew on, around, and under cobbles and boulders that represent the remnants of a submerged paleo-shoreline (Mackey, Sumner, Hawes, & Jungblut, 2017). Some rocks projected out over the surrounding benthic surface to form overhangs and resulted in a gradient in light available to the mats growing under them. Suspended mud-sized sediment was deposited on the entirety of the mat underneath overhangs (Mackey, Sumner, Hawes, & Jungblut, 2017), demonstrating that the water under the rocks was mixed with open lake water, which was the source of the mud. Thus, in contrast to significant light gradients, we assume that no gradients in water chemistry or temperature exist under overhangs. As a result, these microenvironments provide a natural laboratory to test the effects of photosynthetically active radiation (PAR) on BHP production.

A cohesive mat sample (10.5 x 15 cm) was collected from below an overhanging rock that partially shaded the mat from ambient irradiance, as described by Mackey et al. (2017). The mat was cut and delaminated from underlying substratum along the 2001 flood sediment layer. Differences in PAR across the surface of mat unit were modeled on a 3D point cloud reconstruction of the sampled mat and surrounding lake bottom (Mackey, Sumner, Hawes, & Jungblut, 2017). Relative PAR contours (10-80% relative PAR) were defined across the mat unit. We define relative PAR as local PAR relative to ambient PAR, or PAR received by corresponding open water mat subsamples at the same depth. Ambient PAR (100% relative PAR) values of approximately $180 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ were extrapolated for open water top mat samples at 9 m depth, assuming typical January peak incident irradiance of $1200 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ (Howard-Williams, Schwarz, Hawes, & Priscu, 1998), ice thickness of 4 m resulting in 20% light transmission through the ice cover to the water column below (Hawes

1 & Schwarz, 2000), and a vertical extinction coefficient for down welling irradiance of 0.06
2 m^{-1} (Howard-Williams, 1998). Absolute PAR received by subsurface laminae (green and
3 bottom) is less than top mat samples due to adsorption by pigments in the mat, which results
4 in approximately 1-5% transmission of 455 nm and 16-22% transmission of 580 nm light to
5 2.5 mm depth into the mat (Sumner et al., 2016). Given these constraints, BHP results were
6 interpreted in the context of relative PAR values across the transect.

7
8 Microbial mat was subsampled across a transition from a region under the overhanging rock
9 (<10% relative PAR) toward open water (>80% relative PAR). Samples were extracted from
10 flat-lying prostrate mats between pinnacles using sharpened metal rings with areas of 0.5 and
11 0.95 cm^2 , chosen based on the size of the prostrate mat. Pinnacles were avoided due to their
12 relatively complex internal structure. Individual samples (~0.5-1 mm thick) were delaminated
13 according to pigmentation: brown surface layer, green subsurface layer, and an interior layer
14 comprised of a combination of green and purple pigmented communities in addition to
15 degraded organic material. Sample size was chosen to maximize the number of samples
16 collected across the transect, while still being able to detect and quantify BHT and 2-
17 MeBHT, which are the most abundant compounds present. As a result, BHT and 2-MeBHT
18 are the only compounds described across the transect.

20 **2.2.4. Core Samples**

21 A core (~5 cm) was collected from the lower convection cell (31 m depth) using a
22 polycarbonate sampling tube (38 mm diameter). The core included a cohesive pinnacle mat
23 (~1 cm) consistent with actively growing microbial mat, which was underlain by organic
24 material (~1 cm) and sediments (3 cm). Microbial mats at 31 m depth in 2013 have been
25 submerged since the first observations of lake level in 1947, when they would have been at
26 approximately 16 m depth (Castendyk et al., 2016). The core was extruded and sectioned
27 approximately 2 hours after sampling. The samples were then homogenized in a Falcon tube
28 and stored in glass scintillation vials. Samples were placed in the freezer approximately 3.5
29 hours after collection of the core.

31 **2.3 Lipid extraction and analysis**

32 Microbial biomass and sediment core samples were placed in combusted glass centrifuge
33 vials and homogenized. Total lipid extracts (TLEs) were achieved through the extraction of
34 the samples using a modified Bligh and Dyer method (Bligh & Dyer, 1959) as reported by

Matys et al. (2017). Dichloromethane (DCM) was used as a replacement for chloroform. Samples were ultrasonicated (30 min. at room temperature) initially (2x) with a methanol (MeOH)/DCM/phosphate buffer (2:1:0.8, v/v/v) solution, and then (2x) with a MeOH/DCM/trichloroacetic acid buffer (2:1:0.8, v/v/v) solution. Following each extraction, supernatants were transferred to respective separatory funnels (phosphate vs. trichloroacetic acid buffer). The supernatants were subjected to liquid-liquid extractions with DCM and water (1:1). Extracts were combined only after the TCA was removed through the liquid-liquid extraction. The TLEs were stored at -20 °C pending analysis.

The TLE was acetylated with pyridine/acetic anhydride (1:1, v/v) for 1 h at 70 °C. The acetylated TLEs were stored at -20 °C until 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 included a 1200 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) outfitted with an auto sampler and binary pump. The LC was linked to a Q-TOF 6520 mass spectrometer (Agilent Technologies) through an APCI interface (positive ion mode). The APCI parameters were set to the following: 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; fragmentor, 150 V. A Poroshell 120 EC-C18 column (2.1 x 150 mm, 2.7 µm; Agilent Technologies) was set to 30 °C and provided fast and high-resolution separations of small molecules at low pressure. The eluent flow was held constant at 0.19 ml/min. Eluent A comprised MeOH: water (95:5, v/v) and eluent B was pure isopropyl alcohol (IPA). The HPLC gradient was as follows: isocratic flow of 100% eluent A (0-2 min.), a linear ramp of eluent B from 0-20% (2-20 min.), isocratic flow of 20% eluent B (20-30 mins), a linear ramp of eluent B from 20-30% (30-40 mins), a linear ramp of eluent B from 30-0% (40-45 mins), isocratic flow of 100% eluent A for 5 min. Following the run, the column was conditioned for 5 min. at 100% A.

The HPLC-APCI-MS method described has been optimized for the identification and quantification of hopanoids containing extended side chains (BHPs). As such, this manuscript focuses on the discussion of BHP diversity and abundance. BHPs were identified through the accurate mass measurements of protonated molecular ions, fragmentation patterns in MS-MS mode and assessment of relative retention times (Talbot, Summons, Jahnke, & Farrimond, 2003; Talbot, Rohmer, & Farrimond, 2007; Welander et al., 2012). Quantification was

completed through external standard calibration curves of 3 α ,12 α -Dihydroxy-5 β -pregnan-20-one,3,12-diacetate (PD) and authentic bacteriohopane-32,33,34,35-tetrol (BHT), 2-methylbacteriohopane-32,33,34,35-tetrol (2-MeBHT), diplopterol, 2-Mediplopterol, and 35-amino-bacteriohopane-32,33,34-triol (aminoBHT). PD was also used as an internal standard due to its structural similarity to hopanoids and because it elutes prior to BHPs of interest. Calibrations with authentic BHP standards were conducted since they are necessary to account for variations in ionization efficiencies of different BHPs (Wu et al., 2015; Garby et al. 2017).

2.4 Loss on ignition

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. In order to create a comparable dataset with Sutherland and Hawes (2009) and Mackey et al. (2017), the same LOI method was employed. 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.

2.5 Genomic analyses

Several related samples from Lake Vanda were sequenced by the Joint Genome Institute (JGI) for a different project. Assembled metagenomes and coverage files were downloaded from JGI IMG. Metagenomes represent four individual samples: bulk prostrate mat (300022222) and pinnacle subsamples including green (3300030596) and purple (3300020201) subsurface and interior (3300020057) components.

We searched for translated nucleotide sequences for squalene hopene cyclase (SHC) which catalyzes the initial cyclization of squalene, essential for the production of all hopanoids, and a B-12 binding radical S-adenosylmethionine (SAM) protein (HpnP) that catalyzes the methylation of hopanoids at the C-2 position. We retrieved SHC sequences that were annotated with KEGG enzyme EC:5.4.99.17. Because EC:5.4.99.17 is also used to annotate tetraprenyl-beta-curcumen cyclase, retrieved sequences were also blasted against the NCBI database and all sequences that hit non-targeted gene products were removed. Taxonomy was assigned to each sequence based on the best NCBI blast hit. Custom protein BLAST database was created for HpnP using the makeblastdb command in blast+ 2.2.29. The database

1 contained all protein sequences used in the phylogenetic reconstruction of HpnP in Ricci et
2 al. (2015) excluding the two environmental sequences. Each assembled metagenome (bulk
3 prostrate mat and pinnacle subsamples) was blasted against the HpnP database using blastx
4 with an e-value of 1E-30. The sequences retrieved from the HpnP database were blasted
5 against the NCBI database, but the top results only indicated that they were in the radical
6 SAM family which includes non-target protein products. Therefore, the retrieved sequences
7 and database sequences were aligned in MEGA7 (Kumar, Stecher, & Tamura, 2016) with
8 Muscle (Edgar, 2004) using default parameters. The phylogeny of HpnP protein sequences is
9 depicted through a maximum likelihood tree was built in MEGA7 based on the JTT matrix-
10 based model (Jones, Taylor, & Thornton, 1992), with 1000 bootstrap replicates. The analysis
11 involved 138 amino acid sequences with a total of 420 positions. All positions containing
12 gaps and missing data were eliminated. Sequences that fell outside the HpnP clade defined by
13 Ricci et al. (2015) were removed. Phylum level taxonomy was assigned to each remaining
14 sequence based on the nearest relative in the phylogenetic tree.

15
16 We refer to the diversity of SHC and HpnP sequences identified in this study as unique
17 sequences. The term unique sequences indicates that the translated nucleotide sequences
18 identified are SHC and HpnP, but have small differences in the nucleotide sequences because
19 they have different evolutionary histories. If each bacterial species has a single copy of the
20 gene, then the number of unique sequences would indicate the number of species with the
21 gene. However, it is possible that some species may have multiple copies of one unique
22 sequence in their genomes or that different individuals from the same species have slightly
23 different sequences. As a result, we do not correlate the number of unique sequences directly
24 to the number of species with the genes for SHC and HpnP. However, the identification of
25 unique sequences of SHC and HpnP allow us to determine the diversity of phyla of those
26 organisms that have the genes for SHC and HpnP in the microbial mat samples described
27 here.

28
29 The fold-coverage ($\# \text{ reads} * \text{read length} / \text{contig length}$) was used as a proxy for the
30 abundance of each unique sequence. The relative abundance of each unique sequence
31 identified as either SHC or HpnP is defined as $\text{fold coverage individual sequence} / \sum \text{fold}$
32 $\text{coverage protein sequences}$.

33 34 3. RESULTS

3.1 Diversity and abundance of BHPs in whole prostrate mats

All quantitative BHP data were normalized to the organic matter content of each sample, which ranged from 3.9-32.2% (Supplementary Table 2). The abundance and diversity of BHPs varied with depth in whole prostrate mat samples collected at 5 depths, between 11-27 m (Fig. 2). Total BHP (n= 10), the summed concentration of all BHPs identified, varied with depth in no particular trend (mean= 36.3 $\mu\text{g/g}$, standard deviation (SD)= 10.9). Bacteriohopanetrol (BHT; Supplementary Fig. 1 **1a**; m/z 655) and 2-methylbacteriohopanetrol (2-MeBHT; **2a**; m/z 669) were the most abundant compounds in all samples, ranging from 6.5-50 $\mu\text{g/g}$ and 2.5-17.9 $\mu\text{g/g}$, respectively. 2-Methylbacteriohopanepentol (2-MeBHpentol; **2b**; m/z 727) was also identified in all whole mat samples (0.1-0.9 $\mu\text{g/g}$). Ribonylhopane (**1e**; m/z 627) was identified in only one sample from 19 m depth (1 $\mu\text{g/g}$). Aminotriol (**1c**; m/z 714) was detected in low abundance at 15 m (0.1 $\mu\text{g/g}$) and at all depths greater than 19 m (1-3.3 $\mu\text{g/g}$) within the pycnocline.

3.2 Diversity and abundance of BHPs in whole pinnacles

The organic matter content of each pinnacle sample averaged 59.6% TOM (SD= 23.6; n= 3) in small and 42.1% TOM (SD= 30.3; n= 3) in medium sized pinnacles (Supplementary Table 2). BHP abundance and diversity were higher in medium than small sized pinnacles (Fig. 3c). The mean of total BHP was 64.7 $\mu\text{g/g}$ (SD= 16.3; n= 3) in small sized pinnacles. BHPs present included ribonylhopane, BHT, 2-MeBHT, aminotriol, 2-MeBHpentol, and 3-MeAminotetrol (**3d**; m/z 786). Total BHP averaged 118.4 $\mu\text{g/g}$ (SD= 15.7; n= 3) in medium sized pinnacles and included all of the BHPs present in small sized pinnacles in addition to unsaturated BHpentose (**4e**; m/z 941). BHT and 2-MeBHT were the most abundant compounds present in all small and medium sized pinnacles, accounting for up to 96% of the total BHP. Minor compounds accounted for an average of 7.4% of the total BHP in small sized pinnacles and 12.2% of the total BHP in medium sized pinnacles.

3.3 Diversity and abundance of BHPs in pinnacle subsamples

The total organic content of each pinnacle subsample ranged from 41-100% (Supplementary Table 2). BHP abundance and diversity generally decreased with depth into the large pinnacle, which was collected from 19 m depth (Fig. 3a-b, d). Total BHP was 98.8 $\mu\text{g/g}$ in the brown surface sample, 158.6 $\mu\text{g/g}$ in the green subsurface sample, 81.4 $\mu\text{g/g}$ in the purple subsurface sample, and 10.9 $\mu\text{g/g}$ in the pinnacle interior. Seven compounds were identified in the brown surface layer of the pinnacle, 6 in the green subsurface, 6 in the purple

subsurface, and 3 in the pinnacle interior. BHT and 2-MeBHT were the most abundant compounds present in all pinnacle subsamples, ranging from 93-99% of the total BHP.

3.4 Relative abundance of 2-MeBHT across a PAR transect

Sample area was minimized in order to maximize the number of data points generated across the PAR transect. As a result, minor BHP components were not always detected and we describe only the most abundant compounds present, BHT and 2-MeBHT. The 2-MeBHT ratio (2-MeBHT/total BHT) was used to identify the relative contribution of 2-MeBHT in respect to its desmethyl counterpart (BHT) within individual mat layers (Fig. 4). Generally, the 2-MeBHT ratios were lowest in the top mat layer (0.13-0.23) and increased with depth into the mat, with the highest 2-MeBHT ratios measured in the bottom mat layer (0.32-0.44). The Pearson correlation coefficient was calculated in order to measure the strength of the linear relationship between PAR and the 2-MeBHT ratio for each mat layer. The 2-MeBHT ratio and relative PAR values exhibit positive linear relationships in top brown ($r = -0.52$) and middle green mat ($r = -0.89$) layers and showed no particular trend in the bottom mat layer (mean = 0.4, SD = 0.04, $n = 8$).

3.5 Core Data

The organic matter content of core samples ranged from 2.9-20.1% (Supplementary Table 2). Total BHP measured within each 0.5 cm sample generally decreased with depth into the core, from 26.1-11.2 $\mu\text{g/g}$ in the top layers (pinnacle mat and underlying organic material) to 1.4-9.3 $\mu\text{g/g}$ in the sediment below. However, BHP diversity and relative abundance were invariable throughout the core (Fig. 5). BHPs present included BHT, $\Delta^{6/11}$ BHT (4/5a; m/z 653), 2-MeBHT, 2-MeBHpentol, and aminotriol.

3.6 Hopanoid producers in whole prostrate mat and pinnacle subsamples

Using genetic data for Lake Vanda benthic microbial mat samples from JGI IMG, hopanoid producers were identified based on the translated nucleotide sequences of SHC, essential for the cyclization of squalene to produce the basic hopanoid carbon skeleton (Seckler & Poralla, 1986). Samples included: one bulk prostrate mat and pinnacle subsamples (green, purple, and interior) from 19 m depth (Fig. 6). As described in Section 2.1, we assume that the samples from JGI are considered representative of other similar samples from the same depth. The bulk prostrate mat sample comprised 127 unique SHC sequences. The number of unique

SHC sequences increased with depth into the pinnacle mat, from green (93), purple (102), to interior (142). Cyanobacteria (Synechococcales, Nostocales, and Oscillatoriales) made up the majority of potential hopanoid producers in green and purple subsurface regions (36-42%) and 8% in the pinnacle interior. 2-Methylhopanoid producers were identified based on translated nucleotide sequences for HpnP, required for methylation of hopanoids at the C-2 position (Welander, Coleman, Sessions, Summons, & Newman, 2010). We retrieved three unique HpnP sequences from the metagenomic data all of which are phylogenetically placed in the cyanobacterial phylum (Supplementary Fig. 2). The community from the purple laminae contained copies of all three unique HpnP sequences, whereas the communities from the bulk sample and green laminae only contained two, and the interior community contained a single sequence (Supplementary Fig. 2).

4. DISCUSSION

Bacteriohopanepolyol (BHP) diversity and abundance varied at multiple scales in Lake Vanda, with local environmental conditions, mat morphology, and between mat laminae. Due to the absence of a significant exogenous organic matter influx into Lake Vanda (McKnight, Aiken, Andrews, Bowles, & Harnish, 2013) and age constraints on the growth of the mats (Hawes, Sumner, Andersen, Jungblut, & Mackey, 2013; Sumner et al., 2016), the organic contents of benthic microbial mats analyzed are a result of in situ growth rather than sedimentary processes. Consequently, BHP abundance and diversity were interpreted as markers of active microbial communities that reflect and respond to local environmental conditions.

BHP diversity in whole prostrate mat samples reflected ambient environmental conditions, which vary with depth into Lake Vanda. Samples collected from the upper convection cell (UCC; 4-23 m) experienced relatively similar environmental conditions (conductivity, DO, temperature, pH, and nutrients) and exhibited similar BHP profiles, comprising BHT, 2-MeBHT and 2-MeBHpentol (Fig. 2). The lack of variation suggests stable community structure and function across the UCC. However, whole mat BHP analyses described here homogenized and averaged internal mat layers with their own microenvironments, which differ in light, oxygen, and nutrient availability. Therefore, it is unclear as to how the absolute or relative abundance of these compounds change throughout the mat structure with depth in the UCC. Aminotriol was detected in one sample within the UCC (15 m depth) in low concentrations but only in abundance within the pycnocline (23-28 m), amounting to up to 17.9% of the total BHP (Fig. 2). The abundance of aminotriol in the pycnocline implies a

1 shift in bacterial community structure and/or hopanoid production in response to
2 environmental conditions. However, all hopanoids identified (BHT, 2-MeBHT, 2-
3 MeBHpentol, and aminotriol) are produced by diverse bacteria and cannot be unambiguously
4 assigned to a known shift in community structure.

5
6 BHP profiles differ between prostrate and pinnacle mat morphologies. BHP abundance and
7 diversity were higher in pinnacles (Fig. 3) than prostrate whole mat samples (Fig. 2), likely
8 due to bacteria that colonize within and develop the pinnacle morphology. Sumner et al.
9 (2016) described the growth of pinnacles in Lake Vanda as proceeding through several steps:
10 Tufts initiate from random irregularities in prostrate mat, which may grow into small sized
11 pinnacles with annual laminae. As small pinnacles develop to medium sized pinnacles, an
12 increasingly diverse microbial community colonizes the mat interior. The community creates
13 and exploits physical and chemical gradients within the mat, eventually forming enhanced
14 pigment zones characteristic of larger pinnacles. The increasing complexity of the bacterial
15 community and changes in environmental gradients that occur as the pinnacles increase in
16 size correlates with an increase in the abundance and diversity of hopanoids found in the
17 medium sized pinnacles compared to the small pinnacles.

18
19 Changes in BHP abundance and diversity with pinnacle development, from small to medium
20 sized pinnacles, suggests that BHP source organisms and conditions that prompt the
21 production of BHPs may be spatially variable. For this reason, increased sampling resolution
22 of the microbial mats, such as that distinguishable by pigmentation zones in large sized
23 pinnacles (Fig. 3b), is essential to constraining the sources and production of the compounds
24 identified. BHP abundance and diversity (Fig. 3d), the relative abundance of SHC translated
25 nucleotide sequences (Fig. 6), and the number of unique HpnP sequences (Fig. 6) varied with
26 depth into the dissected pinnacle. For example, BHP abundance and diversity were highest in
27 photosynthetically active mat laminae and decreased substantially into the pinnacle interior.
28 However, SHC sequences were most diverse (142 unique sequences) in the pinnacle interior,
29 suggesting that 1) the diversity of SHC sequences were higher but abundance of SHC
30 sequences were lower than photosynthetically active mat laminae or that 2) bacteria capable
31 of hopanoid biosynthesis were not active or did not experience environmental conditions that
32 necessitate the production of BHP in the mat interior.

1 Increased sampling resolution also indicated that the relative abundance of 2-MeBHP
2 increased with depth into the photosynthetically active mat laminae (Fig. 3d), in line with the
3 relative abundance of SHC translated nucleotide sequences from cyanobacteria (Fig. 6), and
4 the number of unique HpnP sequences (Fig. 6) all of which are phylogenetically placed in the
5 cyanobacterial phylum. 2-MeBHP have previously been identified in sessile microbial
6 communities (Summons, Jahnke, Hope, & Logan, 1999; Talbot et al., 2008; Blumenberg et
7 al., 2013) but are particularly abundant in benthic mats subjected to low-light conditions. For
8 example, 2-MeBHP account for up to 42% of the total BHP in Lake Vanda, 60% of the total
9 BHP in Little Salt Spring, FL (Hamilton et al., 2017), and 54.8% of the total BHP in Mars
10 Oasis mat, Antarctica (Talbot et al., 2008). Furthermore, we have identified 2-MeBHpentol
11 and 2-MeBHpentose without detectable desmethyl counterparts (BHpentol and BHpentose),
12 a rare feature. We hypothesize that shade-adapted communities in the pinnacle subsurface
13 produce an abundance of 2-MeBHP in response to the low light environment.

14
15 2-MeBHPs act as membrane rigidifiers (Wu et al., 2015) which, in turn, may influence
16 membrane protein function and transport (Sáenz et al., 2015). Culture experiments have
17 shown that 2-MeBHP are produced in abundance in akinetes formed by the cyanobacterium
18 *Nostoc punctiforme* in response to low light treatments (Doughty, Hunter, Summons, &
19 Newman, 2009). However, akinete-forming cyanobacterial taxa have not been identified in
20 benthic mats from Lake Vanda (Hawes, Sumner, Andersen, Jungblut, & Mackey, 2013;
21 Zhang et al., 2015; Sumner et al., 2016) and so are an unlikely source of 2-MeBHP here. In
22 vegetative cell types of *Nostoc punctiforme*, 2-MeBHP have been shown to localize in the
23 thylakoid membrane (Doughty, Hunter, Summons, & Newman, 2009). Thylakoid membranes
24 host the light-harvesting components for photosynthesis, and their organization and structural
25 dynamics are essential for the success of photosynthetic organisms (Mullineaux & Kirchhoff,
26 2009; Iwai, Yokono, & Nakano, 2014; Stingaciu et al., 2016). It is possible that an abundance
27 of C-2 methylated hopanoids advantageously alters the physical properties of cyanobacterial
28 thylakoid membranes under low light conditions.

29
30 The association between PAR and 2-MeBHP production was assessed through the
31 characterization of the 2-MeBHT ratio for individual mat laminae spanning a PAR transect at
32 a single depth with consistent water chemistry (Mackey, Sumner, Hawes, & Jungblut, 2017).
33 The 2-MeBHT ratio was inversely correlated with relative PAR across the transect in both
34 the top ($r = -0.52$) and middle ($r = -0.89$) mat layers (Fig. 4c). Furthermore, the 2-MeBHT

ratio was consistently higher in the middle mat layer (0.22-0.38) as compared to the top mat layer (0.13-0.23). These values are consistent with results from the large pinnacle, where the 2-MeBHT ratio increased with depth into the structure. Our results suggest that C-2 methylation is advantageous to cyanobacteria in low light environments, which implies an apparent functional role of 2-MeBHP in photic adaptation. To date, no studies have investigated the production of 2-MeBHP over desmethyl counterparts by cyanobacteria that do not produce akinetes, in response to environmental conditions.

Alternate hypotheses that may explain the inverse correlation between the 2-MeBHT ratio and relative PAR should be noted. The increase in the 2-MeBHT ratio may be due to an increase in the abundance of hopanoid producers across or with depth into the mat unit, rather than an increase in C-2 methylation within individual cells. This hypothesis may be supported by the increase in 2-MeBHP abundance and number of unique HpnP sequences identified with depth into the large pinnacle structure. Perhaps it is counterintuitive that the cyanobacteria would increase in number under lower light conditions, but low light adapted species may thrive with lower relative PAR. However, both the production of 2-MeBHP over desmethyl counterparts and an increase in 2-MeBHP producers suggest that the ability to produce 2-MeBHP may be advantageous in this environment. Additionally, while light may be the most prevalent physical variable across the transect, it cannot be ruled out that low light leads to changes in the underlying community and/or nutrient availability which affect 2-MeBHP production.

BHP signatures, including the 2-MeBHT ratio, produced in benthic microbial mats from Lake Vanda may be preserved in sedimentary archives, as exhibited in the analysis of a core collected at 31 m depth (Fig. 5). While total BHP decreased with depth, the relative proportions of BHPs remained relatively stable throughout the core, likely reflecting the recycling and degradation of organic material. This suggests that hopanoids may be useful tools for monitoring environmental change over time in Lake Vanda. However, this would require age constraints on preserved organic material, not available at the time of this study.

5. CONCLUSIONS

Bacteriohopanepolyols (BHPs) are potentially valuable biological and environmental biomarkers due to the regulation of hopanoid production by some organisms in response to particular environmental conditions. BHPs are diverse and abundant in Lake Vanda, varying

with microbial community structure and environmental conditions present. BHP abundance and diversity vary with mat morphology and water depth for those whole mat samples collected along a depth transect, spanning the upper convection cell and pycnocline. The results of this study suggest that cyanobacteria, identified by genomic data as the sole source of 2-MeBHP in Lake Vanda, produce 2-MeBHP in abundance, possibly in response to the low light in this setting. Two lines of evidence support this conjecture. Firstly, the relative abundance of 2-methylbacteriohopanetetrol increased with depth into benthic microbial mats as the irradiance decreased. Second, the relative abundance of 2-methylbacteriohopanetetrol was inversely correlated with photosynthetically active radiation in isolated mat layers across a shading gradient, where other environmental parameters would have varied only slightly. These data should be further investigated in laboratory experiments that examine the production of 2-MeBHP by non-akinetes forming cyanobacteria growing under controlled light regimes. Such experiments are essential in further elucidating the physiological function of 2-MeBHP in cyanobacteria.

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FIGURES

Figure 1.

Map of the McMurdo Dry Valleys, Antarctica, including Lake Vanda (adapted from Sumner et al., 2016). Red circle indicates approximate sampling locality.

Figure 2.

Bacteriohopanepolyol relative (%) and total ($\mu\text{g BHP/g TOM}$) abundance in whole prostrate mat samples collected from 11-27 m depth in Lake Vanda.

Figure 3.

Well-developed pinnacle (Pin.) and prostrate mats (Pr.; a) at 19 m depth in Lake Vanda. Large pinnacles have complex internal structures (b) which include surface, green- and purple-pigmented subsurface, and interior zones. Bacteriohopanepolyol relative (%) and total ($\mu\text{g BHP/g TOM}$) abundance vary with pinnacle size (c) due to differences in bacteriohopanepolyol profiles present throughout individual pinnacle structures (d). Pinnacle size classes were defined by Sumner et al. (2016). Small sized pinnacles contained two or more hyaline-sediment laminae. Medium sized pinnacles were larger than small pinnacles but lacked internal green and purple pigmented areas present in large pinnacles.

Figure 4.

Microbial mats grew under overhanging rocks at 9 m depth in Lake Vanda (a). Mat subsamples (white circles) were collected and analyzed across a photosynthetically active radiation (PAR) gradient (b) which extended from below the rock (0% relative PAR) to ambient irradiance (100% relative PAR). Relative PAR contours (white lines; <20 to >60 %)

were defined by Mackey et al. (2017). 2-MeBHT ratio values (2-MeBHT/BHT + 2-MeBHT) were calculated for top, middle, and bottom mat subsamples and plotted against relative PAR (%; c). The Pearson correlation coefficient (r) was calculated to measure the strength of the linear relationship between relative PAR and the 2-MeBHT ratio for each mat layer.

Figure 5.

Bacteriohopanepolyol relative (%) and total ($\mu\text{g BHP/g TOM}$) abundance in a core collected from 31 m depth in Lake Vanda. The core comprised whole pinnacle mat (1-1.5 cm), underlying organic material (0-1 cm), and sediments below (0 to -3 cm).

Figure 6.

Occurrence of SHC (relative abundance; %) and HpnP (unique sequences) translated nucleotide sequences in whole prostrate mat and green, purple, and interior pinnacle mat subsamples from 19 m depth in Lake Vanda. All unique HpnP sequences belong to cyanobacteria.

SUPPORTING INFORMATION

Figure 1.

Bacteriohopanepolyol structures referred to and described in this study.

Figure 2.

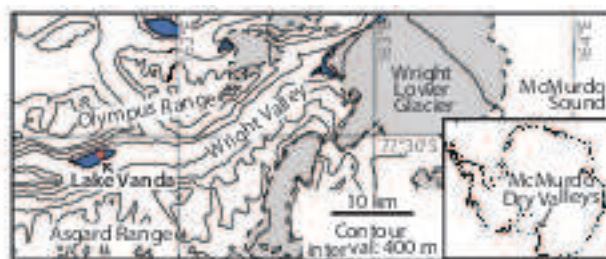
Phylogeny of HpnP protein sequences. Sequences retrieved from the Lake Vanda metagenomic data are indicated in bold.

Table 1.

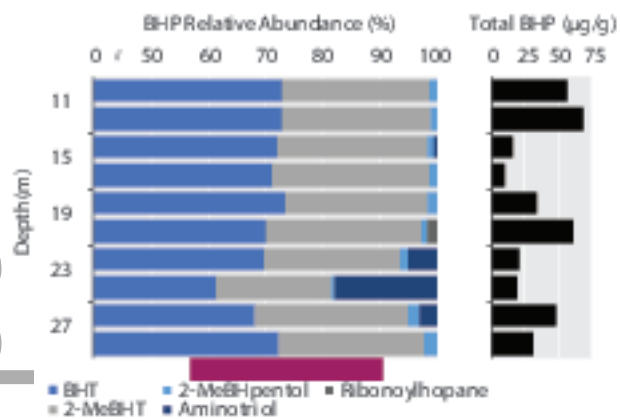
Lake Vanda physical and chemical variables measured in January 2014^a.

Table 2.

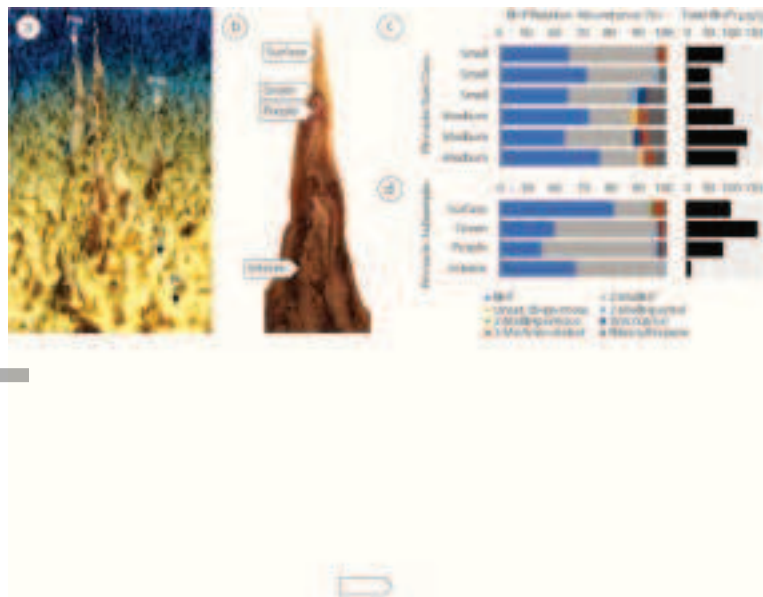
Concentration of bacteriohopanepolyols in benthic microbial mats from Lake Vanda^a.



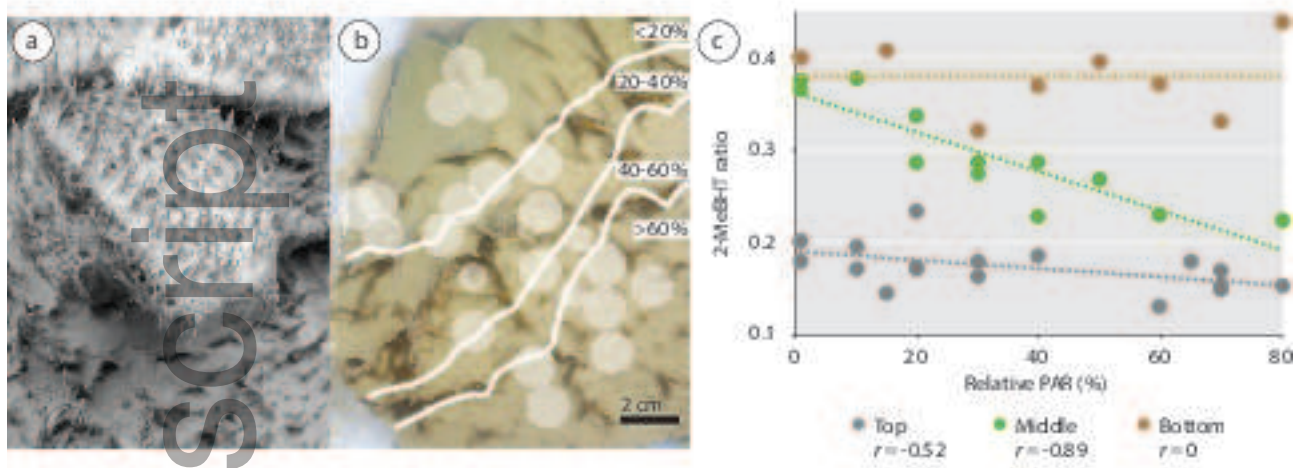
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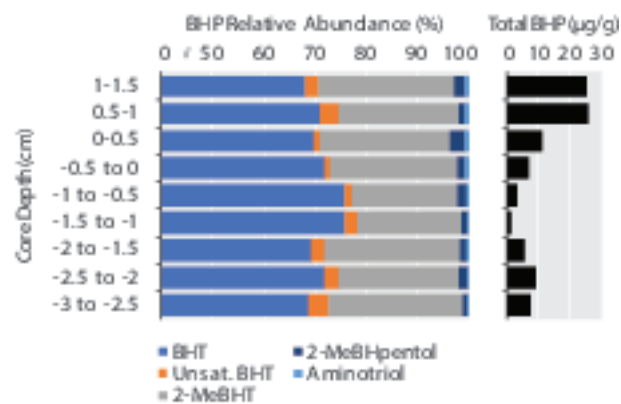
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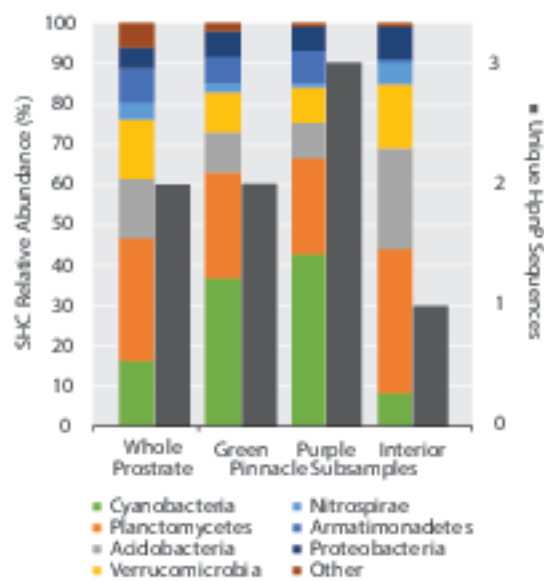
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gbi_12335_f6.tiff