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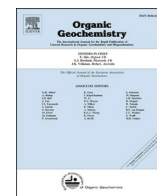
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# Characterization of diverse bacteriohopanepolyols in a permanently stratified, hyper-euxinic lake

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## ABSTRACT

Bacteriohopanepolyols (BHPs) are a diverse class of bacterial lipids that hold promise as biomarkers of specific microbes, microbial processes, and environmental conditions. BHPs have been characterized in a variety of terrestrial and aquatic environments, but less is known about their distribution and abundance in extreme environmental systems. In the present study, samples taken from the water column and upper sediments of the hyper-euxinic, meromictic Mahoney Lake (Canada) were analyzed for BHPs. Analyses show distinct BHP distributions within the oxic mixolimnion, the chemocline, and the euxinic monimolimnion. Bacteriohopanetetrol (BHT) and unsaturated BHT are the dominant BHPs found in the oxic mixolimnion and at the chemocline, whereas a novel BHP (tentatively identified as diunsaturated aminotriol) dominates the euxinic monimolimnion. Along with the novel BHP structure, composite BHPs (i.e., BHT-cyclitol ether and BHT-glucosamine) were observed in the euxinic monimolimnion and sediments, indicating their production by anaerobic bacteria. Complementary metagenomic analysis of genes involved in BHP biosynthesis (i.e., *shc*, *hpnH*, *hpnO*, *hpnP*, and *hpnR*) further revealed that BHPs in Mahoney Lake are most likely produced by bacteria belonging to Deltaproteobacteria, Chloroflexi, Planctomycetia, and Verrucomicrobia. The combined observations of BHP distribution and metagenomic analyses additionally indicate that 2- and 3-methyl BHTs are produced within the euxinic sediments in response to low oxygen and high osmotic concentrations, as opposed to being diagnostic biomarkers of cyanobacteria and aerobic metabolisms.

## 1. Introduction

Bacteriohopanepolyols (BHPs) are a promising class of hopanoid lipid biomarkers used for tracing microbial activity and reconstructing environmental conditions. BHPs and their diagenetic derivatives (i.e., hopanoids) persist over geologic timescales (Brocks et al., 1999; Brocks et al., 2003, 2005; Bednarczyk et al., 2005; van Dongen et al., 2006; Cooke et al., 2008; Handley et al., 2010) and specific structural configurations of BHPs are associated with specific bacterial origins, environmental conditions, and microbial processes. For example, sulfate reducing bacteria (SRB) and aerobic methane oxidizing bacteria have been shown to produce variations of aminoBHPs (Zundel and Rohmer, 1985; Coolen et al., 2008; Blumenberg et al., 2006; Rush et al., 2016).

Additionally, it has been suggested that the ratio of bacteriohopanetetrol (BHT) to its isomer(s) in sediments can be used as a proxy for suboxic-anoxic water column conditions (Sáenz et al., 2011). The presence of specific BHT isomers has more recently been associated with anaerobic ammonium-oxidation (anammox), an important microbial process in the nitrogen cycle (e.g., Rush et al., 2014; Matys et al., 2017; Schwartz-Narbonne et al., 2020; van Kemenade et al., 2022). Many studies now postulate that C-2 methylated hopanoids (originally thought to be indicative of oxygenic photosynthesis; Summons et al., 1999) are produced in response to myriad environmental stressors (e.g., changes in pH, redox, nutrient availability, solar irradiance), combinations of which are still being revealed (see review by Newman et al., 2016 and references therein; Matys et al., 2019a). Clearly, there is still much to be

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discovered in terms of BHP diversity and potential source organisms in under-explored modern environments and especially in endmember-type environments.

Since most microbes cannot be cultured and exact environmental conditions are difficult to mimic, the advancement of culture-independent approaches has increased our understanding of hopanoid source organisms and their potential in situ production through the identification of genes that encode proteins specific to the biosynthesis of BHPs. Specifically, *shc* (squalene-hopene cyclase) is primarily responsible for hopane, and therefore BHP, synthesis (Seckler and Poralla, 1986; Ochs et al., 1992; Kleemann et al., 1994; Perzl et al., 1997; Wendt et al., 1997). Due to the strict specificity and the complex nature of the reactions *shc* catalyzes, the amino acid sequence encoding *shc* is highly conserved among bacterial phyla (e.g., Hoshino and Sato, 2002; Fischer and Pearson, 2007). Consequently, if bacteria are producing BHPs their genome should encode the critical *shc* enzyme. Downstream from the reactions catalyzed by *shc*, the genes *hpnH* and *hpnO* (adenosyl hopane synthase and aminobacteriohopanetriol synthase) have been identified in the synthesis of the (poly)functionalized side chains of BHT and aminotriol, while the genes *hpnP* and *hpnR* (hopanoid C-2 methylase and hopanoid C-3 methylase) are involved in the methylation of the A-ring at the C-2 and C-3 position, respectively (Bradley et al., 2010; Welander et al., 2012; Welander and Summons, 2012; Schmerk et al.,

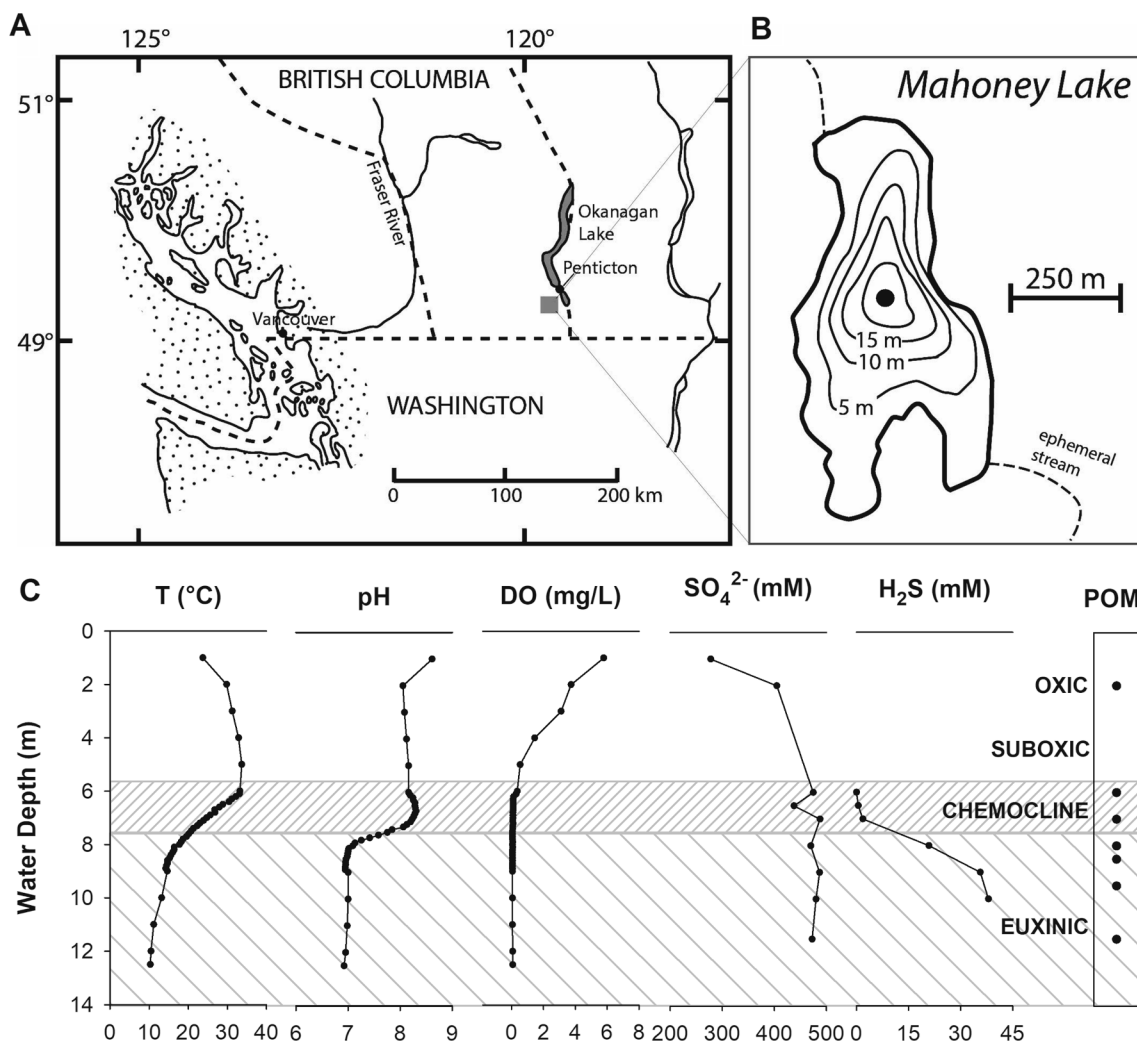
2015). The aforementioned are the main genes that have currently been identified; however, complete biosynthetic pathways for all BHPs have yet to be fully elucidated.

Combining organic geochemical and (meta)genomic approaches to investigate the diversity and biological origins of BHPs in a range of modern environments thus has great potential to further enhance our understanding and interpretation of hopanoids in the geologic record (e.g., Coolen et al., 2008; Pearson et al., 2009; Höfle et al., 2015; Newman et al., 2016; Matys et al., 2017, 2019a, 2019b). To this end, we analyzed water column and sediment samples from a modern, hyper-euxinic, permanently stratified (meromictic) lacustrine system for BHPs, and interrogated previously published metagenomic data for specific genes involved in BHP biosynthesis to determine bacterial origin(s) of observed BHPs.

## 2. Materials and methods

### 2.1. Study area

Mahoney Lake (Fig. 1) is a small (surface area, 11.5 ha; maximum depth, 14.5 m; e.g., Overmann, 1997), saline, meromictic lake in British Columbia, Canada which exhibits photic zone euxinia (anoxic and sulfidic conditions extend into the photic zone). Previous studies have



**Fig. 1.** (A) Geographic location of Mahoney Lake in British Columbia, Canada. (B) Expanded view of Mahoney Lake. The filled circle indicates the water column sampling and sediment coring location. (C) Mahoney Lake water column characteristics including temperature, pH, dissolved oxygen concentrations, sulfate and sulfide concentrations, and particulate organic matter (POM) sample locations. Shaded regions denote the purple sulfur bacteria (PSB) layer at the chemocline and euxinic (anoxic and sulfidic) conditions.

shown it has maintained photic zone euxinic conditions since ca. 11,000 yrs cal BP (Overmann et al., 1993; Coolen and Overmann, 1998). Environments exhibiting photic zone euxinia are rare today, but are thought to have been prevalent at times in the past (e.g., Pancost et al., 2004; Grice et al., 2005). From approximately 6–7 m water depth, a salinity-induced density gradient prevents mixing between the upper (mixolimnion) and lower (monimolimnion) water column. The interface between the oxic mixolimnion and euxinic monimolimnion, referred to as the chemocline, hosts one of the densest populations of anoxygenic phototrophic purple sulfur bacteria (PSB) ever measured (Overmann et al., 1991; Hamilton et al., 2014).

The surrounding geology (i.e., metavolcanic rocks which weather to produce Na–Ca–Mg–SO<sub>4</sub> salts) and active microbial sulfur cycling maintains extremely high concentrations of sulfate (400–500 mM) and sulfide (up to 40 mM) in the monimolimnion (Northcote and Hall, 1983; Gilhooly et al., 2016). For comparison, the concentration of sulfide in the monimolimnion is over 100 times greater than that of the Black Sea (cf., Wakeham et al., 2007), the archetypal example of euxinia, while sulfate concentrations are approximately 10 times greater than modern-day seawater concentrations. Additionally, the sulfate and sulfide concentrations are much higher than those postulated for past ocean conditions (Gill et al., 2011; Wortmann and Paytan, 2012; Reinhard et al., 2013). These chemical characteristics classify Mahoney Lake as an extreme endmember of euxinia and although this impedes direct chemical comparisons to modern euxinic systems, the physical structure and induced biological zonation can provide important information for understanding environmental extremes in terms of potential bio-signatures (i.e., BHPs) produced by microbes involved in biogeochemical cycling and maintaining photic zone euxinia.

Previous genomic studies indicate that each of Mahoney Lake's stratified layers (i.e., mixolimnion, chemocline, and monimolimnion) is taxonomically distinct (Klepac-Ceraj et al., 2012; Hamilton et al., 2014, 2016). For instance, the oxic mixolimnion supports the growth of phytoplankton and obligate aerobic heterotrophic bacteria, mainly cyanobacteria, Alphaproteobacteria, and Actinobacteria (Overmann et al., 1996; Klepac-Ceraj et al., 2012). Below this layer, the chemocline supports chemolithoautotrophic oxidation of sulfide and sulfur by anoxygenic phototrophic PSB from the class Gammaproteobacteria, as well as sulfate reduction by Epsilon- and Deltaproteobacteria, respectively (Overmann et al., 1991; Hamilton et al., 2014). Below the

chemocline, the aphotic, euxinic monimolimnion supports obligate anaerobic chemoautotrophic and heterotrophic sulfur-reducing bacteria, primarily members of Firmicutes and Deltaproteobacteria (Hamilton et al., 2016). Furthermore, the sediments have bacterial communities most similar to the aphotic, euxinic monimolimnion but with a significantly higher abundance of Planctomycetes bacteria (Hamilton et al., 2016).

## 2.2. Water column sampling

Mahoney Lake is an ecological reserve and, as such, sampling permits were obtained from BC Parks in advance of all sampling. Water column samples and measurements were taken from the deepest part of Mahoney Lake (coordinates: 49.289011, -119.582370; Fig. 1) in August 2015. Water column characteristics (Fig. 1) were measured using a Hydrolab Quanta water quality sonde, which was calibrated to conditions in the field. Particulate organic matter (POM) was filtered from 7 water depths (Figs. 1 and 2; one above the chemocline in the oxic mixolimnion, two within the chemocline, and four below the chemocline in the euxinic monimolimnion) using a McLane Research Laboratories, Inc. Large Volume Water Transfer System (WTS-LV). The WTS-LV was fitted with 2-sets of metal screens before a 142 mm diameter, 0.42 µm nominal pore size pre-combusted (450 °C for 4 h) glass fiber filter (Whatman). The WTS-LV was programmed to filter 200 L of water or stop at 1-hour collection time.

## 2.3. Sediment coring and age control

Surface sediment cores were taken from the deepest part of Mahoney Lake in August 2017 using a modified Bolivia and Livingstone piston coring system. The surface cores, containing the sediment-water interface, were expeditiously extruded (2 cm intervals) in the field while being flushed with nitrogen gas and immediately frozen on dry ice. Four samples were analyzed for this project, representing the nepheloid layer (MAH17-1; 0–2 cm), necroid layer (MAH17-3; 4–6 cm), unconsolidated sediment (MAH17-21; 40–42 cm), and consolidated sediment (MAH17-40; 78–80 cm). Charcoal from 83 cm core depth was <sup>14</sup>C-dated at the Keck Carbon Cycle AMS Facility, Earth System Science Department, University of California – Irvine.



**Fig. 2.** (A) POM filter from the chemocline with abundant purple sulfur bacteria (PSB). (B) POM filters prepared for modified Bligh and Dyer extraction. (C) Bligh and Dyer extracts (BDEs) from POM filters. (D) Column chromatographic separation of the BDE from the PSB layer.



## 2.4. Total Organic Carbon (TOC)

Weight percent total organic carbon (%TOC) of bulk OM was determined from portions of POM filters with known areas and homogenized sediment samples of known weight. The samples were acidified in concentrated HCl vapor in a desiccator (sans desiccant) for 8 h to remove inorganic carbon and analyzed with a Costech Elemental Analyzer at the University of Pittsburgh (Pitt). Values of %TOC were calibrated against a certified acetanilide standard (Costech Analytical Technologies, Inc.). Filter samples were run in triplicate and sediments were run in duplicate.

## 2.5. BHP extraction and analysis

Lipid extracts were obtained from freeze-dried POM filters (Fig. 2; one entire 142 mm GFF per depth) and freeze-dried, homogenized sediment samples (ca. 2 g per sample) using a modified Bligh and Dyer extraction (Bligh and Dyer, 1959; Pitcher et al., 2009). Briefly, the filters and sediments were ultrasonically extracted (until color was no longer observed in the extracts) with methanol (MeOH)/dichloromethane (DCM)/phosphate buffer (PB; 50 mM, pH 7.4), (2:1:0.8, v/v/v), respectively. The solvent/buffer mixture was collected after centrifugation and the combined liquid phases were adjusted to a solvent ratio of MeOH/DCM/PB (1:1:0.9, v/v/v) which led to separation between the DCM and MeOH/PB phases. The DCM phase was collected and the MeOH/PB phase was repeatedly extracted (until color was no longer observed in the extracts) with additional volumes of DCM. The combined DCM phases were then reduced under a gentle stream of N<sub>2</sub> and filtered over solvent extracted cotton wool using DCM/MeOH (9:1, v/v). The extracts were subsequently reduced to dryness under a gentle stream of N<sub>2</sub> and stored in the freezer (-20 °C).

Lipid extracts (ca. 5 mg per extract) were separated into two fractions via solid phase extraction (SPE) column chromatography (Talbot et al., 2016). In short, aminopropyl SPE gel was weighed into a combusted, glass SPE column plugged with solvent-extracted cotton wool. The column was preconditioned with hexane, after which the extract (dissolved in a small amount of chloroform) was loaded onto the column (Fig. 2). The first fraction was eluted with diethyl ether/acetic acid (98:2, v/v). The second fraction, containing the BHPs, was eluted with MeOH. The fractions were reduced to dryness under a gentle stream of N<sub>2</sub> and stored in the freezer (-20 °C) until analysis. Immediately prior to analysis, 5 $\beta$ -Pregnane-3 $\alpha$ ,20 $\alpha$ -diol standard was added to each of the BHP-containing fractions (200  $\mu$ l, 0.265 mg/ml; Tokyo Chemical Industry UK Ltd.) and the fractions were acetylated with 250  $\mu$ l of pyridine/acetic anhydride (1:1, v/v). Each fraction was heated on a hot plate at 40 °C for one hour, then left to cool overnight. The following morning, each fraction was reduced to dryness under a gentle N<sub>2</sub> stream and re-dissolved in 500  $\mu$ l of propanol/MeOH (60:40, v/v). This was filtered using a glass syringe and 0.2  $\mu$ m PTFE filter into an LC-MS vial, and the original vial rinsed with another 500  $\mu$ l of propanol/MeOH (60:40, v/v), filtered through the same PTFE filter, and added to the LC-MS vial to make a total of 1 ml. This was reduced to dryness, and re-dissolved in 500  $\mu$ l of propanol/MeOH (60:40, v/v) ready for injection. To avoid loss of acetyl groups during storage, analysis was carried out within one week of acetylation.

BHPs were measured using an Agilent 1260 HPLC coupled to an Agilent 6540 Q-TOF MS equipped with an APCI source operated in positive ion mode at Manchester Metropolitan University (MMU), using a method based on Cooke et al. (2008). The BHPs were analyzed using reverse phase chromatography with an ACE Excel 5 SuperC18 column (5  $\mu$ m particles, 150 mm length, 3.0 mm i.d.). Solvent A was MeOH/water (90:10, v/v), Solvent B was MeOH/isopropanol/water (59:40:1, v/v/v). All solvents were of LC-MS grade (Fisher Scientific, UK). Separation was achieved at 30 °C at 0.5 ml/min using the following gradient profile: Starting at 100% A, linear gradient to 100% B in 25 min, held for 15 min, linear gradient to 100% A in 5 min. The column was backflushed

for 13 min in 100% A, and re-equilibrated for 2 min in 100% A prior to the next injection. Conditions for APCI-MS were: nebulizer pressure 35 psig, vaporizer 400 °C, drying gas (N<sub>2</sub>) 8 L/min and 300 °C, capillary voltage 3.5 kV and corona 8  $\mu$ A. Masses from  $m/z$  100 to 1500 (resolution:  $\geq 20,000$ ; accuracy:  $<0.25$  ppm,  $<2 \times 10^{-4}$  Da) were sampled at a rate of 2 spectra/second and with subsequent data-dependent tandem MS-MS.

BHPs were identified using a combination of MS and MS-MS data. Characteristic base peak and fragment ions (e.g., Talbot et al., 2007) were used to identify known BHPs in samples with high concentrations of BHPs. These identifications were used to define absolute and relative retention times for each BHP for this column-solvent-instrument combination. BHP identification on each datafile was based on these retention times, supported by MS and MS-MS fragment ions where necessary. Compounds are shown in Appendix A and identifying information (observed mass, major fragment ions, and retention time) is reported in Appendix B. The novel BHP was tentatively identified by comparing its major fragment ions to those found in known BHP compounds (i.e., aminotriol; discussed further in Section 3.1.1). Fragment ions from the novel BHP were confirmed by re-running samples using targeted MS-MS mode (target mass 710.5900; Z = 1; isolation window "Narrow, 1.3 Da"; fragmentor 175 V; collision energy 35 V; collision gas N<sub>2</sub>).

Semi-quantitative estimates of BHP concentrations were calculated by comparing peak areas of the extracted ion chromatogram (EIC) for individual BHP base peak ions with the EIC of the internal standard base peak ion ( $m/z$  345.280). Limits of detection and quantification were found using serial dilutions of the acetylated internal standard (from  $4 \times 10^{-2}$   $\mu$ g/ml to  $4 \times 10^{-7}$   $\mu$ g/ml). The base peak area response was linear until  $4 \times 10^{-5}$   $\mu$ g/ml (5000 counts), and the internal standard was detectable at  $4 \times 10^{-6}$   $\mu$ g/ml (300 counts). To ensure confidence when calculating BHP concentrations, compounds with base peak areas  $\leq 10,000$  counts were excluded. Concentrations of BHPs were normalized using typical response factors (8 or 12, depending on the presence of nitrogen atoms in the structure; Cooke et al., 2008; van Winden et al., 2012).

## 2.6. Genomic analysis

Previously published metagenomic datasets from the PSB layer, euxinic monimolimnion, and a surface sediment grab sample were used to examine genes that encode proteins involved in the production of BHPs in bacterial communities in Mahoney Lake. The collection, preservation, extraction of DNA, and analyses of the metagenomic sequencing for these samples has been previously described (Hamilton et al., 2014, 2016). In brief, after collection, samples of water and sediments were immediately frozen on dry ice during transport and stored at -80 °C until DNA extraction. For extraction of genomic DNA, samples were thawed and centrifuged, and DNA was extracted from the resulting pellets using an e.Z.N.A SP Plant Maxi Kit (Omega Bio-tek, Norcross, Georgia) according to the manufacturer's instructions. Gel electrophoresis was used to assess the yield and quality of the extracted DNA.

Genes encoding proteins involved in the production of BHPs in microbial communities in Mahoney Lake included *shc*, which encodes the squalene-hopene cyclase protein, as well as the genes *hpnH* (adenosyl hopane synthase), *hpnO* (aminobacteriohopanetriol synthase), *hpnP* (hopanoid C-2 methylase), and *hpnR* (hopanoid C-3 methylase). Representative sequences of each gene were obtained from the National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/>) and used in a search of each annotated metagenomic data set using the NCBI Basic Local Alignment Search Tool (BLASTX®) (e.g., Altschul et al., 1990; Johnson et al., 2008). Multiple query sequences for BLAST® searches were chosen to sample the diversity of organisms present in a sample. For each functional gene, functional annotation was assigned if each hit met the following criteria: e-value scores less than 1e-25 [except for *hpnR* where the e-value cutoff

was 1e-100 after [Welander and Summons \(2012\)](#)] and quality coverage of at least 85%. Phylum-level affiliations were assigned based on the best BLASTX® hit to each functional gene following the criteria above.

### 3. Results and discussion

#### 3.1. Distribution of BHPs in Mahoney Lake

Overall, a total of 10 BHPs were observed throughout the water column and upper sediments of Mahoney Lake. BHP compounds are given in Appendix A and abundances (ng/L or ng/g sed and µg/g TOC) are listed in [Table 1](#). Abundances are discussed as µg/g TOC to allow for general comparison between the water column and sediments (i.e., abundances in the water column and sediment are converted to the same unit of measure). Abundances in µg/g TOC at 6 m, 8 m, and 9.5 m were calculated from interpolated TOC values. Therefore the abundances at 6 m are likely underestimated, while those at 8 m may be overestimated due to the distinct difference in TOC concentration at the chemocline ([Table 1](#)).

The number and abundance of BHPs varied widely within all samples analyzed ([Fig. 3](#)). The lowest number and abundance of BHPs was found in samples taken from the PSB layer and chemocline (2 BHPs, avg. 2 µg/g TOC), while the highest overall number and abundance was found in the deeper sediment samples (10 BHPs and 725 µg/g TOC).

The general profile of total BHP abundance increasing below the chemocline ([Fig. 3A](#) and [Table 1](#)) is in contrast with marine systems that also exhibit meromictic and/or anoxic/euxinic conditions (cf. [Wakeham et al., 2007](#) for Black Sea water column profiles and [Sáenz et al., 2011](#) for Arabian Sea, Peru Margin, and Cariaco Basin water column profiles). In these marine systems, total BHP abundance generally reached its maximum at or within the chemocline and decreases thereafter with water depth. The opposite trend in total BHP abundance observed in Mahoney Lake is thus likely due to its comparatively unique physical (e.g., depth), chemical (e.g., extremely high sulfate and sulfide concentrations), and biological (e.g., high concentrations of PSB at the chemocline) attributes, which necessarily limits direct comparisons with these well-studied marine systems.

The deepest sediment sample at 78-80 cm blf corresponds to ca. 660 cal BP (based on radiocarbon dating of charcoal at 83 cm core depth), so most BHPs produced in Mahoney Lake are preserved over at least centennial timescales while further studies are needed to fully evaluate preservation over greater timescales. It is also possible BHPs are

produced within the sediments of Mahoney Lake. That said, BHPs have been observed in ancient sedimentary systems, the oldest being of Jurassic age (e.g., [Bednarczyk et al., 2005](#); [van Dongen et al., 2006](#)) and up to 10,000 yrs cal BP in the anoxic sediments of the Black Sea ([Blumenberg et al., 2009](#)) and Ace Lake, Antarctica - a polar, meromictic, saline lake, with euxinic bottom waters and apparent euxinic conditions since ca. 9,400 yrs cal BP ([Franzmann et al., 1991](#); [Rankin et al., 1999](#); [Coolen et al., 2008](#); [Laybourn-Parry and Bell, 2014](#)). Therefore, it is plausible that anoxic/euxinic Mahoney Lake sediments have a similar preservation potential.

##### 3.1.1. Water column BHP distribution

In the oxic mixolimnion (2 m water depth), four BHPs were identified - bacteriohopanetetrol (BHT) and unsaturated BHT were the dominant compounds, comprising >85% of total BHPs, with aminotriol and BHT-cyclitol ether in much lower abundance ([Fig. 3](#)). Several aerobic bacteria are known to produce these BHPs (e.g., [Talbot et al., 2008](#)), including the previously observed cyanobacteria, Alphaproteobacteria, and Actinobacteria in the mixolimnion ([Klepac-Ceraj et al., 2012](#)), therefore they are the most probable bacterial sources.

At the chemocline and within the PSB layer, two BHPs were present, BHT and unsaturated BHT ([Fig. 3](#)). These BHPs are not source-specific because they are produced by several bacteria, and thus are not considered diagnostic biomarkers. PSB are the most abundant bacteria in the chemocline ([Overmann et al., 1991](#); [Hamilton et al., 2014](#)). Despite their overwhelming abundance, previous studies have shown that PSB do not produce BHPs ([Rohmer et al., 1984](#)). Thus, BHT and unsaturated BHT must be sourced from bacteria above the PSB layer and/or by minor bacterial constituents residing within the PSB layer. The very low BHP abundance within the PSB layer (avg. 2 µg/g TOC compared to ca. 13 µg/g TOC above the PSB layer) along with the distinctly different distribution of BHPs below it ([Fig. 3](#)), indicates BHPs from the upper water column are efficiently recycled within the PSB layer at the chemocline. Consequently, BHPs produced in the mixolimnion and within the PSB layer are not exported to the monimolimnion in substantial amounts.

Below the chemocline, within the euxinic monimolimnion, six BHPs were detected ([Fig. 3](#)), with the dominant structure (>65% of total BHPs) being a novel, abundant BHP (5-82% total BHPs), which has a base peak of  $m/z$  710.589 ([Fig. 4](#)). This compound has been tentatively identified as a diunsaturated aminotriol based on its + APCI MS<sup>2</sup> fragmentation pattern ([Fig. 4B](#)) and retention time relative to aminotriol ( $m/z$

**Table 1**

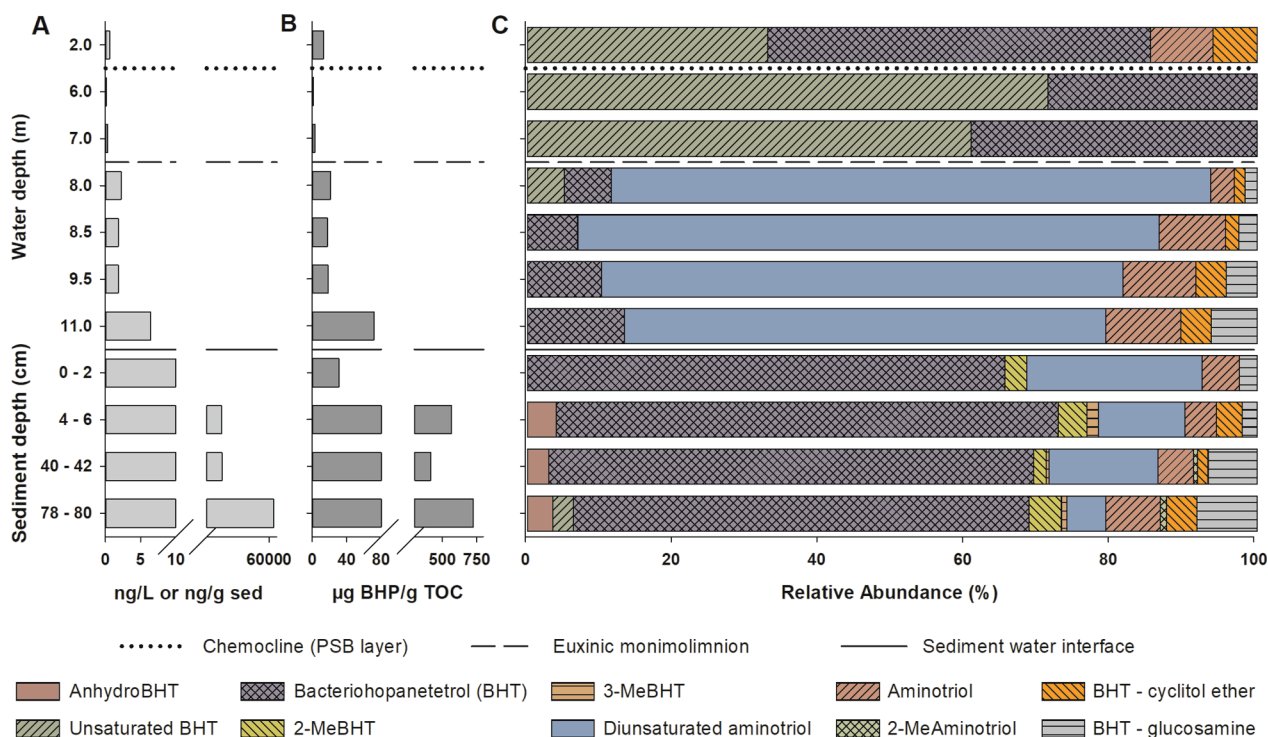
Concentration and distribution of BHPs identified in Mahoney Lake. See Appendix A for compounds.

Abbreviated name	Base peak ( $m/z$ )	µg BHP per g TOC										
		Water column depth (m)							Sediment depth (cm)			
		2	6	7	8	8.5	9.5	11.5	0-2	4-6	40-42	78-80
AnhydroBHT	613 <sup>a</sup>	–	–	–	–	–	–	–	–	23	12	26
Unsaturated BHT	653 <sup>b</sup>	4.3	1.1	1.8	1.1	–	–	–	–	–	–	21
BHT	655 <sup>b</sup>	6.8	0.4	1.2	1.4	1.3	1.9	9.5	21	390	278	453
2-MeBHT	669 <sup>b</sup>	–	–	–	–	–	–	–	1	22	7	32
3-MeBHT	669 <sup>b</sup>	–	–	–	–	–	–	–	–	9	2	6
Diunsaturated aminotriol	710 <sup>a</sup>	–	–	–	17.4	14.4	13.3	47.2	8	67	62	39
Aminotriol	714 <sup>a</sup>	1.1	–	–	0.7	1.6	1.9	7.4	2	25	20	54
2-Me Aminotriol	728 <sup>a</sup>	–	–	–	–	–	–	–	–	–	2	6
BHT-cyclitol ether	1044 <sup>a</sup>	0.8	–	–	0.3	0.3	0.8	3.0	–	20	6	30
BHT-glucosamine	1002 <sup>a</sup>	–	–	–	0.4	0.5	0.8	4.5	1	12	28	59
Total BHPs (ng/L or ng/g sed)		0.6	0.1	0.3	2.2	1.8	1.8	6.5	741	15,180	15,560	64,270
Total Organic Carbon (g/L or g/g sed)		4.5	9.8	1.1	1.0	9.9	9.6	9.0	0.024	0.027	0.037	0.089
		E-05	E-05	E-04	E-04	E-05	E-05	E-05				
Total BHPs (µg/g TOC)		13.0	1.5	3.0	21.1	18.0	18.6	71.6	31	566	418	725

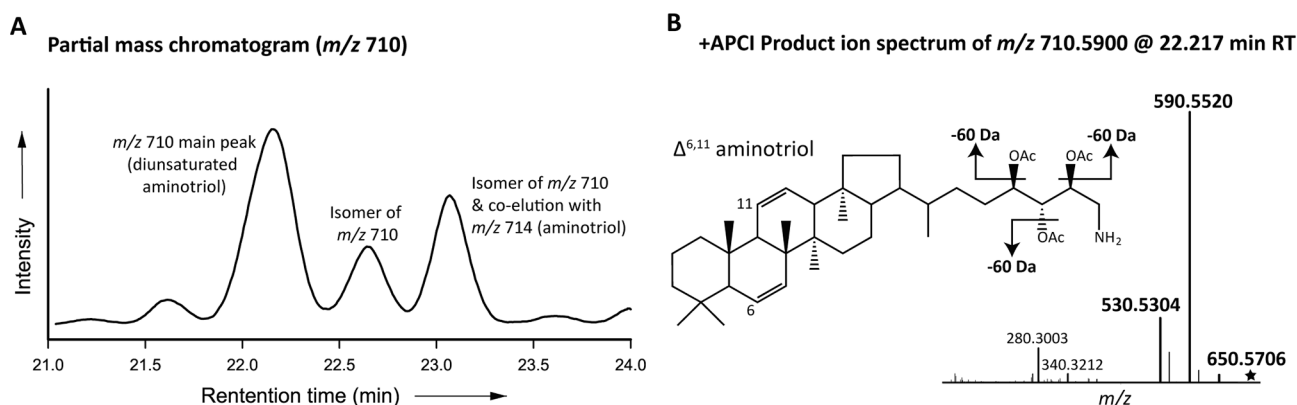
**Bold** values for Total Organic Carbon (TOC) have been interpolated *italicized* values have been calculated from interpolated TOC values.

<sup>a</sup>  $m/z$  = [M + H]<sup>+</sup> in acetylated form.

<sup>b</sup>  $m/z$  = [M + H – CH<sub>3</sub>COOH]<sup>+</sup> in acetylated form.



**Fig. 3.** Water column and sedimentary distributions of bacteriohopanepolyols (BHPs) in Mahoney Lake. (A) Total BHP concentration displayed as ng/L in the water column and ng/g sed in the sediments. Note break in scale from 10 to 500 and change in scale thereafter. (B) Total BHP concentration normalized to organic carbon concentration. Note break in scale from 80 to 300 and change in scale thereafter. (C) Relative abundance of individual BHPs identified in Mahoney Lake.



**Fig. 4.** Identifying information for the proposed diunsaturated aminotriol. (A) Partial mass chromatogram ( $m/z$  710) between 21 and 24 min retention time (RT) from a representative Mahoney Lake acetylated lipid fraction. The three peaks correspond to isomers of the same compound with a base peak of  $m/z$  710, based on the similar  $MS^2$  fragmentation of the three peaks (not shown). (B)  $MS^2$  + APCI product ion spectrum of the main  $m/z$  710 peak (targeted  $m/z$  710.5900) at 22.217 min RT. The  $MS^2$  spectrum has the characteristic fragments ( $m/z$  650.751, 590.552, and 530.530) for the repeated loss of 60 Da corresponding to the sequential loss of three acetylated hydroxyl groups from the proposed diunsaturated aminotriol ( $\Delta^{6,11}$  aminotriol) structure.

$m/z$  714). The proposed diunsaturated aminotriol has fragments ( $m/z$  650.571, 590.552, 530.530) that are 4 Da lighter than those observed in acetylated aminotriol (cf. Talbot et al., 2001), which likely results from double unsaturation of the aminotriol structure, while the loss of 60 Da corresponds to the sequential loss of three acetylated hydroxyl groups (Fig. 4B). In the partial chromatogram of  $m/z$  710 (Fig. 4A), the three eluted peaks likely correspond to isomers of  $m/z$  710 based on  $MS^1$  spectra. The  $MS^1$  spectra show the same base peak and fragmentation for each of the three peaks, except for the latest eluting peak which co-elutes with aminotriol and thus also contains fragments consistent with  $m/z$  714.

The structure with unsaturations at the C-6 and C-11 position (Fig. 4B) is drawn because these are typical positions for unsaturation to

occur in known BHPs (e.g., Talbot et al., 2016 and references therein), but the location of the double bonds may vary (e.g., both double bonds located within the core, both located in the side chain, or one in the core and one in the side chain). Further analysis using different derivatization techniques (e.g., periodic acid treatment followed by sodium borohydride and/or addition with dimethyldisulfide) and/or detailed nuclear magnetic resonance analysis of the isolated compound(s) would confirm its presence and the specific positions of unsaturation. As such, our identification remains tentative. Monounsaturated aminotriol ( $m/z$  712) was not detected in any of the samples.

The much greater concentrations (ca. 21–72  $\mu\text{g/g}$  TOC) within the euxinic monimolimnion relative to the layers above and the presence of a more diverse array of BHPs indicates that they are produced at depth

and likely derive from anaerobic heterotrophic and/or chemotrophic bacteria below the photic zone. The relative abundance of BHT, aminotriol, BHT-cyclitol ether, and BHT-glucosamine increase with depth in the monimolimnion, while the relative abundance of diunsaturated aminotriol decreases. This behavior is likely a result of the balance between production, mineralization, and transport of the BHPs through the water column. That said, the strong correspondence between diunsaturated aminotriol and euxinic water column conditions does indicate its potential as a proxy for euxinia, though more studies are needed to confirm this assertion.

The presence of relatively abundant composite BHPs (BHT-cyclitol ether and BHT-glucosamine) throughout the euxinic monimolimnion is in agreement with previous studies that showed that anaerobic bacteria are major producers of composite BHPs. Specifically, BHT-cyclitol ether and BHT-glucosamine are the major BHPs produced by the anaerobic bacteria *Geobacter sulfurreducens*, comprising 54% and 22% of total BHPs (Eickhoff et al., 2013). In Mahoney Lake, Desulfuromonadales (the order to which *Geobacter sulfurreducens* belongs) are present in high read coverage and are a major component of the sulfur cycle below the chemocline (Hamilton et al., 2016), so it is highly plausible that related bacteria, belonging to the class Deltaproteobacteria and order Desulfuromonadales, are the producers of composite BHPs in the euxinic monimolimnion.

Notably, stereoisomers of BHT were not observed in any of the samples, despite 'late-eluting BHT isomers' being associated with modern marine water column suboxia/anoxia, as well as bacterial anaerobic oxidation of ammonium (anammox) activity (Sáenz et al., 2011; Wakeham et al., 2012; Kharbush et al., 2013; Rush et al., 2014; Matys et al., 2017, 2019; Schwartz-Narbonne et al., 2020; van Kemnade et al., 2022). The lack of BHT isomer(s) supports previous studies which suggest that the isomerization of BHT and ratios of BHT to its isomer(s) are more indicative of anammox activity rather than simply suboxia/anoxia. The genetic potential for anammox was found to be insignificant in Mahoney Lake, which helps explain the absence of BHT isomer(s) even though water column conditions range from oxic-suboxic-euxinic (Hamilton et al., 2016).

### 3.1.2. Sedimentary BHP distributions

Within the sediments of Mahoney Lake, a total of 10 BHPs were detected (Fig. 3). In general, the overall concentration and diversity of BHPs increases with increasing sediment depth. The relative abundance of diunsaturated aminotriol decreases (down to ca. 5% compared to >65% in the water column), while the relative abundance of BHT increases to ca. 62% and remains relatively steady with sediment depth. The increase in BHT in the sediments relative to the water column indicates that they are efficiently exported from the water column and/or that they are also produced in the sediments. The lower relative abundance of aminotriol and diunsaturated aminotriol in the sediments compared to the overlying water column is driven by the marked increase in BHT concentrations compared to the modest increase of aminoBHP concentrations in the sediments (aminotriol concentrations increase from an avg. of 2.5 to 25 µg/g TOC and diunsaturated aminotriol increases from 23 to 44 µg/g TOC between the water column and sediments, respectively; Table 1).

The relative abundance and absolute concentration of composite BHPs in the sediments generally increases with depth (Fig. 3; Table 1), which is somewhat surprising considering that composite BHPs are prone to degradation, so generally do not withstand diagenetic conditions. BHT-cyclitol ether has been observed in sediments up to ca. 10,000 yrs cal BP in the Black Sea (Blumenberg et al., 2009), as well as Ace Lake, Antarctica (Coolen et al., 2008). The anoxic/euxinic nature of the Black Sea, Ace Lake, and Mahoney Lake likely serves to increase the preservation of the composite BHPs in these systems relative to more oxygenated systems. That said, with the current data, it is equally probable that: (1) the composite BHPs continue to be produced in the sediments of Mahoney Lake and/or (2) their export from the water

column has changed over time.

2- and 3-MeBHTs are only observed in sediment samples, so it is plausible they are produced in the sediments (Fig. 3). Methylated BHPs have been widely applied as a diagnostic biomarker of cyanobacteria and oxygenic photosynthesis (e.g., Summons et al., 1999), as well as aerobic metabolisms in methanotrophs and acetic acid bacteria (e.g., Zundel and Rohmer, 1985), but more recent studies have shown that the genes required for methylated BHP biosynthesis are wider ranging, and culture-based studies have suggested that they are produced in response to certain environmental conditions rather than diagnostic of specific bacteria (Welander et al., 2010; Welander and Summons, 2012; Ricci et al., 2014, 2015; Wu et al., 2015; Newman et al., 2016). Specifically, low oxygen and high osmotic concentration are associated with increased abundance of 2-MeBHPs (Kulkarni et al., 2013; Ricci et al., 2014; Newman et al., 2016). Mahoney Lake sediments are euxinic and have high concentrations of osmolytes (i.e., extracellular polysaccharides and excreted small molecules), which may explain the appearance of 2-MeBHT in the sediments.

Moreover, elevated concentrations of the diagenetic products of 2-MeBHPs (i.e., 2-methyl hopanes) have been observed throughout the Proterozoic (Summons et al., 1999). The highest measurements of which coincide with Oceanic Anoxic Events (OAEs), times where Earth's oceans exhibited widespread anoxia and marginal euxinia - including periods of photic zone euxinia (Pancost et al., 2004; Kuypers et al., 2004a, 2004b; Grice et al., 2005; Xie et al. 2005; Knoll et al., 2007). Thus, the occurrence of high 2-methylhopane indices (i.e., the ratio of 2-methylhopanes to methylated and unmethylated hopanes) may very well be explained by anaerobic bacterial production of 2-MeBHT induced by low oxygen and high osmolarity stressors, as observed in the euxinic sediments of Mahoney Lake.

Similar to C-2 methylation of BHPs, C-3 methylation appears to be a requirement for cell survival in late stationary phase of bacterial growth when environmental conditions are adverse (e.g., low nutrient and/or low oxygen concentrations; e.g., Welander and Summons, 2012). Therefore, it is probable that 3-MeBHT in the sediments reflects bacterial response to environmental stressors encountered in the sediment as bacterial cells enter sessile stages.

There was no evidence of terrigenous soil-marker BHPs (i.e., adenosylhopane and related compounds; Cooke et al., 2008, 2009) in any of the samples analyzed. This observation is in contrast with previous studies of lipids extracted from the water column and sediments of Mahoney Lake, which found most lipid biomarkers (i.e., fatty acids and alcohols) in the sediments originated from terrigenous sources, rather than being deposited from sources within the water column (Bovee and Pearson, 2014). That said, a terrigenous contribution to BHPs cannot be completely ruled out given the presence of anhydroBHT, which is a diagenetic product of both adenosylhopane and BHT (Schaeffer et al., 2010; Eickhoff et al., 2014). BHTs are in high abundance in Mahoney Lake, therefore it is most likely that anhydroBHT (which constitutes less than 4% of total BHPs) is a diagenetic product of BHT rather than adenosylhopane.

Changes in the abundances of BHPs downcore in Mahoney Lake may indicate that either BHP production and/or export from the water column changed over time or BHP production and/or preservation within the sediments changed. Distinguishing between these postulated possibilities cannot be fully accomplished with the current data, so remains to be determined. The main inferences are that the majority of sedimentary BHPs are conceivably produced by anaerobic bacteria below the chemocline and are preserved in the sedimentary record up to at least ca. 600 years in Mahoney Lake. Because it is likely that BHPs are also produced within sediments, due caution is warranted when reconstructing strictly water column processes using BHPs from sedimentary archives.



### 3.2. Metagenomic assessment of potential bacterial sources of BHPs

A BLAST® search of previously published metagenomic data from Mahoney Lake for known genes that encode proteins involved in BHP biosynthesis (i.e., *shc*, *hnpH*, *hpnO*, *hpnR*, and *hpnP*) was completed to determine potential BHP producers in Mahoney Lake. Specifically, *shc* is involved in the cyclization of hopanoids from the isoprenoid squalene, *hnpH* is involved in the production of bacteriohopanetetrol (BHT) and aminobacteriohopanetriol (aminotriol), *hpnO* is needed for aminotriol production alone, *hpnP* is involved in the methylation at the C-2 position, and *hpnR* is involved in methylation at the C-3 position (Seckler and Poralla, 1986; Ochs et al., 1992; Kleemann et al., 1994; Perzl et al., 1997; Wendt et al., 1997; Bradley et al., 2010; Welander et al., 2012; Welander and Summons, 2012; Schmerk et al., 2015). Because there are genes involved in BHP biosynthesis that have yet to be identified, including at least one that can also catalyze BHT production (Welander et al., 2012), and due to the inherent potential limitations in metagenomic sequencing coverage (i.e., complete biosynthetic pathways were likely not recovered), the data have been interpreted conservatively. Only potential BHP producers whose genome encode *shc* and at least one other currently known gene required for the synthesis of the observed BHPs (i.e., *hpnH*, *hpnO*, *hpnP*, and *hpnR*) are discussed and *hpnH* and *hpnP* sequences affiliated with archaea (i.e., DPANN group and Thermoplasmata) are omitted from the discussion for this reason and because archaea are not currently known to produce hopanes or BHPs.

Throughout the water column, *shc* and *hpnH* affiliated with Deltaproteobacteria were abundant, which indicates that they are the most likely producers of BHT. Potential producers of aminotriol (including the newly identified diunsaturated aminotriol) belong to the taxonomic affiliations of *shc* and *hpnO* which include Deltaproteobacteria and Planctomycetia at the chemocline, Planctomycetia and Chloroflexi in the euxinic monimolimnion, and Planctomycetia and Verrucomicrobia in the sediments. Evidently, Planctomycetia are a key producer of aminoBHPs in Mahoney Lake, though the lack of anammox genetic markers (Hamilton et al., 2016) suggests that they are produced by other members of Planctomycetia rather than those capable of anammox. Additionally, genes encoding both methanogenesis and aerobic oxidation of methane (AOM) were not found within Mahoney Lake (Hamilton et al., 2016), which precludes methanogens and methanotrophs as potential source organisms of aminoBHPs. Abundant aminoBHPs were only observed in the euxinic monimolimnion, even though bacteria throughout the water column have the genetic potential to produce aminoBHPs. This observation strongly suggests that euxinic conditions are indeed linked to the production of aminoBHPs and especially to diunsaturated aminotriol.

*shc*, *hpnP* and *hpnR* sequences were recovered, which are needed to produce 2- and 3-MeBHPs. These sequences are affiliated with

Deltaproteobacteria and Verrucomicrobia, respectively, suggesting organisms within these phyla produce 2- and 3-MeBHPs in situ (Fig. 5). These two taxa extend the potential source organisms of 2-MeBHPs which have, thus far, included cyanobacteria, Alphaproteobacteria, and Acidobacteria (e.g., Welander et al., 2010; Ricci et al., 2015). The presence of the *hpnR* gene needed for 3-MeBHP production is known in Deltaproteobacteria (e.g., Welander and Summons, 2012), but has not been explored in Verrucomicrobia. That said, the production of methylated BHPs is more likely a bacterial response to environmental conditions, rather than a marker of specific bacteria (Welander and Summons, 2012; Kulkarni et al., 2013; Ricci et al., 2014; Wu et al., 2015; Newman et al., 2016). The production of methylated BHPs as a response to environmental conditions is supported by the observation that even though at the chemocline Deltaproteobacteria and Verrucomicrobia have the genetic potential to produce 2- and 3-MeBHPs, those BHPs are not present (Figs. 3 and 5). Instead, 2- and 3-MeBHPs are only found in the sediments (Fig. 3), where their production by Deltaproteobacteria and Verrucomicrobia is likely stimulated by environmental stressors (e.g., low oxygen and high osmolarity; also discussed in Section 3.1.2.).

### 4. Conclusions

In Mahoney Lake, BHPs are structurally diverse and distributions parallel redox conditions. The euxinic monimolimnion has the greatest diversity and abundance of BHPs in the water column, including a novel BHP that we have tentatively identified as a diunsaturated aminotriol. The diunsaturated aminotriol is likely produced by anaerobic bacteria (Planctomycetia and/or Chloroflexi) and is strongly associated with euxinic water column conditions. These characteristics make it an interesting potential proxy for euxinic conditions, but further studies are required to define the relationships among the environmental distribution, degree of euxinia or anoxia, and preservation potential of the diunsaturated aminotriol, as well as to verify its bacterial source organism(s). Similarly, composite BHPs in Mahoney Lake are mostly sourced from anaerobic bacteria. Our results also provide evidence of sedimentary BHP production, which may obfuscate reconstructions of water column processes from sedimentary archives.

Corroborating previous genomic and culture-based studies, the comparison of genes involved in methylated BHP synthesis and methylated BHP distributions in the water column and sediments of Mahoney Lake showed that 2- and 3-MeBHT are most likely produced in response to low oxygen and high osmotic concentrations within the euxinic sediments. This observation enhances our understanding of 2-methylhopane (diagenetic products of 2-MeBHPs) occurrence in the geologic record.

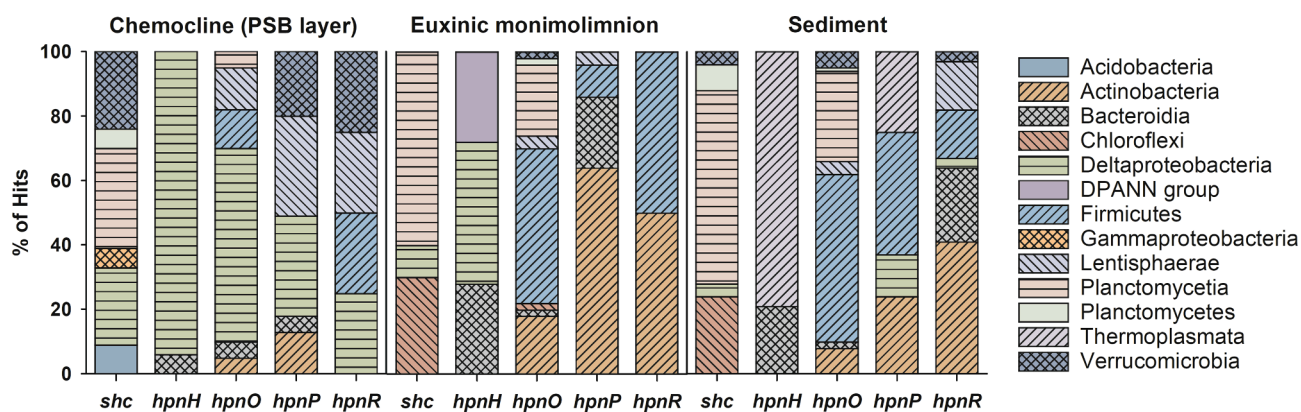


Fig. 5. BLAST® search results showing the taxonomic affiliation(s) of several genes involved in BHP biosynthesis at the chemocline/purple sulfur bacteria (PSB) layer, euxinic monimolimnion, and in a surface sediment grab sample. Genes are *shc* (squalene-hopene cyclase), *hpnH* (adenosyl hopane synthase), *hpnO* (aminobacteriohopanetriol synthase), *hpnP* (hopanoid C-2 methylase), and *hpnR* (hopanoid C-3 methylase).

## Declaration of Competing Interest

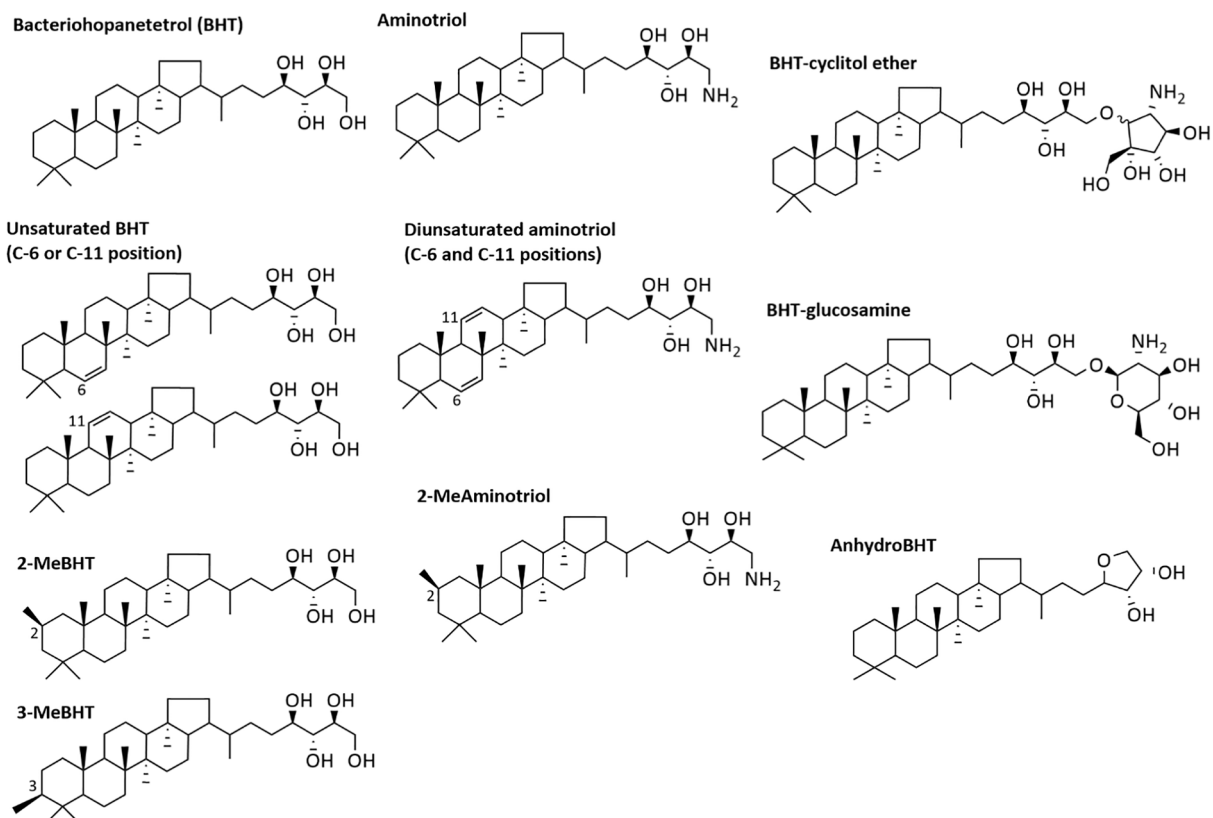
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Bacterioplanepolyol (BHP) compounds observed in the water column and sediments of Mahoney Lake. Structural configurations are based on LC-MS<sup>n</sup> analysis only. The positions of the double bonds in the diunsaturated aminotriol are tentative.



## Appendix B. Identifying information for acetylated derivatives of BHPs reported in Mahoney Lake.

Abbreviated name	Retention time (min)	Nominal mass: Base peak ( <i>m/z</i> )	Observed mass: Base peak ( <i>m/z</i> )	Major fragment ions ( <i>m/z</i> )		
AnhydroBHT	25.7	613	613.487	553.462	493.440	191.180
Unsaturated BHT	22.6	653	653.458	593.436	533.414	
BHT	25.7	655	655.495	595.473	535.451	191.180
2-MeBHT	26.1	669	669.476	609.488	205.194	
3-MeBHT	26.9	669	669.476	609.488	205.194	
Diunsaturated aminotriol (three peaks)	22.6, 23.1, 23.5	710	710.589	650.571	590.552	530.530
Aminotriol	23.5	714	714.535	654.510	594.489	534.461
2-MeAminotriol	24.0	728	728.546	668.526	608.533	
BHT-cyclitol ether	22.1	1002 + 1044	1002.617	330.117		
BHT-glucosamine	22.9	1002	1002.617	942.594	330.118	210.075

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