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BACTERIOHOPANEPOLYOLS ACROSS ENVIRONMENTAL GRADIENTS

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ABSTRACT

The goal of this research is to provide information on the function of Bacteriohopanepolyols (BHPs) in modern bacteria and to give more insight on the use of hopanes as paleoenvironmental indicators. BHPs are bacterial lipids used as biomarkers for oil correlation and as maturity indicators and in paleoenvironmental studies. We investigated BHP production in environmental samples and cultures across a range of geochemical gradients. Through this work, we developed two methods to study BHPs: (1) A method for the quantification of BHPs by (APCI)LC/MS, including determination of BHP response factors, and (2) a method for the analysis of ^{13}C content in individual BHP molecules. These methods were applied to the analysis of BHPs in environmental samples and laboratory cultures of bacteria.

In the Frasassi cave system, Italy, we find evidence for multiple BHP producers in the cave stream waters and sediments. The BHP structures present and the abundances of particular structures suggest the sediments contain sulfur-reducing, BHP-producing bacteria (Blumenberg et al., 2005). Using a ^{13}C labeling experiment we find that autotrophs within a sulfur-cycling community produce BHPs and we propose *Beggiatoa*, a filamentous, sulfur-oxidizing bacteria, as a likely BHP producer in the system. Our analyses show that the ^{13}C label is incorporated into the sugar moiety tail of the BHP structures preferentially indicating that these polar groups turn over biochemically much faster than the isoprenoid hydrocarbon.

We found BHP production in both *Acidithiobacillus thiooxidans* and *ferrooxidans*. *A. thiooxidans* produce BHPs in both cultures and snottite biofilms. In

culture *A. thiooxidans* produces more BHPs with a decrease in pH. The cell count of *A. ferroxidans*, in an acid mine drainage stream, correlates with decrease in pH and an increase in total BHPs. The increase of BHPs with a decrease in pH suggests BHPs are an adaptation to decrease proton transport across cell membranes by lowering permeability. In samples from Rio Tinto, Spain, we find that the iron-oxidizing bacteria, *Leptospirillum* is a major producer of 3Me-BHPs as a member of a biofilm, in a planktonic lifestyle, and in culture. In culture, this bacterium produces more BHPs when fixing nitrogen than when assimilating nitrate.

We find that *Anabaena variabilis*, a freshwater cyanobacterium, produces more BHPs when living as a photoautotroph and less when living as a chemoheterotroph. Notably, when *A. variabilis* is phototrophic, the bacterium produces more BHPs when fixing nitrogen than when assimilating nitrate. These results indicate that BHPs may have a role in protecting the nitrogenase enzyme and the photosystem. Lastly, we find that a seasonal red pigmented biofilm is the major BHP producer in the Little Salt Spring, Florida. Important to paleoenvironment interpretations, this system produces C2 methylated BHPs in large quantities.

These results combined suggest that BHP production structures and abundances change with a change in environment or culture geochemistry. We suggest that BHPs play unknown roles in adaptation to acid tolerance, nitrogen fixation, and photosynthesis.

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Chapter 1

Introduction

Biomarkers

Biomarkers are biomolecules preserved in sediments and are widely used to interpret paleobiology or paleoenvironmental conditions. Proteins, DNA, RNA, and lipids are all biomarkers, but lipids are the only structures sufficiently recalcitrant for early Earth studies. Also, specific lipid biomarkers are linked to physical or chemical conditions in depositional environments, which aid interpretation of paleoenvironments. For example, TEX_{86} is an abundance ratio of GDGT lipids from pelagic archeaen that correlates with the temperature of the waters in which they were grown (Schouten et al., 2007). The ratio of pristane to phytane can reflect whether an aquatic depositional environment was oxidizing or reducing (Didyk et al., 1978). These environmental indicators are useful, but they need to be used with caution, as they were developed by research that focused on a single geochemical parameter within a dataset and may not be applicable in all depositional environments (Turich et al., 2007). Therefore, using multiple biomarker compounds and geochemistry proxies will strengthen and refine paleoenvironmental interpretations.

Hopanes

Hopanes are potentially very informative biomarkers because they are recalcitrant, likely produced by specific bacteria, and possible preservers of environmental information. Hopane lipids are the geologically preserved isoprenoid

hydrocarbons of bacteriohopanepolyols (BHPs) and were first identified by geochemists Albrecht and Ourisson (1969). Hopanes may be the most common geochemical product in the world (Ourisson and Albrecht, 1992) because they are very resistive to degradation and are found in the oldest sedimentary rock records, i.e., as old as 2.7-billion-year-old rocks (Summons et al., 1999; Eigenbrode et al., 2006). Hopanes have been well developed as biomarkers for oil correlation and petroleum system analysis as well as organic maturity indicators (Farrimond et al., 2004; Jahnke et al., 1999). In paleoenvironmental studies, hopanes are limited to use as a presence-absence indicator of bacteria because we are unsure of their phylogenetic origins and function within microbial cells.

Although the functional groups of BHPs are not preserved over geologic time, the isoprenoidal ring structure is, including methylation at C2 and C3 and unsaturated bonds in the rings (Talbot et al., 2007; Zundel and Rohmer, 1985; Rohmer et al., 1984; Simonin et al., 1994). C2 methylation has been interpreted as a cyanobacterial and hence oxygenic photosynthesis biomarker (Summons et al., 1999). But, a recent study shows C2 methylation by a purple sulfur bacterium draws this interpretation into question, Table 1-1 & Table 1-2 (Rashby et al., 2007). The C3 methylation without unsaturated bonds in the rings is currently interpreted as a biomarker for aerobic methanotrophs, Table 1-3 & Table 1-4 (Rohmer et al., 1984; Jahnke et al., 1999; Zundel and Rohmer, 1985; Talbot et al., 2007; Neunlist and Rohmer, 1985; Cvejic et al., 2000). While C3 methylation with an unsaturation is attributed to acetogenic bacteria (Rohmer et al., 1984; Talbot et al., 2003a; Rohmer and Ourisson, 1986).

Hopanes full potential as paleoenvironmental biomarkers is limited by gaps in our knowledge about modern BHPs. We do not understand their distribution throughout the bacterial domain, their biochemical function, or the bacterial physiological response in BHP production to environmental signals. Do bacteria change the amount of total BHPs they produce, or the functional groups attached to the side chain under certain environmental conditions? Or are the BHPs always produced in steady state as a necessary lipid that does not respond to environmental changes? The answers to these questions are necessary in order to interpret changes in the amount of hopanes or the ratio of methylated to non-methylated hopanes found in the rock record.

Bacteriohopanepolyols

Bacteriohopanepolyols (BHPs) are a class of isoprenoid lipids made only by bacteria. BHPs are amphiphilic molecules consisting of a C30-triterpenoid pentacyclic hydrocarbon skeleton with six-carbon side chain (attached at C22) and sixty-three possible functional groups, comprised of alcohols, amino groups, sugar and fatty acids (Talbot et al., 2003, 2004, 2005, 2008). BHPs can also be methylated at either the C2 or the C3 position of the A ring adding geologically preserved structural diversity. BHPs are thought to be sterol functional surrogates in bacteria, providing membrane reinforcement and maintaining membrane fluidity and permeability (Kannenberg and Proralla, 1999). BHPs are produced by both gram positive and gram negative bacteria, but the ability to make BHPs is scattered throughout the bacterial kingdom (Pearson et al., 2008; Welander et al., 2010). Therefore, they are not necessary for bacterial life as sterols are for eukaryotes.

BHPs are interesting both because they are a modern biochemical mystery and because they are precursors to geologic bacterial biomarkers, hopanes. The first unknown is which bacteria produce BHPs. Hopanes were first identified by geochemists without any known biological precursor (Albrecht and Ourisson, 1969). Later, biologists identified the precursors, BHPs, and began to study their production in bacteria (Forster et al., 1973; Langworthy and Mayberry, 1976; Rohmer and Ourisson, 1976). Since then, BHPs have been found in a wide variety of environmental samples (Rohmer et al., 1984; 1988; Summons et al., 1999, Talbot et al., 2003a,b, c; 2007a,b; 2008), although only a limited number of cultured bacteria are known to produce them. These include methanotrophs, cyanobacteria, acetic acid bacteria, and anaerobic photosynthesizers (Summons et al., 1999; Rashby et al., 2008; Rohmer et al., 1984). To resolve the paradox of ubiquitous hopanes but only a few known sources, genetic methods have been developed for use with pure cultures and environmental samples. Through knockout methods these studies have identified the squalene hopene cyclase gene *(shc*), which encodes for the protein (SHC) that converts squalene into hopene, and the gene *hpnP*, which encodes for the protein that adds the methyl group on the second carbon of ring A. (Tippelt et al., 1998; Wendt et al., 1999; Thoma et al., 2004; Welander et al., 2010). Although genetic databases currently available are incomplete, *shc* and *hpnP* genes appear to be rare and unevenly distributed within bacterial families (Pearson et al., 2008; Welander et al., 2010).

BHPs are biochemically intriguing because of their function and location within the cell. BHPs are theorized to serve as membrane rigidifiers (Benvenist et al., 1966; Kannenberg and Poralla, 1982; Bloom and Mouritsen, 1988) based on their geometry

(Ourisson et al., 1982) and their abundance in the membranes of nitrogen-fixing bacteria and extremophiles (Berry et al., 1993; Summons et al., 1999; Joyeaux et al., 2004). BHPs are believed to protect the nitrogenase enzyme from oxygen (Berry et al., 1993), extremophiles from toxic molecules such as hydrogen sulfide, anaerobes from oxygen, and akinetes from nutrient limitation, Table 1-5 (Hartner et al., 2005; Rashby et al., 2008; Doughty et al., 2009). Previously, BHPs were thought only to be produced by aerobic bacteria, but now are known to be produced by several heterotrophic anaerobes (Hartner et al., 2005) and one phototrophic anaerobe (Rashby et al, 2008), adding more unknowns to the function of BHPs.

But BHPs are not essential for bacterial growth, as evidenced by their scattered distribution throughout the kingdom, unlike the ubiquitous eukaryotic sterols, and confirmed by knock out experiments in *Streptomyces* and *Rhodopseudomonas* species, Table 1-5 (Seipke 2009; Welander et al., 2010). Doughty et al. (2009) discovered that 2-methyl BHPs are produced by the akinete, or resting cell phase, of *Nostoc punctiforme*. They documented that the outer membrane has thirty-four more times the concentration of 2-methyl BHP than the vegetative phase. Not knowing the true function of BHPs within the cell hinders further studies to identify which and under what conditions diverse bacteria produce BHPs (Welander et al., 2010; Pearson et al., 2009).

How to Make Hopanes More Useful Biomarkers

Hopanes can become a more developed biomarker by studying the production of BHPs in modern bacteria. Less than one percent of bacteria can be grown in pure culture (Wark, 1999). Hence, the continued screening of cultured bacteria for their production of specific functional groups as well as amount of BHPs produced is important, but cannot

yield a complete survey of bacteria. Therefore, environmental samples are an essential source of information about bacterial BHP production. Charting the distribution of BHPs in modern environments is the first step in understanding the potential for hopane signatures in the rock record.

Identifying the genetic and enzymatic pathway of BHP production would greatly enhance our ability to identify the functions of BHPs. Past studies have identified the genes for cyclization and C2 methylation, but the genes for C3 methylation, side chain addition, and functional group addition are all still unknown. If the genes are identified, genetic knock out studies would be possible to help identify the function of BHPs in bacteria. To date, knock out studies of the *shc* gene have shown that the cells of *Rhodopseudomonas palustris* TIE-1 and *Streptomyces scabies* do not require BHPs to live, but their absence makes the cells grow less vigourously at lower pH (Welander et al., 2010; Seipke, 2009).

We can learn about hopanoid function by documenting changes in BHP production due to environmental gradients. BHP data for cultured bacteria grown under varying conditions and environmental samples across geochemical gradients can give insights to the biochemical response of bacteria to environmental stimuli. Understanding environmental drivers that cause changes in total BHP production, methylation, or functional groups will ultimately and strongly enhance paleoenvironmental interpretations.

Thesis Objectives

The goal of this dissertation is to determine relationships between environmental changes and BHP structures and quantities. I identifed BHP structures and quantified

their abundances across geochemical gradient environments. To do so, I first developed a method that allows us to quantify functionalized BHPs. Prior studies have relied on gas chromatography mass spectrometry (GC/MS) for quantitation, which requires removal of the functional groups of the BHPs, therefore losing valuable information. By using LC/MS methods, I characterized environmental patterns and specific influences on intact BHPs, and provide insights to their functions in geochemically significant settings.

Using this approach, I quantitatively identified the diversity of BHP structures and changes in their production across geochemical gradients in a wide suite of modern environments. I sampled across gradients in order to determine if BHP abundance and structure change with environmental stimuli. First, I analyzed BHPs in the oxygen minimum zone of the Eastern tropical North Pacific, using samples of particulates across the oxycline. Second, I studied non-photosynthetic sulfur cycling biofilms, sampling across oxygen and sulfur gradients. Third, I explored BHPs produced by the sulfur and iron oxidizing acidophilic *Acidithiobacillus* bacteria sampled across a pH gradient in both culture and environmental samples. Fourth, acidic, iron-oxidizing *Leptospirillum* biofilm, planktonic, and cultures were analyzed for BHP production. Fifth, I examined if BHP production is affected by nitrogen fixation in the cyanobacterium, *Anabaena variabilis*, growing as both a phototroph and as a heterotroph.

Key Findings and Future Publications

Intact BHPs can be quantified by LC/MS analysis, which allows us to investigate BHP production across environmental gradients. Quantification on a (APCI)LC/MS system requires steps to maintain and characterize the ionization efficiency over time, such as frequent ionization chamber and corona needle cleaning and frequent calculation

of response factors. The BHP concentration versus depth profile of the oxygen minimum zone of the Eastern Tropical North Pacific indicates that BHP production is increased at both the upper and lower oxyclines. The structures and $\delta^{13}C$ values provide strong evidence for aerobic methanotrophy BHP production.

In the Frasassi cave system, Italy, we find evidence for multiple BHP producers in the cave stream waters and sediments. Anoxic sediments have similar BHP structures and ratios of structures as sulfur reducing *Desulfovibrio* bacteria (Blumenberg et al., 2005). Using a ^{13}C labeling experiment we document that autotrophs within a sulfur-cycling community produce BHPs, and we propose *Beggiatoa* as a significant BHP producer in the system. The ^{13}C label is incorporated into the sugar moiety tail of the BHP structures preferentially, indicating that the polar functional groups are turned over much more rapidly than the isoprenoid hydrocarbon.

A sulfide oxidizing snotitte biofilm and the isolated *Acidithiobacillus thiooxidans* Mu1 strain from the snotitte are compared to iron oxidizing, *Acidithiobacillus ferrooxidans* dominated, Lower Red Eyes acid mine drainage stream sediments. All of the samples contain BHPs. In culture, *A. thiooxidans* Mu1 produces more BHPs with a decrease in pH, while in Lower Red Eyes, the total amount of BHPs is related to both the amount of *A. ferrooxidans* present in the sample and pH. These studies indicate that BHPs may be important in decreasing permeability of protons across cell membranes. In environmental samples from an acid mine drainage site (Rio Tinto, Spain), we find that the iron-oxidizing bacteria *Leptospirillum* produce BHPs both in a biofilm as well as in a planktonic habitat. The biofilm and planktonic findings were confirmed by isolation of the strain into pure culture. In culture, the bacterium produces more BHPs when fixing

nitrogen than assimilating nitrate. Surprisingly, we found *Leptospirillum* to be a major producer of 3Me-BHPs, which is the first non-methanotroph documented to do so. These findings will profoundly impact paleoenvironmental interpretations of hopane data.

Lastly, we find that *Anabaena variabilis*, a freshwater cyanobacterium, produces more BHPs with photoautotrophic rather than chemoheterotrophic metabolism. Also, when *A. variabilis* grows phototrophically, it produces more BHPs when fixing nitrogen than assimilating nitrate. These results indicate that BHPs may be used to protect the nitrogenase enzyme and the photosystem.

We hope to publish these findings in the following journals:

Chpater 2

HL Albrecht, KH Freeman, JL Macalady, and SG Wakeham. **Bacteriohopanepolyols Across the Oxygen Minimum Zone of the Eastern Tropical North Pacific.** *Organic Geochemistry* (in preparation).

Chapter 3

HL Albrecht, JL Macalady, and KH Freeman. **Bacteriohopanepolyols in a unique sulfur-oxidizing environment.** *Organic Geochemistry* (in preparation).

Data from **Chapters 4, 5,** and **6** will contribute to one or more publications in *Applied Environmental Microbiology* or *Geobiology*.

Table 1-1. 2-Methyl Hopanols in pure culture

Type of Bacteria	Name	Strain	Tetra	2Me-Tetra	Penta	2Me-Penta	Reference
Cyanobacteria	*Anacystis Montana*	CCAP 1405/3	+	+			Herrmann et al. (1996a)
Cyanobacteria	*Cyanotece*	RCB4	+	+			Jahnke et al. (2004) Talbot et al. (2003b)
Cyanobacteria	*Gloeobacter* sp.			+			Summons et al. (1999)
Cyanobacteria	*Gloeobacter violaceus*	ATCC 29082	+	+			Summons et al. (1999)
Cyanobacteria	*Synechococcus*	ATCC27144	+	+	+	+	Summons et al. (1999)
Cyanobacteria	*Synechococcus*	ATCC 29534	+	+			Summons et al. (1999)
Cyanobacteria	*Synechococcus*	ATCC 27180	+	+			Summons et al. (1999)
Cyanobacteria	*Synechococcus*	PCC 6907	+	+			Llopiz et al. (1996)
Cyanobacteria	*Calothrix anomala*			+	+		Summons et al. (1999)
Cyanobacteria	*Nostoc muscorum*	B 1452-12b	+	+	+	+	Rohmer et al. (1984) Bisseret et al. (1985)
Cyanobacteria	*Nostoc* sp.	ATCC 27985	+	+	+	+	Rohmer et al. (1984)
Cyanobacteria	*Nostoc* sp.	PCC 6720	+	+	+	+	Zhao et al. (1996)
Cyanobacteria	*Scytonema* sp.	ATCC 29171	+	+			Rohmer et al (1984)
Cyanobacteria	*Oscillatoria amphigranulata*	OSU	+	+	+	+	Jahnke et al. (2004)
Cyanobacteria	*Phormidium*	FPOS4	+	+	+	+	Jahnke et al. (2004)
Cyanobacteria	*Phormidium luridum*	UTEX 426	+	+			Summons et al. (1999) Jahnke et al. (2004)
Cyanobacteria	*Phormidium* sp.	OSS4	+	+	+	+	Summons et al. (1999) Jahnke et al. (2004)
Cyanobacteria	*Prochlorothrix hollandica*	CCAP 1490	+	+			Simonin et al. (1996)
Cyanobacteria	*Chlorogloeopsus fritschii*	ATCC 27193	+	+			Summons et al. (1999) Jahnke et al. (2004)
Methylotroph	Methylobacterium organophilum		+	+			Bisseret et al. (1985)

Table 1-2. 2-Methyl BHPs in pure culture

Type of Bacteria	*Species*	Strain	Me-BHP (m/z)	other BHPs (m/z)	Reference
Cyanobacteria	*Nostoc muscorum*	CCAP 1453	2Me-669	655, 772	Talbot et al. (2008)
Cyanobacteria	*Phormidium luridum*	UTEX 426 P27-3	2Me-669	655	Talbot et al. (2008)
Cyanobacteria	*Nostoc punctiforme*		2Me-669t, 727*	655	Doughty et al. (2009)
Cyanobacteria	*Prochlorothrix hollandica*	CCAP	2Me-869	855	Talbot et al. (2003b)
Cyanobacteria	*Cyanotece*	RCB4	2Me-869	855	Talbot et al. (2003b)
Purple Sulfur Bacteria	*Rhodopseudomonas palustris*	TIE-1	2Me-669, 728	655, 714	Rashby et al. (2007)
N2-Fixing Soil Bacteria	*Bradyrhizobium japonicum*	USDA 110	2Me-diplotetrol	714, 746	Bravo et al. (2001)
N2-Fixing Soil Bacteria	*Beijerinckia indica*	NCIMB 8712	2Me-diplotetrol*	655*, 714, diplopterol	Vilcheze et al. (1994)
N2-Fixing Soil Bacteria	*Beijerinckia mobilis*	DSM 2326	2Me-diplotetrol	714*, diplopterol*	Vilcheze et al. (1994)

* = major abundance

Table 1-3. 3-Methyl Hopanols in pure culture

Type of Bacteria	Name	Strain	Tetra	3Me-Tetra	Penta	3Me-Penta	ring unsaturations	Reference
Methanotroph	*Methylococcus capsulatus*	NCIB 11132	+	+				Zundel and Rohmer (1985)
Methanotroph	*Methylococcus capsulatus*	ATCC 33009	+	+	+	+		Jahnke et al. (1999)
Methanotroph	*Methylococcus capsulatus*	MC	+	+				Rohmer et al. (1984)
Methanotroph	*Methylococcus capsulatus*	TRMC	+	+				Rohmer et al. (1984)
Heterotroph	*Burkholderia cepacia*	Berkeley 382	+	+				Rohmer et al. (1984)
Acetogen	*Acetobacter pasteurianus*	NCIB 6249	+	+			Yes	Zundel and Rohmer (1985)
Acetogen	Acetobacter pasteurianus	NCIB 6429	+	+	+	+	Yes	Rohmer et al. (1984)
Acetogen	Acetobacter pasteurianus	NCIB 6426	+	+	+	+	Yes	Rohmer et al. (1984)
Acetogen	Acetobacter pasteurianus	NCIB 8856	+	+	+	+	Yes	Rohmer et al. (1984)
Acetogen	Acetobacter pasteurianus	NCIB 8620	+	+	+	+	Yes	Rohmer et al. (1984)
Acetogen	Acetobacter pasteurianus	NCIB 8935	+	+	+	+	Yes	Rohmer et al. (1984)
Acetogen	*Gluconacetobacter xylinum*	NCIB 4112	+	+	+	+	Yes	Rohmer et al. (1984)
Acetogen	*Gluconacetobacter xylinum*	R 2277	+	+	+	+	Yes	Rohmer et al. (1984)
Acetogen	*Acetobacter peroxydans*	NCIB 8087	+	+	+	+	Yes	Rohmer et al. (1984)
Acetogen	*Acetobacter peroxydans*	NCIB 8618	+	+	+	+	Yes	Rohmer et al. (1984)
Acetogen	Acetobacter europaeus	DSM 6160	+	+	+	+	Yes	Simonin et al. (1994)

Table 1-4. 3-Methyl BHPs in pure culture

Type of Bacteria	Name	Strain	3Me-BHP	Other BHPs	ring unsaturations	Reference
Acetogen	*Gluconacetobacter xylinum*		3Me-669, 1072, 1074, 1016	655, 653, 651, 667, 665, 1060, 1058, 1056, 1070, 1002	Yes	Rohmer and Ourisson (1986), Talbot et a. (2007)
Methanotroph	*Methylococcus capsulatus*	ATCC 29191	3Me-786, 844	772, 830		Talbot et al. (2003a)
Methanotroph	*Methylococcus capsulatus*	NCIB 11132	3Me-786t, 844t	830*, 772t		Neunlist and Rohmer, (1985)
Methanotroph	*Methylocaldum szegediense*	OR2	3Me-844*, 842t, 786t	830, 828t	Yes	Cvejic et al. (2000)
Methanotroph	*Methylocaldum tepidum*	LK6	3Me-844, 786t	830*, 772		Cvejic et al. (2000)

* = major abundance, t = trace

Table 1-5. BHP response to culturing conditions and knockout studies.

Species	Paramater tested	BHP response	Reference
Alicyclobacillus acidocaldarius	temperature	BHPs increase with an increase in temperature	Poralla and Kannenberg (1980)
Alicyclobacillus acidocaldarius	pH	BHP increase with decrease in pH	Poralla and Kannenberg (1980)
Frankia mycelia	N status	BHPs increase with N2 fixation	Berry et al. (1993)
Frankia mycelia	N status	no change in BHP content with N2 fixation	Nalin et al. (2000)
Nostoc punctiforme	N status	no change in BHP content with N2 fixation	Doughty et al. (2009)
Nostoc punctiforme	akinete formation	BHP increase with akinete formation	Doughty et al. (2009)
Rhodopseudomonas palustris TIE-1	pH	no change in total BHP content, 2Me-BHP increase at low & high pH	Welander et al. (2009)
Rhodopseudomonas palustris TIE-1	photo vs. chemo heterotroph	Increased BHPs with photoheterotroph	Welander et al. (2009)
Frateuria aurantia DSMZ 6220	temperature	BHPs increase with an increase in temperature	Joyeux et al. (2004)
Zymomonas mobilis	temperature	BHPs increase with an increase in temperature	Schmidt et al. (1986)
Zymomonas mobilis	tolerance of ethanol	Increase in BHP content with increase of ethanol	Schmidt et al. (1986) Bringer et al. (1985)
Zymomonas mobilis	tolerance of ethanol	no change in BHP content	Hermans et al. (1991)
Rhodopseudomonas palustris TIE-1		Total BHPs and %2Me-BHPs increase in anaerobic chamber	Rashby et al. (2007)
***shc* Inhibition & Knockout Studies**	**Strain**	**Growth Reponse**	**Reference**
Zymomonas mobilis	ATCC 29191	sensitive to ethanol when BHP inhibited	Sahm et al. (1993)
Rhodopseudomonas palustris	TIE-1	growth as photo-, chemoheterotrophy & photoautotrophy; severe growth defect with acidic and alkaline conditions	Welander et al. (2009)
Streptomyces scabies	87-22	normal growth with tolerance of ethanol, osmotic and oxidative stress, high temperature, and low pH	Seipke and Loria (2009)
Isotope Fractionation Studies	**Strain**	**Fractionation Response**	**Reference**
Methylococcus capsulatus	ATCC 33009	increase temperature = increase fractionation	Jahnke et al. (1999)
Methylococcus capsulatus	ATCC 33009	growth with pMMO = higher fractionation	Jahnke et al. (1999)
Methylococcus capsulatus	ATCC 33009	growth with sMMO = lower fractionation	Jahnke et al. (1999)

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Chapter 2

Bacteriohopanepolyols Across the Oxygen Minimum Zone of the Eastern Tropical North Pacific

Introduction

Bacteriohopanepolyols are functionalized versions of hopanes, pentacyclic triterpenoids that are well-preserved over geologic time (Rhomer et al., 1984). Biologists have documented BHPs in modern bacteria, Figure 2-1 (Forster et al., 1973; Langworthy and Mayberry, 1976; Rohmer and Ourisson, 1976), but the full diversity of species that produce BHPs remains unclear as does, importantly, their cellular functions.

The Eastern tropical North Pacific (ETNP) region is characterized by a pronounced suboxic or oxygen minimum zone (OMZ). In this manuscript suboxic refers to $< 2 – 10$ μM O_2. Low oxygen realms play an important role in ocean C and N cycling (Helly and Levin, 2004). Quantifying their influence on carbon cycling in the ocean is critical for understanding the ocean's role in global element cycling, including sequestration of anthropogenic CO_2 (Fiedler and Lavin, 2006, Paulmier and Ruiz-Pino, 2009). Physical vs. biological explanations for oxygen minima in the ocean have been debated (Richards, 1957), but a consensus has emerged that the lack of oxygen results from oxygen consumption during degradation of sinking particulate organic matter produced in relatively productive surface waters and the absence of advection/diffusion to replenish oxygen (Brandhorst, 1959; Wyrtki, 1962; Kester, 1975; Riley, 1941). Weak

regional winds and subtropical gyre circulation that do not penetrate the ETNP lead to long residence times that enhance O_2 depletion, producing a remarkably large and strong OMZ (Fiedler and Talley, 2006).

Globally, the major oceanic regions that exhibit permanent suboxic include the eastern Pacific, Indian, and western Atlantic Oceans. The OMZ in the eastern Pacific Ocean, North and South, accounts for ~33% of the total OMZ area (7.4×10^5 km^2 using dissolved O_2 <5μM) in the World Ocean (Helly and Levin, 2004). Low oxygen conditions lead to enhanced chemoautotophy (Ward et al., 1989; Taylor et al., 2001) and greater use of NO_3^- as an electron acceptor.

There is a close relationship between climate change and OMZs (Hendy and Pederson, 2006). Global warming may lead to lowered O_2 of the World Ocean (Keeling and Garcia, 2002), expansion of OMZs (Helly and Levin, 2004), and a shift in the dynamic global balance between carbon and oxygen (respiration that releases CO_2 vs. burial that leads to accumulation of O_2) (Berner and Canfield, 1989). If, as suggested by Redfield et al. (1963), the margin of safety against the global ocean going anoxic is small, and indeed, expansion of anoxic regimes has occurred in the past (for example the Cretaceous Oceanic Anoxic Events [OAEs]; Jenkyns, 1980) then understanding the biogeochemical processes cycling carbon, oxygen and other elements within oceanic OMZs is critical to predicting future ocean anoxia.

To strengthen our understanding of the sources and cycling of organic carbon in suboxic marine environments, we investigated microbial agents and their biogeochemical processes using diagnostic molecular signatures of BHPs in the OMZ of the ETNP. Sansone et al. (2001) suggested that methane derived from coastal anoxic bottom waters

is advected into the OMZ region, where it has a long residence time but is ultimately oxidized in midwaters. We hypothesize that microbial agents are important in this oxidation process, and tested for the presence of diagnostic lipid biomarkers of methane oxidizing bacteria, aminotriol, aminotetrol, and aminopentol and 3-methyl-BHPs with the same polar tails (Talbot et al., 2001). Ultimately, the linking of process, ecology and lipid signatures will provide new insights to hopanes and their interpretation as indicators of water column microbial processes.

Originally, BHPs were identified from pure cultures of bacteria by lipid extraction and analysis of the de-functionalized hydrocarbon ring structures using gas chromatography-mass spectrometry, GC/MS (Ourisson et al., 1987; Innes et al., 1997). More recently, liquid chromatography-mass spectrometry (LC/MS) has been applied to the study of BHPs with their functional groups intact (Barrow and Chuck, 1990; Talbot et al., 2001). This approach has enriched our understanding of BHP production through the characterization of diverse functional groups produced by different taxa and within different environments (Talbot et al., 2001; 2003; 2008; 2009).

BHPs are found in a wide variety of environmental samples (Rohmer et al., 1984; 1988; Summons et al., 1999, Talbot et al., 2003a,b,c; 2007a,b; 2008), although only a limited number of cultured bacteria are known to produce them. These include methanotrophs, cyanobacteria, acetic acid bacteria, and anaerobic photoautotrophs (Summons et al., 1999; Rashby et al., 2008; Rohmer et al., 1984). To address the paradox of ubiquitous hopanes but only a few known BHP producers, genetic methods have been developed for use with pure cultures and environmental samples. These studies have identified the squalene hopene cyclase gene *(shc*), which encodes for the protein (SHC)

that converts squalene into hopene, and the gene *hpnP*, which encodes for the protein that adds the methyl group on the second carbon of ring A (Wendt et al., 1997; Thoma et al., 2004; Welander et al., 2010). However, pure cultures are necessary to confirm bacterial sources for specific BHP structures. Based on the incomplete genetic databases currently available, *shc* and *hpnP* genes are rare and unevenly distributed within bacterial families (Pearson et al., 2007; Welander et al., 2010). *shc* gene studies can identify which species or clade is capable of BHP production but cannot provide molecular structure information. Current studies have not assessed the *shc* gene activity under different environmental conditions.

BHPs are hypothesized to serve as membrane rigidifiers (Benvenist et al., 1966; Kannenberg and Poralla, 1982; Bloom and Mouritsen, 1988;) based on their geometry (Ourisson et al., 1982) and their abundance in the membranes of nitrogen-fixing bacteria and extremophiles (Berry et al., 1993; Summons et al., 1999; Joyeaux et al., 2004). BHPs are believed to protect the nitrogenase enzyme from oxygen (Berry et al., 1993), anaerobes from oxygen (Hartner et al., 2005; Rashby et al., 2008), and akinetes from nutrient limitation (Doughty et al., 2009). Previously, BHPs were thought to be produced only by aerobic bacteria, but are now known to be produced also by several heterotrophic anaerobes (Hartner et al., 2005) and one phototrophic anaerobe (Rashby et al, 2008). But BHPs are not essential for bacterial growth, as evidenced by their scattered distribution throughout the kingdom, unlike the ubiquitous eukaryotic sterols, and confirmed by knock out experiments in *Streptomyces* and *Rhodopseudomonas* species (Seipke 2009; Welander et al., 2010). Doughty et al. (2009) propose that 2-methyl BHPs are produced by the akinete, or resting cell phase, of *Nostoc punctiforme*. They documented that the

outer membrane has thirty-four more times the concentration of 2-methyl BHP than the vegetative phase does. Not knowing the function(s) of BHPs within the cell hinders further studies to identify which, and under what conditions, diverse bacteria produce BHPs (Welander et al., 2010; Pearson et al., 2009).

Quantification of intact BHPs with atmospheric pressure chemical ionization liquid chromatography mass spectrometry (APCI-LC/$MS^{n)}$ facilitates our ability to characterize BHP distributions in modern environments and bacteria, and ultimately serves to strengthen interpretation of the geochemical record. APCI-LC/MS enables analysis of large, non-volatile lipids, which are not separable by gas chromatography (GC). In the 1970s, analytical chemists developed this "soft" ionization method in order to characterize the structures of large molecules without fragmentation (Horning et al., 1973; Carroll et al., 1974; Horning et al., 1974). Over the past decade, HPLC/MS^n has been used to identify intact BHPs (Talbot et al., 2003; 2007; 2008; Rashby et al., 2008). Gas chromatography mass spectrometry (GC/MS) and high temperature gas chromatography mass spectrometry (HT-GC/MS) are useful for quantifying de-functionalized BHPs and geologic hopanes (Innes et al., 1997; Welander et al., 2009; 2010). However, de-functionalized BHPs have lost structurally specific information. Although this information is also lost in the sedimentary record due to diagenesis, nevertheless linking biological structures with specific bacteria can provide insight to the functions of BHPs in cells. Structural detail about BHPs provide important evidence for tying their potential metabolic function with the environmental conditions that favor production of certain structures, important for paleoenvironmental reconstruction (Summons et al., 1999; Rashby et al., 2008; Doughty et al., 2009). Here we present the

development of BHP quantitification method. With this method we present evidence for BHP abundance peaks at the redox boundaries of the oxygen minimum zone. BHP structures indicate the occurrence of methanotrophic bacteria in the ETNP with methanotrophic BHP structures and ^{13}C isotopically depleted values. Also, the OMZ shows the co-occurrence of 2-methyl and 3-methyl BHP structures in the chlorophyll maximum indicating the presence of both cyanobacterial and methanotrophic bacteria.

Methods

Sample Collection: ETNP samples were collected in October 2007 on the *R/V Seward Johnson*, by Woods Hole *In-Situ* pumps mounted on hydrographic wire. These large filtering systems pumped an average of 4000 liters of seawater through ashed glass-fiber filters (293 mm, type A/E, nominal pore size 1 μm). Following recovery, the filters were frozen and returned to a shore-based laboratory for lipid extraction.

Three grams of sediment were collected from Bear Meadows Bog, State College, PA a local environment with abundant BHPs. All samples were freeze-dried and extracted using a microscale version of the protocol prescribed by Talbot et al. (2003a). Dichloromethane (DCM) was substituted for chloroform in this procedure. Samples were acetylated with acetic anhydride and pyridine at 60 C for 1 hr. BHPs were characterized using an Agilent 6310 high-pressure liquid chromatography/mass spectrometry (HPLC/MS^n) ion trap with an atmospheric pressure chemical ionization (APCI) chamber.

Lipid Extractions: Lipids were extracted from frozen filters using 2:1 DCM: Hexane by Soxhlet for 12 h. Total lipid extracts were subdivided and a fraction was acetylated (1:1 pyridine: acetic anhydride, heated 1 hr 60°C).

Chromatographic and detection conditions: Reverse-phase HPLC was performed using a Phenomenex Gemini 5 μm C_{18} column (150 mm x 3.0 mm i.d.) and a 5 μm pre-column with the same solid phase. Mobile phase flowed at 0.5 ml/min and was composed of (A) water, (B) methanol, and (C) isopropyl alcohol (0 min A: B: C 10: 90: 0, with a ramp to 25 min 1: 59: 40 and an isocratic flow to 45 min). LC/MS settings follow Talbot et al., 2003: positive APCI mode, mass scanning from 150-1300 m/z, and "Smart" fragmentation setting (i.e., 8000 nA corona voltage, 60 psi nebulizer pressure, dry gas of five l/min, dry temperature of 350 C, vaporizer temperature 490 C). The run was divided into three segments, with target masses of 285 (0-10 min), 1002 (10-17 min) and 655 (17-50 min), all with normal optimization. Auto MS^n settings for two precursor ions were an absolute threshold of 100,000 and a relative threshold of 5%. The most abundant ion is excluded after two measurements and released after 0.5 min. The acquisition parameter had a fragment amplitude 1.0 V, with an average of 5.0 and an isolation width of 3.0 m/z. Auto MS (n>2) settings with one precursor ion were as follows: absolute threshold of 1,000, relative threshold 5%, and fragment amplitude 1.0V.

Standards: Individual BHP compounds from samples collected at Bear Meadows Bog, Rothrock State Forest, PA were fraction-collected following reverse phase separation. Each collected fraction was additionally purified via neutral phase separation on a silica column (150 mm x 3.0 mm, Waters) and an isocratic flow of 80:30 hexanes:isopropyl alcohol (IPA) at 1 ml/min. The fractions were then carefully

transferred, dried and weighed using tin EA cups on a nanogram scale. Compounds collected for use as standards include: BHT (m/z 655), Aminotriol (m/z 714), and BHTriol cyclitol ether (m/z 1002).

Calibration curves and limits of detection: Response factors were determined for the above standards across three orders of magnitude of detection by injection of 1 ng, 10 ng, 20 ng, 50 ng, 100 ng, and 200 ng of each standard compound (Figures 2-2, Table 2-1). Peak areas were normalized to the peak area of the internal standard, pregnanediol (Sigma Aldrich). Normalization to an internal standard accounts for instrument sensitivity changes between injections as well as minor volume inaccuracies introduced during pipeting and injection. Normalized data were used to calculate a linear regression between peak areas of each compound of interest (divided by peak area of pregnanediol) and known mass injected. The equation of this line was used to calculate the amount of compound in environmental samples (Figure 2-2, Table 2-1). Signal-to-noise (S/N) ratios averaged 20 for analyse of pregnanediol for mass ranging from 50 to 200 ng.

Hopanol Derivatization: Total lipid extracts were derivatized to make them GC amenable following Innes et al., 1998. First, the acetyl groups were reductively removed by lithium aluminum hydride, $LiAlH_4$: 10 mg of $LiAlH_4$ in anhydrous tetrahydrofuran, THF, were added to the acetylated BHP and shaken for one hour. Second, the polar BHP was reacted with 30 mg of periodic acid in 1 ml of THF/water 8:1, shaken for one hour, and quenched with water. This reaction cleaves carbon-carbon bonds in vicinal diols to yield aldehydes. Third, the aldehydes were reduced to alcohols with sodium borohydride, $NaBH_4$: 10 mg of $NaBH_4$ in 1 ml of ethanol was added, shaken for 1 hr and quenched with 1 ml KH_2PO_4 (100 mM). Finally, terminal alcohols were acetylated with 100 μl

each of acetic anhydride and pyridine and heated to 60°C for 1 hr. Reaction products were analyzed by GC/MS (Hewlett Packard 5990) for identification.

GC/MS: Hopanols were analyzed on a gas chromatograph-mass spectrometer (HP GC 6890, HP quadrapole 5973) to identify methyl positions based on published mass spectra and chromatograms (Figure 2-4). Hopanols were diluted in 8:2 hexane:DCM and injected on a split/splitless injector and a fused silica capillary column (Agilent J&W DB-5, 60m, 0.32mm, 25μm). They were then heated from 60ºC to 320ºC at 6ºC per minute.

GC/FID: Hopanols were quantified using a gas chromatograph/flame ion detector (HP 5890) to confirm proportions of molecules quantified on the LC/MS. Also, co-elution of the methyl compounds on the LCMS made GC/FID quantification of these molecules necessary. Hopanols were diluted in 8:2 hexane:DCM and injected on a split/splitless injector and a fused silica capillary column (Agilent J&W DB-5, 60m, 0.32mm, 25μm) heated from 60ºC to 320ºC at 6ºC per minute. Quantification is based on peak areas normalized to a derivitized (acetylated) internal standard, cholesterol. Response factors calculated from an acetylated cholesterol dilution series (1 ng/μl to 111 ng/μl).

Hopanol Carbon Isotope Measurement: Hopanol $\delta^{13}C$ values were determined by GC-IRMS. Compounds were separated with a Varian 3400 GC with a split/splitless injector and a fused silica capillary column (Agilent J&W DB-5; 30 m, 0.32 mm, 25 μm). After chromatographic separation, compounds are combusted with nickel and platinum with O_2 in He (1%, v/v) at 1000˚C. A Finnegan Mat 252 monitored the stable isotope ratios and measurements were made relative to a reference gas calibrated with Mix A (n-

C16 to n-C30 alkanes, Arndt Shimmelmann, Indiana University). Sample (SA) isotope values are reported in delta notation relative to VPDB as $\delta^{13}C = [(^{13}R_{SA}/^{13}R_{VPDB}) - 1]$ where $^{13}R = {}^{13}C/{}^{12}C$. Internal standards of squalane and androstane were co-injected. Sample accuracy is ±0.4 ‰ calculated as the average difference between measured and the known value for each of the fifteen standards in Mix A averaged over nine injections. (N=9). Sample precision is ± 0.3 ‰ (1σ), calculated from nine injections of Mix A, a mix of fifteen standards, treated as samples. The standard deviation was calculated for each of the fifteen standards and then the average standard deviation was calculated (eqn. 1). (N=9, N=number of measurements, x = measured value, $\bar{x}$ = actual value).

$$\sigma = \sqrt{(1/N-1)\sum(x-\bar{x})^2} \quad \text{(eqn. 1)}$$

The $\delta^{13}C$ of the added carbon in the acetyl groups was corrected for by using mass balance to determine the acetyl $\delta^{13}C$ with pregnanediol. The $\delta^{13}C$ of pregnanediol was measured with and without acetylation using the nanoEA-IRMS (Polisar et al., 2009) in order to calculate acetyl ^{13}C abundance from the measured values:

$$n_{preg+acet.}F_{preg+acet} = n_{preg}F_{preg} + n_{acetyl}F_{acetyl} \quad \text{(eqn. 2)}$$

n is the number of carbons in the compound. F is the fractional abundance of ^{13}C in the compound ($F = {}^{13}C/({}^{12}C + {}^{13}C)$. Then we applied equation two to calculate ^{13}C of the BHP without the acetyl groups.

$$n_{PL+acet.}F_{PL+acet} = n_{PL}F_{PL} + n_{acetyl}F_{acetyl} \quad \text{(eqn. 3)}$$

Fractional abundance values were converted to delta notation to be comparable to literature values using equations 4 & 5 where $R_{VPDB} = 0.0112372$.

$$R = F/(1+F) \text{ (eqn. 4)}$$

$$\delta = (R_{sample}/R_{VPDB}) -1)*1000 \text{ (eqn. 5)}$$

Errors were assumed to be independent and Gaussian in distribution, and propagated using basic equations for the handling of random errors. (σ = standard deviation of the measured value, PL = polar lipid, acet = acetyl) (Freeman and Pagani, 2005).

$$\sigma_{PL}^2 = \sigma_{PL+acet}^2 + \sigma_{acet}^2 \text{ (eqn. 6)}$$

Thus combining uncertainties from analysis of derivitized hopane with that of acetyl carbon yields an uncertainty of 1.1 ‰ for hopane structures.

Results

BHP Quantitation by HPLC/MS: Calculated response factors differed from previously reported values and range 2.5 orders of magnitude (Table 2-1) (Talbot et al., 2001; Blumenberg et al., 2005). HPLC/MS response was compared with GC/FID quantitation of hopanols, which confirmed a two order of magnitude response difference between hydroxyl functionalized compounds and amine functionalized compounds (Figure 2-3). Response factors also varied slightly from run to run, although the ratios between standards remained constant. Response factors decreased by 20% over 68 hours of analysis time. The signal-to-noise ratio determined by analysis of the standard pregnanediol, injected ten times in repetition, averaged twenty above 20 ng but it drops to five below 20 ng.

Bacteriohopanepolyols in the Equatorial tropical North Pacific: BHPs were present at all sites and at all depths of the water column. Concentrations were greatest at the chlorophyll maximum, and at both the upper and lower oxyclines. We observed the

highest concentration of BHPs at the lower oxycline (53 ng/l water) of Station 1 and the lower oxycline (81 ng/l water) of Station 8. BHP concentrations maximized (17 ng/l water) at the upper oxycline of Station 2 (Figures 2-4, 2-5, & 2-6). Among the he chlorophyll maximum samples, BHPs were twelve times more abundant at Station 1 (12 ng/l water) relative to those at either Stations 2 or 8 (0.6 ng/l water and 0.1 ng/l water). Station 1 has a 400 m suboxic zone (300 – 720 m) but with an interesting bump of oxygen from 80 – 300 m after a very small suboxic depth at 80 m. This oxygen may provide energy for the increase is BHPs.

BHP functional group occurrence and distribution varied through the water column in a similar pattern for all three stations with cyanobacterial specific and non-specific structures above the suboxic zone and in the upper redox zone and methanotrophic specific structures within the suboxic zone (pie charts within Figures 2-4, 2-5, & 2-6). We found the highest diversity of structures in samples from chlorphyll maxima, although concentrations were lower than at either the upper or lower oxycline. Generally, upper oxycline samples contained a greater diversity of structures compared to the lower oxycline samples. Within the suboxic zone, we found relatively low diversity of structures and low average concentration of BHPs.

Differences in functional groups observed were consistent with bacterial families known to inhabit the water column. For example, BHP structures BHpentol (m/z 713), BHTpentose (m/z 943), 2-methyl-BHTpentose (m/z 957) found at the chlorophyll maximum reflect cyanobacterial production (Talbot et al., 2009). Aminotetrol, aminopentol, 3-methyl-aminotetrol (m/z 772), and 3-methyl-aminopentol (m/z 869) were

present at the upper and lower oxycline and throughout the OMZ. These structures are representative of aerobic methanotrophs (Rhomer et al., 1984; Talbot et al., 2001).

$\delta^{13}C$ values of hopanols were measured at station one (Figure 2-4, Table 2-3). Within the oxygen minimum zone (400 & 600 m), hopanols that are hexa- and penta-functionalized were depleted in ^{13}C, with $\delta^{13}C$ values of -45‰ and -49‰, respectively. At the chlorophyll maximum (35 m) $\delta^{13}C$ values for penta-, hexa-, and 3-methyl-hexa-functionalized hopanols were -65‰ -73‰, and -70‰, respectively.

Discussion

BHP Quantitation by HPLC/MS: For analyses by APCI, we found quantification was unreliable below 50 ng ($S/N \leq 5$). The S/N ratio above 50 ng (20) is still low compared to a typical GC/MS signal-to-noise ratio of 160, reflecting poor ionization efficiency of the mostly non-polar BHPs and changing conditions within the APCI chamber over the course of a sample run. BHP polar groups have extremely negative pKas (estimated -3.0 to -3.8) that indicate they are extremely difficult to ionize. Also, the mobile phase gradient used in separation decreases the amount of H^+ ions available for ionization. Notably, the less polar compounds and those that elute earlier are more easily ionized. Signal can also be degraded by co-eluting compounds in the sample other than the compounds of interest (matrix). Lastly, over time, un-ionized material builds up on the corona needle and in the ion chamber. Combined, these factors can cause the ionization efficiency to both decrease and vary over time; variability is detrimental to quantitation, as ionization efficiency is not a constant. Internal standards therefore play an

important role in accounting for changing instrument performance drift over time (Figure 2-2, Table 2-1).

In order to apply response factors to natural samples, several assumptions are necessary. First, we assume response factors are similar for related BHP structures. There are 63 identified BHP functional groups, but we used only four compounds to determine response factors reported in this study. Therefore, we applied response factors for BHT to structures with hydroxyl functional groups in the polar tail, the response factor for aminotriol to all compounds with amine groups and no ring structures, and the response factor of BHTriol cyclitol ether for compounds with cyclic structures containing amine groups. We also assume that variability in instrument performance over the course of several runs is accounted for by normalizing response factors relative to an internal standard. However, BHPs, which elute later during the chromatographic mobile phase gradient, experience reduced exposure to H^+ ions, and likely have lower ionization efficiency than estimated by our methods.

There are many fewer scans per second in MS^3 analysis, and therefore the peak shape is attenuated. Thus, we used MS^3 analysis to confirm identification and then three subsequent MS^1 analyses to quantify peak areas. To improve the accuracy of peak area calculations, we determined if a statistical difference in peak area exists between a MS^1 and MS^3 peak. If peak size and shape are sufficiently robust, all samples could be run on MS^3, which would allow a single analysis for both identification and quantification. In some samples, co-eluting compounds from the matrix were large enough to diminish the signal of the target BHP or make quantitation unrealiable. We quantified the peak if its area contained less than ten percent co-eluting masses in MS^1 mass spectra.

Additional external factors may impact the ionization efficiency (and therefore response factors), including ambient temperature, fluctuations greater than 1°C, electrical surges, and maintenance. Electrical surges, turning off the mass spectrometer for maintenance, or a power outage will drastically affect calibration and ionization. Recalibration can change the response factors considerably. In some samples, co-eluting compounds from the matrix were large enough to diminish the signal of the target BHP.

The best practices for this method are to run an electrically well-insulated instrument in a temperature-controlled room, create a three-point calibration with standards at the beginning and end of each sample set, and clean the corona needle and ionization chamber frequently (every eight samples). BHP structures without calculated response factors have response factors selected based on similar size and chemical structure to the standard BHPs.

Bacteriohopanepolyols in the Equatorial tropical North Pacific. Previous studies have documented BHPs within suspended organic particles in surface waters (Aloson-Saez et al., 2007; Pearson et al., 2008), across the chemocline of the Black Sea (Blumenberg et al., 2007; Wakeham et al., 2007), within hydrothermal vent sediments (Scouten et al., 2003; Blumenburg et al., 2005), in anoxic Black Sea sediments (Thiel et al., 2003 and references therein). Also, hopanols, BHPs with the functional groups removed by periodic acid reduction, have been analyzed in oceanic (Arning et al., 2008). This is the first study of BHPs in suspended particles sampled through the water column of an OMZ at an open ocean site. Lipid profiles indicate bacterial BHP concentrations were highest at both the upper and lower oxyclines of the OMZ, and at the chlorophyll maximum depths (Figures 2-4, 2-5, & 2-6). As observed in the Black Sea, bacterial BHP

abundance is greatest across ocean redox gradients (Blumenberg et al., 2007; Wakeham et al., 2007).

If the BHPs of the water column were intact, the sediments BHP core hopanes would average a ratio of 100 : 0.3 : 1.8 for hopane: 2Me-hopane: 3Me-hopane. C2 and C3 methylated hopanes are often found together in sediments and oils (Eignebrode et al., 2008; Brocks et al., 2003; Farrimond et al., 2004). The ratio of C3 to C2 methylated hopanes is used is a depositional environment indicator with a high ratio (> 1) considered indicative of lacustrine environments (Farrimond et al., 2004). The high C3:C2 ratio found here (6) is unusual for a marine setting and may add new insight into environments with mixed indicators of depositional environment. If there is a high C3:C2 ratio and a depleted carbon isotope ratio but no lacustrine biomarkers our data suggest an interpretation of marine oxygen minimum zone.

Differences in functional groups of BHPs can be indicative of bacterial families although specificity varies for different compounds. Bacteriohopanetetrol (BHT, m/z 655) is the most common BHP and Talbot et al. (2003c) suggest it is not diagnostic for specific bacterial taxa. Aminotriol (m/z 714) is produced by cyanobacteria, purple non-sulfur bacteria, all Type II methanotrophs tested thus far, and some Type I methanotrophs (Neunlist et al., 1985, 1988; Talbot et al., 2007a,b; Talbot et al., 2001 and references therein). BHP structures BHpentol (m/z 713), BHTpentose (m/z 943), 2Me-BHTpentose (m/z 957) were all present at the chlorophyll maximum. These structures are considered specific to cyanobacterial production (Talbot et al., 2009). Aminotetrol (m/z 772) is produced by all methanotrophs analyzed thus far and is present in ammonia-oxidizing bacteria *Nitrosomonas europaea* (Talbot et al., 2007a), purple non-sulfur bacteria

Rhodomicrobium Vannielii (Neunlist et al., 1985), *Rhodopseudomonas palustris* (Talbot et al., 2007a). Aminopentol (m/z 830), so far, has only been detected in Type I methanotrophs. Hopanol studies have indicated that a high proportion of the hexafunctionalised BHP, aminopentol (m/z 830), is typical of Type I methanotrophs (Talbot et al., 2001; Summons and Jahnke, 1992). Contrastingly, Type II methanotrophs contain tetra- and pentafunctinalized BHPs, aminotriol (m/z 714) and aminotetrol (m/a 772) (Neunlist and Rohmer, 1985a,b; Talbot et al., 2001).

In our study, BHPs typical of Type I methanotrophs were present at the upper oxyclines including aminopentol (m/z 830) and 3-methyl-aminopentol (m/z 844). 3-methyl-aminopentol (m/z 844) is present in small amounts are the upper oxycline at all stations and is most abundant at 300 m depth at Station 1. BHPs considered characteristic of Type II methanotrophs are present within the OMZ, including aminotriol (m/z 714), aminotetrol (m/z 772), and 3-methyl-aminotretrol (m/z 786). These structures are all most abundant within the OMZ and at the lower redox zones. As in other studies (Talbot et al., 2008, and references therein), BHTetrol (m/z 655) is ubiquitous, being present at all sites and all depths.

Wakeham et al. (2007) cited the presence of aminotriol (m/z 714), aminotetrol (m/z 772), and aminopentol (m/z 830) as evidence for aerobic methanotrophs in the suboxic portion of the water column of the Black Sea. Blumenberg et al. (2007), studying the northern Black Sea shallow shelf, regarded aminotriol, aminotetrol, and 3Me-aminopentol as evidence for Type I methanotrophs. Type I methanotrophs tend to outcompete Type II methanotrophs at low concentrations of methane and high concentrations of oxygen (Jahnke et al., 19999). Evidence for Type I methanotrophs in

other marine water columns and the relatively low methane and high oxygen concentrations of the ETNP are consistent with our finding BHPs characteristic of Type I methanotrophs. The abundances of compounds indicative for Type I methanotrophs differs at the three stations within the ETNP and this suggests the aerobic methanotrophy community may differ within both the individual water columns and between stations.

There are BHPs methylated at position C2 and at C3 present in the upper oxic zone. C2-methylated BHPs are found in a purple nonsulfur phototroph (Rashby et al., 2007) but these structures are most well known as cyanobacterial biomarkers (Summons et al., 1999), even though no marine cyanobacteria have been found to produce C2-methyl structures (Talbot et al., 2008; Welander et al., 2010). 2Me-BHT has been found in *Bradyrhizobium japonicum* (Talbot et al., 2007a,b) and more recently, in *Leptospirillum ferrooxidans* L3.2 (Albrecht, unpublished, Chapter 5). C2 methyl-hopanols (without the functional group identified) were also found in *Methylobacterium organophilum* (Renoux and Rohmer, 1985), *Methylobacterium* spp. (Bisseret et al., 1985; Knani et al., 1994), and *Bradyrhizobium* spp. (Bravo et al., 2001). C3 methyl-BHPs have thus far been identified in aerobic methanotrophs and some acetic acid bacteria (Rhomer et al., 1984; Talbot et al., 2001; Talbot et al., 2003) and in *L. ferrooxidans* (this work). Therefore the presence of 3-methyl-aminotetrol (m/z 786) and 3-methyl-aminopentol (m/z 844) provides supporting but not definitive evidence for the presence of aerobic methanotrophs at our study sites.

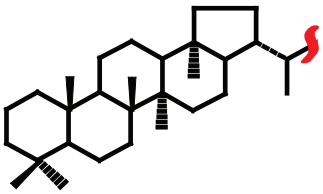
We use $\delta^{13}C$ measurements of hopanols to help confirm that BHP structures were derived from methanotrophic organisms. Because the same hopanol ringed structures are in tetra-, penta-, and hexafunctionalised BHPs, the isotope signal will represent a mixture

from all of these lipids. Methylated structures can represent a smaller set of polar sources, and the 3-methyl forms are of particular interst, given their traditional association with methylotrophic organisms. We found $\delta^{13}C$ = -70 ‰ for 3Me-hexafunctionalised hopanol at 35 m of Station 1, and consider this very low value strongly indicative of an organism using isotopically depleted methane as its main carbon source (Freeman et al., 1990; Talbot et al., 2003c). Although studies are limited, aerobic methanotrophy has been shown to fractionation methane to produce biomass that is depleted in 13C to different degrees by growth phase, ranging from 16 ‰ in stationary phase to 30 ‰ in exponential phase (Summons et al., 1994). Further, hopanols can be up to 10 ‰ depleted relative to biomass (Hayes, 2001). Sansone et al. (2001) determined that there is a flux of methane to the atmosphere (0.77 – 3.0 μmol/m^2/d) with a $\delta^{13}C$ of -41.5 to -46.9 ‰ in the mixed layer. Thus, the highly depleted hopanol values in the oxic zone are consistent with available fractionation data. Sansone et al. (2001) hypothesized that there were two sources for the methane of the ETNP. In the upper water column, they suggested methane is biogenic and more depleted in ^{13}C relative to methane from a deeper, possibly sedimentary source with undetermined origins. A deeper and ^{13}C enriched source is consistent with hopanol $\delta^{13}C$ values of -45 to -49‰ throughout the suboxic zone. Our molecular and isotopic evidence for aerobic methanotrophs suggests that the methane reservoir found by Sansone et al. (2001) is actively consumed in the water column. Such processes may account for the relatively ^{13}C enriched methane observed by Sansone et al. (2001) in the ETNP OMZ.

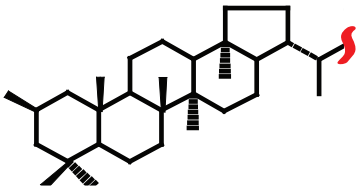
Conclusions

A new method for quantitation of BHPs allowed us to establish BHP patterns for the ETNP and can be applied to other environments. This improves on GC/MS methods used in the past, which could only assess changes in the total amount of BHPs, but not the amounts of hexa-, penta-, or tetra- functionalized compounds.

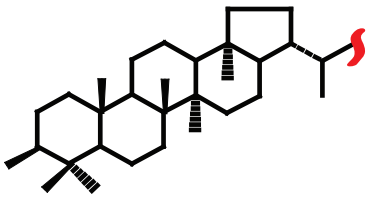
In our samples from the ETNP, BHP abundances are highest at the oxyclines above and below the oxygen minimum zone. Further, both BHP structures and isotopic signatures provide strong evidence for lipids derived from aerobic methanotrophs in surface waters, at the oxyclines and within the OMZ. Evidence for widespread methane-consuming aerobic bacteria indicates that the methane reservoir of the OMZ is more than likely mostly consumed before it reaches the atmosphere, consistent with the ^{13}C-enriched methane values determined by Sansone et al. (2001). BHPs patterns if preserved, would yield ratios of 100 : 0.3 : 1.8 for unmethylated : C2-methyl : C3-methyl hopanes. This is an unusually high ratio of C3 to C2 hopanes for marine environments and may reflect the dominance of methane cycling in this environment.



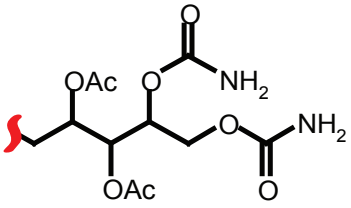
I. Unmethylated



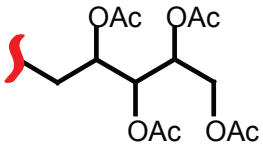
II. 2-Methyl



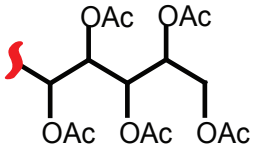
III. 3-Methyl



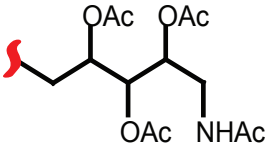
I. m/z 656 Carbamoyl



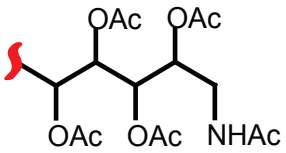
I. m/z 655 BHT
II. m/z 669 2Me-BHT



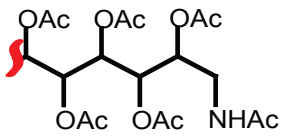
I. m/z 713 BHpentol



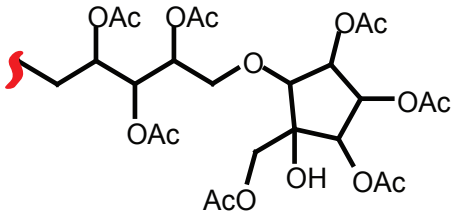
I. m/z 714 Aminotriol
III. m/z 727 3Me-Aminotriol



I. m/z 772 Aminotetrol
III. m/z 786 3Me-Aminotetrol



I. m/z 830 Aminopentol
III. m/z 844 3Me-Aminopentol



I. m/z 943 BHT pentose
II. m/z 957 2Me-BHT pentose

Figure 2-1. The BHP structures of the eastern tropical North Pacific.

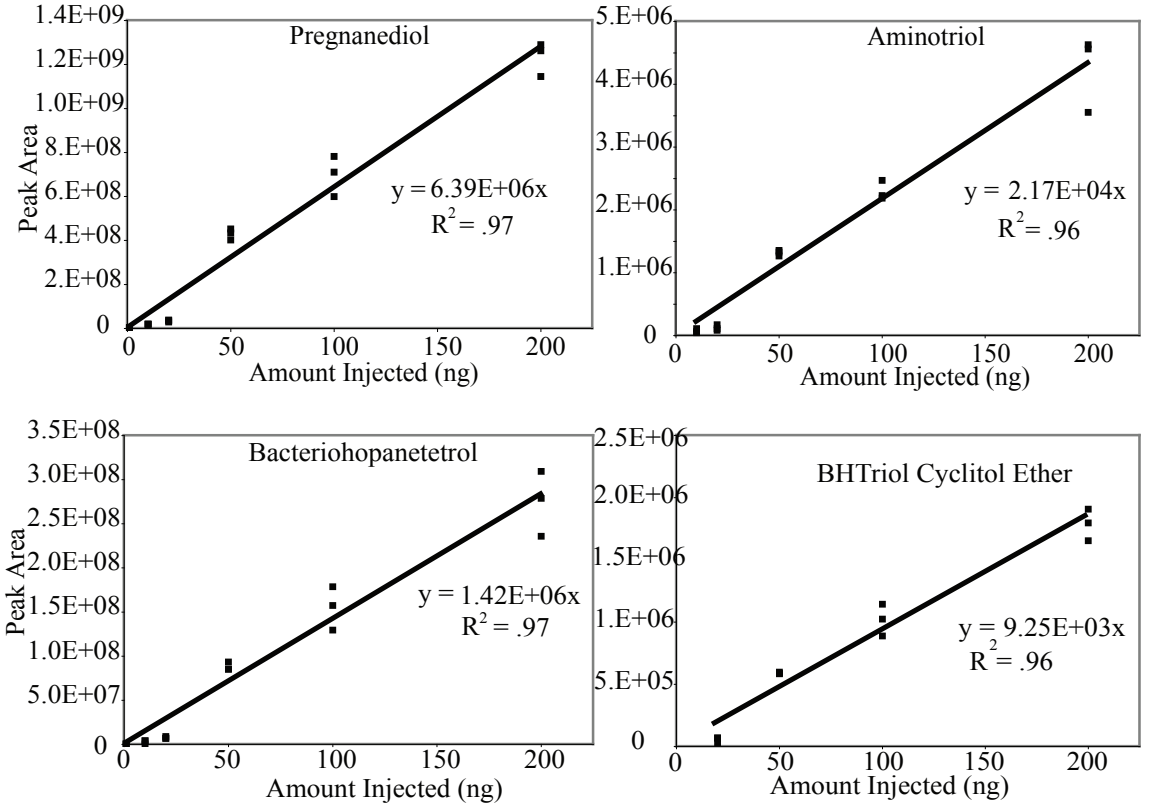


Figure 2-2. Response factors (RRF) of pregnanediol (m/z 285), bacteriohopanetetrol (BHT m/z 655), aminotriol (m/z 714), and BHTriol cyclitol ether (m/z 1002) during the quantitation of the ETNP samples. Fraction collected standards were injected from 1 - 200 ng.

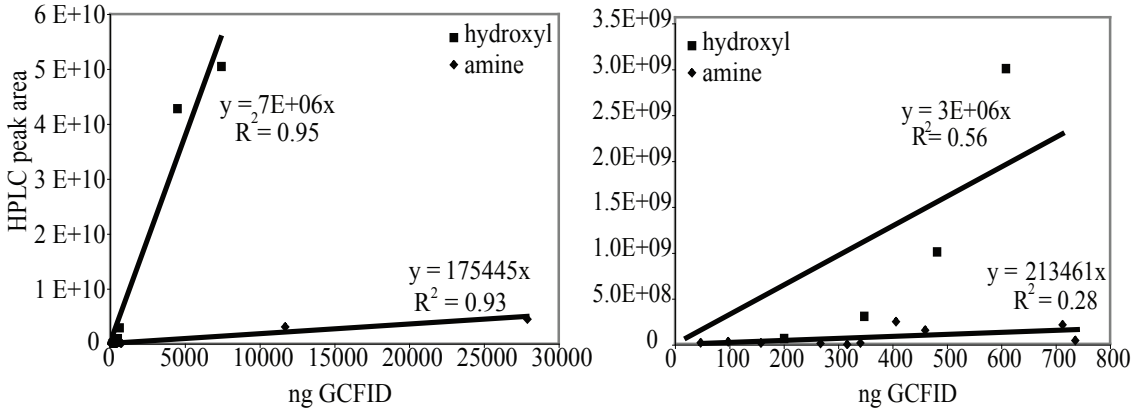


Figure 2-3. Comparison of GCFID and LCMS quantitation. (Left) Samples from the ETNP, Little Salt Spring, Florida and Rio Tinto, Spain. (Right) ETNP samples only. The comparison of GCFID quantities with HPLC peak areas indicate a two order of magnitude difference between hydroxyl and amine containing compounds confirming the HPLC response factors.

Table 2-1. Relative Response Factors

Compound	Relative Response Factor
BHT (m/z 655)	0.0043
Aminotriol (m/z 714)	0.00003
Cyclitol ether (m/z 1002)	0.00001

Table 2-2. ETNP Station 1 Carbon Isotope Values

Depth	Hopanol	$\delta^{13}C$ (‰)	Error (‰)
35m	Hexa	-72.57	1.1
	Penta	-64.87	1.1
	Me-Penta	-68.94	1.1
300m	Penta	-44.87	1.1
	Tetra	-46.46	1.1
400m	Penta	-46.72	1.1
	Tetra	-45.08	1.1
600m	Penta	-45.76	1.1
	Tetra	-49.25	1.1

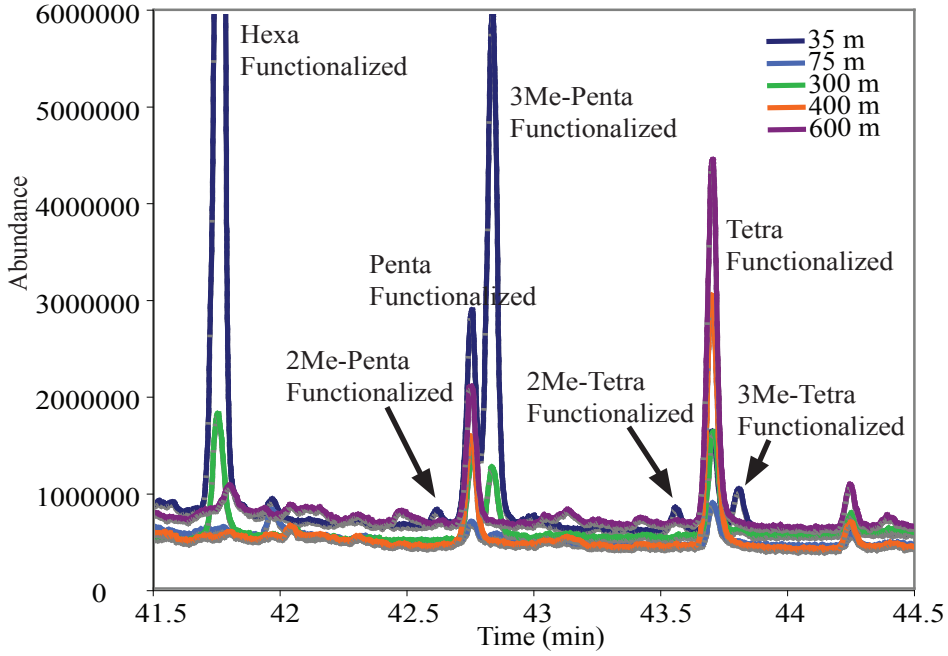


Figure 2-4. Me-hopanol identification of Station 1 ETNP. Stacked GC/MS chromatograms of selected depths. Identification of hopanols based on published mass spectra.

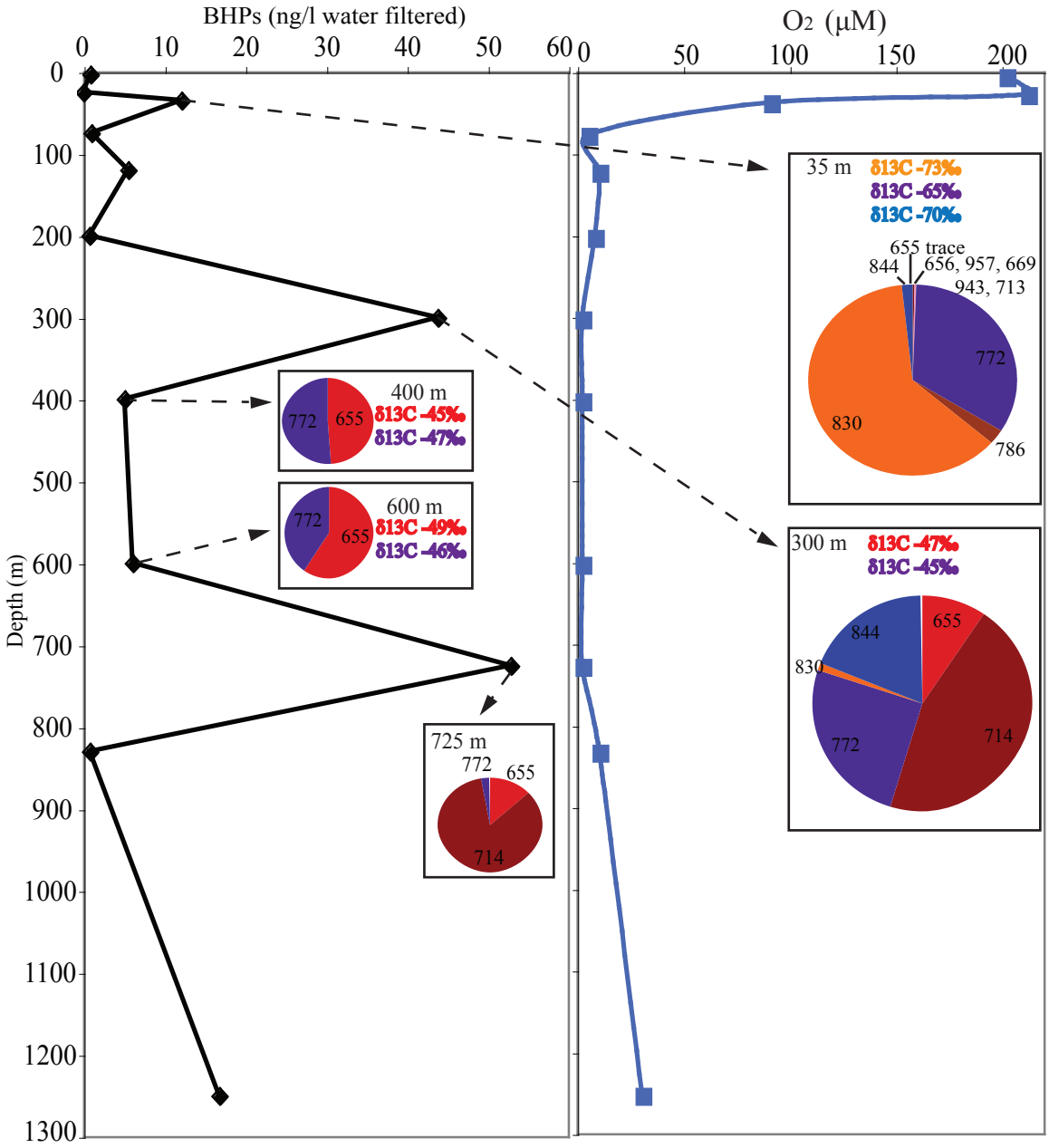


Figure 2-5. Total BHPs (ng/l water filtered) are denoted in black diamonds, oxygen concentration (µM) in blue squares. Pie charts indicate the abundance of BHPs polar side chains. Tetra-functionalized polar side chains are in shades of red. Penta-functionalized side chains are in shades of purple. Hexa-functionalized side chains are shades of orage. Methyl-hexa-functionalized side chains are shades of blue. Methyl-penta-functionalized are shades of brown. Methyl-tetra-functionalized are shades of green. Carbon isotope values of hopanols are reported for depths in the colors of the corresponding combined hopanols.

Station 2

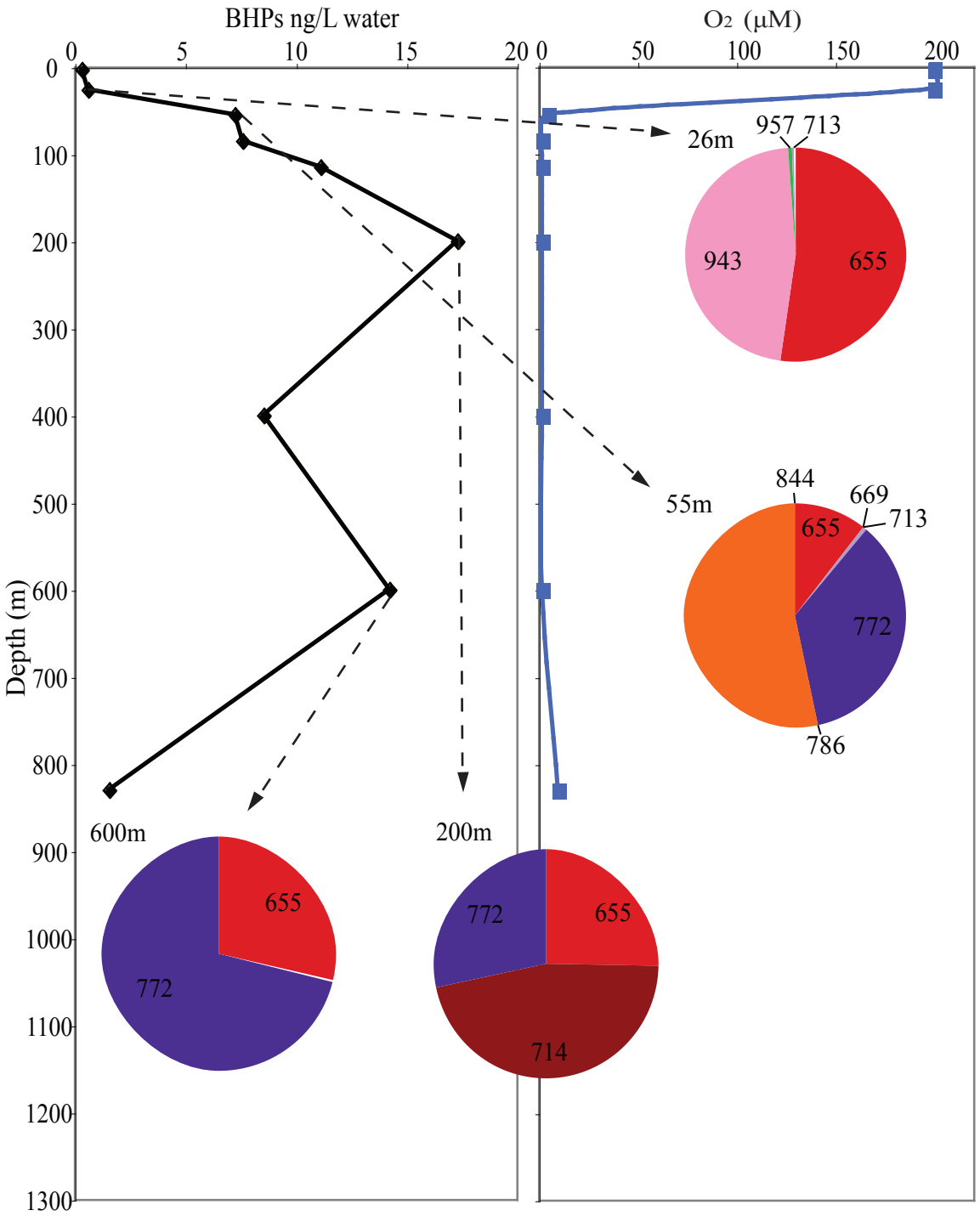


Figure 2-6. Total BHPs (ng/l water filtered) are denoted in black diamonds, oxygen concentration (µM) in blue squares. Pie charts indicate the abundance of BHPs polar side chains. Tetra-functionalized polar side chains are in shades of red. Penta-functionalized side chains are in shades of purple. Hexa-functionalized side chains are shades of orage. Methyl-hexa-functionalized side chains are shades of blue. Methyl-penta-functionalized are shades of brown. Methyl-tetra-functionalized are shades of green.

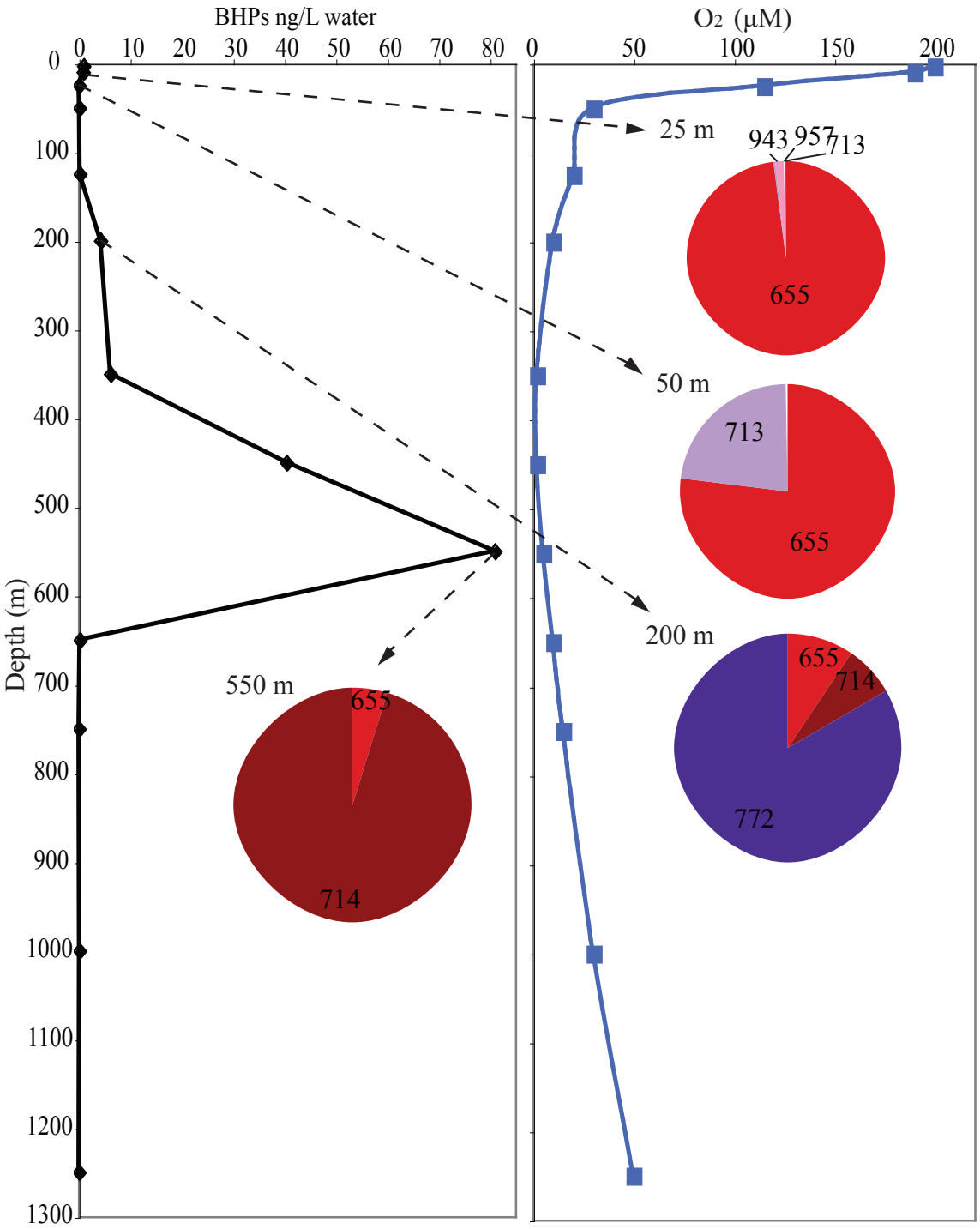


Figure 2-7. Total BHPs (ng/l water filtered) are denoted in black diamonds, oxygen concentration (µM) in blue squares. Pie charts indicate the abundance of BHPs polar side chains. Tetra-functionalized polar side chains are in shades of red. Penta-functionalized side chains are in shades of purple. Hexa-functionalized side chains are shades of orage. Methyl-hexa-functionalized side chains are shades of blue. Methyl-penta-functionalized are shades of brown. Methyl-tetra-functionalized are shades of green.

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Chapter 3

Bacteriohopanepolyols in a unique sulfur-oxidizing environment

Introduction

Bacteriohopanepolyols (BHPs) are bacterial polar lipids consisting of a pentacyclic isoprenoid lipid with a six-carbon side chain (Figure 3-1) (Talbot et al., 2003a, b, c; 2007a, b; 2008). Sixty-three possible functional groups have been identified thus far. Hopanes, geologically preserved hydrocarbon biomarkers derived from BHPs, are found in rocks as old as 2.7 Ga (Brocks et al., 2003; Eigenbrode et al., 2008) and are ubiquitous in organic rich sediments throughout the rock record (Albrecht and Ourisson, 1992). Hopanes serve as excellent biomarkers in oil correlation and thermal maturity studies (Peters and Moldowan, 1991). Yet, only a little is known about the diversity of bacterial sources and the physiological functions of BHPs.

The gene for the cyclase protein that renders squalene into the BHP ring structure is squalene hopene cyclase (*shc*), and it can be used to identify species capable of BHP production (Wendt et al., 1999, Thoma et al., 2004). *hpnP* is the gene for the methylation of the second carbon on ring A in the BHP structure (Welander et al., 2010). Primers have been developed for the amplification and phylogenetic study of both genes (Fischer et al., 2007; Pearson and Rusch, 2009; Welander et al., 2010). Recent studies have found the genes are inconsistently spread throughout the bacterial domain, with representatives

from both sulfur oxidizers and reducers able to produce BHPs (Welander et al., 2010; Fischer et al, 2007; Fischer and Rusch, 2009).

The Frasassi cave system in Italy is a proposed analog of early Earth non-photic sulfur cycling microbial ecosystems (Macalady et al., 2006). The cave system formed in limestone ($CaCO_3$) rocks where oxygenated meteoric waters mix with sulfidic and anoxic groundwaters (Galdenzi et al., 2007). The aphotic, lithoautotrophic microbial ecosystem that inhabits this interface produces sulfuric acid by sulfide oxidation (Macalady et al., 2006). Two morphologically distinct biofilm types utilize the energy from these redox reactions; a sediment-water interface type dominated by *Beggiatoa sp.*, and a streamer morphology that can be dominated by a variety of Proteobacteria including *Thiothrix*, *Thiobacillus baregensis*, *Sulfurovumales*, and *Sulfuricurvales* (Macalady et al., 2008). Both biofilm morphologies also contain δ-Proteobacteria sulfur reducing and disproportionating bacteria (Macalady et al., 2008). The type of biofilm present is controlled by water flow and local sulfide and oxygen geochemistry (Macalady et al., 2008). The *Beggiatoa*-dominated biofilms can grow over a wide range of sulfide and oxygen ratios, while each streamer type is constrained to a smaller range of sulfide and oxygen concentrations (Macalady et al., 2008). *Beggiatoa* dominate biofilms at the sediment-water interface in slow moving water, while the streamer biofilms attach to limestone in faster moving water (Macalady et al., 2008). The cave system thus provides diverse sulfide habitats and a diverse flora of sulfur-cycling organisms. This unusual setting provides an opportunity to investigate the potential sources of BHPs and their function among sulfur-cycling bacteria.

Genetic analyses of environmental samples document the presence of the *shc* gene in the γ-Proteobacteria, which contains many sulfur-oxidizing bacteria (Welander et al., 2010). Although no sulfur-oxidizing bacterium in pure culture is known to produce BHPs, many environmental samples that contain sulfur-oxidizing bacteria also contain BHPs (Pearson and Rusch, 2009; Welander et al., 2010; Wakeham et al. 2007; Schoell et al., 1992; Zang et al., 2005; Thiel et al., 2003). Previous authors have identified BHPs in *Beggiatoa*-dominated biofilms at benthic gas seeps but they assumed the ^{13}C-depleted compounds were produced by methanotrophs (Elvert and Niemann, 2008; Burhan et al., 2002). This is not definitive, though, because heterotrophic *Beggiatoa* could also have produced depleted compounds by growing on ^{13}C-depleted carbon dioxide, likely present in a seep environment (Hayes, 1993). In agreement with these findings, Schoell et al. (1992) studied another marine *Beggiatoa* biofilm and found two hopane isotopic signatures. One group of hopanes was 9‰ enriched in ^{13}C in comparison to the others. They conclude there were two sources of BHP production one methanotrophic and the other chemoheterotrophic source and suggest it may be *Beggiatoa* biomass. Although a source difference is possible, this evidence must be viewed with caution. Hopanes are not likely to represent isotopic signatures from a single source because polar lipids from different organisms can contribute to the same hydrocarbon structure.

Pure cultures of organisms in the *Desulfovibrio* clade produce bacteriohopanetetrol (m/z 655), aminobacteriohopanetriol (m/z 714), and aminobacteriohopanetetrol (m/z 772) in high concentrations of 9.8 – 12.3 mg/g dry cells (Blumenberg et al., 2006). Anoxic environmental samples that contain sulfur-reducers have also been shown to produce BHPs (Michaelis et al., 2002; Thiel et al., 2003;

Blumenberg et al., 2004). Both sulfur-oxidizing and sulfate-reducing organisms are present in the cave environments, and we surveyed samples from different biofilms and different geochemical environments to determine BHP structures and quantities.

We sampled biofilms from a range of hydrogen sulfide (22-801 μmol/l) and oxygen (0.5-25 μmol/l) concentrations as well as conductivity (1,535 – 15,975 μS/cm) to determine BHP structures and abundances across ecologic and geochemical gradients (Table H-1). BHPs are hypothesized to decrease the permeability of membranes (Kannenberg and Poralla, 1999; Haines et al., 2001) and we expect BHP abundances and polarity to increase with an increase in hydrogen sulfide or conductivity. BHP abundance and structural patterns across environmental gradients may also indicate other possible bacteria lipid responses to changes in geochemistry. To help constrain sources for BHP structures, we used a ^{13}C isotope label study. We measured isotopic label transfer into bulk biomass, total lipid extracts (TLE), and both individual polar lipids and hopanols. We employ the structural, abundance and isotopic methods to investigate and characterize BHPs in ecosystems dominated by both sulfur-oxidizing and sulfate-reducing microbes.

Methods

Sample Collection: Biofilm and sediment samples were collected on expeditions into the Frasassi Cave System, Italy, from 2005-2010. Biofilm biomass was collected with sterile pipettes and placed into falcon tubes, stored on ice, and processed within 8-12 hours of collection. For the ^{13}C-label experiments, samples were transferred into falcon

tubes with powdered 100% labeled $H^{13}CO_3^-$. Biomass for the labeling experiment was kept at ambient temperatures for approximately six hours before being frozen. Subsamples of approximately 0.25-0.5 grams dry weight were fixed in 3 volumes of freshly prepared 4% (wt/vol) paraformaldehyde in 1x phosphate buffered saline (PBS) for 3 to 4 h, and stored in a 1:1 PBS/ethanol solution at -20°C for FISH analyses. Samples for clone library construction were preserved in 4 parts RNAlater (Ambion) to 1 part sample (v/v). Most samples for lipid analysis were freeze-dried in Italy. Some lipid samples were preserved in RNAlater, which was washed from the biomass three times with phosphate buffer solution. RNAlater was extracted (using BHP extraction method below) and contains no BHPs at a detection limit of 667 ng/ 1 liter RNAlater.

Sample Characterization: Geochemistry of the water was determined with multimeter and spectrophotometer analysis. A 350i multimeter (WTW, Weilheim, Germany) with multiple sensors was used in the field to measure specific conductivity, pH, and temperature of the water. A portable spectrophotometer (Hach Co., Loveland, CO) was used at the sample site to measure dissolved sulfide and oxygen concentrations using methylene blue for total sulfides (Hach method 690, 5% repeatability) and indigo carmine for oxygen (Hach method 8316, 20% repeatability).

Biofilm biomass was characterized by fluorescent *in situ* hybridization (FISH) microscopy. Samples preserved with paraformaldehyde were analyzed using probes described in Macalady et al. (2008). Images from a Nikon E800 epiflroescence microscope were collected and analyzed using image analysis software NIS Elements AR 2.30, Hotfix (Build 312). We determined cell area, for cells hybridizing to probes, with the object count tool for ten images from each sample. We quantified total DAPI-stained

area. Biofilms were determined to be *Beggiatoa*-dominated if they contained more than 50% *Beggiatoa* by cell area.

Lipid Extractions: Biofilm biomass was freeze-dried, homogenized and weighed, and then extracted using a microscale modification of the protocol prescribed by Talbot *et al*., (2003a). Dichloromethane (DCM) was substituted for chloroform in this procedure. Total lipid extracts (TLEs) were subdivided and an aliquot was acetylated (1:1 pyridine: acetic anhydride, heated 1 hr at 60°C). BHPs were separated by HPLC and identified using MS^n capabilities with an Agilent ion trap mass spectrometer 6310, based on published MS^3 spectra (Talbot et al., 2003a; 2003b; 2003c; 2007a; 2007b; 2008). We quanitfied BHPs based on response factors determined for fraction-collected standards normalized to an internal standard, pregnanediol (Chapter 1; Albrecht et al., in preparation).

Chromatographic and Detection Conditions: Reverse-phase HPLC was performed using a Phenomenex Gemini 5 μm C_{18} column (150 mm x 3.0 mm i.d.) and a 5 μm pre-column with the same solid phase. Mobile phase flowed at 0.5 ml/min and was composed of (A) water, (B) methanol, and (C) isopropyl alcohol (0 min A: B: C 10: 90: 0, with a ramp to 25 min 1: 59: 40 and an isocratic flow to 45 min). LC/MS settings follow Talbot et al., 2003; positive APCI mode, mass scanning from 150-1300 m/z, and "Smart" fragmentation setting (i.e., 8000 nA corona voltage, 60 psi nebulizer pressure, dry gas of five l/min, dry temperature of 350 C, vaporizer temperature 490 C). The run was divided into three segments, with target masses of 285 (0-10 min), 1002 (10-17 min) and 655 (17-50 min), all with normal optimization. Auto MS^n settings for two precursor ions included an absolute threshold of 100,000 and a relative threshold of 5%. Ions are

excluded after two ion scans and released after 0.5 min. The acquisition parameter was a fragment amplitude of 1.0 V, with an average of 5.0 and an isolation width of 3.0 m/z. Auto MS (n>2) settings with one precursor ion were as follows: absolute threshold of 1,000, relative threshold 5% and fragment amplitude 1.0V.

Standard Collection: Initially, individual BHP standards were fraction-collected following reverse phase separation. Each collected fraction was additionally purified via neutral phase separation on a silica column (150 mm x 3.0 mm, Waters) and an isocratic flow of 80:30 hexanes:isopropyl alcohol (IPA) at 1 ml/min. The fractions were then weighed using tin EA cups on a nanogram scale.

Calibration curves and limits of detection: Response factors were determined across three orders of magnitude of detection by the injection of 1 ng, 10 ng, 20 ng, 50 ng, 100 ng, and 200 ng of each standard compound. Peak areas were normalized to the peak area of the internal standard, pregnanediol (Sigma Aldrich). Normalization to an internal standard accounts for instrument drift between injections as well as potential variations during pipeting and injection. Normalized data were used to calculate a linear regression between peak areas of each compound of interest (divided by peak area of pregnanediol) and known mass injected. The equation of this line was used to calculate the amount of compound in environmental samples. Signal-to-noise ratios averaged 20 for analysis of pregnanediol for mass ranging from 50 to 200 ng. Uncertainty calculated from three analyses of the same sample range from ($1\sigma = 14\%$).

Hopanol Derivatization: Total lipid extracts and selected isolated individual structures were derivatized to be GC amenable (following Innes et al., 1998). Acetyl groups were first reductively removed by lithium aluminum hydride, $LiAlH_4$: 10 mg of

$LiAlH_4$ in anhydrous tetrahydrofuran (THF) were added to the acetylated BHP and shaken for one hour. the resulting polar BHP was next reacted with 30 mg of periodic acid in 1 ml of a THF-and-water solution (8:1, THF:water ratio), shaken for one hour, and quenched with water. This reaction cleaves the carbon-carbon bond in vicinal diols to yield aldehydes. Third, aldehyde groups were reduced to form alcohols with sodium borohydride, $NaBH_4$: 10 mg of $NaBH_4$ in 1 ml of ethanol was added, shaken for 1 hr and quenched with 1 ml KH_2PO_4 (100mM). Finally, terminal alcohols were acetylated with 100 μl each of acetic anhydride and pyridine and heated to 60°C for 1 hr. Reaction products were analyzed by GC/MS (Hewlett Packard 5990) for identification.

GC/MS: Hopanols were analyzed on a gas chromatograph-mass spectrometer (Hewlett-Packard GC 6890, MS 5973) to identify methyl positions based on published mass spectra and chromatograms. Hopanols were diluted in an 4:1, hexane:DCM solution and injected on a split/splitless injector and a fused silica capillary column (Agilent J&W DB-5, 60m, 0.32mm, 25μm). They were then heated from 60ºC to 320ºC at 6ºC per minute.

GC/FID: Hopanols were quantified on a gas chromatograph equipped with a flame ionization detector (GC/FID; HP 5890). These analyses helped confirm response factors and quantities determined on the LC/MS. Also, co-elution of the methyl compounds on the LCMS made GC/FID quantification of these molecules necessary. Hopanols were diluted in an 4:1, hexane:DCM solution and injected on a split/splitless injector and a fused silica capillary column (Agilent J&W DB-5, 60m, 0.32mm, 25μm). They were heated from 60ºC to 320ºC at 6ºC per minute. Quantification was determined

using an internal standard and response factors calculated from peak areas for a dilution series of acetylated cholesterol (1ng/μl to 111ng/μl).

Bulk Isotope Methods: ^{13}C-incubated bulk biomass was freeze-dried and homogenized. $\delta^{13}C$ values were measured at the Pennsylvania State University Biogeochemistry Stable Isotope Laboratory using a continuous-flow inlet system that includes a Costech elemental analyzer. Samples were oxidized at 1020ºC over chromium (III) oxide and silvered cobalt (II, III) oxide followed by reduction over elemental copper wire at 650°C. Evolved CO_2 was subsequently passed through a water trap and then a 5 Å molecular sieve gas chromatograph column at 50°C to separate N_2 from CO_2. CO_2 was diluted with ultra high purity He in a Conflo III interface/open split prior to analysis. Isotope ratios were measured on a Thermo Finnegan Delta Plus XP isotope ratio mass spectrometer. $\delta^{13}C$ values were corrected for linearity using an internal standard (NBS caffeine) and then normalized to the VPDB scale using a two-point calibration with international standards (USGS 24 graphite; IAEA CH7 polyethylene; IAEA CH6 sucrose) and internal standards (glycine, caffeine, and modern marine sediment working standard) following the methods of Coplen *et al.* (2006). Sample accuracy is ±0.02‰ calculated as the average difference between measured and the known value for an internal standard (n=9). Sample precision is ±0.05‰ (1σ), calculated from internal standards treated as samples (eqn. 1). (N=9, N=number of measurements, x = measured value, $\bar{x}$ = actual value).

$$\sigma = \sqrt{(1/N-1)\sum(x-\bar{x})^2} \quad \text{(eqn. 1)}$$

Total Lipid Extract and Polar Lipid BHP Carbon Isotope Measurements: Total lipid extracts and individual BHPs were analyzed on a EA-IRMS system modified

for nanogram-scale samples, nano-EA-IRMS (Polissar *et al*., 2009). Briefly, the modifications to the above Costech EA include an autosampler, evacuation and purge lines, narrower bore quartz reactors, and a lower volume water trap. The GC column and thermal conductivity detector were removed from the flow path and the effluent from the EA was transferred directly to a custom trapping system. CO_2 is concentrated by the system that includes a cryogenic/heated trap, a six-way Valco valve, low-flow helium supply, and capillary column. Prior to analysis, samples were pipetted into pre-cleaned, smooth-sided tin capsules and solvent evaporated at room temperature.

Hopanol Carbon Isotope Measurement: The acetylated hopanols $\delta^{13}C$ values were measured by irm-GCMS. Compounds were separated with a Varian 3400 GC with a split/splitless injector and a fused silica capillary column (Agilent J&W DB-5; 30m, 0.32mm, 25μm). After chromatographic separation, compounds were combusted with nickel and platinum with O_2 in He (1%, v/v) at 1000˚C. A Finnegan Mat 252 monitored the stable isotope ratios and measurements were made relative to a reference gas calibrated with Mix A (n-C16 to n-C30 alkanes, Arndt Shimmelmann, Indiana University). Sample (SA) isotope values were reported in delta notation relative to Vee Dee Belemnite (VPDB) as $\delta^{13}C = [(^{13}R_{SA}/^{13}R_{VPDB}) - 1]$ where $^{13}R = {}^{13}C/{}^{12}C$. Internal standards of squalane and androstane were co-injected. Sample accuracy is ±0.3 ‰ calculated as the average difference between measured and the known value for each of the fifteen standards in Mix A averaged over nine injections. (N=9). Sample precision is ± 0.8 ‰ (1σ), calculated from nine injections of Mix A, a mix of fifteen standards, treated as samples. The standard deviation was calculated for each of the fifteen standards and then the average standard deviation was calculated (eqn. 1). The $\delta^{13}C$ of the added

carbon in the acetyl groups was corrected for by using mass balance to determine the acetyl $\delta^{13}C$ with pregnanediol. The $\delta^{13}C$ of pregnanediol was measured with and without acetylation using the nanoEA-IRMS (Polisar et al., 2009) in order to calculate acetyl ^{13}C abundance from the measured values:

$$n_{preg+acet.}F_{preg+acet} = n_{preg}F_{preg} + n_{acetyl}F_{acetyl} \quad \text{(eqn. 2)}$$

n is the number of carbons in the compound. F is the fractional abundance of ^{13}C in the compound ($F = {}^{13}C/({}^{12}C + {}^{13}C)$. Then we applied equation two to calculate ^{13}C of the BHP without the acetyl groups.

$$n_{PL+acet.}F_{PL+acet} = n_{PL}F_{PL} + n_{acetyl}F_{acetyl} \quad \text{(eqn. 3)}$$

Fractional abundance values were converted to delta notation to be comparable to literature values using equations 4 & 5 where $R_{VPDB} = 0.0112372$.

$$R = F/(1+F) \text{ (eqn. 4)}$$

$$\delta = (R_{sample}/R_{VPDB}) -1)*1000 \text{ (eqn. 5)}$$

Errors were assumed to be independent and Gaussian in distribution, and propagated using basic equations for the handling of random errors. (σ = standard deviation of the measured value, PL = polar lipid, acet = acetyl) (Freeman and Pagani, 2005).

$$\sigma_{PL}^2 = \sigma_{PL+acet}^2 + \sigma_{acet}^2 \text{ (eqn. 6)}$$

Thus combining uncertainties from analysis of derivitized hopane with that of acetyl carbon yields an uncertainty of 1.3 ‰ for hopane ring structures.

Genomic Search: Published incomplete genomes of three marine and one freshwater *Beggiatoa* species as well as DNA sequences from environmental samples containing *Beggiatoa* were searched using BLASTp. The JCVI database was searched using the National Center for Biotechnology Information's (NCBI; http:

//www.ncbi.nlm.nih.gov/) BLASTp Basic Local Alignment Search Tool (BLAST) (Altscul *et al*., 1990). The *shc* sequence for *Methylococcus capsulatus* (GI: 77128441) was used to perform a protein query vs. protein database (BLASTp). Freshwater species *Beggiatoa alba* B18LD and marine species *Beggiatoa* sp. "Orange Guaymas", *Beggiatoa* sp. SS and *Beggiatoa* PS incomplete genomes were searched for the *shc* gene.

Statistical Analysis: Standard least squares multiple linear regressions and linear regression were applied to the dataset to assess correlations between BHP concentration and geochemical analysis using JMP 8.0.1.

Results

BHP Abundances and Diversity: *Beggiatoa*-dominated biofilms had BHP abundances from 0-3.50 x 10^5 μg/g TLE. Streamer biofilms contained between 0 and 1.3x 10^5 μg/gTLE. Sediments contained $4.7x10^4$ – $8.7 x10^4$ μg/g TLE BHPs. The PSB biofilm contained $5.1x10^4$ μg/g TLE BHPs. The cyanobacterial biofilm contained $2.1x10^4$ μg/g TLE BHPs. Two of the twenty-one *Beggiatoa*-dominated biofilms had no BHPs detected. Five of the eleven streamer biofilms had no BHPs detected, while all three sediment samples and the cyanobacterial and PSB biofilms had BHPs present. On average, *Beggiatoa*-dominated biofilms (average = $3.6x10^4$, $1\sigma = 3.9x10^4$ μg/g TLE) contained fifteen times more BHPs than in streamer biofilms (average = $2. 3x10^3$, $1\sigma = 3.8x10^3$ μg/g TLE) (2 sample t test, $p = 0.012$). While the sediments contained similar amounts (average = $7.1x10^5$, $1\sigma = 2.1x10^5$ μg/g TLE), but different distributions of BHPs than the *Beggiatoa*-dominated biofilms sampled. Stream sediments without overlying

Beggiatoa-dominated biofilms and sediment from under a *Beggiatoa*-dominated biofilm contained similar abundances and relative proportions of BHPs. The PSB and cyanobacterial biofilms contained half the amount of BHPs than the *Beggiatoa* biofilms and had very different molecular distributions.

Beggiatoa dominated biofilms contained BHTriol cyclitol ether (m/z 1002), aminotriol (m/z 714), BHTetrol (m/z 655), adenosyl (m/z 746), BHTetrol cyclitol ether (m/z 1060), guanidine-substituted BHTriol cyclitol ether (m/z 1086) and a BHP with an unknown polar side chain (m/z 762) (Talbot et al., 2003, 2005, 2007) (Figure 3-2). BHTriol cyclitol ether, aminotriol, and BHTetrol were present in all biofilms, there are inconsistent appearances of adenosyl, BHTetrol cyclitol ether, guanidine-substituted BHTriol cyclitol ether and a BHP with an unknown polar side chain. BHTriol cyclitol ether and aminotriol were the dominant structures. Adensyl, when present (3 instances) was abundant while, BHTetrol cyclitol ether (5 trace appearances), guanidine-substituted BHTriol cyclitol ether (1 small apperance) and the unknown BHP (3 trace appearances) had inconsistent and generally low abundances.

Streamer biofilms with BHPs (n=6) had all structures found in the *Beggiatoa*-dominated biofilms, except there were no instances of guanidine-substituted BHTriol cyclitol ether. In contrast to *Beggiatoa* biofilms, BHTetrol was the most abundant structure in the streamer biofilms that contained BHPs. The three sediment samples contained the structures BHTriol cyclitol ether, aminotriol, BHTetrol, adenosyl, and BHTetrol cyclitol ether. Aminotriol was the dominant structure in two sediments (LV05-4 and RS06-4) while BHTriol cyclitol ether dominanted the other (PC09-45). The cave aquifer effluent to the river contained a cyanobacterial biofilm and purple sulfur bacteria

(PSB) biofilm. Both biofilms contained BHTetrol and BHTriol cyclitol ether in significant abundances and they both were dominated by BHTriol cyclitol ether.

Isotopic Labeling Experiments: Bulk biomass, TLE, and individual polar BHPs and hydrocarbon hopanols all showed incorporation of ^{13}C label in *Beggiatoa*-dominated biofilms (Figure 3-3, Table 3-1). The streamer biofilms also showed ^{13}C incorporation into bulk biomass and TLE, but unfortunately BHP abundances in streamer biofilms sampled for isotope labeling were too low to permit isotope ratio analysis. Intra-molecular mass balance calculations indicated that the sugar moiety of the BHP side chain contains most of the ^{13}C label. We used isotope data for the intact polar BHP and the isolated hydrocarbon, to calculate the $\delta^{13}C$ of carbon in the polar head group (Table 3-1).

$$n_{pl}F_{pl} = n_{hc}F_{hc} + n_{tail}F_{tail} \quad \text{(eqn. 4)}$$

In equation 3, 'n' represents the number of carbons, 'F' represents the isotopic fractional abundance ($F = {}^{13}C/{}^{12}C + {}^{13}C$), 'pl' represents the intact polar lipid BHP, 'hc' represents the associated hydrocarbon hopanol and 'tail' represents the three carbons removed during GC derivatization. We used F instead of δ values because of the high level of ^{13}C enrichment in the experiment (Moran et al., 2007). For BHTetrol (m/z 655), carbon in the polar tails was more enriched in ^{13}C than in the hydrocarbon (Figure 3-3, Table 3-1). In both samples the unlabeled sugar tail $\delta^{13}C$ ratio consistent with carbon fixation using the Calvin cycle mediated by ribulose bisphosphate carboxylase-oxidase (rubisco) and a CO_2 source isotopically similar to atmospheric values (-24 and -26‰) (Hayes, 2001). In contrast, the polar tail carbon in the labeled sample was strongly enriched in ^{13}C (204 and 191‰). Carbon in the polar group is derived from a sugar (Summons et al., 2006), while

the hydrocarbon is derived from the cyclization of the isoprenoid lipid squalene by the squalene-hopene cyclase enzyme (Wendt et al., 1999).

Genomic Search: The *shc* gene was not present in the three marine and one freshwater *Beggiatoa* species that are currently incompletely sequenced.

Discussion

Differences in functional groups of BHPs, can be indicative of bacterial families. However, the structures present in the cave system are common amongst the pure cultures studied thus far and therefore are not diagnostic of bacterial clades (Talbot et al., 2009 and references therein). Although the structures are not specific to individual taxa, the relative abundances of different BHP structures can be used to denote differences in bacterial production. The biofilms and streamers have many structures in common but the *Beggiatoa*-dominated biofilms are dominated by BHTriol cyclitol ether and aminotriol, while the streamers are dominated by aminotriol. The sediments in the cave have a similar assortment of structures as the *Beggiatoa*-dominated biofilms but a larger amount of BHTetrol. Our results show a similar ratio of BHTetrol, aminotriol, and BHTriol cyclitol ether to those observed in three *Desulfovibrio* pure cultures (Blumenberg et al., 2005). We found *Desulfovibrio* species in clone libraries of the Frasassi biofilms, and therefore we suggest a *Desulfovibrio* sulfate reducer is a source of BHPs in sediment samples.

Our data indicate that there is no correlation of BHP concentration or structure to hydrogen sulfide, oxygen concentration, or other geochemical parameters present in the

stream waters (Table 3-2). Therefore, the original hypothesis that BHP abundance will increase with hydrogen sulfide is not supported. These findings are based on lipid ratios to total lipid extract. This result needs to be further tested by the normalization of the BHP concentrations to the amount of biomass in each biofilm produced by the BHP producing bacteria. This requires FISH microscopy to quantify biomass, provided identity of the BHP producing species(s) is known.

^{13}C label incorporation from bicarbonate in the bulk biomass, TLE, and individual BHPs (Figure 3-3 and Table 3-1) strongly implies that an autotroph(s) in the samples is the producer of the BHPs. *Beggiatoa*, the dominant biomass (Macalady et al., 2008) in the label samples, is a diverse genus with significant divergence within the 16S rRNA gene (Gray and Head, 1999). Although the *shc* gene is not present in the two marine and one freshwater *Beggiatoa* species sequenced thus far, this does not imply that all *Beggiatoa* species lack the *shc* gene. For example, the cyanobacterium *Phormidum sp. OSS3* produces BHPs whereas closely related *Phormidium sp. RCO* does not (Talbot *et al*., 2008). We note there is over 10% dissimilarity in the 16S rRNA gene between the freshwater *Beggiatoa alba* and the *Beggiatoa* species found in the cave system. Therefore, even though *Beggiatoa alba* does not produce BHPs, it is still quite possible the cave *Beggiatoa* species do. It has been suggested from indirect evidence in previous studies (Schoell et al., 1992; Burhan et al., 2002) that *Beggiatoa* species are capable of BHP production. *Beggiatoa*-dominated biofilms collected at marine gas seeps also contain BHPs (Arning et al., 2008; Elvert and Niemann, 2008). *Beggiatoa* is difficult to culture, it has not been well-studied biochemically, and many species are still unidentified. In the cave samples, *Beggiatoa*-dominated biofilms and sediments contain

similar absolute abundances of BHPs. Although it is possible some sediment was incorporated in the *Beggiatoa* biofilm samples, the distribution of BHP structures and their relative abundances differ markedly and this seems unlikely. We suggest the BHPs in the *Beggiatoa* biofilms are produced *in situ,* and that *Beggiatoa* is the most likely candidate for their source.

The diversity in abundances and structures of BHPs indicate that there are most likely multiple species producing BHPs. Sediment BHP structures are consistent with a source from sulfur reducing bacteria, possibly *Desulfovibrio* (Blumenberg et al., 2005). Although we have not cloned a sediment sample, in the biofilm samples cloned *Desulfovibrio* is found in small quantities in the biofilm samples (Macalady et al., 2008). Absolute abundances and differences in proportions of BHPs in the *Beggiatoa*-dominated biofilms cannot be accounted for by production of BHPs from underlying anoxic sediments being incorporated into the *Beggiatoa* type biofilm. The ^{13}C label in the *Beggiatoa*-dominated biofilms indicates that an autotrophic bacterium in the biofilm is producing the BHPs. *Beggiatoa* is the major biomass in the biofilms (greater than 50% by cell biomass, Macalady et al., 2008), and by association, we conclude *Beggiatoa* is the most likely dominant BHP producer of the biofilm. The minor BHP structures that are not consistently found throughout all the biofilms and sediments may be produced by separate species. Or, the minor structures may represent a signature of the BHPs produced under environmental conditions that prompt an alternative physiological response (Doughty et al., 2009; Welander et al., 2010).

It is difficult to assign differences in the proportions of BHP structures and abundances to different microbial communities or geochemistry, because we do not know

the function of BHPs. Further, growing evidence suggests bacteria can adjust BHP concentration and polar groups based on a physiological response to an environmental signal. For example, Doughty et al. (2009) found a change in proportion of structures during akinete formation by the cyanobacterium *Nostoc punctiforme.* In other organisms, BHPs increase in abundance as a response to higher temperature (*Frateuria aurantia* and *Alicyclobacillus acidocaldarius*), exposure to ethanol (*Zymomonas mobilis*), or a decrease in pH (*Alicyclobacillus acidocaldarius*) (Joyeaux et al., 2004; Schmidt et al., 1986). BHP content in *Rhodopseudomonas palustris* was constant with pH, but the proportion of 2-methyl BHP structures increased by 50% when pH was reduced (5, 7, & 9) (Welander et al., 2009). In contrast, BHP levels in *Streptomyces scabies* have shown no change in response to oxidative stress, low pH, tolerance to ethanol or high temperature (Seipke and Loria, 2009).

Because they are more strongly labeled, BHP polar tails are apparently synthesized or replaced more frequently than the hydrocarbon ring structures. This indicates that BHP functional groups are capable of being adjusted rapidly in response to changing environmental conditions. The isoprenoid ring structures of the BHPs are very energy intensive to produce (Kannenberg and Poralla, 1999) consistent with our label results that indicate the polar side chain turns over more quickly than the isoprenoid. This may be the reason that Crossman et al. (2001; 2005) did not find incorporation of a carbon label in hopanols even though it was rapidly incorporated in fatty acids. Our labeling results vary in extent of label uptake (3 to 2,000‰), suggesting that some biofilms were more active than others during the six-hour incubation (Table 3-1).

Conclusions

BHPs are found in all of the aphotic, sulfur-cycling bacterial communities of the Frasassi system stream biofilms; *Beggiatoa*-dominated biofilms, streamer biofilms and the anoxic sediments. The observed polar structures are diverse and their distributions indicate multiple BHP-producing species may be present. The presence and ratio of structures in the anoxic sediments are similar to previous studies of BHP production by the sulfur reducing *Desulfovibrio* clade (Blumenberg et al., 2005). The presence of BHPs in appreciable quantities in *Beggiatoa*-dominated biofilms and the incorporation of the ^{13}C label into BHPs suggest that the Frasassi *Beggiatoa* bacteria are BHP producers in this system. Label incorporation is greatest in the polar tail and much less in the isoprenoid hydrocarbon structure. This suggests bacteria may be able to change functional groups relatively rapidly under changing environmental conditions. Future labeling studies that investigate BHP production should use methods to analyze the intact individual BHPs to locate the label in BHPs.

Acknowledgements

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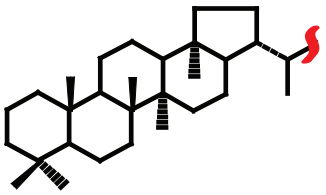
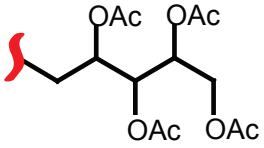
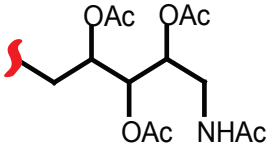


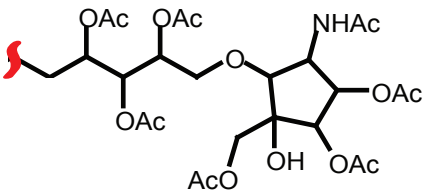
BHP ring structure



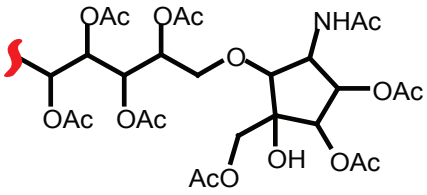
m/z 655 BHT



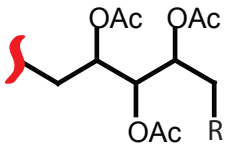
m/z 714 Aminotriol



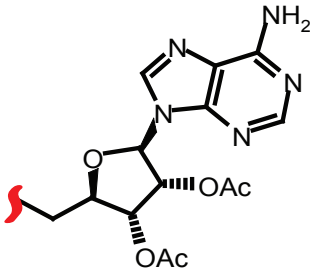
m/z 1002 BHtriol Cyclitol Ether



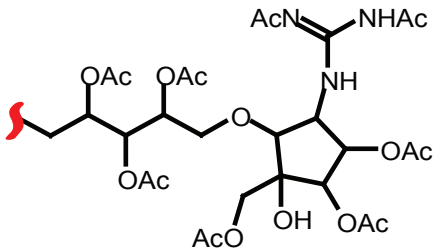
m/z 1060 BHtetrol Cyclitol Ether



m/z 762 Unknown



m/z 746 Adenosyl



m/z 1086 Guanidine-substituted
BHTetrol cyclitol ether

Figure 3-1. Bacteriohopanepolyols of the Frasassi cave system stream waters.

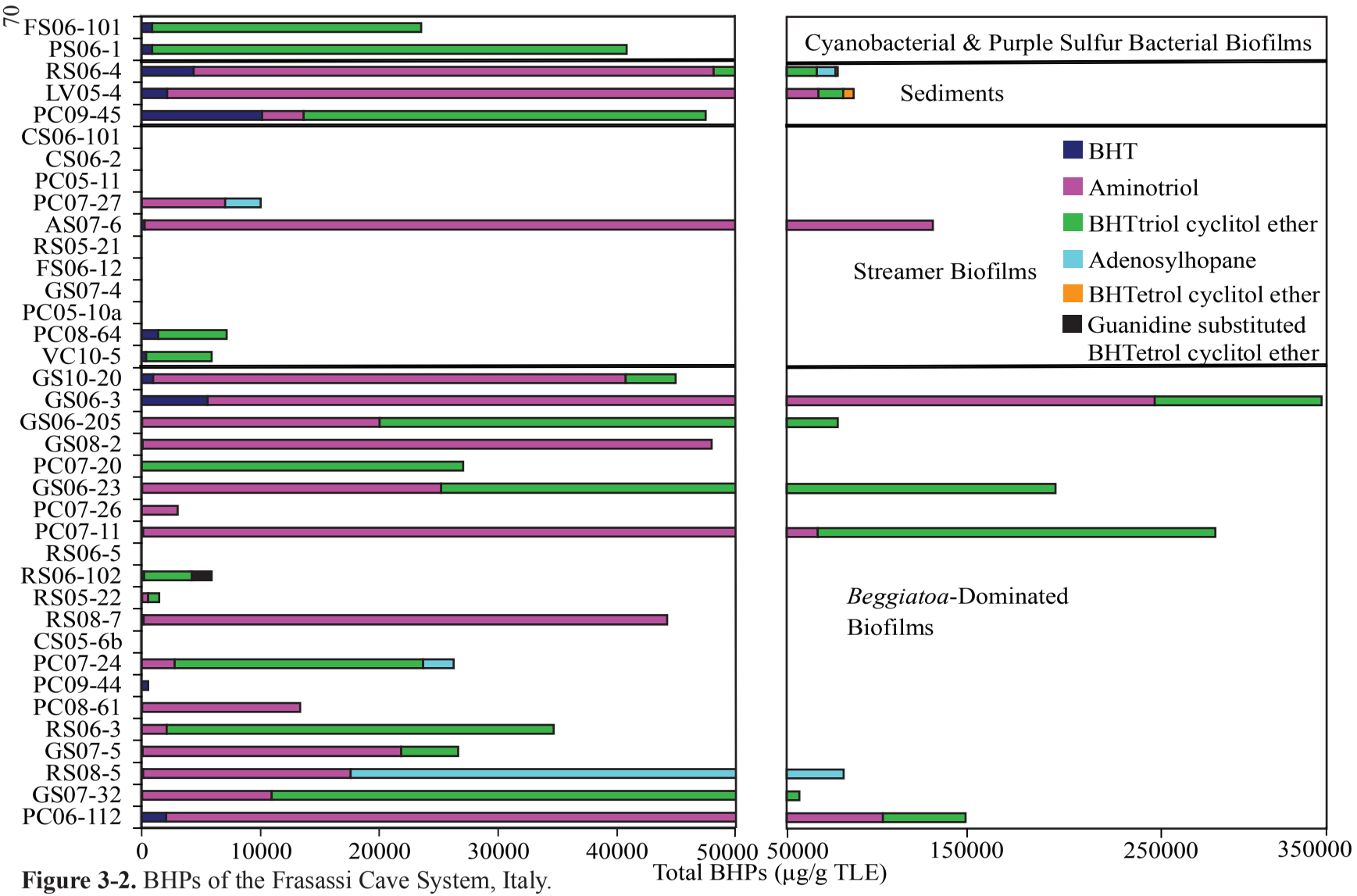


Figure 3-2. BHPs of the Frasassi Cave System, Italy.

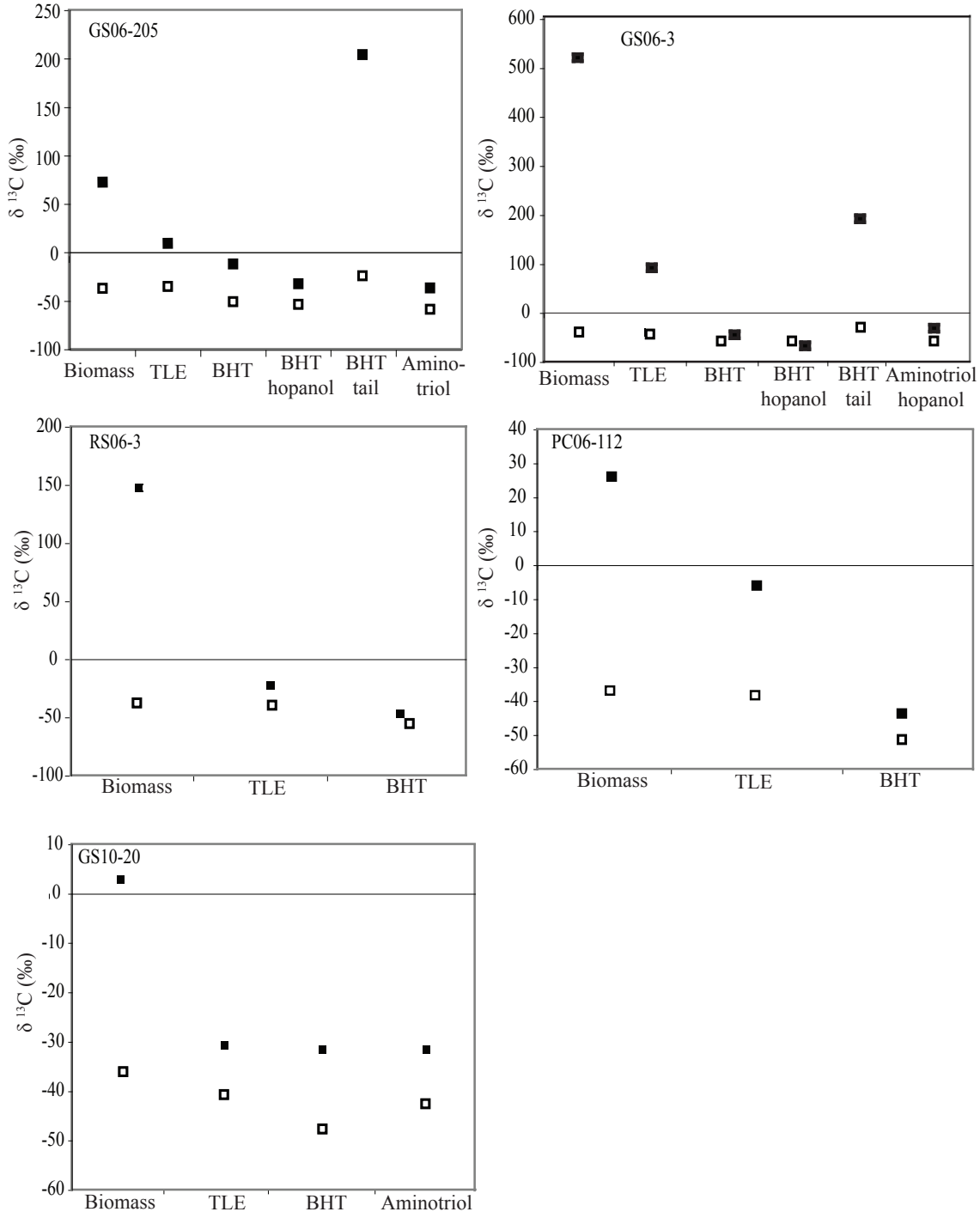


Figure 3-3. The carbon isotopic value of a ^{13}C labeled cave stream biofilms. Biomass refers to bulk biomass measurements on the EA. TLE = total lipid extract measured on the nano-EA. BHT and Aminotriol = fraction collected individual BHPs measured on the nano-EA. Hopanol = fraction collected individual BHP with the tail of the BHP removed and measured on a GC-IRMS.

Table 3-1. The ^{13}C Labeling Experiment Results

Sample Name	^{13}C label	Sample Type	PL (polar lipid) /H(hopanol)	$\delta^{13}C$	Error (‰)
GS06-205	no	BULK		-36.6	0.02
GS06-205	yes	BULK		73.3	0.02
GS06-205	no	TLE		-34.9	1.0
GS06-205	yes	TLE		9.3	1.0
GS06-205	no	655	PL	-50.7	0.8
GS06-205	yes	655	PL	-11.7	0.8
GS06-205	no	655	H	-53.2	0.8
GS06-205	yes	655	H	-32.0	0.8
GS06-205	no	714	H	-56.4	0.8
GS06-205	yes	714	H	-50.1	0.8
GS06-205	no	655	tail	-23.6	1.3
GS06-205	yes	655	tail	204.4	1.3
GS06-3	no	BULK		-38.4	0.02
GS06-3	yes	BULK		517.9	0.02
GS06-3	no	TLE		-40.1	1.0
GS06-3	yes	TLE		91.2	1.0
GS06-3	no	655	PL	-54.8	0.8
GS06-3	yes	655	PL	-44.1	0.8
GS06-3	no	655	H	-57.5	0.8
GS06-3	yes	655	H	-66.2	0.8
GS06-3	no	655	tail	-26.5	1.3
GS06-3	yes	655	tail	191.3	1.3
GS06-3	no	714	H	-60.6	0.8
GS06-3	yes	714	H	-30.9	0.8
GS06-3	no	1002	H	-63.2	0.8
GS06-3	yes	1002	H	no peak	

Table 3-1 Continued.

Sample Name	^{13}C label	Sample Type	PL (polar lipid) /H(hopanol)	$\delta^{13}C$	Error (‰)
PC06-112	no	BULK		-36.8	0.02
PC06-112	yes	BULK		26.2	0.02
PC06-112	no	TLE		-38.2	1.0
PC06-112	yes	TLE		-5.9	1.0
PC06-112	no	655	PL	-51.3	0.8
PC06-112	yes	655	PL	-43.5	0.8
RS06-3	no	BULK		-36.4	0.02
RS06-3	yes	BULK		147.4	0.02
RS06-3	no	TLE		-39.3	1.0
RS06-3	yes	TLE		-21.9	1.0
RS06-3	no	655	PL	-46.9	0.8
RS06-3	yes	655	PL	-53.5	0.8
GS10-20	no	BULK		-35.9	0.02
GS10-20	yes	BULK		3.0	0.02
GS10-20	no	TLE		-40.4	1.0
GS10-20	yes	TLE		-30.6	1.0
gs10-20	no	655	PL	-47.6	0.8
gs10-20	yes	655	PL	-31.5	0.8
FS06-12	no	BULK		-25.2	0.02
FS06-12	yes	BULK		668.3	0.02
CS06-2	no	BULK		-28.3	0.02
CS06-2	yes	BULK		2002.3	0.02
PC06-110	no	BULK		-31.4	0.02
PC06-110	yes	BULK		16.1	0.02

Table 3-2. Statistical Analysis of BHP Concentration and Geochemical Parameters

Data Set	Total BHPs and Conductivity	Total BHPs and Oxygen Concentration	Total BHPs and Hydrogen Sulfide Concentration	Multiple Linear Regression
All Data	$p = 0.83$ $R^2 = 0.00$	$p = 0.05$ $R^2 = 0.14$	$p = 0.57$ $R^2 = 0.01$	$p = 0.30$ $R^2 = 0.14$
Beggiatoa-Dominated Biofilms	$p = 0.70$ $R^2 = 0.01$	$p = 0.10$ $R^2 = 0.16$	$p = 0.26$ $R^2 = 0.08$	$p = 0.07$ $R^2 = 0.39$
Streamer Biofilms	$p = 0.50$ $R^2 = 0.16$	$p = 0.74$ $R^2 = 0.04$	$p = 0.06$ $R^2 = 0.74$	$p = 0.45$ $R^2 = 0.87$

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Chapter 4

Production of bacteriohopanepolyols by *Acidithiobacillus* species in the environment and in culture

Introduction

Bacteriohopanepolyols (BHPs) are bacterial biomarkers consisting of a pentacyclic triterpenoid lipid with a six-carbon side chain with a possible combination of sixty-three different functional groups (Talbot et al., 2003abc; 2007ab; 2008). Hopanes are the geologically preserved hydrocarbons derived from BHPs. They are recalcitrant, found in rocks 2.7 Ga (Brocks et al., 2003) and ubiquitous in the rock record (Albrecht and Ourisson, 1992). Therefore, these compounds could be excellent biomarkers. However, the interpretation of hopanoid-derived biomarkers in the rock record is hindered because identification of the bacterial sources of BHPs and their physiological functions within the cell are poorly understood.

Because of their geometry and location in bacterial membranes, BHPs may have a homologous function to sterols by lowering membrane fluidity (Demel et al., 1976; Poralla et al., 1980; Rohmer et al., 1976; Kannenberg et al., 1980; Kannenberg and Poralla, 1982; Kannenberg et al., 1983). BHPs are known to increase in abundance as a response to temperature (*Frateuria aurantia* and *Alicyclobacillus acidocaldarius*), exposure to ethanol (*Zymomonas mobilis*), and decrease in pH (*Alicyclobacillus acidocaldarius*) (Joyeaux et al., 2004; Schmidt et al., 1986). In contrast many studies have shown no change in BHP content with a change in culturing conditions. For

instance, BHP content in *Rhodopseudomonas* did not increase with a decrease in pH (Welander et al., 2009). Furthermore, in *Streptomyces scabies* BHP concentration did not change in response to oxidative stress (0.3-3.0 μmol H_2O_2), pH (7.3-5.0), exposure to ethanol (1-10% vol/vol) or high temperature (25-42 ºC) (Seipke and Loria, 2009). These studies have revealed that the physiological response of bacterial BHP production to environmental stimuli varies with different organisms.

To resolve the paradox of ubiquitous hopanes but only a few known BHP producers, genetic methods have been developed for use with pure cultures and environmental samples. These studies have identified the squalene hopene cyclase gene *(shc*), which encodes for the protein (SHC) that converts squalene into hopene, and the gene *hpnP*, which encodes for the protein that adds the methyl group on the second carbon of ring A. (Wendt et al., 1999; Thoma et al., 2004; Welander et al., 2010). The *shc* gene is well represented among acidophilic bacteria (Pearson et al., 2007). Consistent with this pattern, BHPs are found in pure cultures of *Acidithiobacillus ferrooxidans*, *Acidimicrobium capsulatus*, *Alicyclobacillus acidocaldarius*, numerous acetic acid bacteria, and thermo-acidophilic bacilli (Rohmer et al., 1984; Langworthy and Mayberry, 1976; Poralla et al., 1984; Hippchen et al., 1981). Despite the mixed results of past studies, the common occurrence of the *shc* gene in acidophilic bacteria suggests BHPs have an important role in adaptation to acidic habitats.

Acidophiles use multiple adaptations to live in low pH including making the cell membrane impermeable to protons, reducing pore size of membrane channels, inhibiting proton influx by creating a Donnan potential, pumping out protons, cytoplasmic buffering, and creating a negative surface charge potential (Baker-Austin and Dopson,

2007; Kar and Dusgupta, 1996). One common physiological change to decrease proton permeability across the membrane is to increase the concentration of terpenoids (lipids derived from five-carbon isoprene units that include sterols and BHPs) and saturated fatty acids (Mykytczak et al., 2010; Baker-Austin and Dopson, 2009). Hopanoids are thought to influence membrane permeability by decreasing water content within the bilayer and by blocking the continuity of water wires through the membrane (Haines et al., 2001). Water wires are a proposed mechanism for proton leakage through lipid bilayers where a transient, transmembrane chain of water molecules forms and passes protons through the membrane (Haines et al., 2001; Brewer et al., 2001; Brookes et al., 1997). Liposome experiments can specifically test the properties of membranes containing triterpenoids (C_{30} isoprenoid lipids e.g. BHPs and sterols). For example, Haines et al., (2001) show that ion permeability is decreased in membranes that have an increase in cholesterol and diplopterol. In contrast, Lande et al., (1995) use a mixture of sterols, spingomyelin and acyl chain saturations and find that decreased membrane fluidity strongly correlates to decreased permeability to water, urea, acetamide, and ammonia but a weak correlation to protons (1995).

In this study, we evaluate the influence of pH on BHP concentration and structures in two species of *Acidithiobacillus* bacteria: *A. thiooxidans,* a sulfur oxidizer in an environmental sample and in pure culture and *A. ferrooxidans,* an iron oxidizer in environmental samples. A physiological response to increase BHPs will potentially decrease water wire formation across the membrane and therefore decrease passive diffusion of ions across the membrane (Benz et al., 1983). A increase in functional groups will potential create a more negative surface charge on the cell (Kar and

Dasgupta, 1996). Therefore we predict an increase in BHP abundance and an increase functional groups of the polar tail with a decrease in pH.

Sample Description

Acidithiobacillus sp. Mu 1, a sulfur-oxidizing acidophilic bacterium, was isolated from a snotitte biofilm in the Frasassi cave system (Jones et al., unpublished). Snotittes form on a cave wall using hydrogen sulfide that is degassing from sulfur-rich groundwater as an electron donor for autotrophy. The sample for lipid analysis was a collection of many small biofilms collected over a one meter squared area of cave wall. In the biofilm metagenomic analysis and fluorescent *in-situ* hybridization (FISH) microscopy reveal *A. thiooxidans* is the dominant species (75%). The biofilm also contains *Acidimicrobium* (7%) related to *Acidimicrobium ferrooxidans*, and an archaeon (18%) related to *Ferroplasma* (Jones et al., in review). The pH of the snotitte biofilm matrix is 0-2. *Shc* gene amplification from the snotitte biofilm found three partial, unique sequences one with a 95% match to *Acidithiobacilus ferrooxidans* ATCC 53993, one from a *Proteobacteria* (clone SDPr_Cl121), and an unknown clade (Jones et al., in review). Further evidence that close relative to *A. ferrooxidans* is that direct sequencing from the PCR product was possible with that *shc* PCR product but no the other two PCR products (Jones et al., in review). The Mu1 strain is an aerobic chemolithoautotroph and can be grown on thiosulfate or solid sulfur at pH 0-5.

Biofilms were sampled across a pH gradient (3.51-2.57) from the Lower Red Eyes site, which is an acid mine drainage (AMD) stream. The sediments contain a diverse

microbial community of iron-oxidizing organisms in mineral crusts along the streambed, including *A. ferrooxidans*, a *Leptospirillum* species, eukaryotic *Euglena* algae, and small amounts of other bacteria. The geochemical and biological setting has been well described by Brown et al. (2011). The Lower Red Eyes microbial community was characterized by fluorescent *in-situ* hybridization (FISH) microscopy (Brown et al., 2011). Briefly, the stream pH decreases from the headwaters (3.51) to downstream (2.57). *A. ferrooxidans* is a more dominant species within the biofilms at the lowest pHs, 65% of total bacteria at pH 2.6. The *A. ferrooxidans* disappears from the biofilms above pH 3.

Methods

Sample Collection: Samples were collected on two expeditions (May, October) to the Lower Red Eyes acid mine drainage in Prince Gallitzin State Forest, Somerset County, PA, USA (Table 4-1), and Frasassi cave system, Italy. Biofilm biomass was collected with sterile pipettes and transferred into falcon tubes. Samples from Lower Red Eyes were kept on ice in the field, frozen, and then freeze-dried. The sample, RS05-24c, from the Frasassi cave was preserved in RNAlater for shipping. RNAlater was subsequently removed by washing the samples three times with phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4). RNAlater was extracted (using BHP extraction method below) and contains no BHPs at a detection limit of 667 ng/ 1 l RNAlater.

Culturing: Because factors such as nitrogen limitation, oxidative stress, growth phase and temperature can potentially affect BHP production, we controlled these variables in order to focus on the effect of pH in culture. The *A. thiooxidans* culture, *Mu1* was grown across a pH gradient at pH 2, 2.5, 3, 3.5 and 4 using a thiosulfate medium originally developed for *Acidithiobacillus thiooxidans* at a temperature of 30°C; shaking at 160 rpm, in aerobic conditions, in the light (Leathen et al., 1956). Harvesting was carried out during the exponential growth phase, based on optical density measurements calibrated using a growth curve established for the culture. The pH of the culture medium was controlled using a phosphate buffer system (pKa = 2.15, 322 g/l NAH_2PO_4, 256 g/l NA_2HPO_4).

Lipid Extractions. Environmental and cultured samples were freeze-dried, homogenized, weighed and then extracted using a microscale modification of the protocol prescribed by Talbot *et al*., (2003a). Dichloromethane (DCM) was substituted for chloroform in this procedure. Total lipid extracts were subdivided and a fraction was acetylated (1:1 pyridine: acetic anhydride, heated 1 hr 60°C). Identification of BHPs was done with an Agilent LC ion trap mass spectrometer 6310 based on MS^3 published spectra (Talbot et al., 2003a; 2003b; 2003c; 2007a; 2007b; 2008). Quantitation of BHPs was based on response factors of fraction-collected standards and normalization to an internal standard, pregnanediol.

Chromatographic and detection conditions: Reverse-phase HPLC was performed using a Phenomenex Gemini 5 μm C_{18} column (150 mm x 3.0 mm i.d.) and a 5 μm pre-column with the same solid phase. LC/MS settings follow Talbot et al., 2003; these settings are positive APCI mode, mass scanning from 150-1300 m/z, and "Smart"

fragmentation setting (i.e., 8000 nA corona voltage, 60 psi nebulizer pressure, dry gas of five l/min, dry temperature of 350 C, vaporizer temperature 490 C). The run was divided into three segments, with target masses of 285 (0-10 min), 1002 (10-17 min) and 655 (17-50 min), all with normal optimization. Auto MS^n settings for two precursor ions were an absolute threshold of 100,000 and a relative threshold of 5%. Ions are excluded after two ions and released after 0.5 min. The acquisition parameter had a fragment amplitude 1.0 V, with an average of 5.0 and an isolation width of 3.0 m/z. Auto MS (n>2) settings with one precursor ion were as follows: absolute threshold of 1,000, relative threshold 5%, and fragment amplitude 1.0V.

Standard Collection: Initially, individual BHP compounds were fraction-collected from Bear Meadows, an acidic bog, sediments (Rothrock State Forest, PA). Each collected fraction was additionally purified via neutral phase separation on a silica column (150 mm x 3.0 mm, Waters) and an isocratic flow of 80:30 hexanes:isopropyl alcohol (IPA) at 1 ml/min. The fractions were then carefully transferred, dried and weighed using tin EA cups on a nanogram scale.

Calibration curves and limits of detection: Response factors were determined across three orders of magnitude by injection of 1 ng, 10 ng, 20 ng, 50 ng, 100 ng, and 200 ng of each standard compound. Peak areas were normalized to the peak area of the internal standard, pregnanediol (Sigma Aldrich). Normalization to an internal standard accounts for instrument drift between injections as well as potential variations during pipeting and injection. Normalized data were used to calculate a linear regression between peak areas of each compound of interest (divided by peak area of pregnanediol) and known mass injected. The equation of the resulting linear fit was used to calculate the

amount of each compound in environmental samples. Signal-to-noise ratios averaged 20 for analysis of pregnanediol for injections containing 50 to 200 ng.

Results

Environmental samples from Lower Red Eyes acid mine drainage and the snotitte biofilm and cultured samples from *Acidithiobacillus thiooxidans* Mu1 produced BHPs (Figure 4-1). The snotitte biofilm contained four BHP structures: bacteriohopanetetrol (m/z 655), bacteriohopanetetrol cyclitol ether (m/z 1002), bacterioaminotriol (m/z 714), and bacterioadenosyll (m/z 746). The total amount of BHPs produced by the snotitte biofilm was 335,000 μg/gTLE (Figure 4-1 & 4-2). *Acidithiobacillus sp.* Mu1 isolated from the snotitte biofilm produced two BHP structures, bacteriohopanetetrol cyclitol ether (m/z 1002) and bacteriohopanepentol cyclitol ether (m/z 1060) (Figure 4-1). The Mu1 cultures showed an increase in total BHPs (1,000 – 6,000 μg/g TLE) with a decrease in pH (4 – 2). Also the ratio of BHTetrol cyclitol ether (m/z 1060) to BHTriol cyclitol ether (m/z 1002) decreased as the pH decreased (1.97 – 0.42) (Figure 4-3).

The ten Lower Red Eyes samples contained bacteriohopanetetrol (m/z 655), bacterioaminotriol (m/z 714), bacteriohopanepentol cyclitol ether (m/z 1060), bacteriohopanetetrol cyclitol ether (m/z 1002), and bacteriopentose (m/z 943) (Figure 4-4). The amount of BHPs in the samples was strongly related to *A. ferrooxidans* population sizes (0 – 65% *A. ferrooxidans* and 0 – 214,000 μg/g TLE). The samples with no detectable *A. ferrooxidans* and the sample dominated by *Euglena* (LPB green) contained no BHPs. The amount of *Ferrovum* bacteria was indirectly proportional to the

amount of *A. ferrooxidans*. A. *ferrooxidans* showed an order of magnitude increase in BHPs below pH 3. This was concurrent with an increase in cell numbers of *A. ferrooxidans* in the biofilms (Figure 4-5 & 4-6).

Discussion

Although *Acidithiobacillus sp.* Mu1 and *Acidithiobacillus ferrooxidans* both have *shc* genes (Jones et al., in review; Welander et al., 2010), this is the first reported analysis of BHPs in an *A. thiooxidans* pure culture. These findings show that acidic environments involving sulfur and iron oxidizing bacteria must be considered when assessing the paleoenvironment of ancient sediments containing hopanes.

Differences in functional groups of BHPs can be indicative of specific bacterial lineages. Pure culture studies (152 species) have shown that bacteriohopanetetrol (m/z 655) is produced by diverse bacterial lineages and is therefore not diagnostic for specific bacterial taxa (Talbot et al., 2008 and references therein). Aminotriol (m/z 714) is produced by cyanobacteria, purple non-sulfur bacteria, all Type II methanotrophs tested thus far and some Type I methanotrophs (Neunlist et al., 1985, 1988; Talbot et al., 2007a,b; Talbot et al., 2001 and references therein). BHT-pentose (m/z 943) is thought to be specific to cyanobacterial production (Talbot et al., 2009). Adenosylhopane (m/z 746) is produced by *Rhodomicrobium vannielli*, *Rhodoblastis acidophilus*, *Rhodopseudomonas palustris* and *Alicyclobacillus acidocaldarius* (Neunlist et al., 1985, Talbot et al., 2007a). BHT cyclitol ether (m/z 1002) is produced by *Acetobacter* spp., *Methylobacterium fujisawaense*, *Rhodopseudomonas* spp., *Rhodoblastus acidophilus* and

Alicyclobacillus acidocaldarius (Talbot et al., 2007a, Neunlist et al., 1988). Finally, BHpentol cyclitol ether (m/z 1060) is produced by *Acetobacter* (acetogen), *Chlorogloepsis* (cyanobacterium), *Frateuria* and *Burkholderia* species (proteobacteria) (Neunlist et al., 1985).

Based on the above pure culture findings and the Mu1 culture results, we interpret that BHTriol cyclitol ether (m/z 1002) and BHTetrol cyclitol ether (m/z 1060) could be contributed by the *A. ferrooxidans* in the Lower Red Eyes environmental samples. Also, BHT pentose (m/z 943) is most likely not specific to cyanobacterial production because it is unlikely that cyanobacteria are growing at the low pH of the Lower Red Eyes samples based on FISH microscopy descriptions of the samples (Brown et al., 2011).

The snotitte biofilm contains more BHP structures and significantly more BHPs than the Mu1 pure culture as a percentage of total lipids. The decrease in structures and the significant decrease in abundance could be a physiological response to the culturing conditions that are different than those of the biofilm such as planktonic habitat, higher pH, warmer temperature, and differing sulfur source. Also, there is evidence that the *Acidimicrobium* species present in the snotitte contains an *shc* gene. Therefore, it could be producing the other structures (BHT, aminotriol, and adenoylhopane) and contributing to the larger abundance of BHPs (Jones et al., in review). The larger abundance of BHP structures could also be a physiological response to lower pH (snotitte pH 0-1 versus cultures pH 4-2) as our results indicate there is a significant increase in BHP abundance at lower pH in the pure cultures.

The Mu1 culture results indicate that increased BHP production indeed may be a physiological response to lowered pH. *A. thiooxidans* shows a significant increase in

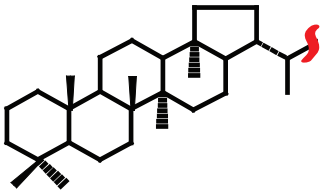
BHPs below a pH of three, concurrent with an increase in BHTtriol cyclitol ether (m/z 1002). BHTriol cyclitol ether (m/z 1002) has nine functional groups with very negative estimated pKas (-3.8 to -3.0) (Figure 4-1 & Figure 4-7). With the extremely negative pKas of the functional groups of both BHTriol and BHTetrol cyclitol ether the functional groups will stay deprotonated at all viable pHs. If the headgroups of the BHPs are placed in the periplasm or in the extracellular environment the negative charge will help balance the charge potential of the cell in the positive acidic environment. Kar and Dasgupta (1996) studied the surface charge density and potential in *Thiobaccilus ferrooxidans* and found that with increased pH surface charge density and surface potential become less positive. This matches our results that less BHPs are produced at increased pH and indicates that decreasing the surface charge potential may be a role of highly functionalized BHPs in acidophiles. The Lower Red Eyes samples show an increase in BHPs at lower pH that is concurrent with an increase in *A. ferrooxidans* and a decrease in *Ferrovum* cells. This indicates that *A. ferrooxidans* is the BHP producer of the system while *Ferrovum* is most likely not producing BHPs. Previous studies have indicated that hopanoid analogues, cholesterol and diplopterol, have been shown to limit water wire formation, and therefore, decrease proton conductance across lipid bilayer membranes (Haines et al., 2001). Highly functionalized BHP structures (BHTriol cyclitol ether and BHTetrol cyclitol ether) make up a significant fraction of BHPs in the Mu1 cultures and Lower Red Eyes samples and may play a role in decreasing surface potential as well as decreasing water wire formation as acid adaptations (Kar and Dsgupta, 1996).

Conclusions

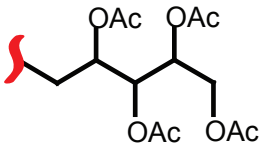
This study presents the first analysis of a pure culture of *A. thiooxidans*, Mu1, and it's BHP production across a pH gradient. The Mu1 strain increases the production of BHPs with a decrease in pH below three while increasing BHTriol cyclitol ether (m/z 1002). We also strongly relate cell numbers of *A. ferrooxidans* with BHP concentrations in the Lower Red Eyes samples suggesting they are the main BHP producers in the system. These results suggest that BHPs are important in bacterial adaptations to acid by decreasing proton permeability through the blocking of water wires and decreasing the charge potential across the membrane by addition of highly functionalized BHPs. Therefore in studies of paleoenvironments, acidophilic iron and sulfur oxidizing organisms should be considered as contributors of significant amounts of hopanoids.

Acknowledgements

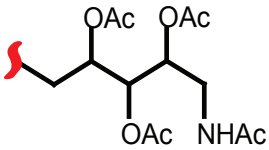
Thanks to Juliana Brown, Dan Jones, Dan Mills and William Burgos for sample collection and geochemical measurements. Thanks to Kara Cahoon for assistance in culturing and Laurie Eccles for assistance with lipid extraction.



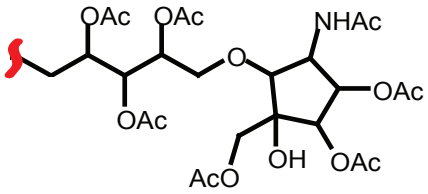
BHP ring structure



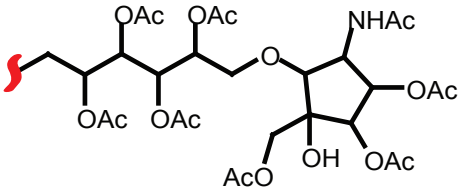
m/z 655 BHT



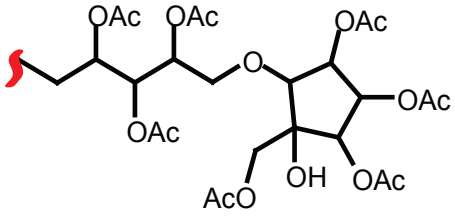
m/z 714 Aminotriol



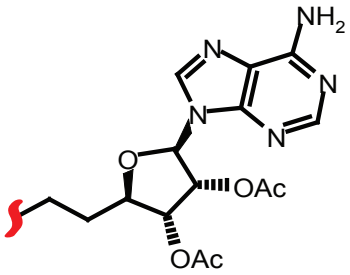
ı/z 1002 BHtriol Cyclitol Ether



m/z 1060 BHtetrol Cyclitol Ether



m/z 943 BHT-pentose



m/z 746 Adenosyl

Figure 4-1. The Bacteriohopanepolyols (BHPs) present in the snotitte biofilm, *A. thiooxidans* Mu1 cultures and Lower Red Eyes surface sediments in this study.

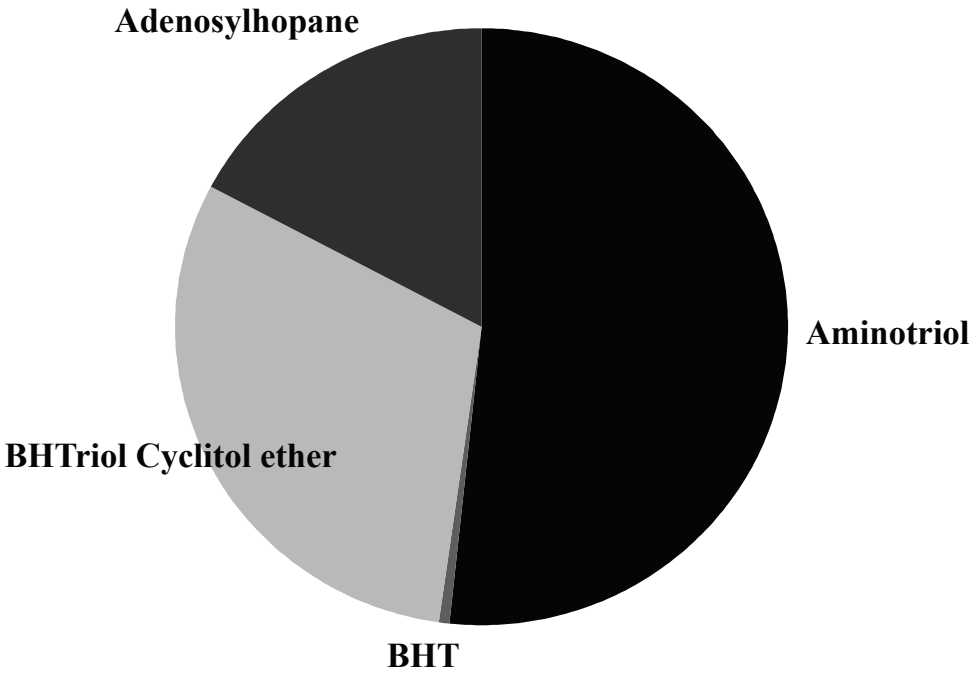


Figure 4-2. The BHP concentration (159,147 µg/gTLE, 16% BHPs of total lipids) and structures present in the snotitte biofilm (RS05-24c) of Frasassi cave system, Italy.

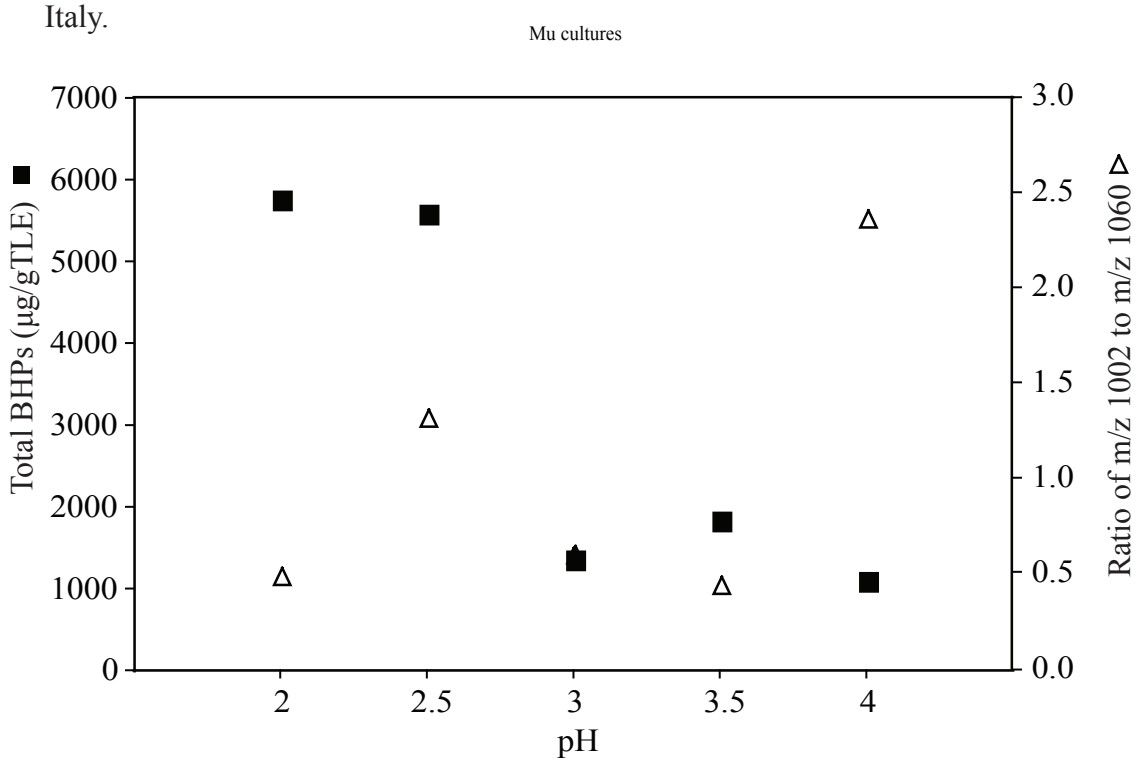


Figure 4-3. The *Acidithiobacillus sp.* Mu1 total BHP concentration (squares) and ratio of BHtriol cyclitol ether (m/z 1002) and BHTetrol cyclitol ether (m/z 1060) (diamond) in relationship to the pH of the culture.

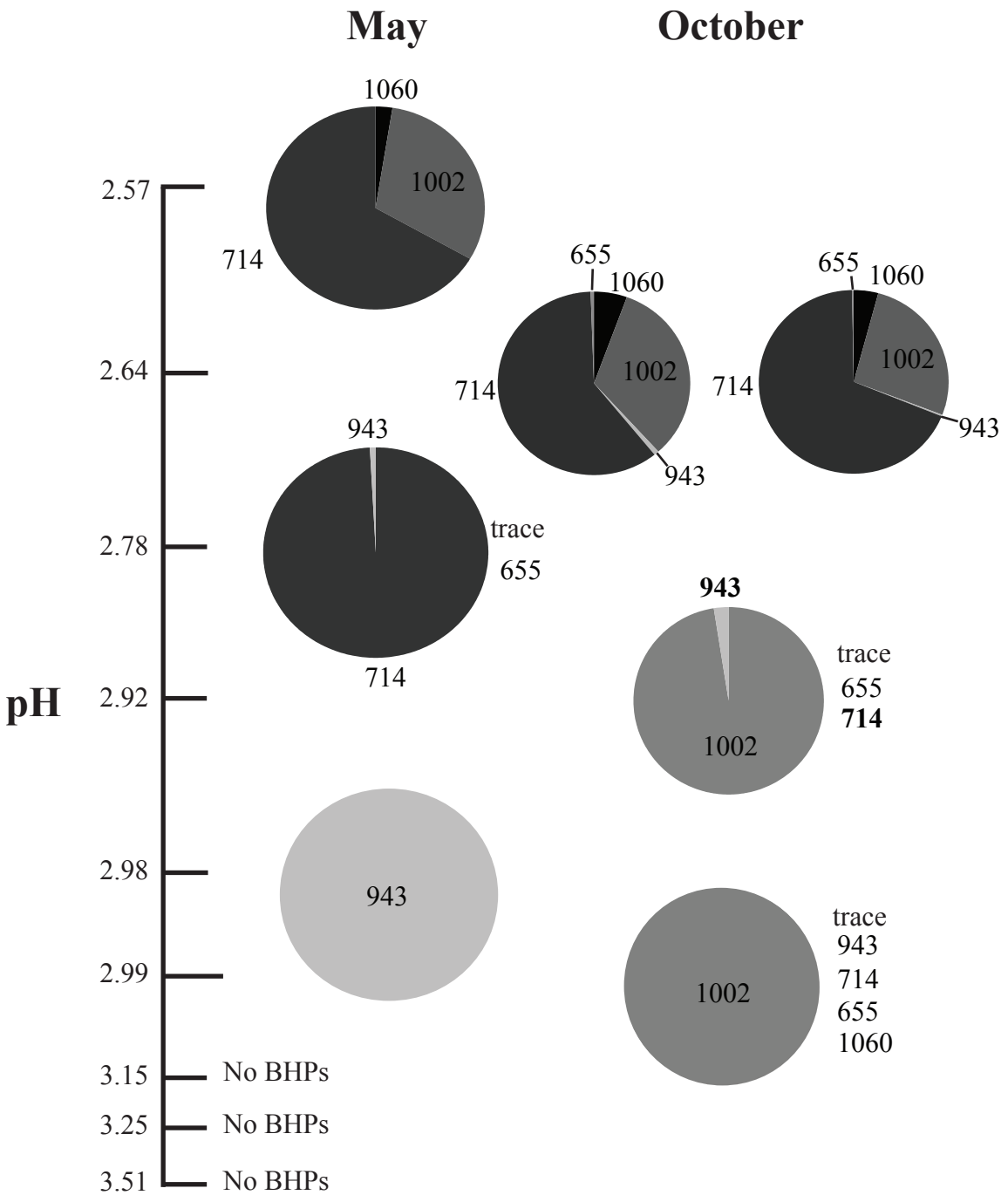


Figure 4-4. BHP structures (m/z) and abundances in the biofilms of the Lower Red Eyes Acid Mine Drainage Site. Samples are organized by month sampled and pH of the surrounding waters.

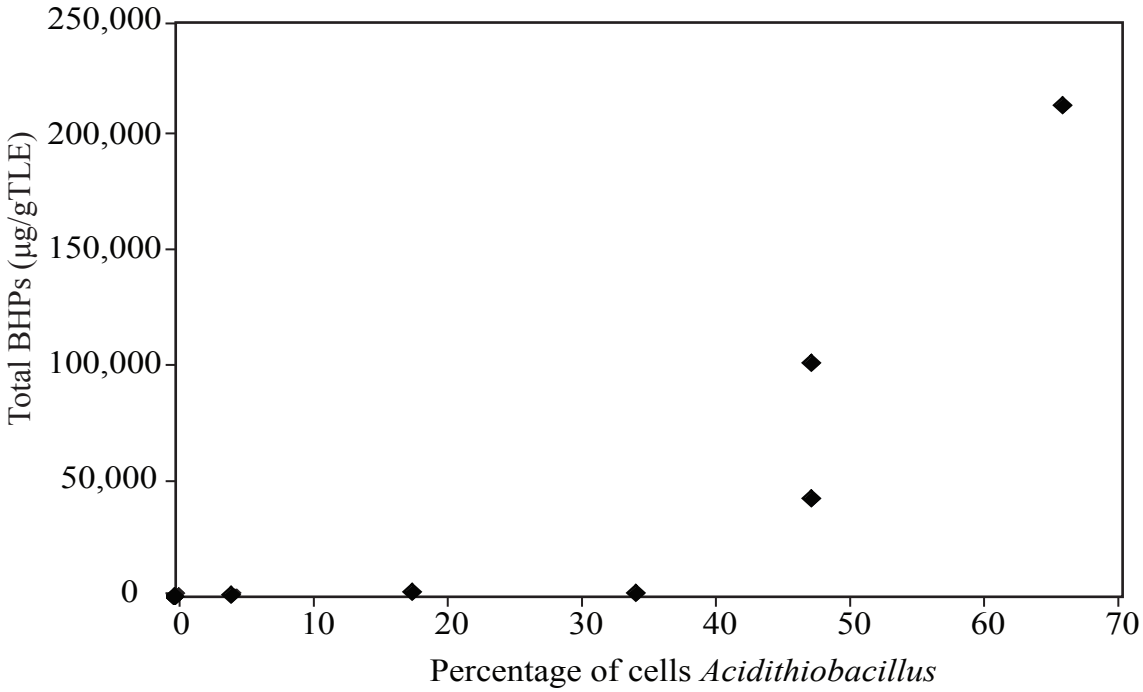


Figure 4-5. Total BHPs in relationship to the percentage of *Acidithiobacillus* cells in the Lower Red Eyes acid mine drainage biofilms. Cells counted by fluorescent *in situ* hybridization (Brown et al., 2011).

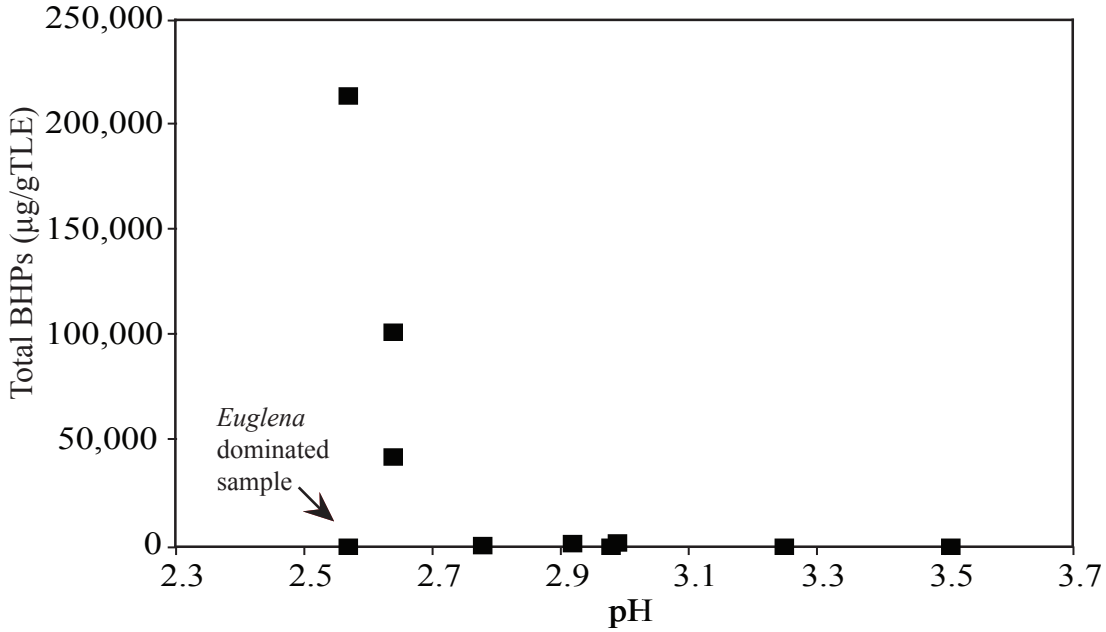
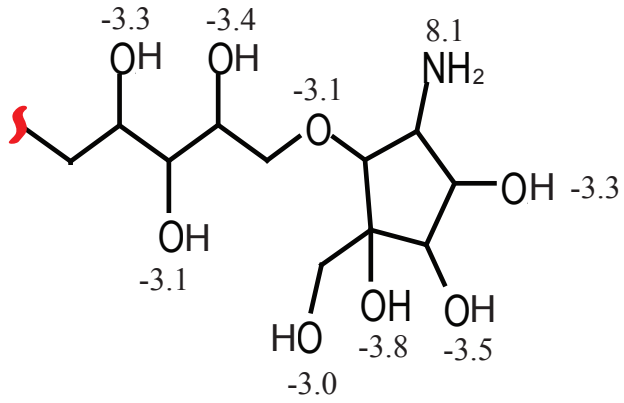
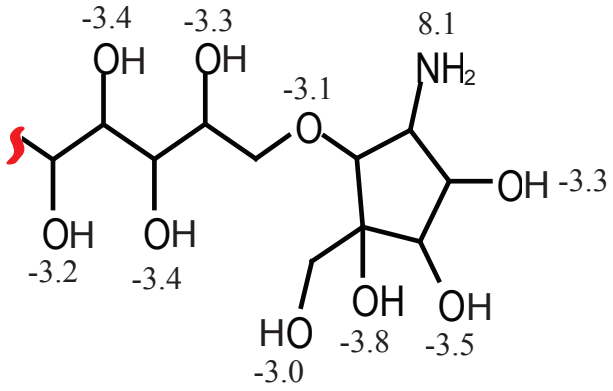


Figure 4-6. The relationship between total BHPs and pH in the Lower Red Eyes acid mine drainage biofilms.



m/z 1002 BHtriol Cyclitol Ether



m/z 1060 BHtetrol Cyclitol Ether

Figure 4-7. The estimated pKas of BHTriol Cyclitol Ether and BHTetrol Cyclitol Ether.

Table 4-1. Lower Red Eyes samples.

Sample Site	Date Sampled	distance from emergence (m)	% A. thiobaccilus of total cells	total Fe (mg/L)	Ferrous_Fe2 (mg/L)	pH
BP	May-09	10.36	0	514.46	548.73	3.41
SPA	May-09	13.56	0	507.75	421.66	3.15
LPA	May-09	26.82	0	460.83	316.24	2.98
SPB	May-09	50.6	4.2	398.27	256.41	2.78
LPB	May-09	58.83	65.8	340.18	230.2	2.57
BP	Oct-09	10.36	0	486.78	399.2	3.51
SPA	Oct-09	13.56	0	424.22	329.26	3.25
LPA	Oct-09	26.82	17.6	353.71	218.37	2.99
SPB	Oct-09	50.6	34.2	201.88	97.81	2.92
LPB	Oct-09	58.83	47.2	231.45	110.89	2.64

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Chapter 5

Production of bacteriohopanepolyols by *Leptospirillum* in the environment and in culture

Introduction

Bacteriohopanepolyols (BHPs) are bacterial biomarkers consisting of a pentacyclic triterpenoid lipid with a six-carbon side chain that is functionalized by sixty-three possible functional groups (Talbot et al., 2003abc; 2007ab; 2008). Hopanes are the geologically preserved hydrocarbons derived from BHPs. They are recalcitrant, found in rocks 2.7 Ga (Brocks et al., 2003) and ubiquitous in the rock record (Albrecht and Ourisson, 1992). Therefore, they have the potential to be excellent biomarkers, but biomarker analysis is hindered because we are still identifying the bacterial sources of BHPs and their physiological function within the cell.

Because of their geometry and their location in bacterial membranes, BHPs may play a role analogous to sterols by lowering membrane fluidity (Demel et al., 1976; Poralla et al., 1980; Rohmer et al., 1976; Kannenberg et al., 1980; Kannenberg and Poralla, 1982; Kannenberg et al., 1983). BHPs are known to increase in abundance as a response to temperature (*Frateuria aurantia* and *Alicyclobacillus acidocaldarius*), exposure to ethanol (*Zymomonas mobilis*), and decrease in pH (*Alicyclobacillus acidocaldarius*) (Joyeaux et al., 2004; Schmidt et al., 1986). BHP content in *Rhodopseudomonas* did not increase with a decrease in pH (Welander et al., 2009). In contrast, BHP concentration in *Streptomyces scabies* did not change in response to

oxidative stress (0.3-3.0 μmol H_2O_2), pH (7.3-5.0), exposure to ethanol (1-10% vol/vol) or high temperature (25-42 ºC) (Seipke and Loria, 2009). These studies have revealed that the physiological response of bacterial BHP production to environmental stimuli varies with different organisms. It is interesting to note that Berry et al., (1993) have shown that BHP production increases with nitrogen fixation in *Frankia* spp. *Frankia* produce an internal vesicle (nitrosome) housing the oxygen-sensitive nitrogenase enzyme. The nitrosome when isolated, contained BHPs, which may serve to lower the oxygen permeability of the nitrosome membrane. Therefore, we are interested in BHP production in nitrogen fixing bacteria under nitrogen fixation and nitrogen assimilation conditions. The goal of this work is to investigate BHP production in *Leptospirillum ferrooxidans* L3.2 in varying growth conditions; biofilm, planktonic, anoxic, oxic, nitrogen assimilative and nitrogen fixating.

Leptospirillum ferrooxidans L3.2*,* an extremely acidophilic iron oxidizing bacterium, was isolated from a biofilm in the Tinto River, Spain (Parro and Moreno-Paz, 2003). The biofilm is at the headwaters of an acidic stream (pH=2) that drains an iron-rich rock formation. *Leptospirillum* species are common in acid mine drainage sites and biohydrometallurgy reactors (Bond et al., 2000; Shrenck, 1998). They are gram negative, with helical curved rod morphology (Pvovarova et al., 1981), and are members of the Nitrospira lineage (Hippe, 2000; Lane et al., 1992). *L. ferrooxidans* L3.2*.* isolate is a nitrogen-fixing chemolithoautotroph and related isolates can be grown at a pH range from 1.1-1.7 (Breed and Hansford, 1998; Parro and Moreno-Paz, 2003). *L. ferrooxidans* L3.2. is found in the Tinto River in locales with a pH range of 0.8 to 2.4, although it is

dominant in the lower pH biofilms (Gonzalez-Toril et al., 2003). It is also thought to be present in the nearby lake 3.2 in a planktonic lifestyle (Parro, unpublished).

Microarray studies of the *L. Ferrooxidans* L3.2 strain have been performed on environmental samples in both biofilm and planktonic growth habitats and on nitrogen fixation and assimilation cultures (Parro and Moreno-Paz, 2003; Moreno-Paz et al., 2010). These studies show an up regulation of genes involving cell envelope modification, energy production, chemotaxis, pumping mechanisms, and sensing and signal transduction when the strain is fixing nitrogen (Parro and Moreno-Paz, 2003). The environmental biofilm samples show an up regulation of genes involved in acid fermentation, cell wall structures, motility and quorum sensing, while the planktonic samples show a more active TCA cycle and ABC transport systems, used for phosphate and potassium transport (Moreno-Paz et al., 2010). Moreno-Paz et al. (2010) theorize that the biofilm matrix protects *Leptospirillum* cells from the high salt concentration of the water (more than 70 g/L of SO_4^{2-}) and therefore they do not need to up regulate cation transporters. They also suggest that the biofilm cells experience an upregulation of acid fermentation metabolism because of acetate production in the biofilm whereas the planktonic cells are using the TCA cycle.

Previous studies have identified the squalene hopene cyclase gene *(shc*), which encodes for the protein (SHC) that converts squalene into hopene, and the gene *hpnP*, which encodes for the protein that adds the methyl group on the second carbon of ring A. (Wendt et al., 1997; Thoma et al., 2004; Welander et al., 2010). The *shc* gene is well represented among acidophilic bacteria, including a *Leptospirillum* species, although no *Leptospirillum* species has been analyzed for BHPs (Pearson et al., 2007). BHPs and the

shc gene have been found in *Acidithiobacillus ferrooxidans*, *Acidimicrobium capsulatus*, *Alicyclobacillus acidocaldarius*, numerous acetic acid bacteria and thermo-acidophilic bacilli (Rohmer et al., 1984; Langworthy and Mayberry, 1976; Poralla et al., 1984; Hippchen et al., 1981). The common occurrence of the *shc* gene and BHPs in acidophilic bacteria suggests it has an important role in membrane permeability. Decreasing membrane permeability of protons is an adaptation of acidophilic bacteria to growth at low pH (Baker-Austin and Dopson, 2007).

Acidophiles use multiple adaptations to live in low pH including making the cell membrane impermeable to protons, reducing pore size of membrane channels, inhibiting proton influx by creating a Donnan potential, pumping out protons, and cytoplasmic buffering (Baker-Austin and Dopson, 2007). One common physiological change to decrease membrane permeability is to increase the concentration of terpenoids (lipids derived from five-carbon isoprene units that include sterols and BHPs) and saturated fatty acids (Mykytczak et al., 2010; Baker-Austin and Dopson, 2009). Hopanoids are thought to influence membrane permeability by decreasing water content within the bilayer and by blocking the continuity of water wires through the membrane (Haines et al., 2001). Water wires are a proposed mechanism for proton leakage through lipid bilayers where a transient, transmembrane chain of water molecules forms and passes protons through the membrane (Haines et al., 2001; Brewer et al., 2001; Brookes et al., 1997). Liposome experiments can specifically test the properties of membranes containing triterpenoids (C_{30} isoprenoid lipids e.g. BHPs and sterols). For example, Haines et al., (2001) show that ion permeability is decreased in membranes that have an increase in cholesterol and diplopterol. In contrast, Lande et al., (1995) use a mixture of sterols, sphingomyelin and

acyl chain saturations and find that decreased membrane fluidity strongly correlates to decreased permeability to water, urea, acetamide, and ammonia but a weak correlation to proton permeability (1995).

Methods

Sample Collection: Samples were collected on an expedition to Rio Tinto, Spain in May 2010. Biofilm biomass was collected with sterile pipettes into falcon tubes. Water filters were collected by peristaltic pump through two ashed 42 mm glass fiber filters (Whatman A/D 3 μm and A/E 1 μm). Samples were preserved in the field upon collection in RNAlater. RNAlater was washed from the biomass and filters with three rinses of phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4). Further, RNAlater was extracted (using BHP extraction method below) and contains no BHPs at a detection limit of 667 ng/1 RNAlater.

Culturing: Because other factors such as oxidative stress, growth phase, and temperature potentially have been shown to affect BHP production in previous studies, we controlled these variables in order to focus on the effect of nitrogen fixation. *L. ferrooxidans* L3.2. was cultured at Spain's Centro de Astrobiologia in the Parro Lab. Cultures were grown in a low phosphate Mackintosh medium at 30°C; shaking at 160 rpm, under aerobic conditions, in the light (Mackintosh, 1978; Parro and Moreno-Paz, 2003). Harvesting was restricted to the exponential phase, based on optical density at 600 nm referenced to a growth curve established for the culture.

Lipid Extractions. Environmental and culture samples were freeze-dried, homogenized, weighed, and then extracted using a microscale modification of the protocol prescribed by Talbot *et al*., (2003a). Dichloromethane (DCM) was substituted for chloroform in this procedure. Total lipid extracts were subdivided and a fraction was acetylated (1:1 pyridine: acetic anhydride, heated 1 hr 60°C). Identification of BHPs was done with an Agient LC ion trap mass spectrometer 6310 based on MS^3 published spectra (Talbot et al., 2003a; 2003b; 2003c; 2007a; 2007b; 2008). Quantitation of BHPs was based on response factors of fraction-collected standards and normalization to an internal standard, pregnanediol.

Chromatographic and detection conditions: Reverse-phase HPLC was preformed using a Phenomenex Gemini 5 μm C_{18} column (150 mm x 3.0 mm i.d.) and a 5 μm pre-column with the same solid phase. LC/MS settings follow Talbot et al., 2003; briefly, positive APCI mode, mass scanning from 150-1300 m/z, and "Smart" fragmentation setting (i.e., 8000 nA corona voltage, 60 psi nebulizer pressure, dry gas of five l/min, dry temperature of 350 C, vaporizer temperature 490 C). The run was divided into three segments, with target masses of 285 (0-10 min), 1002 (10-17 min) and 655 (17-50 min), all with normal optimization. Auto MS^n settings for two precursor ions were as follows: absolute threshold 100,000, relative threshold 5%, exclude after two ions and release after 0.5 min. The acquisition parameter had a fragment amplitude 1.0 V, with an average of 5.0, and an isolation width of 3.0 m/z. Auto MS (n>2) settings with one precursor ion were as follows; absolute threshold of 1,000, relative threshold 5%, and fragment amplitude 1.0V.

Initially, individual BHP standards were fraction collected following reverse phase separation. Each collected fraction was additionally purified via neutral phase separation on a silica column (150 mm x 3.0 mm, Waters) and an isocratic flow of 80:30 hexanes:isopropyl alcohol (IPA) at 1 ml/min. The fractions were then weighed using tin EA cups on a nanogram scale.

Calibration curves and limits of detection: Response factors were determined across three orders of magnitude of detection by injection of 1 ng, 10 ng, 20 ng, 50 ng, 100 ng, and 200 ng of each standard compound. Peak areas were normalized to the peak area of the internal standard, pregnanediol (Sigma Aldrich). Normalization to an internal standard accounts for instrument drift between injections as well as potential variations during pipeting and injection. Normalized data were used to calculate a linear regression between peak areas of each compound of interest (divided by peak area of pregnanediol) and known mass injected. The equation of this line was used to calculate the amount of compound in environmental samples. Signal-to-noise ratio is twenty for analysis of pregnanediol for mass range of 50 – 200 ng.

Hopanol Derivatization: Total lipid extracts were derivatized to make them GC amenable following Innes et al., 1998. The polar BHP was reacted with 30 mg of periodic acid in 1 ml of THF/water 8:1, shaken for one hour, and quenched with water. This reaction broke all diols and created aldehydes. Second, the aldehydes were reduced to alcohols with sodium borohydride, $NaBH_4$: 10 mg of $NaBH_4$ in 1 ml of ethanol was added, shaken for 1 hr and quenched with 1 ml KH_2PO_4 (100 mM). Finally, terminal alcohols were acetylated with 100 μl each of acetic anhydride and pyridine and heated to

60°C for 1 hr. Reaction products were analyzed by GC/MS (Hewlett Packard 5990) for identification.

GC/MS: Hopanols were analyzed on a gas chromatography mass spectrometer (HP GC 6890, HP quadrapole 5973) to identify methyl positions based on published mass spectra and chromatograms (Figure 2-4). Hopanols were diluted in 8:2 hexane:dcm and injected on a split/splitless injector and a fused silica capillary column (Agilent J&W DB-5, 60m, 0.32mm, 25μm). They were then heated from 60ºC to 320ºC at 6ºC per minute.

Results

Both the biofilm, filamentous nacimiento, that served as the innoculum for *L. ferrooxidans* L3.2 and the lake 3.2 water filters contained four BHP structures, including two methylated forms: bacteriohopanetetrol (m/z 655), 3-methyl-bacteriohopanetetrol (m/z 669), bacteriohopanetetrol pentose (m/z 943) and 3-methyl-bacteriohopanetetrol pentose (m/z 957). Methyl identification was confirmed by GC/MS analysis. The water filters from lake 3.2 also contained bacteriotriol cyclitol ether (m/z1002) and bacterioaminotriol (m/z 714) (Figure 5-2 & 5-3). The *Leptospirillum* specific structures found in the pure culture *L. ferrooxidans* L3.2 were similar in relative proportions in both pure cultures and the filamentous nacimiento biofilm sample (Figure 5-2).

The cultured biomass was not sufficient to quantitatively analyze with good accuracy. The peaks were significantly above the base line but not significantly above the noise. Therefore, we provide the data with reservations for drawing conclusions about any possible patterns within the data. The culture produced all four hopanoid structures

when grown under both nitrogen assimilation and nitrogen fixation conditions. (Table 5-1).

We found that the lake 3.2 water filters had large quantities of BHPs in both the oxic (4,000 μg/g TLE) and anoxic water (76,000 and 91,000 μg/g TLE). In the filter samples, the majority of BHPs were structures not present in the *L. ferrooxidans* L3.2 cultures or the filamentous nacimiento biofilm sample (aminotriol, m/z 714 and BHTriol cyclitol ether, m/z 1002). However, if comparing the structures found in *L. ferrooxidans* L3.2 cultures only, the BHPs increased by an order of magnitude in the anoxic waters (oxic 500 μg/g TLE, anoxic 6,000 and 7,000 μg/g TLE).

Discussion

This is the first reported analysis of BHP production in a *Leptospirillum* species either in pure culture or in environmental samples. Interestingly, C3 methylated BHP structures are present in both cultures and environmental samples. The methylated ring structures are well preserved on a geologic timescale and according to theory, C2 structures are produced by cyanobacteria and *Rhodospeudomonas* and C3 structures by methanotrophs and acetic acid bacteria (Summons et al., 1999; Rohmer et al., 1984; Talbot et al., 2001; Rashby et al., 2008). Therefore, our finding of methylated structures in an unexpected microbial taxon may have important implications for early earth studies, and could challenge previous interpretations of the biomarker (Summons et al., 1999; Talbot et al., 2009).

In past studies, both BHT-pentose (m/z 943) and Me-BHT-pentose (m/z 957) have been assumed to be specific to cyanobacteria (Talbot et al., 2009). Our findings indicate they may be less diagnostic than previously thought. The anoxic pit lake waters contain BHPs not produced by the *L. ferrooxidans* L3.2 cultures (aminotriol m/z 714 and BHTriol cyclitol ether m/z 1002). Most likely, these are contributed by other bacterial species but these structures are common and therefore not diagnostic (Talbot et al., 2008 and references therein). Aminotriol and BHTriol cyclitol ether structures are also found in sediment samples dominated by *Acidithiobacillus ferrooxidans* from the Lower Red Eyes acid mine drainage stream (Chapter 4). Also BHTriol cyclitol ether is a structure produced by *Acidithiobacillus thiooxidans* in pure culture (Chapter 4). Therefore, the other dominant species of Rio Tinto, *Acidithiobacillus ferrooxidans* (Parro and Moreno-Paz, 2003), may be the producer of these structures.

The filamentous nacimiento biofilm and *L. ferrooxidans* L3.2 cultures are major producers of BHPs and 3Me-BHPs. The culture produces three times the amount of BHPs compared to the related biofilm. The exact structures and similar proportions of structures produced in both the biofilm and pure culture indicates that the *L. ferrooxidans* L3.2 is the dominant BHP producer in the biofilm.

This study indicates that *L. ferrooxidans* is capable of producing BHPs in a variety of settings and nutrient conditions: planktonic, biofilm, nitrogen assimilation, and nitrogen fixation. Because BHPs are energetically expensive to synthesize (Kannenberg and Poralla, 1999), BHPs must be playing an important role in *Leptospirillum* cells. BHPs are theorized to decrease membrane permeability by decreasing the probability of water wire formation within the membrane (Kannenberg and Poralla, 1999; Haines et al.,

2001). They may also decrease the charge potential across the membrane by adding negatively charged functional groups to the outer membrane (Kar and Dasgupta, 1996). BHPs may be performing other physiological roles that have not been identified. They are not essential for bacterial growth in *Rhodopseudomonas palutris* TIE-1 and *Streptomycies scabies* as shown by knock out studies but no *shc* mutant has been created in acidophilic bacteria (Welander et al., 2009; Seipke and Loria, 2009).

It is unclear whether *L. ferrooxidans* L3.2 produces more BHPs when fixing nitrogen. In future experiments, we would expect more BHP production in nitrogen fixation cultures, which would agree with results that nitrogen fixation cultures up regulate cell envelope modification genes (Parro and Moreno-Paz, 2003). These results would also agree with an earlier study by Berry et al., (1993) in which the concentration of BHPs increased with nitrogen fixation in a *Frankia* species and was a large proportion of the nitrosome membrane (75%). *Leptospirillum* species are not known to produce a nitrosome but could be increasing BHPs in their outer membrane to limit oxygen permeability, and therefore to protect the nitrogenase enzyme.

The strain-specific BHP structures (BHT, 3Me-BHT, BHT-pentose, and 3Me-BHT-pentose) are present in the filamentous nacimiento biofilm (30,000 μg/gTLE) in two orders of magnitude greater concentration than in the oxic planktonic sample (500 μg/gTLE) and one order of magnitude more than the anoxic planktonic samples (6,000 and 7,000 μg/gTLE). This is consistent with the microarray study showing that cell wall structure genes experience upregulation in the biofilm sample in comparison to planktonic samples (Moreno-Paz et al., 2010) although we cannot normalize to biomass

of *Leptospirillum* in these samples. Therefore the differences may be caused by percentage of biomass contributed by *Leptospirillum*.

It is also interesting that the BHP concentration is an order of magnitude greater in the anoxic waters of lake 3.2 (76,000 and 91,000 μg/gTLE) than the oxic waters (4,000 μg/gTLE) (Figure 5-3). A few obligate anaerobes and facultative anaerobes do produce BHPs although it is not common among anaerobic bacteria tested to date (Hartner et al., 2005; Fischer et al., 2005; Sinninghe Damste et al., 2004; Talbot et al., 2003a; Neunlist et al., 1985; 1988; Renoux and Rohmer, 1985). Again, we have not quantified the microbial community of the lake and cannot normalize the total BHP concentration to percentage of biomass contributed by BHP producers. Therefore, the change in BHP concentration may be a change in the microbial community as opposed to a physiological response to the anaerobic environment.

Previous studies have indicated that hopanoid analogues, cholesterol and diplopterol, limit water wire formation, and therefore, decrease proton conductance across lipid bilayer membranes (Haines et al., 2001). BHPs have not been tested specifically because they are difficult to collect in large quantities. Our results support the theory that BHPs are important to acidophilic bacteria adaptation to acid because there are BHPs present in all the sampled extremely acidic environments of Rio Tinto and other extremely acidic environments (Chapter 4 and Appendix F). In the pure cultures studied with this method thus far the acidophilic cultures produce an order of magnitude greater percentage of BHPs in total lipids (*Anabaena variabilis* 0.01-0.5%, *Acidithiobacillus thiooxidans* Mu1 1.1-5.8%, and *Leptospirillum ferrooxidans* L3.2 5.9-12.9%) (Chapter 4 & 6).

Conclusions

In this study we analyzed the BHP content of *L. ferrooxidans* L3.2 an iron oxidizing bacteria isolated from the Rio Tinto, Spain acid mine drainage system. The *L. ferrooxidans* L3.2 produces 3Me hopanoids under all culture conditions tested and in both biofilm and planktonic growth habitats. The finding of methylated structures may have important implications for early earth studies, as it would challenge previous interpretations of the biomarker (Summons et al., 1999; Talbot et al., 2009). When comparing the structures produced by the *L. ferrooxidans* L3.2 pure culture (BHT, 3Me-BHT, BHT-pentose, 3Me-BHT-pentose) in environmental samples *L. ferrooxidans* in the filamentous nacimiento biofilm produces two orders of magnitude more BHPs than when grown in an oxic planktonic habitat and one order of magnitude more BHPs when grown in an anoxic planktonic habitat. This suggests a larger *L. ferrooxidans* biomass in the biofilm and anoxic waters samples or that BHPs play a role in adaptation to biofilm and anoxic habitats. Lake 3.2 waters most likely have multiple BHP producers in the oxic and anoxic waters based on the BHP structures present, that are not present in the *L. ferrooxidans* L3.2 pure cultures (Aminotriol and BHTriol cyclitol ether).

Acknowledgements

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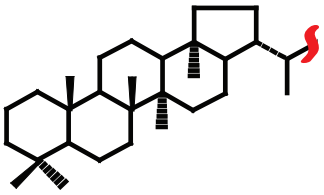
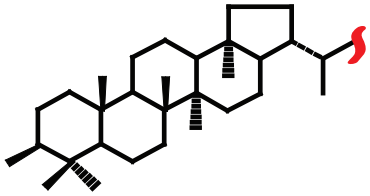
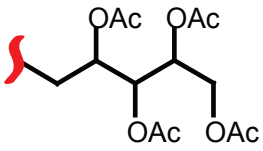


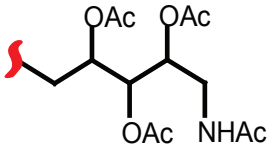
I. Unmethylated BHP
ring structure



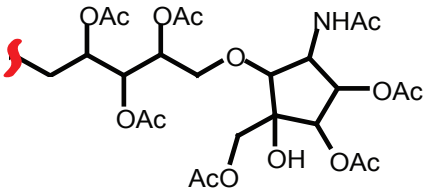
II. 3-Methyl BHP
ring structure



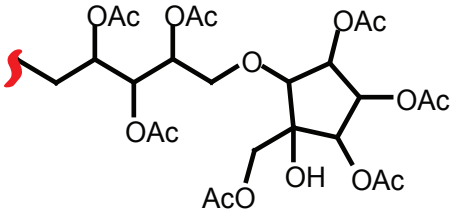
I. m/z 655 BHT
II. m/z 669 3Me-BHT



I. m/z 714 Aminotriol



ı/z 1002 BHtriol Cyclitol Ether



I. m/z 943 BHT-pentose
II. m/z 957 3Me-BHT-pentose

Figure 5-1. The BHP structures identified in Rio Tinto, Spain iron oxidizing biofilms, open water, and *Leptospirillum ferrooxidans* L3.2 culture.

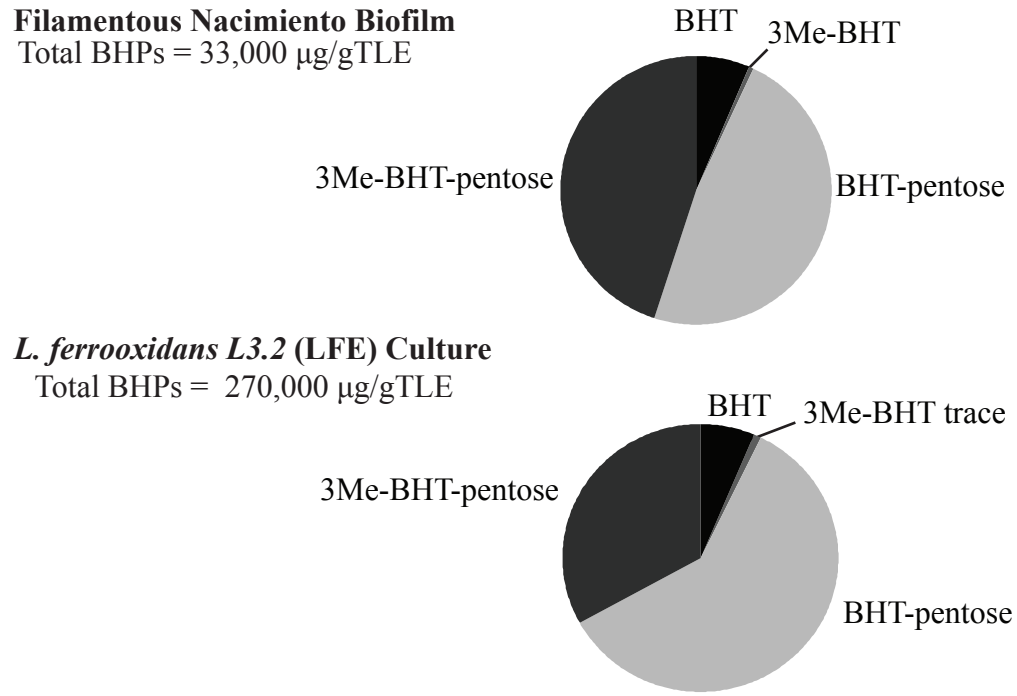


Figure 5-2. BHPs from a Rio Tinto, Spain, iron oxidizing acidic acid mine drainage biofilm (Filamentous Nacimiento) and the isolated *L. ferrooxidans L3.2* culture from the biofilm. Distribution and total abundance indicates the *Leptospirillum* sp. dominates the BHP production of the Filamentous Nacimiento biofilm.

Table 5-1. The *L. ferrooxidans* L3.2 cultures grown with (CSA) and without (SSA) a nitrogen source.

Sample	Total BHPs (µg/g TLE)	655 (µg/g TLE)	669 (µg/g TLE)	943 (µg/g TLE)	957 (µg/g TLE)
CSA-2010 (140910)	59,429	692	trace	49,180	9,557
SSA-2010 (150710)	128,499	6,934	trace	96,814	24,751
CSA-2004	19,277	3,585	197	9,322	6,175
SSA-2004	252	ND	ND	163	89

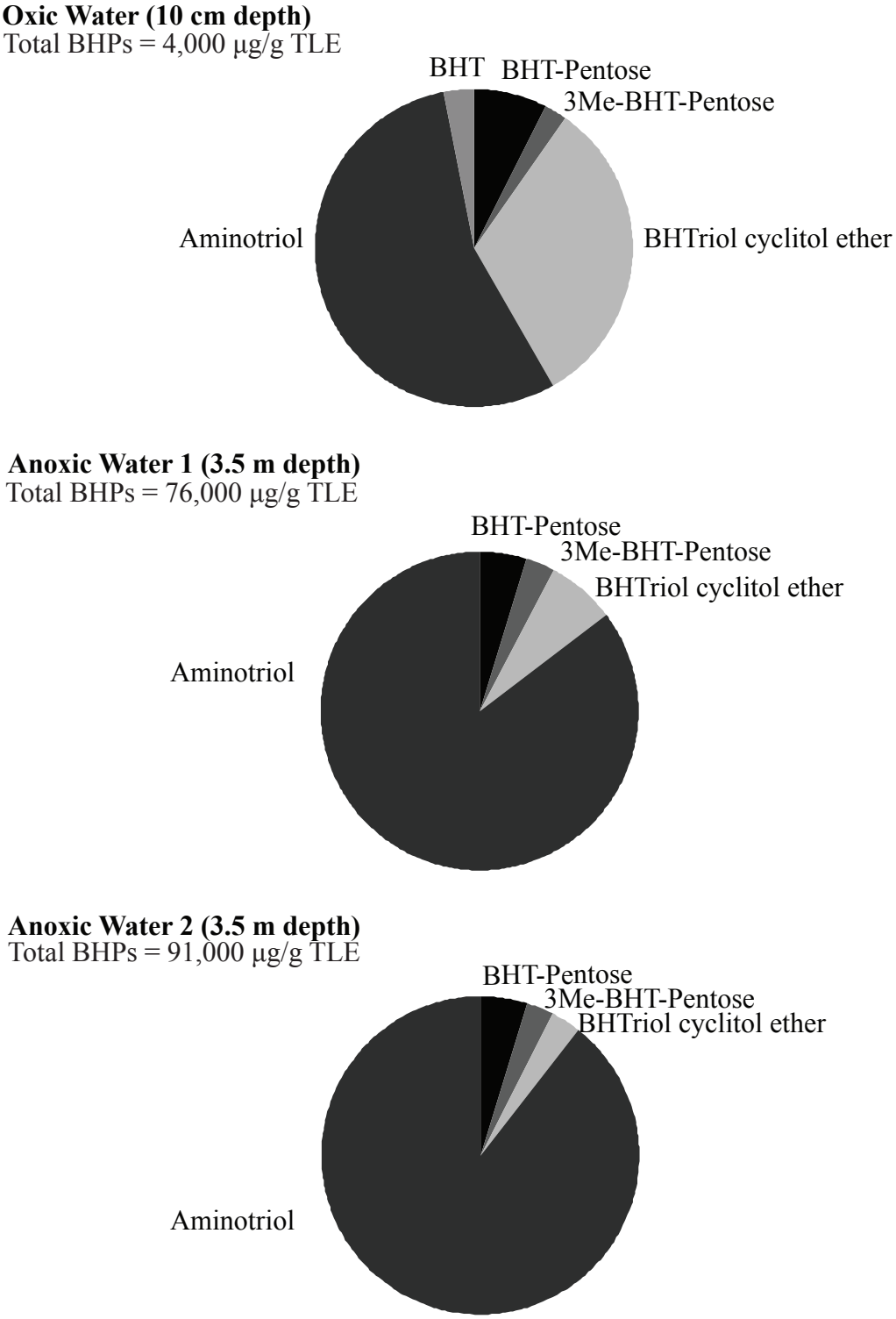


Figure 5-3. BHPs of lake 3.2 Rio Tinto, Spain. Samples were collected with glass fiber filters (A/E and A/D).

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Chapter 6

Bacteriohopanepolyol Production in *Anabaena variabilis* cultures

Introduction

BHPs are bacterial biomarkers consisting of a pentacyclic triterpenoid lipid with a six-carbon side chain that is functionalized by sixty-three possible functional groups (Talbot et al., 2003abc; 2007ab; 2008). Hopanes are the geologically preserved hydrocarbons derived from BHPs. They are very recalcitrant, found in rocks as old as 2.7 Ga (Brocks et al., 2003) and ubiquitous in the rock record following their apperance (Albrecht and Ourisson, 1992). Therefore, they have the ability to be excellent biomarkers, but biomarker analysis is hindered because we are still identifying the bacterial sources of BHPs.

BHP production in cyanobacteria is of particular interest because 2α-Me-hopanes have been used as biomarkers indicative of cyanobacteria and oxygenic photosynthesis timing back to 2.7 billion years ago (Summons et al., 1999; Brocks et al., 1999). Summons et al. (1999) found that 2β-Me-BHPs are produced by nine out of forty-three cyanobacterial cultures tested. Cyanobacteria produced 2β-Me-BHPs in sufficient enough quantities to be the dominant geologic producer of 2α-Me-hopanes (the diagenetic product of 2β-Me-BHPs) even though 2β-Me-BHPs have also been found in Type II methanotrophic bacterium (Renoux and Rohmer, 1985) and in soil nitrogen fixing bacteria (Vilcheze et al., 1994). Several studies have based interpretations of the

beginning of oxygenic photosynthesis on the presence of 2α-Me-hopanes (Brocks et al., 2003; Farrimond et al.2004; Canfield, 2005). More recently 2β-Me-BHPs have been found in additional soil nitrogen fixing bacteria (Bravo et al., 2001) and the anoxygenic photoautotroph *Rhodospeudomonas palustris* (Rashby et al., 2008). In contrast, Talbot et al. (2008) performed a much larger survey of BHP production by cyanobacteria which included marine species and found that there are no modern marine cyanobacteria producing 2β-Me-BHPs. These studies have caused doubt with the interpretation of 2α-Me-hopanes as a biomarker for oxygenic photosynthesis and have shown the need for more investigation into cyanobacterial BHP production.

BHPs are energetically expensive molecules for bacteria to produce (Kannenberg and Poralla, 1999). The ring structure is a squalene molecule, a C_{30} isoprenoid that is cyclised into the hopanoid rings in a cascade reaction that is one of the most complicated one-step reactions known (Corey et al., 1993). This reaction is guided by the squalene-hopene cyclase (SHC) enzyme, a membrane bound protein (Wendt et al., 1997). The squalene-hopene cyclase gene (*shc*) has been identified and is widely distributed throughout marine and freshwater cyanobacterial genera (Pearson et al., 2005; Pearson et al., 2007; Welander et al, 2010). Also, Welander et al. (2010) have identified the gene for C2 methyl addition, *hpnP,* which is present in some cyanobacteria, acidobacteria, and α-Proteobacteria. The gene(s) for C3 methyl addition, tail addition, and functionalization of the tail are yet unknown.

Consistent with the distribution of *shc* genes in cyanobacteria, BHPs have been found in members of the cyanobacteria genera *Anabaena, Chlorogloeopsis, Oscillatoria, Prochlorothrix, Cyanothece, Nostoc, and Synechocystis*, including *Anabaena variabilis*

(Rohmer et al., 1984; Talbot et al., 2008 and references therein). Cyanobacterial taxonomy is complicated by past misidentification on the basis of morphology and is in the process of being reassigned using 16S rRNA analysis (Nubel et al., 1997). Eighty-four pure culture isolates have been studied for BHP production and seventy-four have been found to produce BHPs (Talbot et al., 2008 and references therein) out of hundreds of isolated species (170 isolates in the Pasteur culture collection alone).

In order to use BHPs to their full potential as a biomarker, scientists are working to determine their physiological function. Because of their geometry and their location in bacterial membranes, BHPs may have a homologous function to sterols, which lower membrane fluidity and permeability (Demel et al., 1976; Poralla et al., 1980; Rohmer et al., 1976; Kannenberg et al., 1980; Kannenberg and Poralla, 1982; Kannenberg et al., 1983). In support of this theory studies have shown that BHPs increase in abundance as a response to temperature (*Frateuria aurantia* and *Alicyclobacillus acidocaldarius*), exposure to ethanol (*Zymomonas mobilis*), and a decrease in pH (*Alicyclobacillus acidocaldarius*) (Joyeaux et al., 2004; Schmidt et al., 1986). BHP content in *Rhodopseudomonas palustris* did not increase with a decrease in pH, but the proportion of 2-methyl BHP structures did increase by 50% (Welander et al., 2009). Welander et al. (2009) also show an increase in total BHPs with photoheterotrophic growth (35 μg/mg dry biomass) as compared to chemohetertrophic growth (24 μg/mg dry biomass). Doughty et al. (2009) found that BHP production increased three to four fold in akinetes as opposed to vegetative cells of the cyanobacterium *Nostoc punctiforme*, with a relative increase of 2-MeBHP lipids. In contrast, BHP concentration in *Streptomyces scabies* did

not change in response to oxidative stress (0.3-3.0 μmol H_2O_2), pH (7.3-5.0), exposure to ethanol (1-10% vol/vol) or high temperature (25-42 ºC) (Seipke and Loria, 2009).

These above described studies show contrasting results and indicate that BHPs may have different physiological functions in different bacterial species. Additional studies of bacterial BHP producers under changing culture conditions are necessary to improve understanding of these differing physiological functions. *Anabaena variabilis* ATCC 249713 is a filamentous freshwater cyanobacterium that is valuable for this type of study due to several physiological characteristics. *A. variabilis* can grow as an oxygen producing, aerobic or anaerobic phototroph or as an aerobic heterotroph. Importantly, *A variablis* has been shown to produce a single bacteriohopanepolyol (BHP) structure, bacteriohopanetetrol (BHT m/z 655) (Rhomer et al., 1984; Talbot et al., 2003a).

A. variablis is capable of nitrogen fixation by heterocystic cells that consist of triple membranes and is unique in having three sets of the genes used to produce the nitrogenase enzyme (Thiel et al., 1995). This bacterium can also produce an akinete, a dormant cell type that protects the cell during times of dessication, cold temperatures, or nutrient deprivation (Adams and Duggan, 1999). Berry et al., (1993) showed that BHP production increases with nitrogen fixation in *Frankia* spp.. *Frankia* spp. produces an internal vesicle, analogous to the heterocyst in *A. variabilis*, to protect the nitrogenase enzyme from oxygen by reducing oxygen permeabilty. Analysis of the nitrosome vesicle of *Frankia* showed that it is made of BHPs (76%) as opposed to the heterocyst cell walls the cyanobacterium *Anabaena cylindrica,* which is made of glycolipids (Lambein and Wolk, 1973). Therefore, we are interested in whether BHP production in *A. variabilis* changes under nitrogen fixation and nitrogen assimilation conditions.

In this study, we investigate the changes in BHP production in *A. variabilis* as a function of nitrogen availability (nitrogen fixation, 0 mmol $NaNO_3$, nitrate limitation, 8.8 mmol $NaNO_3$, and maximum nitrate availability, 17.6 mmol $NaNO_3$), phototrophy versus heterotrophy and reduced versus oxidized conditions. Studying *A. variabilis* under these culturing conditions will allow us to determine if BHP production changes with the physiological changes inherent with the changes of nitrate availability as *Frankia* spp. does (Berry et al., 1993) or phototrophy and heterotrophy as in *Rhodopseudomonas palustris* (Welander et al., 2009). Additionally, hopanoid production under varying redox conditions is of interest as a proxy for production in early earth reducing oceans (Canfield, 2005).

Methods

Culturing: *Anabaena variabilis* ATCC 249713 was grown in 300 ml of BG-11 medium with 0, 8.8 and 17.6 mmol $NaNO_3$, shaking at 160 rpm, and at room temperature. Aerobic photosynthetic cultures were grown in a New Brunswick Scientific Innovas 4340 illuminated incubator shaker (400-700 nm, 8.8 Watts/cm^2). Redox shock cultures are reduced medium inoculated with cells grown under aerobic conditions. Redox shock photosynthetic cultures were grown in anaerobic medium degassed with N_2, and headspace of N_2:CO_2 50:50 in the same light box. Chemoheterotrophic cultures were grown with 10 mmol glucose addition to the medium, in the dark. Redox shock chemoheterotrophic cultures were grown in anaerobic medium degassed with N_2, and headspace of N_2:CO_2:O_2 50:40:10. Optical density at 600 nm was used to determine the

growth curves (Figure 6-1). All cultures were harvested during late exponential growth (average 0.25 OD_{600}). Cell morphology was checked for akinete and heterocyst formation (Nikon eclipse 80i).

Lipid Extractions: The cultured biomass was freeze-dried, homogenized, weighed, and then extracted using a microscale modification of the protocol prescribed by Talbot *et al.*, (2003a). Dichloromethane (DCM) was substituted for chloroform in this procedure. Total lipid extracts were subdivided and a fraction was acetylated (1:1 pyridine: acetic anhydride, heated for 1 hr at 60°C). Identification of BHPs was done with an Agient LC ion trap mass spectrometer 6310 based on MS^3 published spectra (Talbot et al., 2003a; 2003b; 2003c; 2007a; 2007b; 2008). Quantitation of BHPs was based on response factors of fraction-collected standards and normalization to an internal standard, pregnanediol.

Chromatographic and detection conditions: Reverse-phase HPLC was performed using a Phenomenex Gemini 5 μm C_{18} column (150 mm x 3.0 mm i.d.) and a 5 μm pre-column with the same solid phase. LC/MS settings follow Talbot et al., 2003; these settings are positive APCI mode, mass scanning from 150-1300 m/z, and "Smart" fragmentation setting (i.e., 8000 nA corona voltage, 60 psi nebulizer pressure, dry gas of five l/min, dry temperature of 350 C, vaporizer temperature 490 C). The run was divided into three segments, with target masses of 285 (0-10 min), 1002 (10-17 min) and 655 (17-50 min), all with normal optimization. Auto MS^n settings for two precursor ions were an absolute threshold of 100,000 and a relative threshold of 5%. Ions are excluded after two ions and released after 0.5 min. The acquisition parameter had a fragment amplitude 1.0 V, with an average of 5.0 and an isolation width of 3.0 m/z. Auto MS (n>2) settings with

one precursor ion were as follows: absolute threshold of 1,000, relative threshold 5%, and fragment amplitude 1.0V.

Standard Collection: Initially, individual BHP compounds were fraction collected from acidic bog sediment samples (Bear Meadows Rothrock State Forest, PA) in reverse phase (Talbot et al., 2003). Additionally, each collected fraction was additionally purified via neutral phase separation on a silica column (150 mm x 3.0 mm, Waters) and an isocratic flow of 80:30 hexanes:isopropyl alcohol (IPA) at 1 ml/min. The fractions were then carefully transferred, dried and weighed using tin EA cups on a nanogram scale.

Calibration curves and limits of detection: Response factors were determined across three orders of magnitude of detection by injection of 1 ng, 10 ng, 20 ng, 50 ng, 100 ng, and 200 ng of each standard compound. Peak areas were normalized to the peak area of the internal standard, pregnanediol (Sigma Aldrich). Normalization to an internal standard accounts for instrument drift between injections as well as potential variations during pipeting and injection. Normalized data were used to calculate a linear regression between peak areas of each compound of interest (divided by peak area of pregnanediol) and known mass injected. The equation of this line was used to calculate the amount of compound in environmental samples. Signal-to-noise ratios averaged 20 for analyses of pregnanediol for mass ranging from 50 to 200 ng.

Results

Growth curves measured by optical density indicate that all cultures (aerobic and redox shocked, photosynthetic and heterotrophic) were grown to similar densities (Figure 6-1). The cultures with a nitrate concentration of 8.8 mmol were considered nitrogen limited. The presence of ammonia or nitrate represses heterocyst formation (Golden and Yoon, 1998). Nitrogen limited cultures had nitrate available for growth (8.8 mmol, 18.8:1 N:P) but produced heterocyst cells to fix nitrogen when biomass consumed the available nitrate. Nalewajko and Murphy (2001) found that an *Anabaena* sp. isolated from Lake Biwa had no change in growth rate over a range of N:P ratios from 1000:1 to 10:1. Our cultures are grown well within their range with N:P ratios from 37.5:1 to 0:1. Therefore, there are no significant difference in the growth curves to differentiate between nitrogen fixation and assimilation (Figure 6-1). No akinetes were detected by light microscopy under these growth conditions. We confirmed past studies that reported *A. variabilis* produces one BHP, bacteriohopanetetrol, BHT (m/z 655) (Rohmer et al., 1984; Talbot et al., 2003). All cultures grown in this study produce BHT (Figure 6-2). Less BHT was produced by the chemoheterotrophic (4 – 400 μg/g TLE) than photosynthetic cultures (900 – 5,500 μg/g TLE). Both photosynthetic and chemoheterotrophic cultures showed that more BHT was produced in the aerobic media (400 – 5,500 μg/g TLE) than in the redox shocked cultures (30 – 1,800 μg/g TLE). Aerobic photosynthetic cultures showed that there was more BHT produced as the *A. variabilis* is fixing nitrogen (2,300 – 5,500 μg/g TLE). Photosynthetic redox shocked cultures indicated that nitrate limited (1,800 μg/g TLE) and nitrogen fixation cultures (1,600 μg/g TLE) produced more BHT than the

nitrate assimilation cultures (900 μg/g TLE). However in contrast to the aerobic photosynthetic cultures the nitrogen limited cultures produced more than the nitrogen fixation cultures. In the heterotrophic cultures, both aerobic and redox shocked, the nitrogen fixation cultures (aerobic 400, redox shocked 30 μg/g TLE) produced more BHT than the nitrate limited (aerobic 200, redox shocked 4 μg/g TLE) and nitrate assimilation cultures (aerobic 300, redox shocked 10 μg/g TLE), which show no differences.

Discussion

The results indicate that BHT production is increased when *A. variabilis* is grown in aerobic media, photoautotrophically, fixing nitrogen. Past studies have shown an increase in BHPs to protect the nitrogenase enzyme in nitrosomes (Berry et al., 1993). During nitrogen fixation the heterocyst cell is formed approximately every ten cells in the filament and consists of a thick layer of laminated glycolipids to lower permeability of oxygen to protect the nitrogenase (Lambein and Wolk, 1973; Krepski and Walton, 1983; Golden and Yoon, 1998). In a past study of *A. cylindrica* heterocysts, cell walls are primarily composed of glycolipids (Lambein and Wolk, 1973). BHT's role is unclear but *A. variabilis* may be using BHT to lower the permeability of the heterocyst even further to oxygen, especially since it is producing oxygen while photosynthesizing.

The decrease in BHT production with heterotrophic growth is similar to the results of a previous study by Welander et al. (2009), which showed that BHP production in *Rhodopseudomonas palustris* decreases when grown heterotrophically. However, Welander et al. (2009) also showed that a mutant strain of *R. palustris* does not need

BHPs to grow photosynthetically, chemoheterotrophically, or photoheterotrophically. *R. palustris* and *A. variabils* both use photosystem I for photosynthesis. Many photosystem I bacteria produce BHPs and contain the *shc* gene (Talbot et al., 2008; Welander et al., 2010). In contrast, *Chloroflexi* and *Chlorobi* all use chlorosomes for photosysthesis and appear to lack both BHP production and the *shc* gene (Rohmer et al., 1984; Talbot et al., 2008; Pearson et al., 2007; Welander et al., 2010). We propose that BHPs may have a role in protecting cells from the oxygen radicals produced by the splitting of water during photosynthesis in the Hill and Mehler reactions (Hill, 1939; Mehler, 1951; Whitehouse et al., 1971).

Hill reaction (where A is an oxidant): $H_2O + A \Rightarrow AH_2 + ½O_2$

Mehler reaction: $H_2O + O_2 \Rightarrow H_2O_2 + ½O_2$

These radicals cause photoinhibition if not efficiently removed or blocked from photosystem I (Storz and Imlay, 1999; Exss-Sonne et al., 2000; Yousef et al., 2003). We propose that BHT may form a protective boundary to the radicals. Lande et al. (2001) showed that BHP-like molecules reduced permeability to radicals in liposome experiments. The reduced medium in the *A. variabilis* redox shock experiments could have absorbed the radicals and therefore suppressed BHP production in comparison to the matching aerobic experiments. Radicals are not produced during the heterotrophic growth and therefore these cultures may only be producing BHPs for protection of nitrogenase in heterocyst nitrogen fixing cells or another unknown function.

Conclusions

A. variabilis ATCC 249713 produced BHT in varying quantities (4-5,500 μg/g TLE) depending on environmental conditions. The results indicate that the BHT in these experiments played a significant role in nitrogen fixation and photosynthetic growth. The heterocyst cells may be using BHT in the triple membrane to protect the nitrogenase enzyme from oxygen in nitrogen fixation. In photosynthetic cultures, BHPs may be used to protect the photosystem from radical damage. Future studies should investigate BHP production during photoheterotrophic growth and akinete formation in *A. variabilis* and other cyanobacteria.

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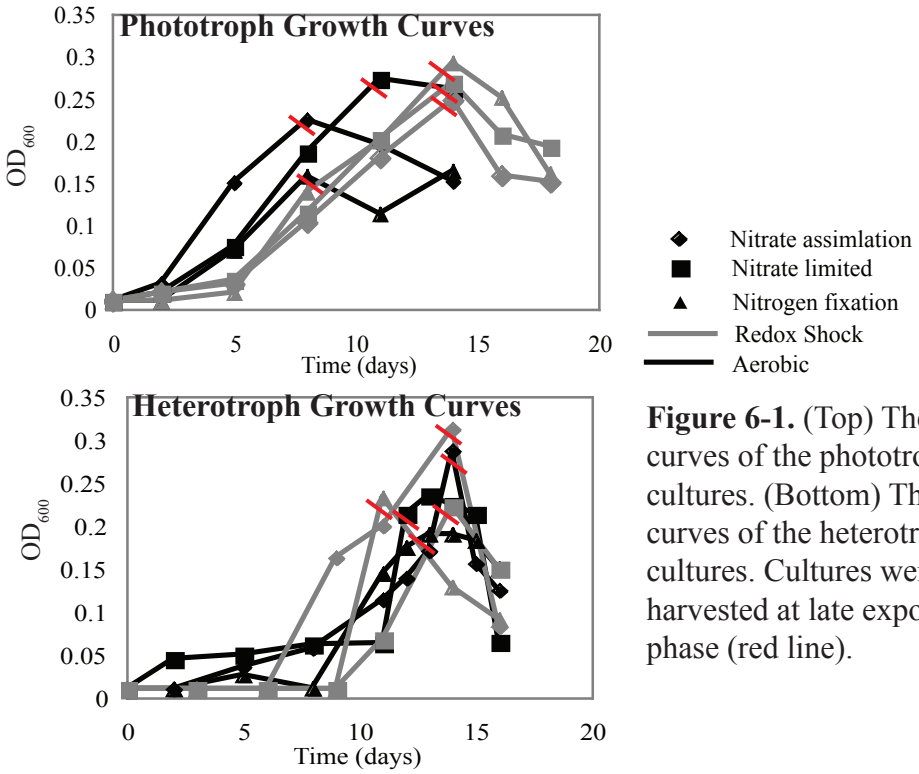

Figure 6-1. (Top) The growth curves of the phototrophic cultures. (Bottom) The growth curves of the heterotrophic cultures. Cultures were harvested at late exponential phase (red line).

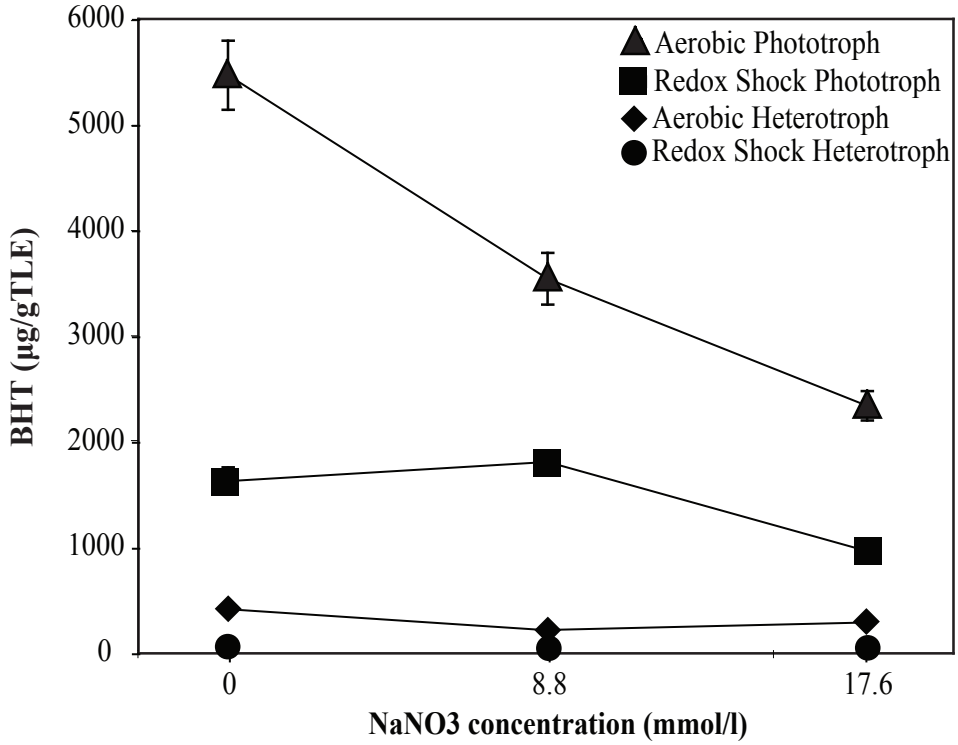

Figure 6-2. The concentration of BHT in *Anabaena variabilis* cultures. Three cultures were grown under each growth condition at each nitrate concentration. Zero nitrate is indicative of nitrogen fixation cultures. 8.8 mmol/l indicates nitrate limited cultures. While, 17.6 mmol/l nitrate indicates normal nitrate concentration.

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Appendix A

Bacteriohopanepolyols of Guaymas Basin Sediments

Introduction

Deep Sea hydrothermal vents are unique and diverse communities discovered in 1977. The vents are formed at seafloor spreading centers and have extreme pressure and steep chemical and thermal gradients. These communities are based on microbial primary production. The discovery of these vents has inspired new theories of life's origins.

The Guaymas basin in the Gulf of California contains a complex system of hydrothermal vents emanating from the Juan de Fuca spreading ridge. The hydrothermal vents are unique in that they are buried beneath hundreds of meters of organic-rich sediments deposited by planktonic and terrestrial organic matter (Balinski et al., 1989). Hot sulfidic waters originating at the spreading ridge permeate through the organic-rich sediments and release petroliferous hydrocarbons (Calvert, 1966; Simoneit, 1983).

The large supply of organic carbon is the source of a thriving and diverse microbial community. Studies have identified and cultured aerobic mesophilic hydrocarbon degraders in the top 30 cm of sediments (Bazylinski et al., 1989; Teske et al., 2002). Sulfate reduction, nitrification, denitrification, and sulfur oxidation metabolisms have all been observed (Canganella F., 1998; Campbell et al., 2001; Teske et al., 2002; Dhillon et al., 2003; Dick and Tebo, 2010). Meanwhile, the sediment water interface is covered in *Beggiatoa*-dominated biofilms (Nelson et al., 1989).

Bacteriohopanepolyols (BHPs) are triterpenoid lipids specific to bacteria with sixty-three possible functional groups (Talbot et al., 2001). BHPs degrade to hopanes, geologically preserved hydrocarbons. Hopanes are very recalcitrant and have been used to indicate bacterial life in the early earth. However, the lack of information about which bacteria produce BHPs, and their function within the cells, causes BHPs to be not useable for specific pale-environmental and paleo-community structures. Studying environments such as the Guaymas Basin to understand the BHP production will provide insight into hopanes for early Earth studies.

Methods

Sample Collection: Sediment samples were collected on two different trips to the basin for lipid analysis (kindly provided by S Wakeham via EA McCliment and Zengler). Guaymas basin sediment samples were collected on the *R/V Atlantis – DSV Alvin* in May 2002. Following recovery, the cores were frozen and returned to a shore-based laboratory for lipid extraction.

Lipid Extractions. Biomass was freeze-dried, homogenized, weighed, and then extracted using 2:1 DCM: Hexane by soxhlet for 12 h. Total lipid extracts were subdivided and a fraction was acetylated (1:1 pyridine: acetic anhydride, heated 1 hr 60°C). Identification of BHPs was done with an Agilent 6310 high pressure liquid chromatography/mass spectrometry (HPLC/MS^n) ion trap with an atmospheric pressure chemical ionization (APCI) chamber. Peak were identified based on MS^3 published spectra (Talbot et al., 2003a; 2003b; 2003c; 2007a; 2007b; 2008). Quantitation of BHPs

was based on response factors of fraction-collected standards and normalization to an internal standard, pregnanediol.

Chromatographic and detection conditions: Reversed-phase HPLC was performed using a Phenomenex Gemini 5 μm C_{18} column (150 mm x 3.0 mm i.d.) and a 5 μm pre-column with the same solid phase. LC/MS settings follow Talbot et al., 2003; these settings are positive APCI mode, mass scanning from 150-1300 m/z, and "Smart" fragmentation setting (i.e., 8000 nA corona voltage, 60 psi nebulizer pressure, dry gas of five l/min, dry temperature of 350 C, vaporizer temperature 490 C). The run was divided into three segments, with target masses of 285 (0-10 min), 1002 (10-17 min) and 655 (17-50 min), all with normal optimization. Auto MS^n settings for two precursor ions were an absolute threshold of 100,000 and a relative threshold of 5%. Ions are excluded after two ions and released after 0.5 min. The acquisition parameter had a fragment amplitude 1.0 V, with an average of 5.0 and an isolation width of 3.0 m/z. Auto MS (n>2) settings with one precursor ion were as follows: absolute threshold of 1,000, relative threshold 5%, and fragment amplitude 1.0V.

Standard Collection: Initially, individual BHP standards were fraction-collected following reverse phase separation. Each collected fraction was additionally purified via neutral phase separation on a silica column (150 mm x 3.0 mm, Waters) and an isocratic flow of 80:30 hexanes:isopropyl alcohol (IPA) at 1 ml/min. The fractions were then weighed using tin EA cups on a nanogram scale.

Calibration curves and limits of detection: Response factors were determined across three orders of magnitude of detection by injection of 1 ng, 10 ng, 20 ng, 50 ng, 100 ng, and 200 ng of each standard compound. Peak areas were normalized to the peak

area of the internal standard, pregnanediol (Sigma Aldrich). Normalization to an internal standard accounts for instrument drift between injections as well as potential variations during pipeting and injection. Normalized data were used to calculate a linear regression between peak areas of each compound of interest (divided by peak area of pregnanediol) and known mass injected. The equation of this line was used to calculate the amount of compound in environmental samples. Signal-to-noise ratio is twenty for analysis of pregnanediol, for a mass range of 50 – 200 ng.

Results

The Guaymas Basin sediments contain BHPs in the upper nine cm in the McCliment sediments and the upper five inches in the Zengler sediments (Figure A-1). The Zengler sediments contain less BHPs (62,000 ug/gTLE all depths) than the McCliment sediments (104,000 ug/gTLE all depths). The Zengler sediments show a steady decrease in total BHPs from the sediment water interface (49,000 ug/gTLE) to lower sediment (five to seven inches) with no BHPs detected. The McCliment sediments have the highest concentration of BHPs in the top three centimeters (78,000 ug/gTLE), a low from three to six centimeters (10,000 ug/gTLE), an increase from six to nine centimeters (16,000 ug/gTLE), and no BHPs detected from nine to fifteen centimeters.

The Guaymas Basin sediment samples contain bacteriohopanetetrol (m/z 655), aminotriol (m/z 714), and aminotetrol (m/z 772). The Zengler sediments and the top McCliment sediment sample show a similar distribution of BHPs, while the lower (three

to nine centimeters) McCliment sediments show a similar distribution of BHPs. No methyl BHP structures are found.

Discussion

Differences in functional groups of BHPs can be indicative of bacterial families. The Guaymas Basin sediments contain bacteriohopanetetrol (m/z 655), aminotriol (m/z 714), and aminotetrol (m/z 772). Bacteriohopanetetrol (m/z655) is the most common BHP and is therefore not diagnostic for specific bacterial taxa (Talbot et al., 2008 and references therein). Aminotriol (m/z 714) is produced by cyanobacteria, purple non-sulfur bacteria, all Type II methanotrophs tested thus far, and some Type I methanotrophs (Neunlist et al., 1985, 1988; Talbot et al., 2007a,b; Talbot et al., 2001 and references therein). Aminotetrol (m/z 772) is produced by all methanotrophs analyzed thus far and is present in ammonia-oxidizing bacteria *Nitrosomonas europaea* (Talbot et al., 2007a), purple non-sulfur bacteria *Rhodomicrobium Vannielii* (Neunlist et al., 1985) and *Rhodopseudomonas palustris* (Talbot et al., 2007a).

Considering the species with these structures identified thus far, it is reasonable to conclude that aerobic methanotrophs are the major source of BHPs in the Guaymas Basin. Past studies have used these structures as evidence for aerobic methanotrophy as well. Wakeham et al. (2007) determined aminotriol, aminotetrol, and aminopentol as evidence for aerobic methanotrophy in the suboxic portion of the water column of the Black Sea. Meanwhile, Blumenberg et al. (2006), studying the northern Black Sea shallow shelf, found aminotriol, aminotetrol, and 3Me-aminopentol as evidence for Type

I methanotrophs. While 3Methyl-BHPs are common among methanotrophs, they are not produced by all methanotrophs (Rhomer et al., 1984; Talbot et al., 2001). In concurrence with our findings, studies have shown the presence of methane, and that its source is both biogenic and thermogenic (Lapham et al., 2008). Also, studies have identified an overabundance of methanotrophic species in a 16S rDNA clone libraries (Lloyd et al., 2010; Dick and Tebo, 2010) and Teske et al., (2002) did not identify 3Methyl-hopanols in Guaymas Basin sediments. According to a brief study of methanotrophs by Talbot et al., (2001) the proportion of tetra- and penta- functionalized compounds is most consistent with Type I methanotrophs.

The Zengler sediments show a consistent set and proportion of BHPs, indicating that the bacterial community producing BHPs is consistent within the top five inches of the core. Contrastingly, the McCliment sediments show a shift in BHP production below three centimeters. This could be a shift in the microbial community of BHP producers or a physiological change in functional group production of the same methanotrophic community. The decrease in total abundance of BHPs with depth is probably due to the lack of aerobic methanotrophic biomass. Lloyd et al. (2010) show that the rate of methane oxidation significantly decreases below five centimeters in similar Guaymas Basin sediment cores. As they are buried, the BHPs are most likely degraded to hopanes, which are not detected by the LC/MS analysis.

Conclusions

Guaymas Basin is a unique organic-rich spreading ridge. This environment provides insight into a possible early Earth situation. BHPs are bacterial-specific lipids that are recalcitrant in their geologic hydrocarbon form, hopanes. By studying the BHPs of the Guaymas Basin, we can identify possible hopane signatures for similar environments in early Earth. Guaymas Basin has no methylated BHPs, but relatively high amounts of BHPs compared to other environments. The BHP functional groups suggest they are produced by aerobic methanotrophs in the Zengler sediments and the top of the McCliment sediments. The lower McCliment sediments indicate a shift in the microbial community of BHP producers or a physiological change in functional group production of the same methanotrophic community. This is difficult to distinguish until more is known about bacterial production of BHPs.

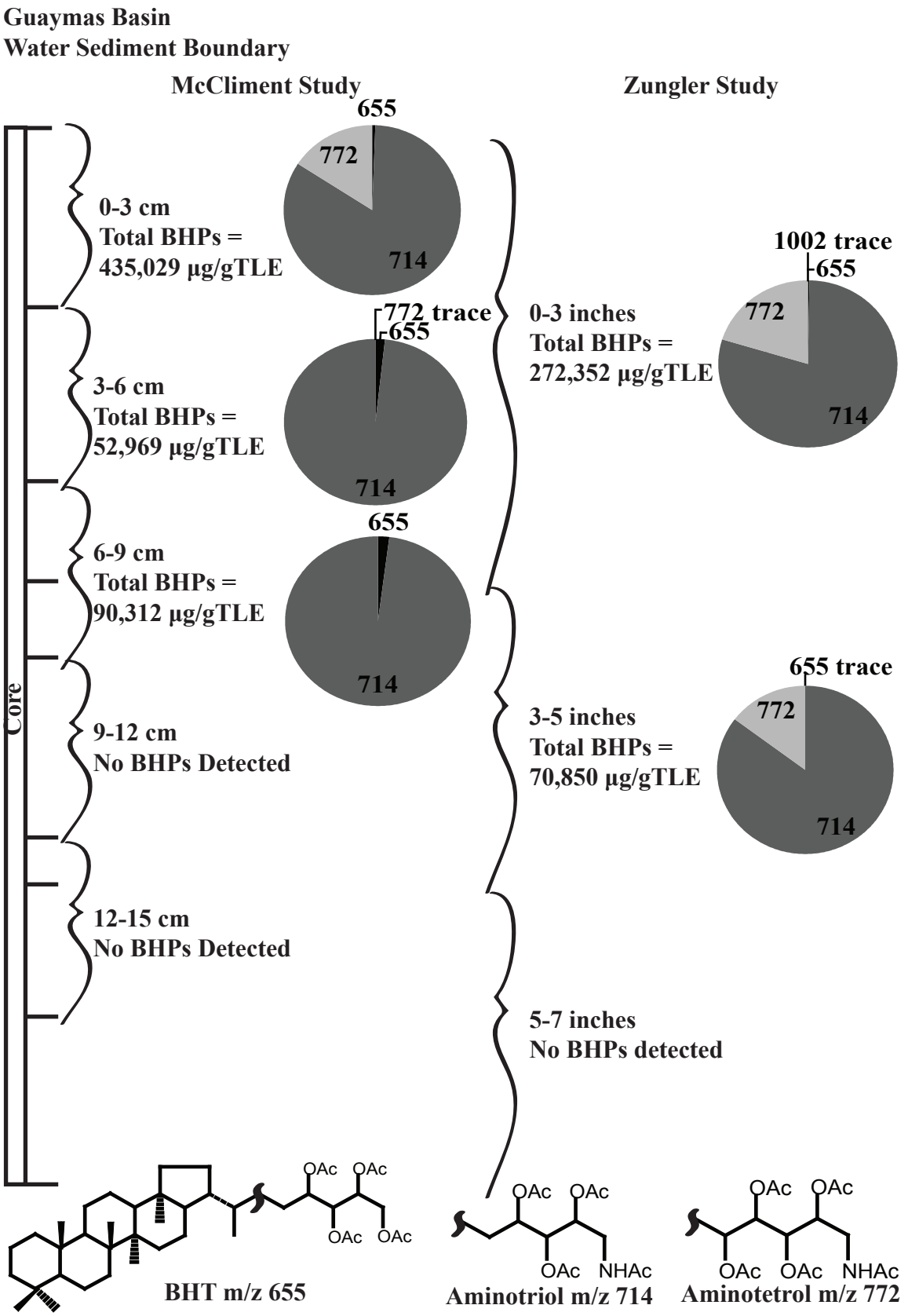

Figure A-1. BHP structures and abundances through two sediment cores.

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Appendix B

Bacteriohopanepolyols from a Marine Sinkhole

Introduction

Sulfide-rich, oxygen-poor environments are widespread in the earth's subsurface and were prevalent at the earth's surface during critical intervals in the earth's past. Modern microbial communities in sulfidic niches have the potential to shed light on the biogeochemistry and biosignatures of anoxia and euxinia in earth history. Karst terrains, including caves and sinkholes, provide rare windows into microbially-dominated, sulfidic subsurface environments that are otherwise difficult and expensive to access. Little Salt Spring is a cover-collapse, karst-solution sinkhole fourteen miles from the Gulf of Mexico in Sarasota, FL. This sinkhole has proven to be the most valuable North American archaeological site because of the anoxic bottom waters that preserve artifacts (Alvarez- Zarikian et al., 2005; Clausen et al., 1975; Clausen et al., 1979; Haynes, 2009; Peres and Simons, 2006). These artifacts have shown that the sinkhole has been continually occupied by North Americans since approximately 5000 yr ago, with artifacts dated to 12,000 yr BP (Clausen et al., 1979; Gifford and Koski, 1994). Importantly, however, the microbial diversity of the sinkhole has never been classified.

Little Salt Spring is formed through Miocene and Paleocene carbonates (Fleury et al., 2006). The sinkhole is a 72 m deep, hourglass-shaped profile with a 78 m diameter circular opening at the surface. The upper basin slopes at twenty-five degrees down to 12

m depth where there is a circular opening 25 m in diameter. Here is an overhanging wall followed by an inverted funnel, down to a 60 m diameter base (Clausen et al., 1979).

The geochemistry of the Little Salt Spring waters has varied with sea level changes through time. In the past, with lower sea level, the sinkhole was a freshwater cenote that attracted early settlers when surface water was scarce (Clausen et al., 1979). Approximately 5000 yr BP, a rise in sea level introduced heavily mineralized water to the sinkhole through the lower aquifers and ended human occupation of the area because of the degraded water quality (Alvarez-Zarikian et al., 2005).

Currently, Little Salt Spring is in a flat sandy area five meters above sea level with hydrologic inputs from rain, runoff, and surficial and intermediate groundwater (Alvarez – Zarikian et al., 2005). Evaporation, a small surface outflow, and groundwater seepage contribute to water loss (Alvarez-Zarikian et al., 2005). The inputs and losses of the sinkhole vary seasonally but with isotopic values (δD & $\delta^{18}O$) suggesting the intermediate aquifer as the main water source (Alvarez-Zarikian et al., 2005). The waters are slightly basic (pH average 7.6) sodium chloride dominated with a high sulfate concentration (639 mg/l) (Alvarez-Zarikian et al., 2005). Also, there is a sulfidic groundwater entrance at 30 m. This groundwater could be sourced from the Upper Floridian aquifer, which contains sulfate-rich waters (Katz, 1992).

Results

Microscopy of the live biofilms was preformed to identify the morphology of the biofilm and individual species. The white biofilm reveals clusters of microbial filaments

with holdfasts and dendritic colonial structures similar to those described in the 1960s for *Thiobacterium*, a sulfur-oxidizing genus with undetermined phylogeny (Lackey and Lackey, 1961). The red biofilm shows unusual and interesting spiky structure at both the macro and micro structures.

The Little Salt Spring samples have BHPs in each biofilm sampled (Figure B-1). The red biofilm contains the largest concentration of BHPs (366,939 μg/gTLE). Concentrations of BHPs decrease in the brown floc (224,592 μg/gTLE) and white biofilm (4,841 μg/gTLE). All three samples contain a small proportion of BHT (m/z 655) and 2 Me-BHT (m/z 669) and large proportions of anhydro-BHT (m/z 855) and 2Me-anhydro-BHT (m/z 869). The brown floc material and the white biofilm also contain trace amount of BHT cycltiol ether (m/z 1002). The methyl structures are a large proportion of structures in the biofilms samples (red and brown 60%, white 40%). The C2 methyl identification in all samples has been confirmed by hopanol GC/MS analysis.

Two 16S rDNA libraries were constructed to characterize the diverse bacteria of the Little Salt Springs biofilms (Figure B-2, B-3, B-4).

Conclusions

The little salt spring seasonal red biofilm is a large source of BHPs and C2 methyl BHPs (anhydro-BHT and 2Me-anhydro-BHT). If the BHPs are produced by the *Leptolyngbya* species it will be the first identified species of that clade to produce BHPs and another cyanobacterial source of C2 methyl hopanes to the rock record. Which would support Summones et al. (1999) who claim C2 methyl hopanes are a cyanobacterial

biomarker. The BHPs present in the brown floc and white biofilm most likely are detrital material from production by the red biofilm (anhydro-BHT and 2Me-anhydro-BHT) although there may be minor contributions mad *in situ* (BHT, 2Me-BHT, and cycltiol ether). Hopanes preserved from this environment are 40:60 hopane:C2 methyl hopane.

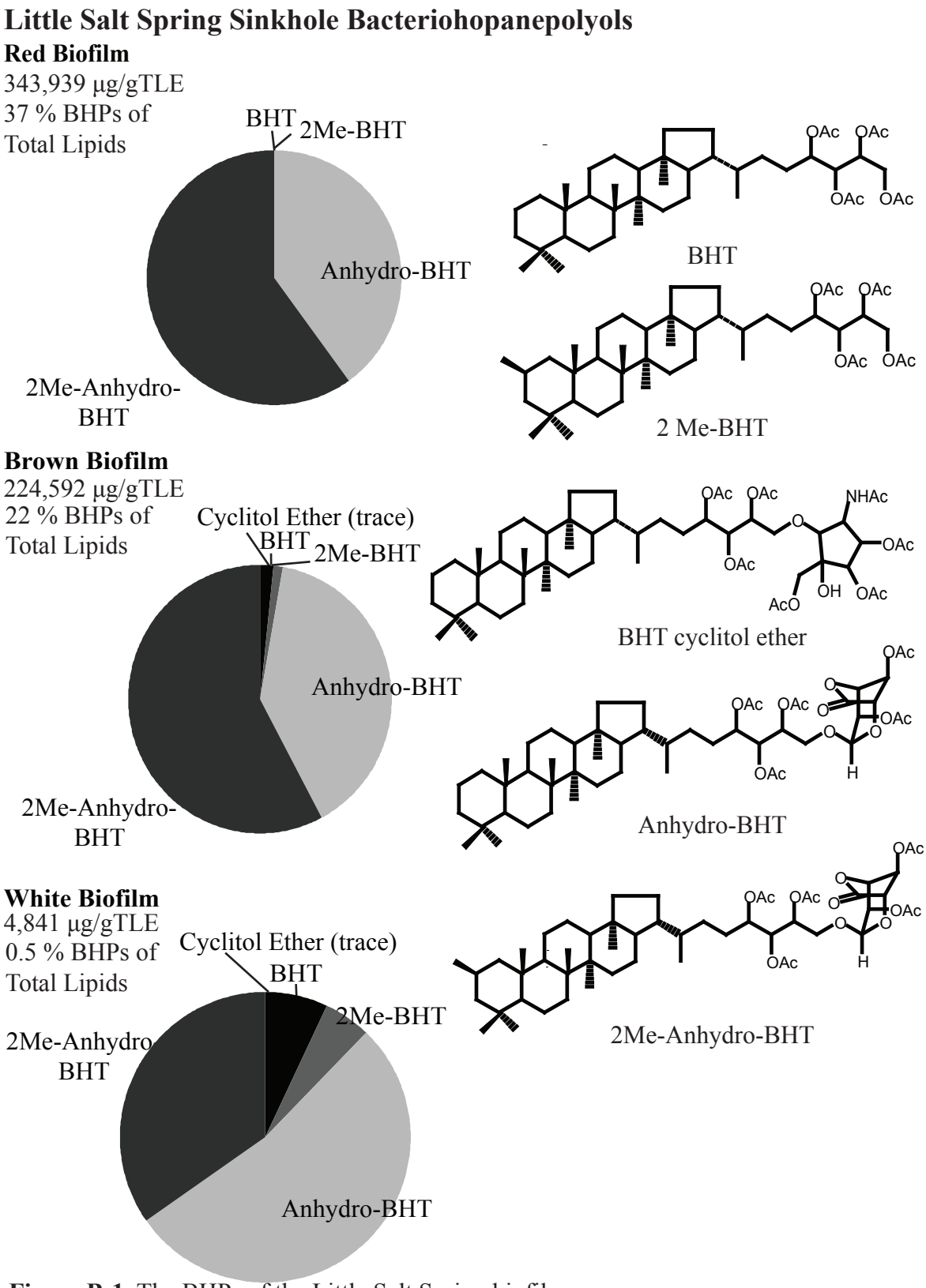

Figure B-1. The BHPs of the Little Salt Spring biofilms.

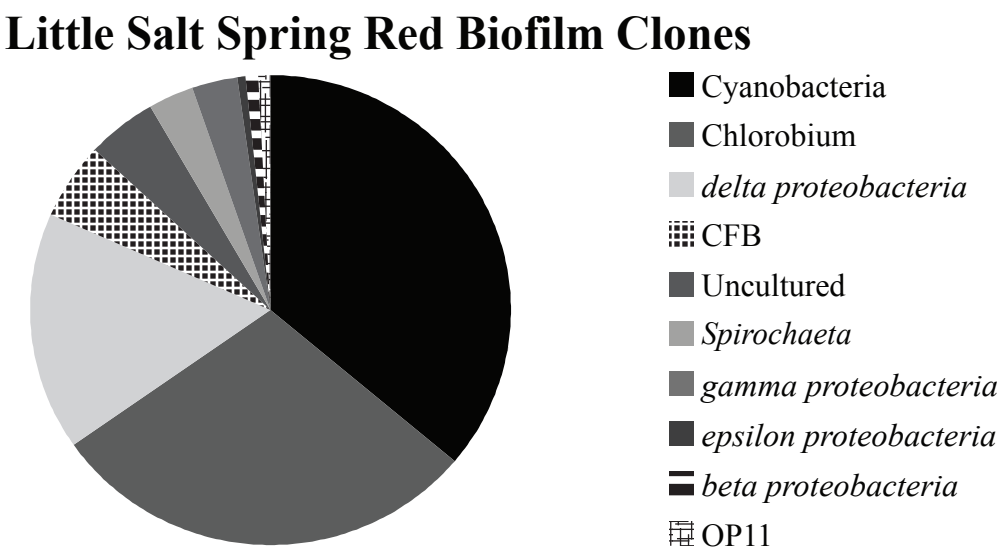

Figure B-2. The 16S rRNA clone library results of the seasonal red biofilm.

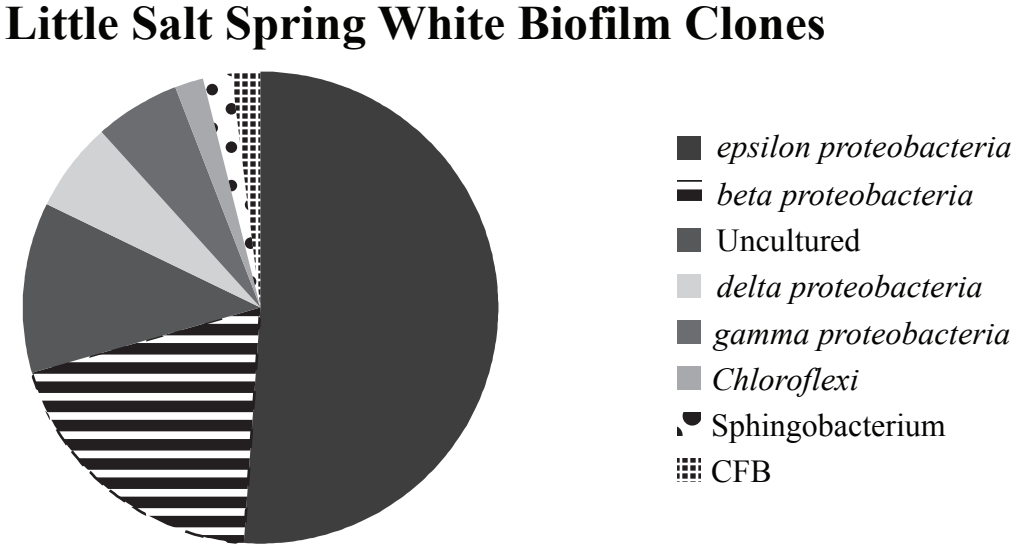

Figure B-3. The 16S rRNA clone library results of the white biofilm surrounding the input of a sulfidic water.

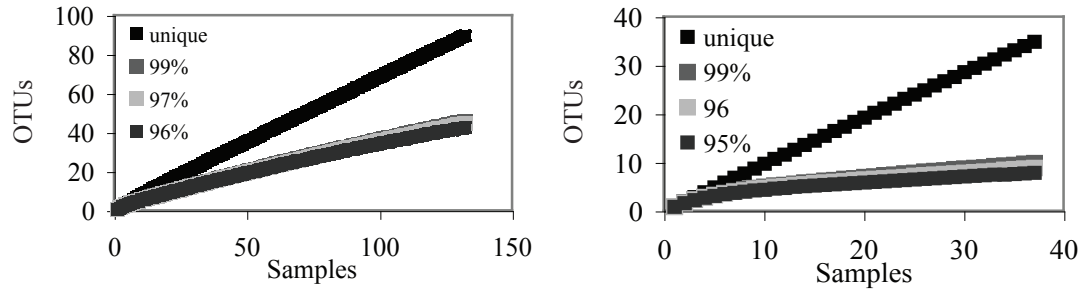

Figure B-4. (a) The rarefaction curve of the red biofilm and **(b)** the white biofilm.

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Appendix C

BHP Extraction

This extraction method is an adapted and micro-scaled version of Talbot et al. (2003).

Supplies

Teflon tubes
2 sets of 4 ml vials
1 set of turbo-vap tubes
1 set of Teflon cap insert
large glass syringes (20 or 30 ml with 4 inch stainless steal flat end tips) or glass pipettes
DCM
MeOH
DCM extracted DI water
Acetic anhydride
Pyridine
Isopropoyl Alcohol

Method

1.) Freeze – dry samples
2.) Homogenize using motar and pestle
3.) Clean enough 60 ml Teflon tubes and caps for each sample and a blank.
    a. Rinse three times with DCM and MeOH.
    b. Label lids tube A.
4.) Weigh amount of sample being extracted with samples in the Teflon tubes (A).
    a. Pure Cultures: 10 mg minimum
    b. Soils: 3 g homogenized
    c. Biofilms: 100 mg minimum
    d. Sartorius Scale (1 mg to 50 g)
5.) Add extraction solution to tubes (make sure to add water first to break static and trap sample from floating out of the tube); cap tightly.
    a. 4 ml water
    b. 10 ml MeOH
    c. 5 ml DCM
6.) Sonicate 1 hr at 35 °C
7.) While sonciating,
    a. rinse out another set of teflon tubes
        i. label tube B.

b. Clean 20 ml glass syringes and stainless steal tip with DCM and MeOH
c. Clean and label turbo-vap tubes for each sample.
d. Clean and prepare 2 sets of 4ml vials.
    i. 1st set with Teflon insert in cap, label for acetylation
    ii. label 2nd set for TLE.
e. Weigh vials for TLE
    i. Mettler-Toledo (0.01 mg to 0.1 mg range)
    ii. Use 4 ml vial as a "weigh blank"

8.) Shake in any of the shakers (New Brunswick Scientific I2400, 200 rpm 1 hr)
9.) Centrifuge at 12,000 rpm for 15 min (Beckman Coulter, Avanti J-E)
10.) Decant solution to tube B (do not remove any of sample, so error on the side of leaving a little solution behind). Use clean large syringes or glass pipette.
11.) To Tube A add another aliquot of solution:
    a. 4 ml water
    b. 10 ml MeOH
    c. 5 ml DCM
12.) Repeat both sonication and shaking
13.) Meanwhile, separate aqueous and organic phases in Tube B and rinse
    a. Add 5 ml DCM
    b. Shake
    c. Add 5 ml water gently (pour down the side)
    d. Invert gently
14.) Centrifuge for 5 min to separate layers and break up emulsion
15.) Pipette DCM from bottom of tube B and transfer to turbo-vap tube.
16.) Add a fresh 5 ml of DCM to tube B, cap.
17.) Invert
18.) Centrifuge (5 min) if necessary to separate layers and break emulsion.
19.) Pipette DCM layer to turbo-vap tube.
20.) Repeat steps 16 through 19.
21.) Archive (or dispose) of extraction residue
22.) Put turbo-vap tubes with TLE in turbo-vap if there is down time, to minimize time at the end of extraction.
23.) When Tube A is done shaking centrifuge for 15 min
24.) Pipette solution to tube B.
25.) If you are extracting a soil sample or small sample size repeat extraction again.
    a. Add solution.
    b. Sonicate and shake.
26.) Meanwhile, repeat phase separation (steps 13-21) on second extraction.
27.) Put turbo-vap tubes with TLE in turbo-vap if there is down time, to minimize time at the end of extraction.
28.) When third extraction is done shaking, centrifuge 15 min.
29.) Pipette solution from third extraction to tube B.
30.) Meanwhile, repeat phase separation (steps 13-21) on second extraction.
31.) Turbo-vap the TLE to just dry.
32.) Transfer TLE to 4 ml vial using a few microliters of DCM and MeOH for each rinse.
33.) Evaporate TLE in 4 ml vial to dry.
34.) Re-weigh on Mettler-Toledo scale.
    a. Use "weigh blank" to adjust for scale differences
35.) Resuspend TLE extract in 1 ml of DCM: MeOH 2:1.

36.)Remove ½ of TLE or appropriate amount to 4 ml vial for acetylation.
37.)Evaporate to dryness.
38.)Add 200 µl acetic anhydride and 200 µl pyridine, cap tightly.
39.)Put in drying oven for 1 hr.
40.)Evaporate to dryness under N_2.
41.)Pipette into 2 ml LC/MS tube and rinse with DCM (4 rinses).
42.)Evaporate to dryness.
43.)Add appropriate amount of acetylated standard
44.)Resuspend in appropriate amount of MeOH:IPA 60:40, for HPLC analysis.

Appendix D

LC/MS Analysis

Optimization of Mass Spectrometry

To optimize the mass spectrometer for best ionization and fragmentation, directly infuse BHPs with syringe. I infused *Anabaena variabilis* acetylated TLE at 0.3 ml/hr with a 500 μl syringe. *Anabaena variabilis* was chosen because it contains large amounts of a single BHP, bacteriohopanetetrol. Then, adjust MS parameters until ionization, trapping, and fragmentation are optimized. Further improvements in this method would be to optimize with more fraction collected BHPs.

Chromatographic and Detection Conditions

Reversed-phase HPLC was performed using a Phenomenex Gemini 5 μm C_{18} column (150 mm x 3.0 mm i.d.) and a 5 μm pre-column with the same solid phase. LC/MS settings follow Talbot et al., 2003; briefly, positive APCI mode, mass scanning from 150-1300 m/z, and "Smart" fragmentation setting (i.e., 8000 nA corona voltage, 60 psi nebulizer pressure, dry gas of five l/min, dry temperature of 350 C, vaporizer temperature 490 C). The run was divided into three segments, with target masses of 285 (0-10 min), 1002 (10-17 min) and 655 (17-50 min), all with normal optimization. Auto MS^n settings for two precursor ions were as follows: absolute threshold 100,000, relative

threshold 5%, exclude after two ions and release after 0.5 min. The acquisition parameter had a fragment amplitude 1.0 V, with an average of 5.0, and an isolation width of 3.0 m/z. Auto MS (n>2) settings with one precursor ion were as follows; absolute threshold of 1,000, relative threshold 5%, and fragment amplitude 1.0V.

Initially, individual BHP standards were fraction collected following reverse phase separation. Each collected fraction was additionally purified via neutral phase separation on a silica column (150 mm x 3.0 mm, Waters) and an isocratic flow of 80:30 hexanes:isopropyl alcohol (IPA) at 1 ml/min. The fractions were then weighed using tin EA cups on a nanogram scale.

Relative Response Factor (RRF) Equation

compound peak area/pregnanediol peak area = slope * amount injected (ng) + Y-intercept

or

amount injected = (((compound peak area/pregnanediol peak area) – Y-intercept)/slope)

Quantity Calculation

For each analysis, the peak areas of identified BHP peaks are divided by the pregnanediol peak area from the same run. This number is then used in the above RRF equation to determine the ng of compound in the injection. Multiply by concentration of injection to adjust for dilution (for instance multiply by 5 for an injection of 10 out of 50). Then multiply by fraction of TLE used for analysis (for instance multiply by 2 if ½ of the

TLE is used). Then divide by g of TLE in sample (the amount weighed in the original 4 ml TLE vial). Which yields ng/g TLE. If appropriate divide by one-thousand to yield μg/g TLE.

Calculation of Averages and Errors

There are three analyses for each sample. A standard average and standard deviation are used to calculate the value for each sample. If there are multiple replicates, for instance with culturing experiments, standard propagation of errors equations are used.

Appendix E

Hopanol Analysis

This method has been added to and micro-scaled from Innes et al. (1997)

Supplies

1 set of 8 ml vials
1 set of 8 ml teflon cap insert
3 sets of 4 ml vials
3 sets of 4 ml teflon cap insets
1 set of 2 ml GC/MS autosampler vials
glass pipettes
Hexane
DCM
Tetrahydrofuran
Extracted DI water
Acetic anhydride
Pyridine
KH_2PO_4
$LiAlH_4$
$NaBH_4$

Method

If starting with fraction collected acetylated BHP then start with step 1. If starting with unacetylated compounds or the TLE then start with step 7.

1.) Prepare 8 ml vial and 3, 4 ml vials for each sample all with Teflon inserts into cap.
2.) In an 8 ml vial add 3 ml anhydrous THF and 30 mg $LiAlH_4$. In vial from fraction collection add 1 ml of anhydrous THF rinse vial and then slowly pipette by drops into $LiAlH_4$ solution. This removes the acetyl group.
3.) 1 hr in shaker (New Brunswick Scientific I2400, 100 rpm).
4.) To quench reaction, add 2 ml extracted KH_2PO_4 slowly (it will release H_2 gas).

   a. Make 100 mM solution of KH_2PO_4
      i. 1.36 g KH_2PO_4 into 100 ml of DI water
   b. then extract with DCM.
5.) Wait for bubbling to stop. Add more KH_2PO_4 if necessary to quench the reaction.
6.) Extract this solution 3 times:
   a. 3 ml DCM:Hex 2:8
   b. shake
   c. Pipette upper phase to new 4 ml vial
7.) Evaporate under N_2.
8.) Add 30 mg H_5IO_6 (periodic acid) and 300 μl 8:1THF:DI water. This breaks all diol bonds.
9.) Shake 1 hr (Jenn's lab).
10.) Quench reaction with 1 ml DI water.
11.) Extract this solution 3 times:
   a. 3 ml DCM:Hex 2:8
   b. shake
   c. remove top solution to new 4 ml vial
12.) Evaporate under N_2.
13.) Add 10 mg $NaBH_4$ and 300 μl ethanol. This changes the aldehydes into and alcohol.
14.) 1 hr in shaker (New Brunswick Scientific I2400, 100 rpm).
15.) To quench reaction, add 2 ml extracted KH_2PO_4 slowly (will release H_2 gas).
16.) Wait for bubbling to stop. Add more KH_2PO_4 if necessary to quench the reaction.
17.) Extract this solution 3 times:
   a. 3 ml DCM:Hex 2:8
   b. shake
   c. remove top solution to new 4 ml vial
18.) Evaporate under N_2.
19.) Add 100 μl of acetic anhydride and 100 μl of pyridine, cap tightly. (Adds acetyl group to alcohol.)
20.) Put in drying oven for 1 hr.
21.) Evaporate under N_2.
22.) Transfer into GC/MS vial with DCM (4 rinses).
23.) Add appropriate concentration of standard (androstane).
24.) Evaporate under N_2.
25.) Resuspend in Hexane at appropriate concentration.
26.) Analyze on GC/MS using published mass spectra.

Appendix F

BHPs in Rio Tinto Biofilms Not Dominated by *Leptospirillum*

Table F-1. BHPs in Rio Tinto Biofilms Not Dominated by *Leptospirillum*

Sample	Total BHT cycltiol ether (µg/g TLE)	% Error	655	1060	pH	Estimated % *Lepto.*	Estimated % *Acidi.*
Gal - A	42676	14	Trace		2.49	<10	?
Gal - B	3270	9	Trace		2.53	0	100
Gal - C	5732	8	Trace		2.35	0	80
AG-3A	79724	40	Trace	Trace	2.52	?	?
AG-3B	263202	26	Trace	Trace	2.46	?	?

Appendix G

ETNP BHP Data

	Depth (m)	Total BHPs (μg/g TLE)	Total BHPs (ng/L water)	O2 (μmol/L)	3Me-hopanols (ng/L water)	2Me-hopanols (ng/L water)	%Lipids of L Water Filtered	%BHPs of TLE
Station 1								
	3	59	0.85	200			0.0014	0.0059
	25	1	0.00	210			0.0027	Trace
	35	1519	12.13	90	0.517	0.0041	0.0008	0.1519
	75	187	1.01	5	0.00013		0.0009	0.0187
	120	464	5.53	10			0.0011	0.0464
	200	253	0.74	8			0.0003	0.0253
	300	9592	43.85	2	0.00012		0.0004	0.9592
	400	762	5.12	2			0.0004	0.0762
	600	1592	6.13	2			0.0002	0.1592
	725	6270	52.92	2			0.0008	0.6270
	830	210	0.83	10			0.0004	0.0210
	1250	1324	16.85	30			0.0005	0.1324
Station 2								
	3	32	0.35	200			0.0011	0.0032
	26	52	0.63	200			0.0012	0.0052
	55	1697	7.26	5			0.0004	0.1697
	85	1120	7.62	2			0.0007	0.1120
	115	1884	11.13	2			0.0006	0.1884
	200	550	17.33	2			0.0032	0.0550
	400	1558	8.56	2			0.0005	0.1558
	600	2578	14.25	2			0.0006	0.2578
	830	127	1.58	10			0.0005	0.0127
Station 8								
	3	36	1.01	200			0.0028	0.0036
	10	28	0.91	190			0.0032	0.0028
	25	13	0.10	115			0.0008	0.0013
	50	14	0.17	30			0.0012	0.0000
	125	37	0.25	20			0.0007	0.0037
	200	98	4.28	10			0.0004	0.0098
	350	2793	6.27	2			0.0007	0.2793
	450	18341	40.45	2			0.0006	1.8341
	550	11949	81.00	5			0.0007	1.1949
	650	48	0.26	10			0.0005	0.0048
	750	8	0.06	15			0.0008	0.0008
	1000	35	0.19	30			0.0005	0.0035
	1250	11	0.08	50			0.0007	0.0011

Table G-2. ETNP Individual BHP Data

	Depth (m)	655 (ng/L water)	% Error	656 (ng/L water)	% Error	669 (ng/L water)	% Error	713 (ng/L water)	% Error	714 (ng/L water)	% Error	772 (ng/L water)	% Error	786 (ng/L water)	% Error	830 (ng/L water)	% Error	844 (ng/L water)	% Error	943 (ng/L water)	% Error	957 (ng/L water)	% Error
Station 1																							
	3	0.85	28							Trace													
	25	Trace								Trace													
	35	Trace		0.0264	30	0.00066	18	0.00	22			4.04	10	0.31	26	7.50	14	0.22	14	0.0284	7	0.00437	19
	75	0.54	27	0.1217	2							0.14	13	0.21	15								
	120	0.78	31	0.0015	8			0.00	12			1.27	5			2.01	8	1.46	8				
	200	0.54	25					0.20	22														
	300	4.17	13							19.78	16	11.10	5			0.54	28	8.26	7				
	400	2.51	5									2.62	3										
	600	3.42	5									2.71	10										
	725	6.84	29							44.58	12	1.50	13										
	830	0.83	13							Trace													
	1250	0.42	24							Trace		16.43	32										
Station 2																							
	3	0.35	5																				
	26	0.33	109					0.32	111											0.2955	18	0.00394	112
	55	0.77	19			0.00002	41	0.04	24			2.58	32	0.00	27	3.85	14	0.008	21				
	85	4.82	7							0.12	3	2.68	0										
	115	6.45	15							0.09	18	4.58	9										
	200	4.38	17							8.02	11	4.93	9										
	400	4.43	2							0.06	28	4.07	17										
	600	4.07	23							0.06	29	10.12	27										
	830	0.66	15							Trace		0.92	4										
Station 8																							
	3	1.01	4																				
	10	0.91	10																				
	25	0.10	57					0.00	131											0.0018	18	0.00002	126
	50	0.13	37					0.04	29														
	125	0.25	18																				
	200	0.41	6							0.31	6	3.56	5										
	350	1.77	11							4.50	3												
	450	1.66	22							38.78	6												
	550	3.28	10							Trace													
	650	0.26	10							Trace													
	750	0.06	2							Trace													
	1000	0.19	20																				
	1250	0.08	37																				

Appendix H

The Frasassi Cave Biofilm BHP Data

Table H-1. The Frasassi Cave Biofilm BHPs.

Sample	Biofilm Type	Total BHPs (µg/g TLE)	% Lipids of Biomass	% Hopanoids of TLE	H_2S (µmol/l)	O_2 (µmol/l)	Conductivity (µS/cm)
PC06-112	*Beggiatoa*	149,449	10.9	14.9	322	0.2	2,930
GS07-32	*Beggiatoa*	57,061	5.9	5.7	289	0.8	2,300
RS08-5	*Beggiatoa*	81,725	4.4	8.2	106	1.2	1,782
GS07-5	*Beggiatoa*	26,616	6.2	2.7	204	1.1	2,290
RS06-3	*Beggiatoa*	34,678	4.7	3.5	52	2.0	1,535
PC08-61	*Beggiatoa*	13,339	4.7	1.3	341	2.0	2,860
PC09-44	*Beggiatoa*	545	11.8	0.1	357	5.2	2,550
PC07-24	*Beggiatoa*	26,255	30.2	2.6	550	2.5	3,270
CS05-6b	*Beggiatoa*	ND			189	2.6	1,823
RS08-7	*Beggiatoa*	44,205	3.5	4.4	170	2.6	1,784
RS05-22	*Beggiatoa*	1,482		0.1	200	2.6	1,823
RS06-102	*Beggiatoa*	5,878	4.4	0.6	198	2.8	2,020
RS06-5	*Beggiatoa*	ND			51	3.1	1,535
PC07-11	*Beggiatoa*	288,236	5.3	28.8	366	3.4	3,150
PC07-26	*Beggiatoa*	3,026	18.6	0.3	477	3.9	3,280
GS06-23	*Beggiatoa*	199,488	9.3	19.9	155	4.1	2,060
PC07-20	*Beggiatoa*	27,034	12.5	2.7	439	15.6	3,270
GS08-2	*Beggiatoa*	47,939	2.4	4.8	62	15.9	1,737
GS06-205	*Beggiatoa*	78,367	7.2	7.8	151	16.2	2,080
GS06-3	*Beggiatoa*	347,322	1.5	34.7	22	25.0	1,587
GS10-20	*Beggiatoa*	44,931	3.7	4.5			
VC10-5	Streamer	5,894	1.3	0.6			
PC08-64	Streamer	7,134	3.1	0.7	366	2.5	2,860
PC05-10a	Streamer	423		0.0	150	0.6	2,460
GS07-4	Streamer	Trace	5.0	0.0	285	0.8	2,300
FS06-12	Streamer	ND			411	1.5	2,740
RS05-21	Streamer	ND			200	2.6	1,823
AS07-6	Streamer	131,308	2.2	13.1	801	3.1	10,640
PC07-27	Streamer	10,013	10.9	1.0	470	3.9	15,975
PC05-11	Streamer	ND			357	5.2	2,550
CS06-2	Streamer	1	3.3	0.0	34	12.6	1,565
CS06-101	Streamer	ND	2.0		253	15.3	2,340
PC09-45	Black Sediment	47,446	1.8	4.7	357	5.2	2,550
LV05-4	Black Sediment	87,257	0.1	8.7	352	8.0	2,510
RS06-4	Black Sediment	78,353	0.6	7.8	51	3.1	1,535
PS06-1	Purple Sulfur Bacteria	40,821	0.8	4.1	378	2.6	1,880
FS06-101	Cyanobacteria	23,501	0.8	2.4	378	2.6	1,880
PC05-24	Gypsum Wall Paste	ND					

Appendix H-2. Frasassi cave system stream biofilm BHP data.

Sample	Biofilm Type	655 (µg/g TLE)	% Error	714 (µg/g TLE)	% Error	1002 (µg/g TLE)	% Error	746 (µg/g TLE)	% Error	762 (µg/g TLE)	% Error	1060 (µg/g TLE)	% Error	1086 (µg/g TLE)	% Error
PC06-112	*Beggiatoa*	2,052	10	101,442	14	45,955	14								
GS07-32	*Beggiatoa*	34	20	10,901	22	46,126	37								
RS08-5	*Beggiatoa*	134	3	17,459	11	Trace		64,132	23			Trace			
GS07-5	*Beggiatoa*	89	25	21,738	3	4,789	24								
RS06-3	*Beggiatoa*	12	4	2,075	5	32,590	2								
PC08-61	*Beggiatoa*	62	6	13,277	3	Trace		Trace		Trace		Trace			
PC09-44	*Beggiatoa*	545	2	Trace		Trace									
PC07-24	*Beggiatoa*	13	10	2,783	6	20,881	35	2,576		2	13				
CS05-6b	*Beggiatoa*														
RS08-7	*Beggiatoa*	162	24	44,043	3	Trace						Trace			
RS05-22	*Beggiatoa*	51	7	486	8	945	14								
RS06-102	*Beggiatoa*	214	9			4,003	2							1,661	27
RS06-5	*Beggiatoa*														
PC07-11	*Beggiatoa*	118	1	67,119	12	220,998	32					Trace			
PC07-26	*Beggiatoa*	16	20	3,010	41	Trace						Trace			
GS06-23	*Beggiatoa*	42	1	25,143	4	174,303	6								
PC07-20	*Beggiatoa*	6	0			27,026	21			2	19				
GS08-2	*Beggiatoa*	91	25	47,848	19										
GS06-205	*Beggiatoa*	47	4	19,998	17	58,322	26								
GS06-3	*Beggiatoa*	5,542	17	249,076	8	92,704	19								
GS10-20	*Beggiatoa*	949	7	39,789	5	4,192	10								
VC10-5	Streamer	393	5	Trace		5,501	4								
PC08-64	Streamer	1,402	8	Trace		5,732	1	Trace							
PC05-10a	Streamer	1				0	35								
GS07-4	Streamer			Trace		Trace									
FS06-12	Streamer														
RS05-21	Streamer														
AS07-6	Streamer	250	5	131,058		trace						Trace			
PC07-27	Streamer	4	45	7,034	4	trace		2,975	36	trace					
PC05-11	Streamer														
CS06-2	Streamer	0.42	5			0.31	18								
CS06-101	Streamer														
PC09-45	Black Sediment	10,138	24	3,485	7	33,822	7								
LV05-4	Black Sediment	2,151	31	65,491	3	13,791	16					5,825	3		
RS06-4	Black Sediment	4,365	10	43,760	6	18,666	1	10,296	14			1,266	9		
PS06-1	Purple Sulfur Bacteria	11,642				39,945									
FS06-101	Cyanobacteria	745	2			20,404	1								
PC05-24	Gypsum Wall Paste														

Appendix I

Acidithiobacillus thiooxidans Mu1 BHP Data

Table I-1. *Acidithiobacillus thiooxidans Mu1* culturesgrown in triplicate across a pH gradient.

pH	% TLE of Biomass	% BHPs of TLE	Total BHPs (μg/g TLE)	655 (μg/g TLE)	% Error	1002 (μg/g TLE)	% Error	1060 (μg/g TLE)	% Error	Ratio 1060/1002
4	10.95	1.11	1,110	292	20	575	12	242	26	1.97
3.5	10.83	1.84	1,843	50	20	1,298	25	495	17	0.38
3	4.80	1.37	1,369			904	1	465	13	0.52
2.5	10.04	0.62	5,569			3,218	17	2,351	22	1.11
2	3.29	5.74	5,742			4,046	17	1,695	18	0.42
Snotitte Biofilm										
pH	% TLE of Biomass	% BHPs of TLE	Total BHPs (μg/g TLE)	655 (μg/g TLE)	% Error	1002 (μg/g TLE)	% Error	746 (μg/g TLE)	% Error	
0-1	0.45	33.5	335,237	1154	9	173,660	7	58,103	12	

Appendix J

Red Eyes BHP Data

Table J-1. Red Eyes Cell Counts and Geochemistry

Month	Location	% otherEUB	% otherBET	% FERRI643	% otherGAM	% THIO1	totalFe	Ferrous Fe2	pH	temp C	cond (mS)	sulfate (μmol)
Oct	LPB	29	1.4	0.8	5.3	47.2	231.45	110.89	2.64	13.6	5.08	2050
Oct	LPB	29	1.4	0.8	5.3	47.2	231.45	110.89	2.64	13.6	5.08	2050
Oct	SPB	37.3	3.3	6.8	7.5	34.2	201.88	97.81	2.92	14	2.91	1650
Oct	LPA	38.2	-1.3	31.2	8.1	17.6	353.71	218.37	2.99	13.5	3.91	2750
Oct	SPA	34.7	20.2	29.5	8.5	0	424.22	329.26	3.25	10.9	4.36	2950
Oct	BP	49.7	24.7	5.8	14.9	0	486.78	399.2	3.51	12.7	4.2	3150
May	LPA	7.6	11.8	77.9	2.4	0	460.83	316.24	2.98	16.8	4.6	3600
May	LPB	17	6.1	3.5	1.6	65.8	340.18	230.2	2.57	14	4.81	3200
May	SPB	12.3	0	81.3	2.2	4.2	398.27	256.41	2.78	17.1	4.63	3289
May	LPB green	17	6.1	3.5	1.6	65.8	340.18	230.2	2.57	14	4.81	3200

Table J-2. Red Eyes BHP Data

Month	Location	Total BHPs (μg/g TLE)	%BHPs of total lipids	% Lipids of Biomass	1060 (μg/g TLE)	% Error	1002 (μg/g TLE)	% Error	943 (μg/g TLE)	% Error	714 (μg/g TLE)	% Error	655 (μg/g TLE)	% Error
Oct	LPB	42,879	4	1	2,397	16	14,063	5	331	4	25,880	2	208	3
Oct	LPB	102,006	10	9	4,305	13	27,039	15	430	10	70,065	1	166	19
Oct	SPB	1,654	0	1			1,573	8	40	4				
Oct	LPA	1,998	0	1	Trace		1,998	6	Trace		Trace		Trace	
Oct	SPA	0	0	28										
Oct	BP	0	0											
May	LPA	10	0	3					10	27				
May	LPB	214,255	21	0	5,587	1	65,574	10			#####	2		
May	SPB	821	0	4					7	17	814	1	Trace	
May	LPB green	0	0	4										

Appendix K

Leptospirillum BHP Data

Table K-1. *Leptospirillum* cultures, biofilms, and planktonic samples.

Sample	% Lipids of Biomass	% Hopanoids of Lipids	Total BHPs (µg/g TLE)	655 (µg/g TLE)	% Error	669 (µg/g TLE)	% Error	943 (µg/g TLE)	% Error	957 (µg/g TLE)	% Error	1002 (µg/g TLE)	% Error	1060 (µg/g TLE)	% Error	714 (µg/g TLE)	% Error
LFE culture	22.18	26.67	266,698	17,185	13	1,858	8	159,234	50	88,421	7						
CSA-2010 (140910)*	5.00	5.94	59,429	692		trace		49,180	0	9,557	17						
SSA-2010 (150710)*	6.67	12.85	128,499	6,934		trace		96,814	5	24,751	1						
CSA-2004*	34.67	1.93	19,277	3,585	22	197	25	9,322	24	6,175	15						
SSA-2004*	41.21	0.03	252					163	38	89	30						
Filamentous Naciamiento biolfilm	1.99	3.33	33,285	2,130	16	207	17	15,966	49	14,982	37						
Lake 3.2 (Dam10-0.1-1 m) (A/E + A/D filters)		0.37	3,712	117	7			275	6	85	5	1,187	8	Trace		2,047	10
Lake 3.2 (Dam10-3.5-2 m) (A/E + A/D filers)		7.60	76,009	Trace		Trace		3,656	5	2,296	10	5,277	5	Trace		64,780	10
Lake 3.2 (Dam10-3.5-3 m) (A/E + A/D filters)		9.06	90,643	Trace		Trace		4,212	8	2,605	16	2,691	16			81,135	26

*Biomass was to low to yield good quantification.

Appendix L

Anabaena variabilis BHP Data

Table L-1. *Anabaena variabilis* cultures grown with different metabolisms and a range of nitrate availability.

	$NaNO_3$ (mmol/l)	BHT (μg/g TLE)	% Error
Phototroph	0.0	5,500	6
Phototroph	8.8	3,500	7
Phototroph	17.6	2,300	7
Redox Shock Phototroph	0.0	1,600	9
Redox Shock Phototroph	8.8	1,800	5
Redox Shock Phototroph	17.6	900	3
Heterotroph	0.0	400	14
Heterotroph	8.8	200	14
Heterotroph	17.6	300	10
Redox Shock Heterotroph	0.0	30	12
Redox Shock Heterotroph	8.8	4	11
Redox Shock Heterotroph	17.6	10	12

Appendix M

Guaymas Basin Sediment BHP Data

M-1. Guaymas Basin Sediment BHP Data

Depth	Total BHPs (µg/g TLE)	%BHPs of TLE	655 (µg/g TLE)	% Error	714 (µg/g TLE)	% Error	772 (µg/g TLE)	% Error	1002 (µg/g TLE)
0-3 inches	272352	27.24	734	12	216175	11	55442	20	Trace
3-5 inches	70850	7.08	Trace		60458	15	10392	18	
5-7 inches	0	0.00			Trace				
0-3 cm	435029	43.50	2032	2	364317	47	68680	11	
3-6 cm	52969	5.30	420	30	52549	25	Trace		
6-9 cm	90312	9.03	1048	10	89264	5			
9-12 cm	0								
12-15 cm	0								

Appendix N

The Little Salt Spring BHP Data

Table N-1. The Little Salt Spring BHP Data

Sample	% Lipids of Biomass	% Hopanoids of Lipids	Total BHPs (µg/gTLE)	655 (µg/g TLE)	% Error	669 (µg/g TLE)	% Error	855 (µg/g TLE)	% Error	869 (µg/g TLE)	% Error	1002 (µg/g TLE)
Red Mat	11.73	36.69	366939	481	22	510	22	146179	0	219769	15	
Brown Floc	3.40	22.46	224592	3625	27	2803	24	88277	6	129888	23	Trace
White Mat	14.02	0.48	4841	331	26	258	20	2572		1680	13	Trace

Curriculum Vitae
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Education

2011 **Pennsylvania State University**, University Park, PA
Ph.D. Geosciences and Biogeochemistry

2006 **University of Utah**, Salt Lake City, UT
Stable Isotopes in Ecology, Summer Course

2003 **University of Pennsylvania**, Philadelphia, PA
B.A., Biology; Minor, Urban Education

Research Experience

2008 **Skidaway Oceanographic Institute,** Skidaway, GA
Trained by Stuart Wakeham on polar lipid fatty acid extraction and analysis

2006 **University of Newcastle**, Newcastle, England
Trained by Helen Talbot in biohopanoid extraction and LC/MS analysis

2007 **Eastern Tropical North Pacific**: Scientific cruise on the *RV Seward Johnson* to collect samples for PhD research

2006 **Frasassi Cave, Italy:** Field site for PhD samples

Awards and Achievements

2009, 2010 Shell Research Award

2007 Hiroshi Ihmoto Graduate Fellowship in Geosciences

2006 CZEN (Critical Zone Exploration Network) International Student Fellowship

2006 BRIE (Biogeochemical Research Initiative in Education) Research Credit Card

2006-9 Kyrnine Award

2005 Charles F Knopff Sr. Memorial Scholarship

Peer Reviewed Publications

Peragine A, Yoshikawa M, Wu G, **Albrecht HL**, Poethig RS (2004) SGS3 and SGS2/SKE1/RDR6 are required for juvenile development and the production of transacting siRNAs in *Arabidopsis*.Genes and Development. **18**(19): 2368-79

Selected Presentations with Published Abstracts

Albrecht HL, Freeman KH, and Macalady JM. Environmental controls on hopanoid distributions: Field and Culture Studies (talk) Astrobiology Conference, Santa Clara, CA. April 19th, 2008.

Albrecht HL, Freeman KH, and Macalady J. Ecological, environmental, and physiological controls on hopanoid distributions. (poster) International Meeting of Organic Geochemists, Bremen, Germany, 2009.