EXPLORING THE DISTRIBUTION AND PHYSIOLOGICAL ROLES OF BACTERIAL MEMBRANE LIPIDS IN THE MARINE ENVIRONMENT

By

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Submitted on May 21, 2010, in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the Massachusetts Institute of Technology and the Woods Hole Oceanographic Institution.

ABSTRACT

Lipids have a legacy in the geologic record extending back to the Archaean. Since the phylogenetic diversity of life is reflected in the structural diversity of biomolecules, lipid biomarkers that are shown to be diagnostic of certain organisms that carry out specific biochemical processes or that are demonstrated to have unique physiological roles can be used to trace the biogeochemical influence of bacteria in modern and ancient environments. In this thesis I explore the application of two classes of bacterial membrane lipids as biomarkers for marine biogeochemical processes in marine environments: ladderanes and hopanoids. Through the detection of ladderane lipids – biomarkers for anaerobic ammonium oxidizing (anammox) bacteria – I demonstrate the presence and distribution of anammox bacteria in a subterranean estuary. Through a survey of hopanoids in marine environments and cultured marine cyanobacteria I show that hopanoids are ubiquitous in the oceans and that their presence in ancient marine sediments could provide information about biogeochemical processes in past environments. Based on novel results demonstrating that hopanoids are resistant to extraction by non-ionic detergent, I propose that they may play a role in lipid ordering and the formation of putative lipid rafts in hopanoid-producing bacteria.

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No, I don't like work. I had rather laze about and think of all the fine things that can be done. I don't like work—no man does—but I like what is in the work—the chance to find yourself. Your own reality—for yourself, not for others—what no other man can ever know. They can only see the mere show, and never can tell what it really means.

- Joseph Conrad, Heart of Darkness

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CHAPTER 1 – INTRODUCTION

Lipids have a legacy in the geologic record dating back to nearly 3 billion years before present (Brocks and Pearson, 2005). Since the phylogenetic diversity of life is reflected in the structural diversity of biomolecules, organic "chemical fossils" preserved from ancient environments can be used to reconstruct the natural history of life throughout Earth's history. Lipid biomarkers diagnostic of bacteria that carry out specific biochemical processes or that are demonstrated to have unique physiological roles can be used to trace the biogeochemical influence of bacteria in modern and ancient environments.

In this thesis, I explore the application of two classes of bacterial membrane lipids as biomarkers for marine biogeochemical processes in marine environments: ladderanes and hopanoids (Fig. 1). Ladderanes are a unique class of lipids that have been identified in bacteria capable of anaerobic ammonium oxidation (anammox) (Sinninghe Damste et al., 2005), and can be used to trace the distribution of anammox bacteria in the environment due to their unique role in the physiology of anammox bacteria. Hopanoids are pentacyclic isoprenoids produced by some bacteria and have been dubbed bacterial "sterol surrogates." Understanding the biological role of hopanoids is of particular interest to understanding the evolutionary history of bacteria since geologically stable products of hopanoids have been

detected as molecular fossils preserved in ancient sedimentary rocks dating back to more than 2.5 billion years before present (Brocks et al., 1999; Rasmussen et al., 2008).

Organic compounds that are preserved in the geologic record have the potential to inform us of biological processes over the course of Earth's history – at least, the part of it that is recorded in the sedimentary record. The ability of such biomarkers to provide information about past environments lies primarily in their restriction to distinct groups of organisms or ecological niches, or in the knowledge of their biological function. In the one sense, the best biomarker would be one that is specific to a particular species and, in this context, DNA been referred to as the "ultimate biomarker." In fact, fossil DNA has been found in sediments up to 10,000 years old (Coolen et al., 2004), however, its preservation is likely limited to sulfidic water columns and on longer time scales DNA is not preserved in detectable amounts. Therefore, in many cases we must rely on more refractory compounds to serve as biomarkers. Fossil lipids are found in rocks that are billions of years old, however they are generally less specific than some of the more bio-informative molecules, such as proteins and nucleic acids. Nonetheless, lipid biomarkers of varying phylogenetic specificity have been identified for a range of organisms (Simoneit, 2002; Brocks et al., 2003; Brocks and Pearson, 2005).

One of the primary goals in marine biogeochemistry is to understand the relationships between life and the chemistry of the ocean, and ultimately what role these relationships have served in authoring the course of evolution. As such, we are interested not only in the type of organisms that were present but more generally in the biochemical pathways and physiological innovations that have emerged and evolved through time. In this sense an ideal biomarker would be, for instance, an enzyme associated with a specific biochemical pathway, such as the nitrogenase enzyme that facilitates nitrogen fixation. However, proteins are rapidly degraded in the environment, and therefore fail to meet the criteria of a biomarker. Another approach would be to identify more refractory structural components of an enzyme that are preserved in the geologic record, such as certain porphyrins. By way of example, the enzyme superoxide dismutase (SOD), which is critical in protecting cells from damage from the superoxide radical, is found in all aerobic and facultative aerobic organisms (McCord et al., 1971), and it is thought to have evolved in concert with the evolution of oxygen-based metabolism (McCord, 2000). SOD is made up of a porphyrin core, and presumably the unique structure of the SOD porphyrin could be used to trace the emergence of oxygenic photosynthesis and the oxygenation of Earth's surface. Unfortunately, intact porphyrins are not well preserved in sediments from the Archean when the evolution of oxygenic photosynthesis is thought to

have occurred, so they fail to meet the essential criteria required to address questions on a time scale relevant to the emergence of molecular oxygen on Earth's surface.

Elucidating the biochemical or physiological role of a lipid biomarker represents another avenue towards reconstructing the emergence and change in the predominance of specific biochemical pathways through the geologic record. Lipid biomarkers that have unique physiological or biochemical associations may also serve as useful markers in modern environments, especially in cases where 16S rRNA phylogeny may provide a less certain proxy for function. Ladderane lipids are an excellent example of this. The ladderane is thought to be a primary component in the membrane of a novel bacterial organelle called the anammoxosome that is unique to organisms capable of anaerobic ammonium oxidation (anammox) in which N_2 is produced from nitrate and ammonium (Sinninghe Damste et al., 2002; van Niftrik et al., 2004). The occurrence of anammox can be unambiguously demonstrated by the presence of ladderanes, which are unique in that they posses a linearly concatenated cyclobutane structure that has never before been observed in any natural living membrane (Damsté et al. 2002). Since ladderanes are thought to play a key role in the physiology of the anammox process they represent a direct proxy for this biochemical pathway.

The bacteriohopanepolyols (BHPs), a class of lipids known more generally as hopanoids, represent another promising candidate for development as bacterial biomarkers. Hopanoids have been purported as "the most abundant natural products on Earth" (Ourisson and Albrecht, 1992) and they are found in many bacteria, and in particular in cyanobacteria (Rohmer et al., 1984; Llopiz et al., 1996; Simonin et al., 1996; Carpenter et al., 1997; Summons et al., 1999). While there is some evidence to suggest that certain structural motifs in BHPs may be unique to cyanobacteria – demonstrating the potential for these compounds to be developed as biomarkers – very little is known about the structural diversity and abundance of BHPs in marine cyanobacteria or in marine environments. This gap in our knowledge is primarily the result of long-standing analytical limitations that have prevented the rapid characterization of intact BHPs in environmental samples or cells in culture. The chemotaxonomic information contained in the polar side chain of cyanobacterial BHPs may provide unique group-specific information that could be used to reconstruct recent changes in the abundance and ecological structure of cyanobacterial communities.

Hopanoids also have the potential to inform us about the evolution and ecology of bacteria through deep time in Earth's history. Although the chemotaxonomic information contained in the polar side chain of the BHP is lost during sedimentary diagenesis, the presence of additional methyl groups

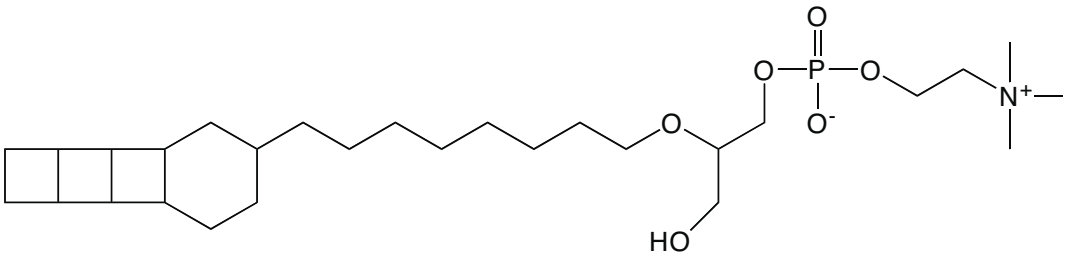
on the hopane ring structure are preserved and may be phylogenetically informative. Functionalized precursors of fossil hopanes containing an extra methyl group on the A ring at the C-2 position are found in many cyanobacteria (Bisseret et al., 1985; Summons et al., 1999) and, as such, the 2-methylhopanoids have been proposed as a biomarker for prokaryotic oxygenic photosynthesis (Summons et al., 1999). Indeed, 2-methylhopanoids represent the earliest evidence for the existence of marine cyanobacteria 2.7 billion years ago (Brocks et al., 1999). Consequently, information about the biological function and ecological distribution of 2-methylhopanoids in the modern ocean could provide crucial information about the environmental conditions that lead to the evolution of oxygenic photosynthesis. Ultimately, understanding the taxonomic distribution, environmental distribution, or biological role of hopanoids will shed light on their significance in the sedimentary record (Fig. 2).

In the first part of this study (Chapter 2) I investigated the presence of ladderanes in sediment from a novel subterranean estuarine environment in order to elucidate the distribution of anammox bacteria with respect to nitrogen speciation and redox gradients. I demonstrate that anammox bacteria are present at redox transitions within subterranean estuaries and could represent an important pathway for fixed nitrogen removal from

terrestrial ground water delivered to coastal marine environments through subterranean ground water discharge.

In Chapter 3 I outline methods for extracting and analyzing intact bacteriohopanepolyols (BHPs) by high performance liquid chromatography mass spectrometry (HPLC-MS). In chapters 4 and 5 I investigate the phylogenetic distribution and potential physiological role(s) of hopanoids in marine cyanobacteria with an eye towards refining their application as bacterial or environmental biomarkers in the marine sedimentary record. I report observations of the intracellular distribution and cellular abundance of hopanoids in *Crocosphaera watsonii*, a marine nitrogen fixing cyanobacterium. Hopanoid cellular abundance does not appear to be linked to nitrogen limitation, arguing against their proposed role in the physiology of nitrogen fixation. However, based on novel results demonstrating that hopanoids are resistant to extraction by non-ionic detergents, I propose that they may play a role in lipid ordering and the formation of putative lipid rafts in hopanoid-producing bacteria. In Chapter 6, I explore the distribution of hopanoids in a globally representative selection of samples from marine and proximal marine environments. My results indicate that hopanoids are ubiquitous in the oceans. I discuss the implications of their distribution and structural diversity for understanding their significance in the geologic record.

Ladderane: phosphocholine monoether (PC-monoether)



Hopanoid: bacteriohopanetetrol (BHT)

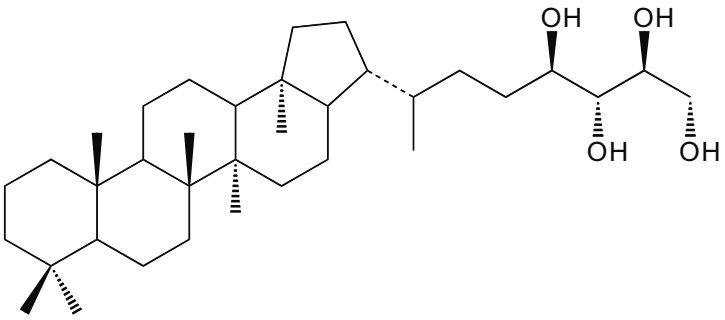


Figure 1. Representative structures of a ladderane and a hopanoid.

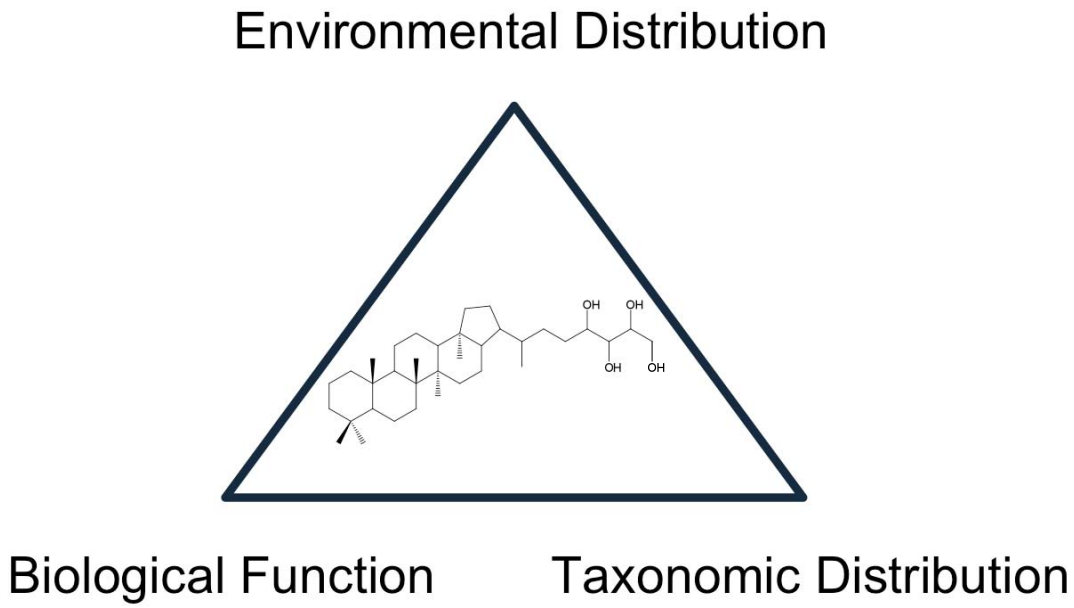


Figure 2. Conceptual cartoon depicting the three principal facets that are essential to understanding a biomarker's significance in the geologic record.

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CHAPTER 2* - DISTRIBUTION OF AMMONIA OXIDIZING MICROBIAL COMMUNITIES IN A SUBTERRANEAN ESTUARY DETERMINED BY LIPID BIOMARKERS AND FUNCTIONAL GENES

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ABSTRACT

The traditional paradigm that rivers and terrestrial run-off are the major contributors of nutrients to coastal waters is being challenged in light of work that has emerged over the past decade suggesting that nutrient fluxes originating from coastal aquifer subterranean estuaries can equal or even exceed that of other terrestrial sources. Within a coastal aquifer where organic carbon is scarce and ammonia is abundant, bacteria capable of anaerobic ammonium oxidation (anammox) and aerobic ammonium oxidizing archaea (AOA) and bacteria (AOB) may play a large role in the removal of fixed nitrogen. We investigate the presence of anammox bacteria and AOA/AOB in a coastal groundwater system (Waquoit Bay, MA USA) using lipid biomarkers and functional gene analysis. From the distribution of

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ladderane phospholipids and *amoA* genes, biomarkers for viable anammox and AOA/AOB, respectively, we show the coexistence of both anammox and AOA/AOB in association with redox transition zones within the aquifer. The distribution of ladderane core lipids and crenarchaeol, in contrast, provided insight into the historical distribution and temporal stability of anammox and crenarchaeota, and indicate that these communities have migrated vertically through time in response to changing pore water redox conditions. Our observations show that anammox and AOA/AOB may form an important and temporally persistent component of the nitrogen cycle in subterranean estuaries.

1. INTRODUCTION

Nitrogen is an essential element in the growth and survival of all living organisms. Since most organisms depend on the presence of fixed nitrogen species such as ammonium and nitrate for growth, understanding the processes controlling the availability of fixed nitrogen in the environment is critical to understanding biochemistry and ecology of the carbon cycle. In coastal marine environments, where nitrogen is often the limiting nutrient in primary productivity, anthropogenic activities can generate large fluxes of fixed nitrogen often leading to eutrophic conditions resulting in the formation of toxic algal and cyanobacterial blooms (Howarth et al., 2000). Rivers and non-point source runoff have traditionally been viewed as the most important

terrestrial sources of fixed nitrogen (Valiela et al., 2000), however it has recently been recognized that nutrient input from groundwater discharge to coastal waters can equal or in some cases exceed nutrient input from rivers (Taniguchi et al., 2002; Slomp and Van Cappellen, 2004; Kroeger et al., 2007; Kroeger and Charette, 2008).

The interface between fresh groundwater and saltwater within coastal aquifers is a biogeochemically active zone that can act as a major chemical sink or source for coastal waters (Charette and Sholkovitz, 2002; Charette et al., 2005; Windom et al., 2006; Beck et al., 2007; Hays and Ullman, 2007; Kroeger and Charette, 2008). In the past decade studies have shown that strong redox gradients can be generated by hydrographic mixing processes and that active chemical cycling can occur within such environments, termed "subterranean estuaries" (Moore, 1999; Charette and Sholkovitz, 2002; Testa et al., 2002; Windom and Niencheski, 2003; Charette et al., 2005). Furthermore, redox gradients can be maintained by the interaction of different sources of fresh groundwater in sediments overlying the salinity transition zone (Kroeger and Charette, 2008). The coexistence of high concentrations of ammonium, in close proximity to suitable oxidants such as manganese, nitrate, nitrite, and dissolved oxygen suggests the potential for removal of fixed nitrogen by microbially mediated ammonium oxidation pathways. Organic matter is typically in very low abundance within

subterranean estuaries, and so chemoautotrophs utilizing ammonia in their metabolisms are expected to be favored in these environments.

One such pathway is the **an**aerobic **amm**onium **ox**idation (anammox)(Mulder et al., 1995; Strous et al., 1999). This pathway carries out the direct conversion of nitrite and ammonium to dinitrogen (N_2) gas in a single biochemical pathway, rather than multiple independent pathways and associated consortia that were previously thought to be the only mechanism for N_2 formation. The existence of an anammox pathway was first demonstrated fifteen years ago in a wastewater treatment facility (Mulder et al., 1995) and has since been identified in a variety of natural marine systems including anoxic basins (Dalsgaard et al., 2003; Kuypers et al., 2003), oxygen minimum zones associated with high productivity upwelling regions (Kuypers et al., 2005; Thamdrup et al., 2006; Woebken et al., 2007), marine sediments (Thamdrup and Dalsgaard, 2002; Freitag and Prosser, 2003; Rysgaard et al., 2004) and estuarine sediments (Risgaard-Petersen et al., 2004). Anammox-mediated removal of fixed nitrogen is now thought to represent one of the dominant terms in nitrogen removal from many marine upwelling systems, and by some estimates may be responsible for up to 50% of nitrogen loss from the oceans (Gruber and Sarmiento, 1997; Codispoti et al., 2001; Brandes et al., 2007). Anammox is presently unaccounted for in mass balance estimates of coastal nitrogen cycling and, in particular,

subterranean estuaries (Kroeger and Charette, 2008; Spiteri et al., 2008). Given the low organic carbon concentrations in subterranean estuarine environments (Charette et al., 2005; Kroeger and Charette, 2008), it is most likely that chemoautotrophic processes such as anammox would be more prevalent than heterotrophic denitrification, which is suggested as the dominant pathway for nitrogen removal in the Arabian Sea (Ward et al., 2009). The presence of anammox in subterranean estuarine environments could have significant implications for the current accounts of the coastal nitrogen budget.

It has also been shown that anammox may exist in close association with ammonia oxidizing bacteria (AOB) (Sliekers et al., 2002; Thamdrup and Dalsgaard, 2002), which can enhance anammox activity by providing a source of nitrite while removing dissolved oxygen which inhibits the anammox reaction. Anammox bacteria might also coexist with the recently discovered ammonia oxidizing archaea under similar conditions (AOA; (Konneke et al., 2005; Wuchter et al., 2006). In the Black Sea, for instance, AOA are abundant at the chemocline (Coolen et al., 2007) and have been estimated to supply roughly 50% of the nitrite to anammox in the water column (Lam et al., 2007). Recently, the presence of AOA and AOB was demonstrated in a subterranean estuary and their relative abundances were shown to vary with changes in salinity (Santoro et al., 2008).

In this study, we investigated the presence and distribution of anammox bacteria and AOA/AOB within a subterranean estuary in northeast America through the detection of lipid biomarkers and functional gene analysis. Anammox species can be unambiguously identified by the presence of ladderanes (Figure 1), which are unique in that they posses a linearly concatenated cyclobutane structure that has not been found in any other organism (Sinninghe Damste et al., 2002; Sinninghe Damste et al., 2005). The ladderanes are thought to be the principal lipids in the membrane of an organelle called the anammoxosome in which the anammox reaction is carried out (van Niftrik et al., 2004). Since the biophysical properties of ladderanes are thought to be essential to the physiology of the anammox pathway (Sinninghe Damste et al., 2002) their detection provides a robust means for establishing the presence of viable communities of anaerobic ammonium oxidizers.

We investigated the distribution of two classes of ladderane lipids. The first is an intact phospholipid (Fig 1: V), which is a membrane-bound lipid and degrades rapidly upon cell death (Boumann et al., 2006; Jaeschke et al., 2009). The second class of ladderanes, which we refer to as "core" lipids (Fig. 1: II-IV), represent components of the hydrocarbon tail of the intact phospholipids that are released when the ladderane phospholipids are degraded. Ladderane core lipids are more stable than intact ladderane

phospholipids and are likely to resist degradation for some time following cell death, whereas the presence of the ladderane phospholipid is thought to be diagnostic of viable or recently deceased cells (Jaeschke et al., 2009). The presence and distribution of AOA can be inferred from crenarchaeol (Figure 1: I) (Sinninghe Damste et al., 2002), which is a membrane lipid that seems to be uniquely synthesized by ammonia-oxidizing crenarchaeota as shown by good correlations to AOA abundance in soils and marine pelagic environments (Leininger et al., 2006)(Wuchter PhD Thesis, Royal NIOZ) and its presence in cultures of AOA (Wuchter et al., 2006; de la Torre et al., 2008; Schouten et al., 2008; Pitcher et al., 2010). This lipid-based assessment is supported in this study by functional gene analysis of ammonia-monooxygenase subunit A (*amoA*) of AOA (Francis et al., 2005; Mincer et al., 2007). Our results show, for the first time, that ladderanes are present in *permeable* sediments at similar depths as bacterial and archaeal *amoA* genes and crenarchaeol. This represents the first direct evidence for anammox in a subterranean estuary and demonstrates the potential importance of AOA/AOB and anammox acting independently and syntrophically as a pathway for nitrogen removal in subterranean environments.

2. MATERIALS AND METHODS

2.1 Study Site and Sediment Sampling

Waquoit Bay is a partially enclosed bay on the south shore of the inner coast of Cape Cod, Massachusetts (Fig. 2). Since the soil is permeable in this area, freshwater runoff mostly penetrates the surface and flows to sea as groundwater and as a result groundwater is the primary source of freshwater to the bay. Waquoit Bay itself is underlain by low permeability marine sediment, however, from the tidal zone inland there is sandy soil overlying a freshwater aquifer. Salt water from the bay penetrates the aquifer beneath the bay, flows inland, circulates back to the Bay beneath a fresh groundwater plume and discharges along a narrow band in the intertidal zone (Michael et al., 2003). Sediment samples were taken from three sites (PZ7, PZ6, and PZ11) on a transect perpendicular to the shore (Fig. 2). Sediments were collected from depth using a pulse auger to core down to 7 m. There is a 0.3 m uncertainty in the reported depth, since that is the depth range over which each sample is collected. Sediments were kept frozen at -80º C until analysis.

2.2 Pore water nutrient, pH, and Eh analysis

Groundwater profiles from the same location as the sediment cores were obtained with a drive-point piezometer system called Retract-A-Tip (AMS, Inc.; Charette and Allen, 2006). Briefly, the stainless steel peizometer was driven to the depth of interest. Samples were pumped through Teflon

tubing using a peristaltic pump. Samples for nutrients were collected into 30 ml acid cleaned scintillation vials after passage through a Pall Aquaprep 0.2 μm capsule filter and stored frozen until analysis. Basic water properties including salinity, pH, dissolved oxygen, and redox potential (Eh) were recorded using a YSI 600XLM multiprobe and 650MDS handheld computer. Back in the laboratory combined NO_3^- and NO_2^-, and NH_4^+ were quantified on a Lachat QuickChem 8000 Flow Injection Analyzer using standard colorimetric techniques.

2.3 Ladderane Core Lipid and Crenarchaeol Analysis

Sediment samples were freeze-dried and extracted (~40g dry weight) by ultrasonication three times each in methanol, methanol/dichloromethane (1:1, v/v), and dichloromethane. Sediment was removed by centrifugation and solvent was dried using rotary evaporation. Total lipid extracts (TLEs) were then saponified in 1N sodium hydroxide in methanol. After addition of water, the saponified TLE was separated into a neutral fraction (containing crenarchaeol) and a fatty acid fraction (containing ladderane core lipids) by extraction into dichloromethane at pH 12 and pH 2, respectively. Fatty acids were derivatized with diazomethane to produce the methyl esters (FAMEs). Polyunsaturated fatty acids were removed by eluting the fatty acid fraction using dichloromethane over a column packed with silver nitrate-impregnated alumina oxide. Ladderane core lipids were detected and quantified according

to methods described in Hopmans *et al*., (2006) using high performance liquid chromatography coupled to positive ion atmospheric pressure chemical ionization mass spectrometry (HPLC/APCI-MS^2) in Selected Reaction Monitoring (SRM) mode. Quantification of the ladderane lipids was achieved using an external standard curve with two authentic standards heptyl-[3]-ladderane FAME and heptyl-[5]-ladderane FAME. Concentrations for the pentyl-[5]-ladderane FAME detected in samples was estimated using the heptyl-[5]-ladderane FAME standard curve. Crenarchaeol was measured and quantified by methods reported in Hopmans *et al.,* (2000) and Schouten et al. (2007) using high performance liquid chromatography coupled to positive ion atmospheric pressure chemical ionization mass spectrometry (HPLC/APCI-MS^2) in Selected Ion Monitoring (SIM) mode. Quantification was achieved by comparison of peak areas with an external standard curve of an authentic crenarchaeol standard. We performed replicate extraction and analysis of ladderane core lipids in sediments at PZ6 from depths 1.1, 2, 2.3, 2.7, 2.9, 3.2, and 4.4 m. Average error between replicates was 34% of the maximum measured value. Reported values are the average of replicate measurements.

2.4 Ladderane monoether-phosphocholine analysis

The intact ladderane phosphocholine (PC) monoethers were extracted and analyzed by methods described in Boumann et al. (2006) and Jaeschke et al. (2009). Briefly, freeze dried sediments (~40g dry weight) were extracted

using a modified Bligh Dyer protocol (Sturt et al., 2004) with phosphate buffer. The resulting extracts were dried by rotary evaporation and kept frozen at -40° C until analysis. Samples were analyzed using HPLC coupled to positive ion Electrospray ionization mass spectrometry (HPLC/ESI-MS^2) in Selected Reaction Monitoring (SRM) mode (Jaeschke et al., 2009). Quantification was achieved by comparison of peak areas with an external standard curve of an authentic PC-monoether standard.

2.5 DNA extraction and Real-time PCR

DNA was extracted from ~1mL of wet sediment using a MoBio soil extraction kit. Gene distribution throughout the core depth (15-30cm intervals) was obtained using real-time PCR on an iCycler thermocycler (Bio-Rad, Hercules, CA). Triplicate DNA extractions of each sample were diluted to 10ng/µl as measured on a Nanodrop 1000. The amplification cocktail contained 1X colorless master mix (Promega, Madison, WI), 10 nM dNTPs (Promega), 0.5X SBYR-Green I (Invitrogen, Carlsbad CA), 20 nM fluorescein (Sigma), 1 mM $MgCl_2$ (Promega), forward and reverse primer (300-1000 nM) and 1.25 units of GoTaq Flexi polymerase (Promega). Archaeal *amoA* was quantified using primers and amplification protocols describe by Francis *et al.,* (2005).

3. RESULTS

3.1 Pore water profiles

Pore water profiles of nitrate+nitrite and ammonium, dissolved oxygen, redox potential, and salinity from site PZ6 are shown in Figure 3. Down core pore water chemistry is characterized by (in order with increasing depth from the top of the water table): 1) an oxic and nitrate-rich surface layer, 2) an anoxic and ammonium-rich plume, 3) a mid-depth oxic nitrate+nitrite-rich zone, and 4) a deep salinity transition zone, with increasing ammonium concentrations. These features are delineated by three gradients in redox potential that occur between 1 - 2 meters, 2.5 - 4 meters, and 5.5 - 7 m. In the discussion that follows we will refer to these as upper redox transition zone (URTZ), middle redox transition zone (MRTZ) and deep redox transition zone (DRTZ). Ammonium concentrations range between 10-40 μM in the upper anoxic zone between the URTZ and MRTZ and 20-25 μM below the DRTZ, and maximum nitrate+nitrite concentrations range between 150-200 μM. A comparison of summer pore water profiles for the previous three years at site PZ6 indicates that the redox gradients and relative trends in nutrient concentrations have been persistent, but that the MRTZ and DRTZ migrated vertically by up to 1 meter between Fall 2005 and Summer 2006 (Fig. 4). This variability is driven by seasonal excursions in the depth of the water table (our results are reported as depth below the beach face rather

than normalized to the water table depth), which itself is driven by seasonal and interannual variability in rainfall recharge to the aquifer. The freshwater ammonium plume bounded by the URTZ and the MRTZ is thought to originate from groundwater recharge through a nearby wetland.

3.2 Ladderane and crenarchaeol profiles

The concentration of three ladderane FAMEs (core lipids), the ladderane phosphocholine (PC) monoether, and crenarchaeol (Fig. 1) were measured in coarse-grained sediments from a core taken at site PZ6 through the Waquoit Bay subterranean estuary. Ladderane core lipids, ladderane monoether PC monoether, and crenarchaeol were detected in all of the samples we measured, and in most instances, all compound classes reach maximum concentrations at similar depths (Fig. 3). Ladderane core lipid concentrations are reported relative to sediment dry mass and are presented as the sum of all three measured ladderane core lipids. The concentrations of the ladderane PC monoether, are also reported for core PZ6 and reported relative to sediment dry mass (Fig. 3). In Figure 3, crenarchaeol abundances are presented as the integrated LC-MS peak area normalized to sediment dry mass. We reanalyzed a subset of samples from PZ6 with a quantitative standard curve in order to report concentrations (Fig. 3).

All three ladderane core lipid FAMEs were present in all of the samples, with average relative percent abundances as follows: II = 14%, III = 38%, and IV = 48%. These relative abundances are in good agreement with those found for core lipids in cultured anammox bacteria (Rattray et al., 2008). Summed ladderane core lipid concentrations – reported as pg ladderane / g sediment dry weight - ranged from roughly 3 pg/g up to nearly 800 pg/g. The maximum concentrations observed are comparable to concentrations observed in sediments from the Irish Sea, and Gullmarsfjorden, Sweden (Hopmans et al., 2006; Jaeschke et al., 2009), which is noteworthy given that the coarse-grained sands in the subterranean estuary have much lower surface area than the fine-grained marine sediments in the North Sea. If we assume that there is a similar relationship between lipid abundance and cell count, then this perhaps suggests that more of the available surface area in the subterranean estuarine sediments is occupied with anammox cells, and that cell density is therefore higher than in the marine sediments. Maximum ladderane core lipid and crenarchaeol concentrations occur at 0.2 m, 2 – 2.3 m, 3.5 m, and 6.7 m. Ladderane core lipid and crenarchaeol concentration maxima occur at the same depths except between 2-2.3 m where the peak in crenarchaeol occurs 0.3 meters below the peak in ladderanes. While the deepest lipid concentration maximum matches up well with the depth of the salinity transition zone and associated DRTZ

and the maximum value at 2-2.3m matches up well with the URTZ, the maximum value observed at 3.5 m does not appear to match up with the depth of the MRTZ when this core was sampled in 2006. However, as we will elaborate on in the discussion, this may be due to depth variability in the pore water redox profiles.

Data for the ladderane PC-monoether profile at PZ6 are shown in Figure 3. The sample depth resolution for the PC-monoether profile is much lower than for the ladderane core lipid profile from this core, and it appears to miss features that may be associated with the DRTZ and the MRTZ. However, a concentration maximum is observed between 1.4-1.7 m, corresponding exactly with the depth of the URTZ.

3.3 amoA and 16S qPCR Profiles

Profiles of bacterial and archaeal amoA and archaeal 16S gene fragments quantified by real-time PCR are shown in Figure 5. These data are from sediments at site PZ6, however, these samples were taken in 2005, one year prior to samples analyzed for lipids at this site. For this reason, we only make qualitative comparisons in the down core trends in profiles of lipids and qPCR data at site PZ6. Both bacterial and archaeal amoA show similar trends, with maximum values occurring at 1-1.2m and at 3m, and minimum

values between 1.8-2.1m. Maxima in *amoA* gene abundance correspond well to the URTZ and MRTZ at this site when it was sampled in 2005.

4. DISCUSSION

Three redox transitions zones occur in the pore waters of the Waquoit Bay subterranean estuary (URTZ, MRTZ, and DRTZ). The depth of these zones may vary from year to year in response to changes in the water table and changes in the relative flux of ground water from different sources. For instance, the depth of all three redox transition zones at PZ6 remained stable over two years, from 2004-2005, but in 2006 the MRTZ and DRTZ migrated vertically by nearly one meter (Fig. 3). Nevertheless, during the course of our observations at this location, the three redox transition zones have been persistent features of this environment and a sink for fixed nitrogen (Kroeger and Charette, 2008). Anammox are well-suited for such a dynamic environment as they are reversibly inhibited by oxygen and, therefore, can survive vertical migration in the position of the redox transitions zones (Strous et al., 1997).

The presence of viable anammox bacteria in the subterranean estuary is demonstrated by the detection of the ladderane PC-monoether at PZ6. The ladderane PC-monoether profile shows that anammox is most abundant within the URTZ. The vertical extent of this peak in anammox abundance appears to be roughly 0.5 m based on the sharp decrease in concentration from 1.7 to 1.4 m, suggesting that anammox is limited to a fairly narrow band with respect to the depth range over which the redox transition zone occurs.

We expected anammox bacteria to be most abundant where ammonia and nitrate coexist and oxygen is suitably low to prevent inhibition of the anammox process. In support of this, the peak in abundance is positioned just below the oxycline and at the depth where ammonia concentration begins to increase. Anammox has relatively low abundance at depths corresponding to the MRTZ at the time pore water was sampled in the summer of 2006. A slightly elevated concentration of the PC-monoether at 4.0 meters indicates a moderate abundance of anammox at the depth of the MRTZ for the years prior to 2006. Taken together, these two observations could suggest that these samples were collected shortly after the migration of the MRTZ, such that the anammox community had not yet relocated to the shallower depth of the redox transition zone. Due to the low depth resolution of the PC-monoether profile in the deeper portion of the core, we cannot determine from the PC-monoether data whether anammox is present or absent within the DRTZ.

We show the presence and distribution of AOA and AOB within the upper 4 meters at PZ6 through quantitative amplification of archaeal and bacterial *amoA* gene fragments. Downcore trends in the qPCR profiles of *amoA* show that maximum abundance of AOA and AOB coincide with depths corresponding to the URTZ and MRTZ at the time pore waters and sediment were sampled for these analyses in 2005. Both AOA and AOB are most

abundant towards the upper part of the depth range of the URTZ – bacterial and archaeal *amoA* both reach their maximum values where the highest Eh values in this depth range are observed. AOA and AOB show a local maximum in abundance centered around 3 m within the MRTZ, but are far less abundant than within the URTZ. Archaeal *amoA* abundance is relatively constant from 2.4-3.7 m and suggests that AOA populations are distributed more diffusely across a larger depth range within the MRTZ than within the URTZ. This could be explained if the MRTZ had exhibited more temporal depth variability than the URTZ prior to sampling, placing pressure on ammonia oxidizing populations to migrate along with the depth where optimal growth conditions occur.

The profiles for *amoA* and ladderane PC-monoether match up well. A comparison of the depth of maximum abundance of anammox and AOA/AOB relative to the redox gradient within the URTZ and MRTZ suggests that anammox and AOA/AOB overlap, but that they may reach maximum abundances at different depths. However, given the low depth resolution of the ladderane PC-monoether profile in this depth range, we cannot rule out the possibility that much higher concentrations occur and we are simply missing a peak in anammox abundance within the URTZ. At the MRTZ, it appears that both anammox and AOA/AOB are locally prevalent but less abundant than within the URTZ. These observations are consistent with the

idea that production of nitrite and removal of oxygen by AOA/AOB may extend the depth range over which anammox bacteria can remain active, allowing them to extend further into the oxic end of the redox transition zones.

In contrast to the ladderane PC-monoether and *amoA* genes, ladderane core lipids and crenarchaeol both have the potential to be preserved in the environment long after the cells they were associated with have died. This means that the depth profiles of these lipids reflect a time-integrated accumulation of biomass of anammox and crenarchaeota. We observed distinct peaks in concentration in both profiles, indicating that either the fossil contribution is relatively small compared with the contribution from living cells or else that, on average, the depth distribution of anammox and crenarchaeota has been stable over the time scale that ladderane core lipids and crenarchaeol are preserved. Given the variability we observe in the pore water profile, the former seems to be most likely.

The ladderane core lipid profile shows three regions of high concentration within the subterranean estuary at depths that correspond to the URTZ, MRTZ, and DRTZ, in addition to high concentration in a surface sample that had a soil-like consistency. The broad depth range over which ladderane core lipids are detected most likely reflects the range over which the redox transition zones and region of maximum anammox activity have

migrated over time. Anammox populations near the DRTZ appear to have remained relatively stationary in comparison with anammox populations from the URTZ and MRTZ, which have spanned nearly 2 m depth. The large peak in ladderane core lipid concentration at 3.5 m depth could be indicative of abundant viable anammox at this depth. There is, however, an apparent depth offset between this peak in ladderane core lipid concentration and the MRTZ centered at ~2.8 meters (Fig. 3). As we argued in the case of the PC-monoether, this offset could be because of a recent migration in the depth of the redox transition zone. The possibility that a shoaling of the MRTZ occurred shortly prior to sampling such that anammox bacteria had not yet had sufficient time to re-locate is supported by fact that we do not see a local concentration maximum for either the ladderane core lipids or PC-monoether (Fig. 3) at the depth of the MRTZ in 2006, and that we do observe elevated concentrations of both lipid classes at depths corresponding to a deeper MRTZ prior to 2006.

The crenarchaeol profile shows a steady decline in concentration from the surface to depth, punctuated by maxima in concentration at depths that roughly correspond to the three redox transition zones. Crenarchaeol and the ladderane core lipid profiles show a remarkable similarity through most of the core, except at 2.2m where the two profiles diverge, indicating that crenarchaeota and anammox have occupied the same range of depths, and

that their distribution has been largely determined by the position of the redox transition zones. A close spatial relationship between crenarchaeol and ladderanes was also observed in the water column of Black Sea, by Wakeham et al (2007), where AOA and AOB are suspected to provide roughly half of the nitrite consumed by anammox (Lam et al., 2007). Crenarchaeol concentrations are on the order of 100 times higher than ladderane FAMEs in both the subterranean estuary sediments as well as filtered particulate matter from the Black Sea (Wakeham et al., 2007)(Fig. 5). If a similar relative abundance of AOA versus anammox bacteria exists at the chemocline of the Black Sea and within the redox transition zones of the Waquoit Bay subterranean estuary, then perhaps this indicates a general stoichiometric relationship driven by syntrophic interactions between these two communities in such environments in general.

The co-occurrence of both aerobic ammonium oxidizing bacteria (AOB) and AOA with anammox has been observed in the Namibian Upwelling system (Woebken et al., 2008) and in the Black Sea (Lam et al., 2007). The potential for syntrophy between aerobic ammonium oxidation and anammox was recognized soon after the discovery of anammox bacteria with the invention of a novel sewage treatment process known as the Completely Autotrophic Nitrogen removal Over Nitrate (CANON) process, where anammox and aerobic ammonium oxidizing bacteria (AOB) are cultivated in

co-culture, with AOB providing nitrite and removing oxygen, and anammox converting ammonium and nitrite to N_2 gas (Sliekers et al., 2002). However, within the subterranean estuary it remains to be determined if anammox and AOA/AOB coexist under a competitive or a mutualistic relationship. Clearly anammox and AOA/AOB both have a demand for ammonium, which would indicate a competitive relationship. However, anammox could benefit from the production of nitrite and consumption of oxygen by AOA/AOB. Within the redox transition zones of the subterranean estuary AOA/AOB could increase the ecological range of anammox and enhance anammox activity, by simultaneously drawing down oxygen levels in the oxic end of the chemocline and supplying nitrate towards the anoxic end of the chemocline, thereby extending the conditions favorable for anammox activity. Do anammox bacteria provide a complimentary benefit to AOA/AOB? The distribution of anammox and AOA/AOB determined by detection of the ladderane PC-monoether and amoA genes indicates that these communities overlap, but do not reach maximum abundance at the same depth. However the presence of AOA/AOB may still extend the spatial range of anammox within the subterranean estuary, thereby increasing the importance of anammox as a sink for fixed nitrogen in this environment. Certainly the presence of anammox in the well-aerated surface soil sample implies that anammox is

thriving in microenvironments that are possibly facilitated by syntrophic interactions with AOA/AOB.

5 CONCLUSIONS

Presently we demonstrate the presence of anammox, AOA/AOB through the detection of ladderane PC-monoether and archaeal and bacterial *amoA*. Our results suggest that anammox and AOA/AOB are associated with three redox transition zones within the subterranean estuary, and could potentially play an important role in the nitrogen cycle of the subterranean estuary. The distribution of ladderane core lipids and crenarchaeol – presumably vestiges of fossil biomass - indicates that the vertical distribution of anammox and crenarchaeota has been fairly constant over the time period that these lipids have accumulated. Additional work in this environment will need to address the quantitative importance of anammox and aerobic ammonium oxidization in terms of absolute rates of nitrogen removal and relative to other processes including dissimulator nitrate reduction (DNRA) and aerobic nitrification.

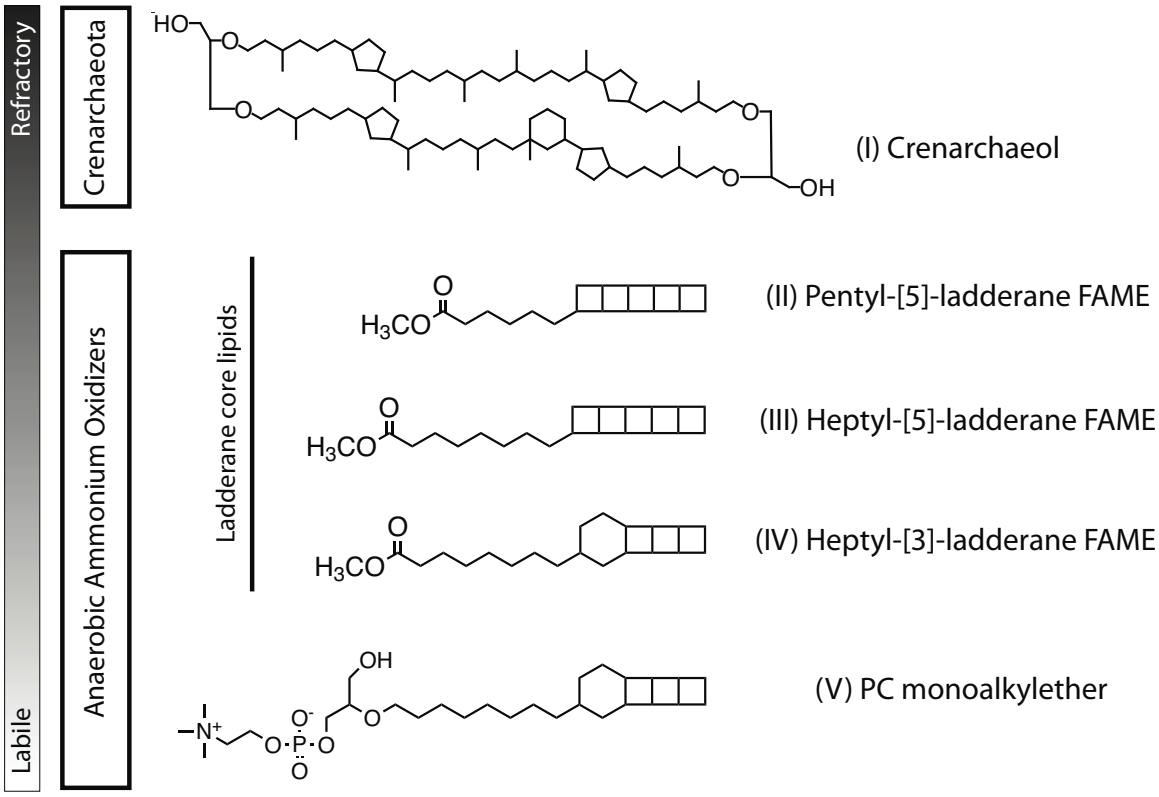

Figure 1. Structures of lipid biomarkers measured in this study. The gradient bar on the left illustrates the relative stability of these compounds, going from labile (least stable) to refractory (most stable).

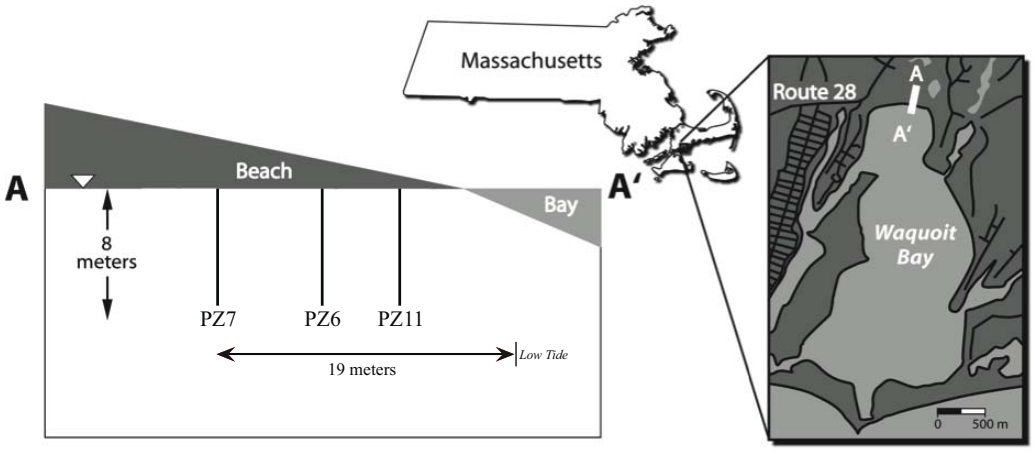

Figure 2. Map showing the location of Waquoit Bay in Falmouth, Massachusetts, USA and location of the core site at the head of the bay. The cartoon on the left depicts the location of site PZ6 with respect to low tide. Lipid profiles from sites PZ7 and PZ11 are shown in Appendix A2-2.

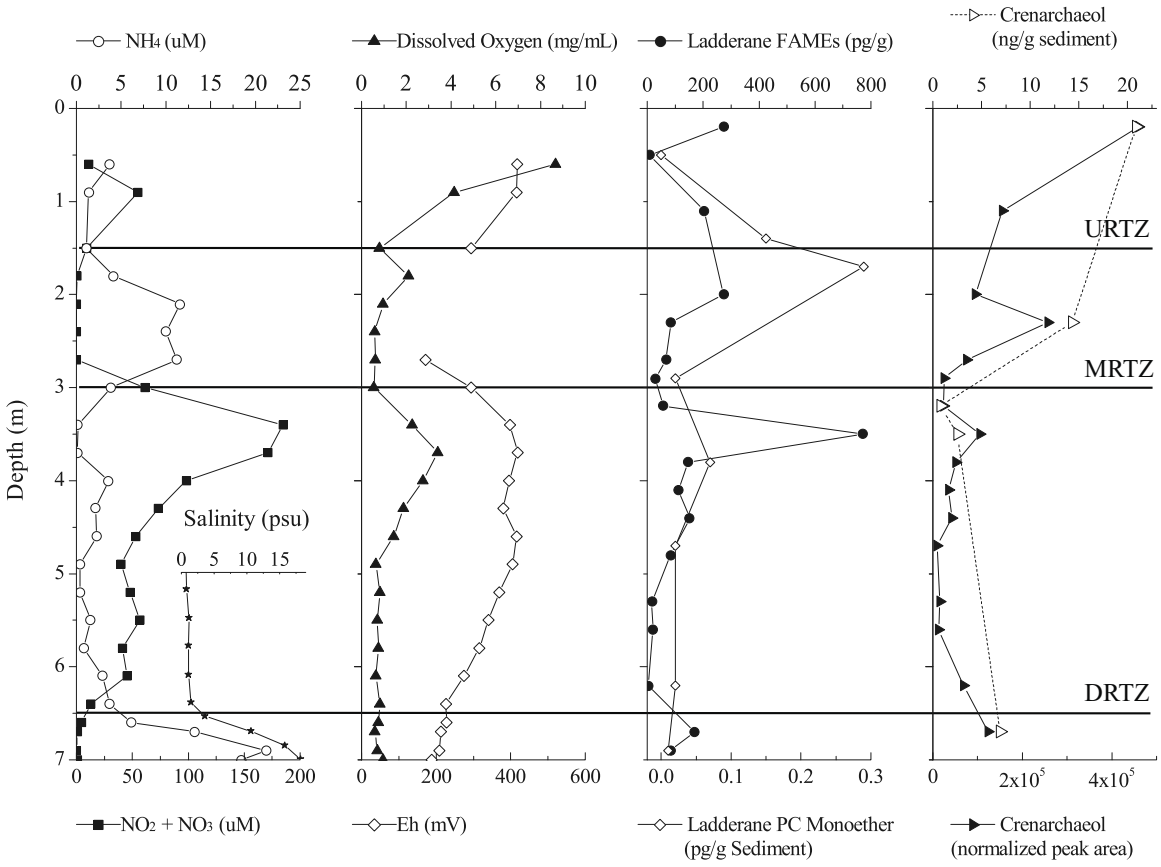

Figure 3. Expanded plot of pore water data and lipid concentrations for PZ6, including ladderane PC-monoether concentrations. Going from left to right, axis show A) Ammonia and nitrate+nitrite concentrations and salinity B) dissolved oxygen and Eh, C) ladderane core lipids and ladderane PC-monoether, and D) Crenarchaeol abundance reported as peak area normalized to sediment mass and a subset of samples analyzed quantitatively and reported as ng/g sediment. Three lines are shown to indicate the position of the three redox transition zones.

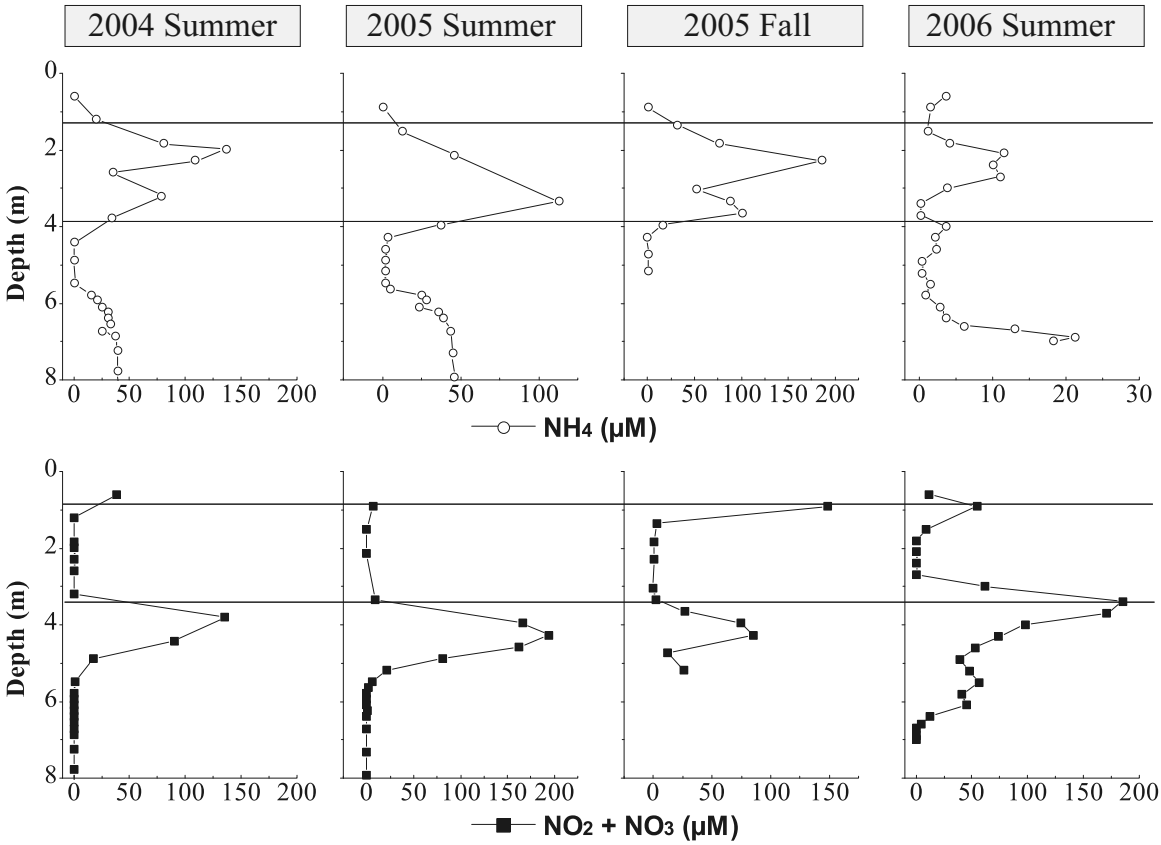

Figure 4. Pore water concentrations of ammonia (top) and nitrate+nitrite (bottom) sampled at four time points over three years at site PZ6. Horizontal lines indicate the general position of the transition from nitrate+nitrite-rich to ammonia-rich water at the URTZ and MRTZ.

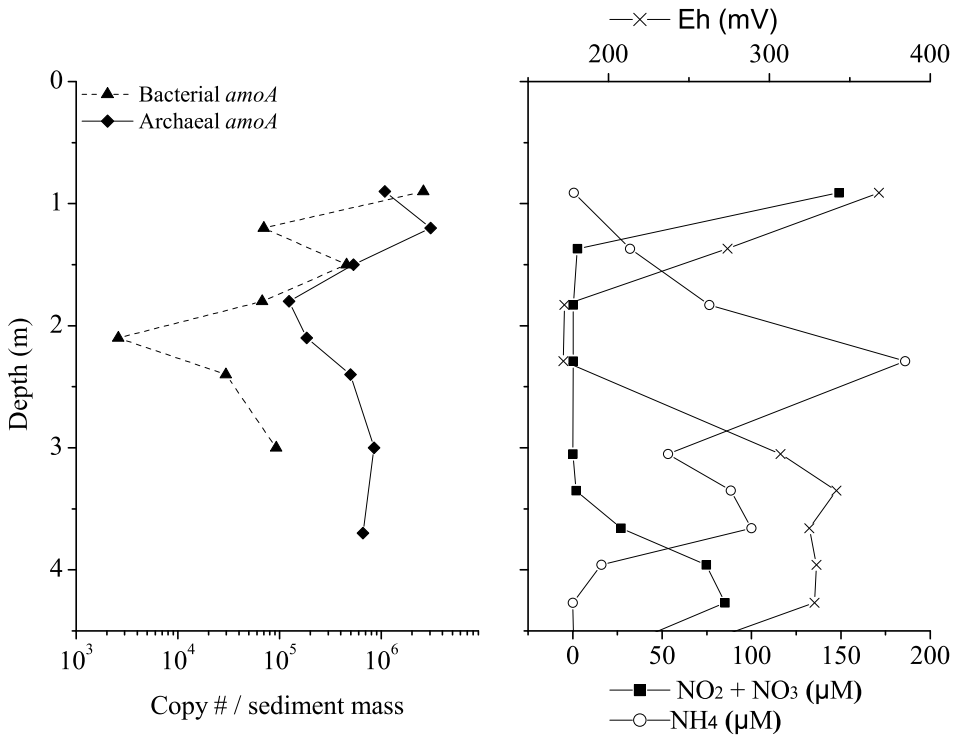

Figure 5. Sediment qPCR profiles of Bacterial and Archaeal *amoA* (left axis) compared with pore water profiles of ammonia and nitrate-nitrite concentration and Eh (right axis).

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CHAPTER 3 – METHODS FOR THE EXTRACTION AND ANALYSIS OF BACTERIOHOPANEPOLYOLS

1. INTRODUCTION

Our extraction and analysis of bacteriohopanepolyols was based on methods developed by Helen Talbot and Paul Farrimond at Newcastle University over the past 10 years. We have made some modifications to the extraction protocol, which we describe in detail below. Additionally, we have prepared an authentic standard of bacteriohopanetetrol (BHT) allowing us to report quantitative values for BHPs in environmental samples.

2. EXTRACTION OF BACTERIOHOPANEPOLYOLS

2.1 Preparation of materials

All glass implements (vials, pipettes, beakers etc…) combusted at 450° C for 8 hours in order to eliminate any organic contaminants. Reusable glassware (e.g. beakers) was washed thoroughly with detergent and distilled water following use and prior to combustion. Syringes, spatulas, and forceps were rinsed three times each in hexane, dichloromethane (DCM), and methanol (MeOH) in order to remove any organic contaminants prior to use.

2.2 Modified Bligh-Dyer protocol for extraction of BHPs

Extraction of bacteriohopanepolyols (BHPs) was carried out according to the method of Bligh and Dyer (Bligh and Dyer, 1959) with some

modifications, including the replacement of chloroform with DCM. The sample is placed in a combusted glass vial or round-bottom flask and brought up in a mixture of MeOH/DCM/water (2:1:0.8 v/v). The sample is sonicated in an ultrasonic bath for 1 hour at 30° C and then fixed to a shaker table and shaken for 3 hours at room temperature. Following sonication and shaking, the sample is transferred to a combusted glass centrifuge vial and pelleted by centrifugation at 1000 rpm for 10 minutes. The supernatant is then decanted with a Pasteur pipette and transferred to a separatory funnel. DCM and water are added in a 1:1 ratio to achieve a mixture of MeOH/DCM/water of 1:1:0.9, which results in separation of aqueous (water and methanol) and organic (DCM) phases. The lower organic phase (DCM) is removed by separatory funnel into a glass vial and labeled as the total lipid extract (TLE). The residual aqueous phase is extracted with DCM twice and the resulting organic phases are added to the TLE fraction. The TLE is kept frozen at -40° C until further use.

2.3 Optimization of Bligh Dyer Protocol.

Settling on an extraction protocol ultimately must involve a compromise between extraction efficiency and time efficiency. We carried out a few basic variations on the Bligh Dyer protocol that has previously been reported for the extraction of hopanoids from environmental samples and cultures, in order to determine if any added benefit would be gained by a

more rigorous extraction. Talbot and Farrimond (2007) for instance shake their samples overnight. Michelle Allen (PhD Thesis, UNSW) replaces the water in the extraction mixture with a 1% solution of trichloroacetic acid (TCA), which has previously been reported as providing the most optimal yield when extracting archaeal cells (Nishihara and Koga, 1987). We also consider the possible effect that freeze-drying might have on BHP yield, since some, but not all, environmental samples used in this study were freeze-dried and this could potentially lead to a bias. By comparing three variations on the extraction procedure described above, we address three aspects of the extraction procedure: 1) the effect of freeze-drying samples prior to extraction; 2) duration of shaking during extraction, and 3) the yield resulting from replacing water with 1% TCA (Table 1).

We prepared 4 equal aliquots of fresh biomass of *Crocosphaera watsonii* (WH8501) and extracted each aliquot with four variations on the Bligh Dyer protocol as listed in Table 1. The results of the extraction yield for BHP cyclitol ether (BHP-CE) and bacteriohopanetetrol (BHT) are shown in Figure 1. These results indicate that freeze-drying samples prior to extraction results in a minimal change in yield in pure biomass. However, the effect of freeze-drying on sediments are yet to be tested. Increasing the extraction time (shaking time), also did not result in a significant change in yield. Interestingly, the extraction with 1% trichloroacetic acid in replacement of

water, showed a marked decrease in yield. These results indicate that the basic procedure (BD1) provides the best optimization for time and extraction efficiency.

Taking extraction conditions used for BD1 in the previous experiment, we extracted an environmental sample repeatedly to determine if this is necessary to achieve maximal recovery, as is reported by Talbot and Farrimond (2007) (Table 2). We also investigated whether "inextricable" BHPs are released by direct acetylation of extracted residue, as is reported by Herrmann et al., (1996). A dried intact sample of deep sea sponge (kindly provided by Dr. Kate Hendry, WHOI) was extracted by the Bligh-Dyer protocol as described above (section 2.1) and the TLE was labeled as extraction "stage 1" (Table 2). The extracted residue was then re-extracted by sonicating for one hour and then shaking for one hour in 2:1 DCM/MeOH. This extract was labeled as "stage 2." The sample was then ground to a fine powder by mortar and pestle. A third extraction was performed on the ground sample by the Bligh-Dyer protocol and labeled as "stage 3." Finally the residue was rinsed with DCM and blown down to dryness. A final extraction was performed by directly acetylating the extracted residue in 1:1:1 acetic anhydride/pyridine/DCM at 70 °C for 1 hour. This extraction was labeled "stage 4." The results of this sequential extraction experiment indicate that one extraction is sufficient to obtain over 90% of the BHPs that can be

retrieved through multiple extractions. We therefore maintain that a single extraction by the method of Bligh and Dyer, with modifications described above, is suitable for extracting BHPs from environmental samples.

2.4 Preparation of total lipid extracts (TLEs) for analysis by HPLC-APCI-MS

TLEs from Bligh-Dyer extraction typically contain some components, such as salts, that can make it difficult to concentrate TLEs to levels suitable for the detection of BHPs in environmental samples. This is because the organic phase from the Bligh-Dyer extraction has some methanol in it, allowing salts to carry over into the TLE. Therefore, all TLEs are subjected to a final step to remove salts and other water soluble components. The TLE transferred to a 1.5 ml glass vial and reduced to dryness under N_2 gas. 0.5 ml of hexane-extracted Milli-Q water is added and the vial is sonicated in an ultrasonic bath for 10 seconds. 0.5 ml of DCM is then added and the sample is shaken vigorously. The DCM and water are allowed to separate and the DCM phase is removed by Pasteur pipette and transferred to a new vial. The DCM extraction is repeated an additional two times. The TLE is then reduced to dryness under N_2 gas. The TLE is acetylated with 1:1:0.2 pyridine/acetic anhydride/DCM (240 ul total volume) for 1 hour at 70 °C and left at room temperature overnight. Following acetylation, the acetylated TLE is reduced to dryness under N2 gas and brought up in 0.5 ml of 60:40:20 MeOH/propan-2-ol (IPA)/DCM, and filtered through an 0.45 μm Millipore

Millex syringe filter to remove particles that could clog the LC column. Finally, the sample is blown down to dryness under N_2 gas, and brought up in a known volume of 60:40:20 MeOH/propan-2-ol (IPA)/DCM for analysis by HPLC-MS.

3. ANALYSIS OF BACTERIOHOPANEPOLYOLS

3.1 HPLC-APCI-MS method and instrument conditions

BHPs were analyzed using an HPLC-APCI/MS method described by Talbot et al. (2003). Briefly, separation of BHP compounds was achieved on a reverse-phase Grace Prevail C_{18} column (3µm, 150 x 2.1 mm) at room temperature. The solvent method began with 90% A and 10% B ramping to 59% A, 1 % B, and 40% C at 45 minutes, where A = methanol, B = water, and C = propan-2-ol. Analyses were performed on a Finnigan Surveyor LC coupled to an LTQ-MS, setup with an APCI source running in positive ion mode with the source temperature set to 400° C. LTQ-MS conditions were optimized by running an auto tune program while infusing an acetylated extract of *Rhodopseudomonas palustris* and tuning to the molecular ion corresponding to acetylated bacteriohopanetetrol (m/z 655).

Data was acquired in three scan events resulting in three sets of mass spectra (MS^1, MS^2 and MS^3): Scan 1 was a full mass spectrum scan; Scan 2 was programmed to isolate masses from MS^1 for CID and full spectrum scan

from a list of known BHP molecular ions using a dynamic exclusion protocol; Scan 3 was programmed to select the most abundant ion from the MS^2 for CID and full spectrum scan. Fragmentation was achieved by collision induced dissociation (CID) set to 15% intensity (arbitrary units specific to the instrument).

3.2 Structural identification of BHPs

Structural identification was based on retention time and characteristic fragmentation patterns determined from extracts of known composition (*R. palustris*, *C. watsonii*). BHPs have characteristic fragmentation patterns that have been reported in detail (Talbot et al., 2003; Talbot et al., 2003; Talbot and Farrimond, 2007; Talbot et al., 2008). The loss of acetic acid, resulting in a mass loss of 60, can be diagnostic of the number of derivatized hydroxyl and amino groups. Compounds with an ether-linked side chain exhibit a detectable fragment associated with the either-linked group. Adenosylhopane and related structures are characterized by the loss of adenosine resulting in an m/z 611 fragment. Hopanoids are also identified by ring-C fragmentation yielding an m/z 191 or 205 ion for des-methyl and methyl BHPs, respectively. However, due to the limits in the mass range that the ion trap can collect in scan 1, the products of ring-C fragmentation cannot be observed for higher mass compounds such as the composite BHPs.

3.3 Quantification of BHPs

Isolation of BHT authentic standard

Acetylated BHT was isolated from *R. Palustris* by preparative reversed phase HPLC using an Agilent 1200 Series LC coupled to a 6130 Quadrupole mass spectrometer. 2 liters of dense *R. Palustris* biomass was extracted according to the modified Bligh and Dyer protocol described above. The resulting total lipid extract (TLE) was saponified in 0.5 N KOH in methanol at 70 °C for 2 hours. Following saponification, water was added to the to achieve a 1:1 methanol/water mixture, and BHPs were collected in a neutral fraction extracted in DCM at pH ~ 12. The neutral fraction, weighing 58.9 mg, was concentrated down to 1 ml (60:40:20 methanol/propan-2-ol/dichloromethane) in preparation for preparative HPLC isolation. The TLE was separated on a reversed phase Agilent Eclipse XDB-C_{18} column (5µm, 4.6 x 150 mm), running a solvent scheme slightly modified from the standard BHP method in order to enhance separation of BHT from aminotriol and other closely eluting compounds. The solvent scheme was as follows: A = 100%, B = 0 % at 0 min, A = 40%, B = 60 % at 10 min, A = 60 % B = 40 % at 40 minutes, where A = 90:10 methanol/water and B = 59:1:40 methanol/water/propan-2-ol. Acetylated BHT was collected in fractions with a fraction collector running in mass-dependent mode triggered by m/z 655 within a specified retention time range. After fractions from 20 consecutive

runs were collected and pooled, the isolated material dried and weighed on a high precision balance (Sartorius micro pro11) and found to have a mass of 141 μg. We then analyzed the acetylated BHT fraction by the standard BHP LC-MS method in order to determine purity (Fig. 2). The molecular ion corresponding to BHT acetate (m/z 655) made up 81% of the total peak area of the total ion current chromatogram. Therefore, we estimated the purity of BHT acetate in this fraction as 81% when making standard concentration curves.

Standards for quantification

Initially Quantification was achieved by an external authentic standard of acetylated glucosyl sitosterol (Matreya cat. # 1117; steryl glucoside)(Fig. 3). However, after we enriched bacteriohopanetetrol (BHT) for use as an authentic standard, subsequent quantification was based on an external standard curve of acetylated BHT (Fig. 4A). 3α,12α-Dihydroxy-5β-pregnan-20-one 3,12-diacetate (PD) was chosen as an internal standard because of its structural similarity to hopanoids and because it has a retention time that does not overlap with the range of BHP retention times. All three standards exhibited linearity in response factor over 4 orders of magnitude (Fig. 3; Fig. 4 A and B). Concentrations for individual BHPs in each sample run were calculated relative to the peak area of the PD internal standard based on the difference in response factor between BHT and PD,

which was estimated from the ratio of the slopes of the two standard curves. Given the large degree of variability of response factor between different head group structures, quantitation is only considered accurate for BHT and BHpentol. BHPs containing amine groups, or composite structures will likely have different response factors. However, in order to plot the relative abundance of other compounds along with BHT, concentrations are reported for these compounds from the BHT external standard, but these values are only reported as a means for comparison.

Normalization of BHP quantitative values

Concentration can be reported in a number of ways depending on the sample type and the sort question being asked. Ideally, we could report concentration as normalized to total organic carbon (TOC) values, since this would allow us to compare concentrations across a range of diverse sample types (sediments, cultures, suspended particulate matter). However, TOC values were not available in many of the samples that we acquired. Where available, we report concentration relative to TOC. Total lipid extract mass was recorded in most samples, although TLE mass does not always correlate with TOC values, especially between samples from different environments. In sediments, we report concentrations relative to sediment dry mass, in water samples we report concentrations relative to volume filtered, and in culture samples we report concentrations relative to cell count and TLE mass.

Table 1: Variations on the Bligh-Dyer extraction are listed. Four equal aliquots of fresh cultured cells of *C. watsonii* were extracted by the four variations. BD1 is the standard method that we employ. In BD2 we replaced water with a 1% trichloroacetic acid (TCA) solution. In BD3 we lengthened the shaking time during extraction to 24 hours. In BD4 all conditions were the same as BD1 except the sample was freeze-dried prior to extraction.

Sample	Sample preparation	Sonication time (hours)	Shake time (hours)	Solvent mixture
BD1	wet	1	3	MeoH/DCM/Water - 2:1:0.8
BD2	wet	1	3	MeoH/DCM/1% TCA (w/v) - 2:1:0.8
BD3	wet	1	24	MeoH/DCM/Water - 2:1:0.8
BD4	freeze-dried	1	3	MeoH/DCM/Water - 2:1:0.8

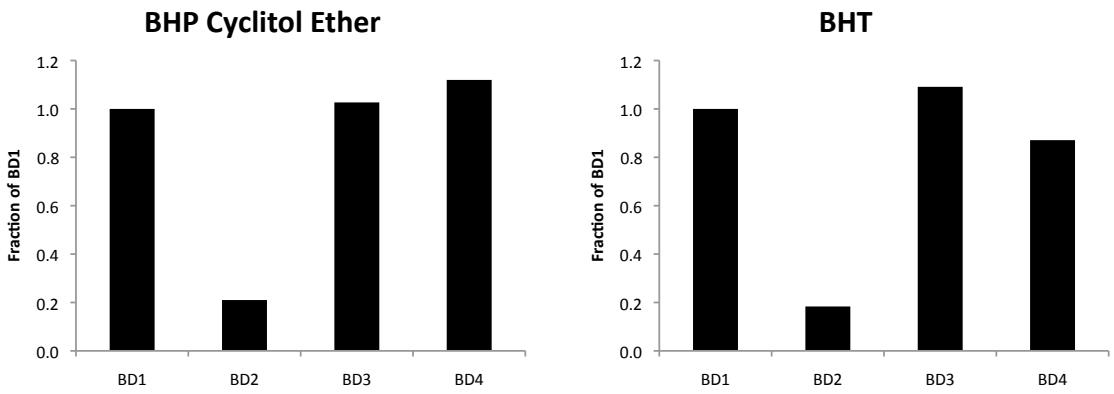


Figure 1: The yield of extractions BD1-BD4 (Table 1) for BHP cyclitol ether and bacteriohopanetetrol (BHT) reported as the fraction relative to the yield from BD1.

Table 2: Results of a four-stage sequential extraction of a deep-sea sponge sample, showing the percent of each of four BHPs extracted in each stage.

Extraction method	Extraction stage	Percent of total extracted in extraction stage			
		Ia	Ib	Ik	Il
Bligh and Dyer	1	97.5%	100.0%	91.1%	100.0%
2:1 DCM/methanol	2	0.6%	0.0%	0.0%	0.0%
Bligh and Dyer	3	0.9%	0.0%	3.1%	0.0%
Acetylation of residue	4	1.0%	0.0%	5.8%	0.0%

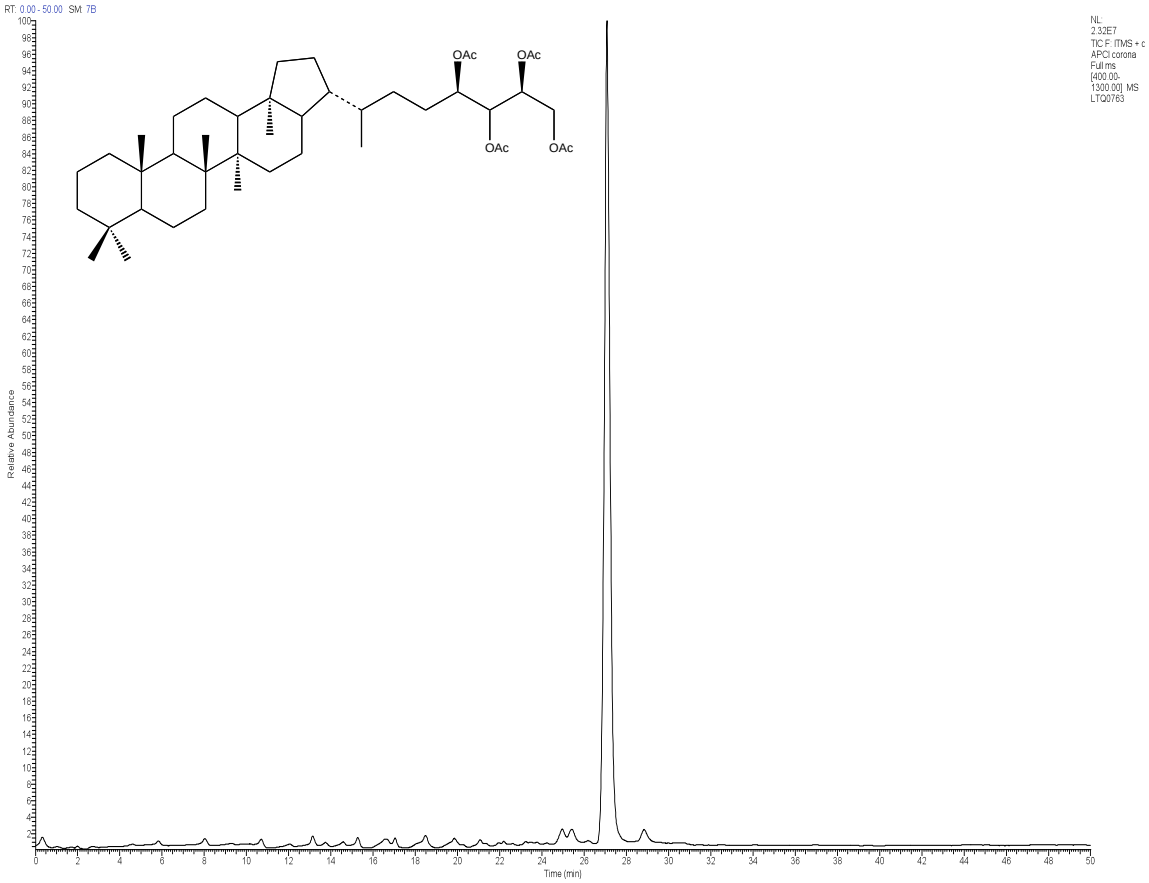


Figure 2: Total ion current chromatogram of BHT acetate fraction enriched by preparative HPLC from an extract of *Rhodopsudomonas palustris*.

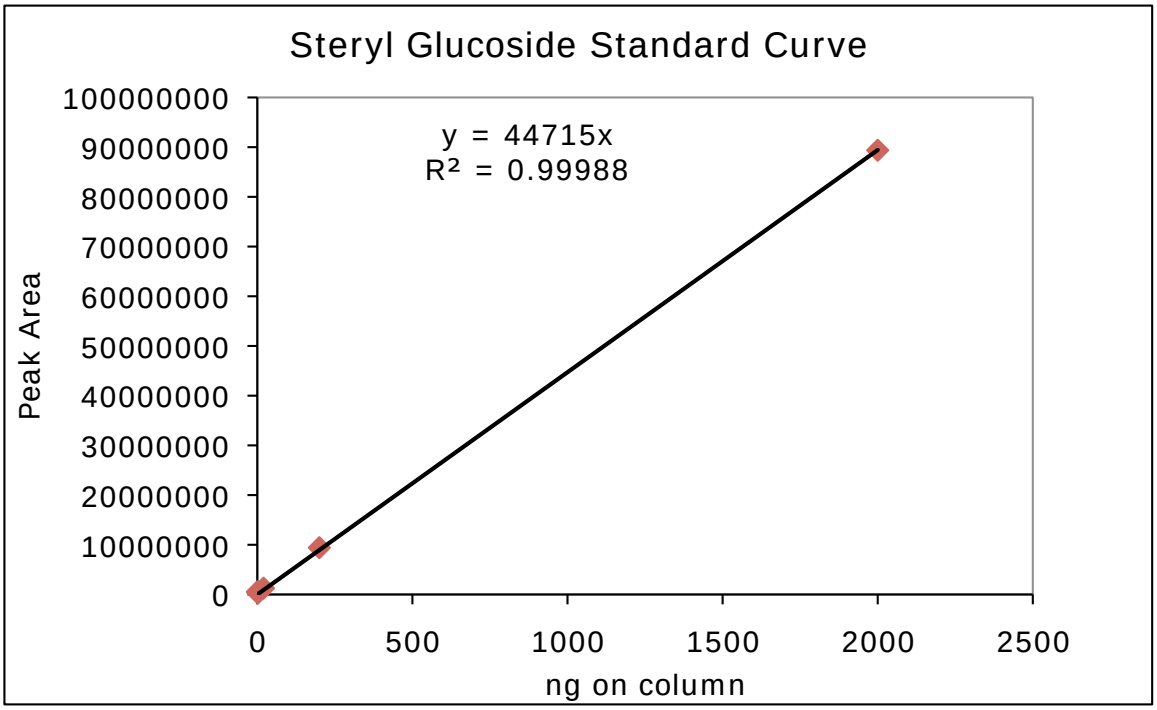
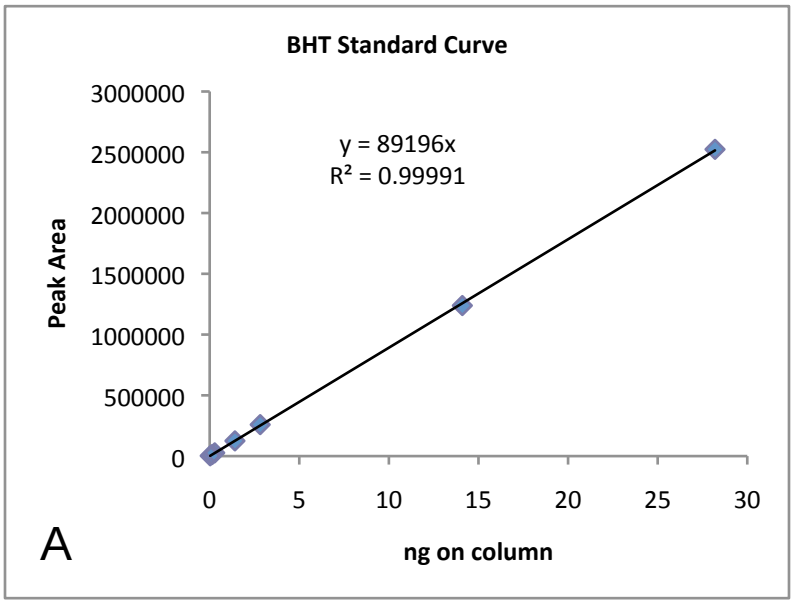


Figure 3: Standard curve of acetylated glucosyl sitosterol (steryl glucoside) plotted as peak area vs. ng of compound on column.



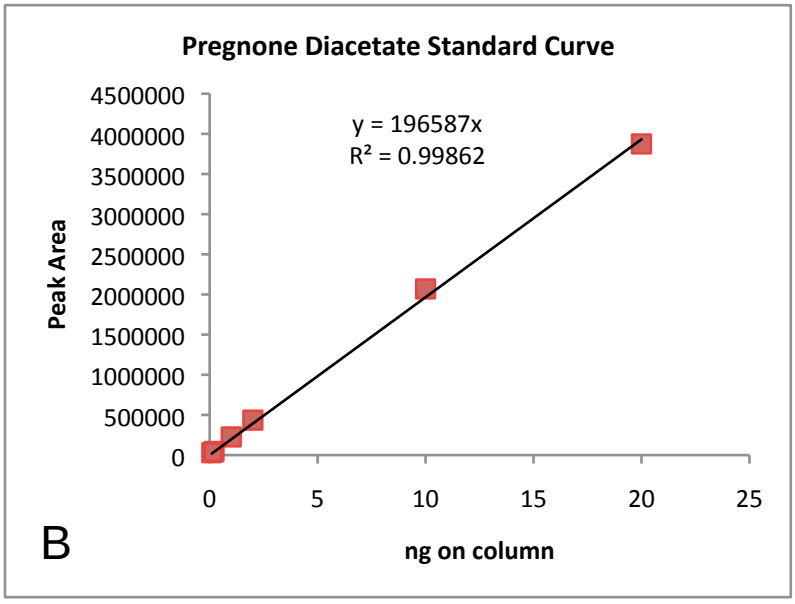


Figure 4: Standard curves of A) acetylated BHT and B) pregnone diacetate plotted as peak area vs. ng of compound.

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CHAPTER 4 - PROBING THE STRUCTURAL DIVERSITY, PHYLOGENETIC DISTRIBUTION, AND BIOLOGICAL ROLE OF BACTERIOHOPANEPOLYOLS (BHPS) IN MARINE CYANOBACTERIA

ABSTRACT

Cyanobacteria are key players in the global carbon and nitrogen cycles and they are thought to have been responsible for the rise of atmospheric oxygen during the Archaean. There is evidence to suggest that hopanoids may be diagnostic of cyanobacteria in the environment, and therefore could serve as biomarkers for cyanobacteria in past and present environments. However, very little is known about the distribution or physiological roles of hopanoids in marine cyanobacteria. Our survey of a diverse range of marine cyanobacterial cultures, and cyanobacterial enrichment cultures indicates that hopanoid production may be unique to the nitrogen-fixing cyanobacteria in marine environments. *Crocosphaera watsonii* (WH8501) is found to have abundant hopanoids that appear to be enriched in the outer membrane. We propose that hopanoids in *C. watsonii* could serve to reduce membrane permeability thereby protecting the cell from oxygen and antimicrobial toxins in the environment.

1. INTRODUCTION

Cyanobacteria are arguably one of the most important groups of organisms in the ocean. They were the putative originators of oxygenic photosynthesis during the Archean (Brocks et al., 1999), which was pivotal in the biogeochemical evolution of the early Earth, and presently they are responsible for a significant component of the primary production on Earth's surface (Goericke and Welschmeyer, 1993). Of particular interest are the nitrogen fixing cyanobacteria, which are thought provide up to half of the fixed nitrogen in the open ocean, fueling the bulk of new primary production through the addition of bioavailable nitrogen to the euphotic zone (Carpenter and Romans, 1991; Lipschultz and Owens, 1996; Capone et al., 1997; Gruber and Sarmiento, 1997; Karl et al., 1997). Up until recently, it was thought that nitrogen fixation was only important in oligotrophic environments, however, new evidence suggests that coastal nitrogen fixation may represent a significant and heretofore overlooked source of fixed nitrogen to the global N-budget (Brandes et al., 1998; Sigman et al., 2005). In the past six years it has also become evident that the diversity of marine nitrogen-fixing cyanobacteria is much greater than previously thought, and that unicellular nanoplankton such as *C. watsonii* may equal or surpass the rates of N_2 production estimated for *Trichodesmium sp.* (Zehr et al., 2000; Zehr et al., 2001; Falcon et al., 2002; Falcon et al., 2004; Montoya et al., 2004). These

recent advances highlight how little is known about the environmental and ecological controls on nitrogen fixation in the oceans.

Previous surveys of hopanoids in cultured bacteria indicate that hopanoid production is widespread among cyanobacteria (Rohmer et al., 1984; Summons et al., 1999; Talbot et al., 2008). Furthermore, given their predominance in many marine environments, cyanobacteria could make a significant contribution to the hopanoid inventory of marine sediments. If unique cyanobacterial hopanoid structures are proven to exist, then hopanoids could serve as useful markers for cyanobacterial ecology through the geologic record.

1.1 Cyanobacterial bacteriohopanepolyols – do any unique ones exist?

Presently, 56 strains of cyanobacteria in culture have been screened for the presence of hopanoids and many of those have had their complete intact BHP compositions characterized by either combined NMR and GC-MS (Gelpi et al., 1970; Bisseret et al., 1985; Renoux and Rohmer, 1985; Jurgens et al., 1992; Herrmann et al., 1996; Llopiz et al., 1996; Simonin et al., 1996; Zhao et al., 1996), or HPLC-APCI/MS (Talbot et al., 2003; Talbot et al., 2008). A total of 25 distinct BHPs have been detected in cyanobacterial cultures, and diversity within a particular strain can range from 1-8 compounds. However, only one BHP (35-O-ß-3,5-anhydro-galacturonopyranosyl BHP and its 2-

methyl homologue; Simonin et al., 1996) has been found exclusively in cyanobacteria, and only occurs in *Prochlorothrix hollandica* (Simonin et al., 1996; Talbot et al., 2008). From these emerging trends, it appears that BHP side chain structures are not likely to serve as diagnostic markers for cyanobacteria. Nonetheless, from a physiological standpoint, the remarkable structural diversity of BHPs in cyanobacteria may hint at a similarly diverse range of functions within the cell. Consequently, comparing the structural diversity between marine and non-marine cyanobacteria might provide clues to the biological role(s) of hopanoids in cyanobacteria.

Structural variations in the hopanoid ring structure may provide another source of taxonomic information. Methylation of the A-ring at C-2 has frequently been observed in non-marine cyanobacteria and in marine cyanobacterial mats from Shark Bay, Australia (M. Allen PhD Thesis, 2007, UNSW). Based on these associations, the 2-methylhopanoids have been applied widely as a molecular marker for the contribution of cyanobacteria to organic carbon in oils, source rocks, and sediments (Summons et al., 1999; Farrimond et al., 2004; Kuypers et al., 2004), and may provide the earliest evidence for cyanobacteria in the geologic record (Brocks et al., 1999; Eigenbrode et al., 2008), although this claim has recently been debated (Rasmussen et al., 2008). However, 2-methylhopanoids have also been identified in organisms other than cyanobacteria (Renoux and Rohmer, 1985;

Rashby et al., 2007), and a recent study indicates that the biosynthetic capacity for C-2 methylation may be more broadly distributed among bacteria than was previously believed (Welander et al., 2010). But given the paucity of marine bacteria that have been screened for 2-methylhopanoids, or for the biosynthetic capacity for C-2 methylation, the possibility that cyanobacteria are a major source of 2-methylhopanoids in the marine realm remains uncertain.

The geological records of marine sediments and oils frequently have abundant 2-methylhopanes (Summons and Jahnke, 1990; Summons et al., 1999; Talbot et al., 2003; Farrimond et al., 2004). Geochemical records from the Cretaceous period indicate that the oceans experienced punctuated intervals of widespread water column euxinia, termed the ocean anoxic events (OAEs) (Schlanger and Jenkyns, 1976). A disruption in the marine nitrogen cycle during the OAEs is proposed to have lead to the emergence of nitrogen fixation as the major source of new nitrogen to the upper oceans (Kuypers et al., 2004). Studies of sediments deposited during the Cretaceous OAEs report a marked rise in the relative abundance of 2-methylhopanes, which has been interpreted as evidence for a rise in the abundance of nitrogen-fixing marine cyanobacteria. The $\delta^{13}C$ of 2-methylhopanes changes in concert with $\delta^{13}C$ of dissolved inorganic carbon (Kuypers et al., 2004) indicating that input from a terrestrial source is unlikely and implicating a

marine bacterium as the most likely source. The absence of 2-methylhopanoids in any of the marine cyanobacteria that have been screened raises a paradox for the significance of 2-methylhopanes in ancient marine sediments. Is the apparent absence of C-2 methylation among marine cyanobacteria an artifact of the relatively small percentage of cultures that have been studied, or are 2-methylhopanoids unrelated to cyanobacteria in the marine realm? A third and presently un-testable possibility is that marine cyanobacteria have lost the biosynthetic capacity for C-2 methylation over time.

The biosynthesis of hopanoids can be traced in the environment and in bacteria with fully sequenced genomes by screening for genes coding for squalene-hopene cyclase (*sqhC*), which catalyzes the cyclization of squalene to form the hopanoid ring structure (Wendt et al., 1997). Of the marine cyanobacteria that have been sequenced, very few appear to have the *sqhC* gene (Pearson et al., 2007). The most abundant cyanobacteria in the present-day oceans, members of the genus *Prochlorococcus* and *Synechococcus*, do not posses *sqhC*, and therefore are presumed to lack the capacity for hopanoid synthesis. This has been confirmed in a handful of cultured strains of *Prochlorococcus* and *Synechococcus* (Talbot et al., 2008). In line with this, cyanobacterial *sqhC* genes appear to be completely absent from global ocean genome (GOS) database (Pearson and Rusch, 2009), raising the possibility

that cyanobacterial hopanoid production may be rare or even non-existent in the oceans. On the other hand, observations from the GOS database represent only a snapshot of the upper 3 m of a single ocean transect and, therefore, may miss transient events such as cyanobacterial blooms or spatial heterogeneity of cyanobacterial communities. Furthermore, the marine cyanobacteria that have been sequenced represent a fraction of the strains available in culture and, in turn, the marine cyanobacteria that have been cultured likely represent a small fraction of the naturally occurring diversity of marine cyanobacteria that have yet to be cultured (Hugenholtz and Pace, 1996). Despite the absence of hopanoids in many marine cyanobacteria, hopanoids have been detected in *C. watsonii* and *Trichodesmium erythrism* (Talbot et al., 2008), which are both capable of nitrogen fixation and widespread in the oceans.

1.2 Ecology and physiology of hopanoids in marine cyanobacteria

The role of hopanoids in bacteria remains a mystery. They have been cited as bacterial "sterol surrogates," (Ourisson et al., 1987) and are thought to play a role in regulating membrane permeability or fluidity (Kannenberg and Poralla, 1999). As we will discuss in Chapter 5, the analogy to sterol function may imply a more nuanced role in the regulation of membrane partitioning and lipid ordering. Nonetheless, the apparently exclusive association among cultured marine bacteria of hopanoids with marine nitrogen fixing

cyanobacteria raises the possibility that they may play a unique role in the physiology and ecology of nitrogen-fixing cyanobacteria in the oceans.

Hopanoids have been implicated in regulating membrane permeability in several cultured bacteria. In *Zymomonas mobilis,* hopanoids are implicated in ethanol tolerance and proposed to reduce diffusion of alcohols across the membrane (Flesch and Rohmer, 1987). *Streptomyces coelicolor* only produce hopanoids when they are grown aerially and it has been suggested that hopanoids play a role in preventing loss of water and cell desiccation (Poralla et al., 2000). Hopanoids are also proposed to serve as a barrier to oxygen diffusion in the nitrogen-fixing bacterium *Frankia spp* (Berry et al., 1993; Kleemann et al., 1994). However, a subsequent study demonstrated that the cellular abundance of hopanoids in Frankia vesicles is not related to the nitrogen status of the cell, raising the possibility that hopanoids may serve other roles in the cell (Nalin et al., 2000). Recently, mutants of *Rhodopseudomonas palustris* lacking the capacity to synthesize hopanoids were shown to be more sensitive to extreme pH conditions than wild type cells containing hopanoids, prompting speculation that hopanoids may serve to reduce permeability of the membrane to protons (Welander et al., 2009), consistent with the suggestions of Haines (2001)

As a permeability barrier, hopanoids could be critical to managing the ecological stresses faced by nitrogen-fixing cyanobacteria. It is not completely

understood how nitrogen-fixing cyanobacteria such as *C. watsonii* can carry out nitrogen fixation in an oxygen-rich environment while preventing oxygen inhibition of the nitrogenase enzyme. Obviously the internal production of oxygen through photosynthesis represents one problem. *C. watsonii* circumvents this problem by performing nitrogen fixation during the night when photosynthesis does not occur (Church et al., 2005). However, limiting the diffusion of oxygen into the cell from the environment presents another significant challenge. In this case, hopanoids could serve to reduce the permeability of the membrane to dissolved oxygen, as is proposed in the case of the *Frankia* vesicle envelope (Berry et al., 1993).

The presence of antimicrobial compounds in the environment may represent another challenge for nitrogen fixing cyanobacteria in particular. Fresh water cyanobacteria, particularly those capable of nitrogen fixation and that occur in dense blooms, are known to be abundant sources of antimicrobial compounds (Burja et al., 2001), many of which directly target and inhibit the photosynthetic apparatus of other phototrophic organisms (Dahms et al., 2006). Many of the anti-photosynthetic compounds that have been identified are lipophilic and could diffuse readily through the membrane bilayer. Given the proposed role of hopanoids in reducing the permeability of *Z. mobilis* membranes to alcohols, it is possible that they might also have an

effect on reducing the permeability of membranes to lipophilic antimicrobial compounds in the environment.

The intracellular distribution of hopanoids has implications for the role of hopanoids in cyanobacteria. Cyanobacteria posses an outer, inner (cytoplasmic), and thylakoid membrane, the latter being associated with the photosynthetic apparatus. There is a small body of literature discussing the intracellular distribution of hopanoids, suggesting that hopanoids may be enriched in either the outer membrane/cell wall or the thylakoid membrane in some cyanobacteria (Jurgens et al., 1992; Simonin et al., 1996). The outer membrane in gram-negative bacteria is thought to play a particularly important role as a permeability barrier to non-polar or large molecules in the environment (Nikaido, 1989). Some bacteria are capable of cell differentiation and, in response to environmental stress, can form cells that are enveloped in a thicker protective cell envelope. Two examples of such cell types are akinetes, formed by some cyanobacteria (Meeks et al., 2002), and spores formed by some gram-positive bacteria. It was recently demonstrated that akinetes are enriched in hopanoids (Doughty et al., 2009) and, therefore, may be involved in protecting cells when they are exposed to environmental stresses. In another study, sporulenes, which are novel class of polycyclic terpenoids with close structural similarities to hopanoids, were found to be enriched in the cell envelope of spores formed by *Bacillius subtilis* suggesting

a protective role analogous to that proposed for hopanoids in akinetes (Bosak et al., 2008).

In this chapter we survey a diverse selection of cultured marine cyanobacteria and enrichment cultures for the presence and structural diversity of BHPs. We also address four questions surrounding the role of hopanoids in *C. watsonii*:

1) How abundant are hopanoids relative to the total lipid content?
2) Does BHP abundance vary between day and night and under growth on different nitrogen substrates?
3) Are BHPs enriched in distinct membrane fractions isolated from the cell?
4) Does *C. watsonii* have a higher tolerance to hydrophobic anti-photosynthetic compounds than non-hopanoid producing cyanobacteria?

2. MATERIALS AND METHODS

2.1 Cultures

The list of cultures surveyed in this study is shown in Table 1. Strains designated with the prefix WH were isolated and maintained by Dr. John Waterbury and Freddy Valois at WHOI. *Cyanothece* ATCC 51142 was obtained from the American Type Culture Collection (ATCC) and maintained by Freddy Valois at WHOI. Strains designated with the prefix MIT were isolated and maintained in the laboratory of Dr. Sallie Chisholm at MIT. All cultures were anemic. 50 ml of fresh biomass of each strain was pelleted by centrifugation and extracted according to the method of Bligh and Dyer (Chapter 3). BHP analyses were carried out according to methods described in Chapter 3.

2.2 Enrichment cultures

Enrichment cultures were prepared from samples collected from marine neritic environments in Bermuda, Cape Verde, and Falmouth, Massachusetts. Bermuda tide pool enrichments were inoculated with filamentous cyanobacteria and water collected from tide pools on the north coast of Ferry Point Park, St. George's, Bermuda (32° 22' 10.09" N x 64° 42' 30.99" W). The Bermuda Carbonate Biofilm was inoculated with a piece of carbonate rock covered by a green biofilm collected from a protected inlet to

the north of St. George's Harbor, Bermuda (32° 23' 20.53" N x 64° 40' 37.83" W). The Bermuda Spanish Point Mat enrichments were inoculated with an algal mat collected from the intertidal zone of Spanish Point beach, Bermuda (32° 18' 20.96" N x 64° 48' 52.79" W). The Sao Vicente Coralline Algae enrichment was inoculated with material scraped from the surface of a coralline algal community collected in the intertidal zone of a beach on the northeast coast of Sao Vicente, Cape Verde (16° 54' 28.33" N x 24° 54' 14.43" W). The Woodneck Beach Mat enrichment was inoculated from a cyanobacterial mat collected from the intertidal zone of Woodneck Beach, Falmouth, Massachusetts (41° 34' 32.79" N x 70° 38' 21.30").

Samples for enrichment were initially collected in sterile 8 ml polycarbonate culture tubes and transported back to the laboratory in seawater at ambient temperature from the source environment. An initial enrichment was achieved by inoculating 50 ml of SOX medium (a 5x dilution of SO medium) with sample biomass collected from the field transferred by flame-sterilized glass pipette tip. Enrichments were incubated at 28 °C with a 14 hour light cycle. After sufficient growth had occurred (~2 weeks), a second enrichment was carried out by inoculating 50mL of standard SO medium with biomass from the first enrichment. Transfer to a third stage enrichment with SO medium was carried out after ~2 weeks. After ~ 2 weeks growth in the third transfer enrichment cells were harvested for extraction by centrifugation. An

enrichment from the Woodneck Beach Mat was also performed with SNAX (5x dilution of SN medium; see appendix) and SN medium in the same fashion as described above.

2.3 Media and experimental conditions for growth experiments with C. watsonii (WH8501) and Synechococcus (WH8102).

C. watsonii (WH8501) was grown in either SN, SO, or SNH_4 medium and incubated at 28 °C with a 14 hour light cycle. *Synechococcus* (WH8102) was grown on SN medium and incubated at 26 °C with a 14-hour light cycle. All glassware was washed with detergent, rinsed seven times in tap water and three times in milliQ filtered water. Glassware, pipettes, and media were sterilized by autoclave.

2.3a – Day vs. night cellular abundance of BHPs in C. watsonii (WH8501) during growth on SN, SO, or SNH_4 medium.

To observe the effects of nitrogen source and limitation, and diel cycle on the cellular abundance of BHPs in *C. watsonii*, batch cultures were grown on SN, SO, or SNH_4 medium and sampled during the middle of the light and dark cycles, respectively. Each treatment (e.g. SO-light, SO-dark, etc…) was grown in triplicate 25 ml batch cultures in 40 ml Pyrex glass culture tubes. Cells were harvested when cell densities reached between 3 – 5 x 10^6 cells/ml. Cell counts for all treatments were performed immediately prior to sampling

the “light cycle” treatments, and consequently cell counts for the “night cycle” treatments represent an underestimate due to cell division that occurred over the 12 hour interval between the cell count and harvesting of cells. Dividing cells were visible and abundant in all batch cultures suggesting that cells were still in the exponential growth stage. However, growth curves were not recorded for these cultures, therefore we cannot be certain what growth stage the cells were in. Cells were harvested by filtration through a combusted Whitman 25 mm GFF with nominal pore size of 0.7 um. Filters were frozen immediately after filtration and stored at -40 °C until extraction. Extraction and analysis of BHPs was performed according to methods outlined in chapter 3.

2.3b – DCMU growth inhibition of C. watsonii (WH8501) and Synechococcus (WH8102)

In order to compare the growth inhibiting concentration of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) on *C. watsonii* and *Synechococcus*, batch cultures of these two strains were treated with varying amounts of DCMU during exponential growth. Batch cultures were grown on SN medium (25 ml) in 40 ml Pyrex culture tubes. Phycoerytherin and chlorophyll a absorbance were measured on a Turner Designs TD700 fluorometer to generate growth curves. DCMU (Sigma-Aldrich, D2425) was dissolved in 100% ethanol. DCMU in ethanol was added to batch cultures to achieve

DCMU concentrations ranging from 0.005-100 uM. The DCMU concentration in ethanol was adjusted such that addition to batch culture media resulted in an ethanol concentration of 0.1% (v/v). One batch culture with 0.1% ethanol and no DCMU was used as a control for *C. watsonii* and *Synechococcus* WH8102, to establish that ethanol toxicity did not contribute to growth inhibition.

2.4 Membrane separation protocol

Batch culture cells of *C. watsonii* were harvested by centrifugation and rinsed in phosphate buffer (pH 7.8) three times. Harvested cells were resuspended in phosphate buffer and homogenized by passing through a pre-cooled (4 °C) French pressure cell. Cells and homogenized cell material were kept on ice or within a refrigerated (4 °C) room throughout the procedure. Membrane fractions were prepared by separation of homogenized cells by ultracentrifugation on a sucrose step gradient according to methods outlined by Moslavac et al (2005) with minor modifications made by Doughty et al., (2009). The sucrose step gradient consisted of three layers which were (from bottom to top) 56%, 28%, and 14% sucrose (w/w) with 0.1% TWEEN detergent to aid in separation of the thylakoid and cytoplasmic membrane fractions (Doughty et al., 2009). The cytoplasmic membrane fraction (F2) and thylakoid membrane fraction (F3) were collected as bands within the gradient, and the

outer membrane fraction (F4) was collected as the pellet formed at the bottom of the gradient.

3 RESULTS AND DISCUSSION

3.1 BHP distribution in cultured marine cyanobacteria

Of the 16 of axenic marine cyanobacterial strains that we screened for BHPs, only *C. watsonii*, *T. erythraeum*, and four strains of *Cyanothece sp.* produced detectable BHPs (Table 1). All of these organisms are capable of performing nitrogen fixation. The BHP content of *C. watsonii* and *T. erythraeum* previously reported by Talbot et al. (2008) and our results are in close agreement. *C. watsonii* and *Cyanothece sp*. produced BHP cyclitol ether (Fig 1; If; BHT-CE) as the major compound (Table 2). T. erythraeum also produced BHT-CE in addition to a BHT-CE with an unsaturation in the ring structure at an undetermined position (Table 2; (Fig. 1; IVf; unsatBHT-CE).

Trichodesmium sp. and unicellular nitrogen-fixing cyanobacteria related to *C. watsonii* are thought to be widespread in the many regions of the upper ocean (Carpenter and Romans, 1991; Capone et al., 1997; Orcutt et al., 2001; Zehr et al., 2001; Falcon et al., 2002; Falcon et al., 2004; Montoya et al., 2004; Brandes et al., 2007). It is therefore surprising that no cyanobacterial *sqhC* genes are detected in surveys of the Venter GOS database (Pearson and Rusch, 2009). This may partly be explained by the fact that samples for GOS were screened for cells smaller than 0.8 μm, whereas *C. watsonii* and *Trichodesmium sp.* are generally larger than 1 μm. Another possibility is that

nitrogen-fixing cyanobacteria typically form in temporally sporadic bloom events and a single transect of the ocean may miss short-lived bloom events.

Our survey suggests that hopanoid production is rare in marine cyanobacteria overall, but ubiquitous in the nitrogen-fixing marine cyanobacteria that we have screened. The low structural diversity of BHPs in marine cyanobacteria studied so far combined with the fact that the BHPs that have been detected are relatively common among bacteria (Talbot et al., 2003; Talbot and Farrimond, 2007; Talbot et al., 2008) indicates that hopanoid structure alone is not likely to provide a fruitful basis for developing marine cyanobacterial biomarkers. In particular, the absence of 2-methylhopanoids leaves the question of their source in the oceans unanswered. If structure is any indication of function then the low structural diversity of BHPs in marine cyanobacteria compared with non-marine cyanobacteria (and bacteria in general) may suggest that hopanoids have a more limited range of intended functions in marine diazotrophic cyanobacteria. However, caution should be exercised in making any strong conclusions from such a small sampling of the natural diversity of marine cyanobacteria that have yet to be cultured. Despite the relatively small number of cultures that have been screened, the phylogenetic diversity of cyanobacteria at the Order level has been fully screened, and hopanoids have been identified in at least one representative strain from Croococcales,

Nostocales, Oscillatoriales, Prochlorales, Stigonematales, and Pleurocapsales (Talbot et al., 2008 and references therein). In the present study of marine cyanobacteria, however, we have not screened cyanobacteria from the Orders Nostocales and Stignonematales (Table 1), and this represents a gap in our understanding of the phylogenetic diversity of hopanoids in marine cyanobacteria.

3.2 BHP distribution in marine cyanobacterial enrichment cultures

A total of 7 BHPs were identified in the marine cyanobacterial enrichment cultures, but the number of compounds in each sample ranged from 2 – 6 (Table 2). BHT (Fig 1; Ia) and lactoneBHP (Fig 1; Ik) were present in all of the samples. With the exception of the neritic coralline algae enrichment culture from Sao Vicente, BHT typically made up a small percentage of the total BHP (3-8%). This is in line with the abundance of BHT observed in cultured bacteria, including *C. watsonii*. Similarly, lactoneBHP was less than 10% of the total BHP except in the Woodneck Beach mat enrichments were it ranged from 70-95% of total BHP. BHT-CE and unsatBHT-CE were the dominant compounds in the enrichment cultures from Bermuda. UnsatBHT-CE, which was present in all of the tide pool enrichments cultures, has been identified in *Trichodesmium sp*.

In contrast to the low structural diversity observed in cultured marine cyanobacteria, marine enrichment cultures exhibited relatively high structural diversity of BHPs. We cannot say with any certainty that the BHPs we observe are produced by cyanobacteria in the enrichment cultures since some heterotrophic bacteria are also likely to be present. However, this result does show that marine microorganisms associated with phototrophic communities can be a rich source of diverse BHPs. Since all but one of these enrichments were prepared using SO medium (no fixed nitrogen source) the growth of nitrogen fixing cyanobacteria is favored. Interestingly, we do observe a 2-methylhopanoid in the Woodneck Beach Mat enrichment with SN medium, but not in the corresponding SO enrichment from this sample. This could suggest that the organism producing 2-methylBHT in this environment may not thrive under nitrogen limiting conditions.

3.3 *Diel variations in the cellular abundance of BHPs in C. watsonii (WH8501) under nitrogen-limiting and nitrogen-replete conditions.*

We detected two BHPs in *C watsonii*: BHT and BHT-CE, the latter being 100 times more abundant than the former. Based on quantitation using BHT as an external standard, we estimate that BHT-CE is about 5 - 10 % of the total lipid extract by mass. Response factors can vary considerably (by up to an order of magnitude) for different compounds. Therefore, since we do not have an authentic standard for BHT-CE, our estimate of absolute cellular

abundance should not be considered accurate within more than an order of magnitude. Additionally, the TLE mass represents the membrane lipid mass in addition to the mass of other non-membrane cellular components that are solvent extractable. Nonetheless, BHT-CE is the largest peak in the total ion current chromatogram suggesting that BHT-CE is a major component in the membranes of *C. watsonii*.

Figure 2 shows the cellular abundances for *C. watsonii* for the different experimental treatments. Overall, the relative abundance of BHT and BHT-CE did not exhibit significant variability for cells grown on SO (no fixed nitrogen source) and SN (nitrate-amended) medium. BHT-CE normalized to TLE mass was slightly lower in cells grown in SO sampled at night. But when BHT-CE is normalized to cell count there is no significant difference between cells sampled at day and night. This indicates that the cellular mass of TLE increased at night, but that cellular abundance of BHT-CE remained constant. The absence of any significant change in BHP abundance between cells grown on SO and SN suggests that BHP abundance is not regulated in response to nitrogen limitation, in line with observations made by Nalin at al., (2000) in *Frankia sp*. While this does not disprove the idea that the primary role for BHPs is as diffusion barrier to oxygen, it does not provide any support for the hypothesis. If the sole purpose of hopanoids in *C. watsonii* were to help protect nitrogenase from oxygen inhibition, then there would be

no reason for cells to produce hopanoids when nitrogen fixation is not performed. Of course, it should be kept in mind that we are assuming that cells grown on SO medium must perform nitrogen fixation in order to meet their cellular nitrogen requirements. However, we have not actually measured the activity of nitrogenase in these cultures to confirm this assumption. An additional complicating factor is that we cannot be certain that the cultures were in exponential growth phase in the absence of a growth curve. Nonetheless, in spite of these uncertainties, the fact that BHT-CE is a significant component of the cell under N-replete conditions suggests that hopanoids are essential even when nitrogen fixation is not performed. Interestingly both BHPs were significantly more abundant in cells sampled at night that were grown on SNH_4 (ammonium amended) medium, and BHT was nearly an order of magnitude more abundant in SNH4 cells sampled at day and night. We cannot presently explain the physiological basis for these trends, but they indicate that the cellular abundance of BHT is dynamic and can vary quite considerably.

3.4 Distribution of BHPs in membrane fractions isolated from C. watsonii (WH8501)

Three membrane fractions (F2-F4) were isolated from homogenized cells of *C. watsonii* (Figure 3). F1 corresponds to the upper layer of the sucrose gradient, which contained the cell homogenate prior to separation by

ultracentrifugation. Since there are presently no established markers for the inner, outer and thylakoid membranes of *C. watsonii*, we have no means to confirm the identity or degree of enrichment of the fractions. Nonetheless, the identity of membrane fractions resulting from this separation protocol has been well-established with cyanobacteria in previous studies (Moslavac et al., 2005; Doughty et al., 2009). At the very least the three fractions are can be considered as distinct – albeit operationally defined by density – components of the membranes.

BHT and BHT-CE show different distribution patterns between the three membrane fractions. BHT is nearly evenly distributed, with a slight enrichment in F4 (outer membrane). BHT-CE on the other hand is enriched by nearly 10-fold in F4, suggesting that it is predominately associated with the outer membrane. Given relatively the high cellular abundance of BHT-CE in *C. watsonii*, if it is primarily associated with the outer membrane, then it could comprise a considerable fraction of outer membrane lipids. This possibility lends support to the hypothesis that BHT-CE may serve to reduce membrane permeability to protect the cell from environmental toxins.

3.5 Growth inhibition of C. watsonii watsonii (WH8501) and Synechococcus (WH8102) by DCMU

3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) is a small hydrophobic compound that inhibits electron transport in photosystem II (PSII) by binding to and blocking the plastiquinone binding site on PSII (Metz et al., 1986). Cyanobacteria, especially nitrogen-fixing cyanobacteria, have been recognized as important natural sources of antimicrobial compounds, particularly anti-photosynthetic compounds with similar mechanisms to DCMU (Dahms et al., 2006). Cyanobacteria that occur in dense blooms under nitrogen-limiting conditions, such as *C. watsonii*, are likely to produce such toxins, and to be exposed to toxins produced by other potential competitors. Two possible strategies for resisting the toxicity of antimicrobial compounds are prevention of cellular uptake of the toxin through membrane barrier properties or, in the case of enzymatic inhibitors such as DCMU, by modification of the binding site of inhibition through genetic mutation (Galloway and Mets, 1982; Gadkari, 1988). It is, therefore, possible that some cyanobacteria have developed physiological strategies for preventing antimicrobial toxins from diffusing through the cell membrane. The outer membrane is thought to serve as a permeability barrier to hydrophobic compounds (Nikaido, 1989), however, recent studies have demonstrated that it is also possible that some hydrophobic compounds may pass through specialized channels in the outer membrane (Hearn et al., 2009). Nonetheless, in the case of *C. watsonii*, hopanoids may provide the

basis for reduce membrane permeability, making cells more resistant to toxins like DCMU.

As a first step towards testing this hypothesis, we compared the concentration of DCMU required to inhibit growth in *C. watsonii* (WH8501) and a non-hopanoid producing unicellular marine cyanobacterium, *Synechococcus* (WH8102). The results of this experiment indicate that the growth inhibiting concentration of DCMU is 20-fold greater for *C. watsonii* than for *Synechococcus* (Figures 4 and 5). While our results certainly indicate that *C. watsonii* has a higher resistance than *Synechococcus* to DCMU and similar toxins, it does not prove that the composition of the cell membrane is the basis for this difference; we are essentially comparing "apples with oranges" in terms of cell physiology. Nonetheless, this result provides a step towards addressing the possibility that the membranes in *C. watsonii* are particularly impermeable to lipophilic antimicrobial toxins. The development of a mutant lacking the capacity to synthesize hopanoids would provide the basis for conducting a controlled experiment to test whether the presence or absence of hopanoids affects DCMU tolerance.

SUMMARY AND CONCLUSIONS

- Hopanoid production in marine cyanobacteria appears to be limited to diazotrophs among the cultures studies to date.

- 2-methylhopanoids are not produced by marine cyanobacteria in culture that have been screened to date. 2-methylBHT is present in an enrichment culture from Woodneck Beach Mat, suggesting that a marine cyanobacterial source is possible, though not ruling out other marine bacteria as potential sources. Given the small number of cultured marine cyanobacteria that have been cultured and screened for hopanoids, there is still a strong possibility that marine cyanobacteria have been a major contributor to marine sedimentary 2-methylhopanes.

- BHP structural diversity in cyanobacterial enrichment cultures from marine neritic environments indicate that despite low diversity in cultured marine cyanobacteria, there may be more diverse sources of BHPs from as yet uncultured marine cyanobacteria in the environment.

- The cellular abundance of BHPs in *C. watsonii* is relatively high, compared with many cyanobacteria, and suggests that hopanoids could serve a role in regulating membrane permeability. Our results showing the apparent enrichment of BHPs in the outer membrane also supports a potential role as a permeability barrier to environmental toxins (e.g. O_2 and antimicrobial compounds). However, high cellular abundance during growth on fixed N substrates indicates that hopanoids are

crucial even when nitrogen fixation is not being carried out. This suggests that hopanoids serve a purpose that is not limited to the physiology of nitrogen fixation.

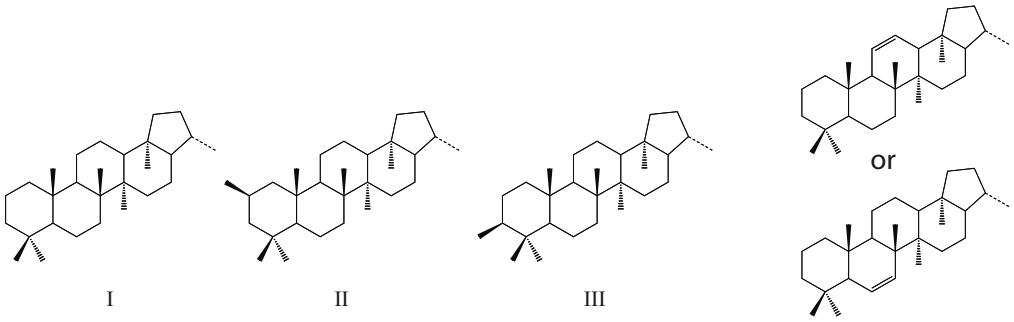
- As a permeability barrier, hopanoids may be essential to the ecology of diazotrophic marine cyanobacteria. One such role may be in the resistance to antimicrobial toxins produced by *C. watsonii* or by competing organisms in the environment. This possibility is supported by the observation that *C. watsonii* has a higher resistance to DCMU than a non-hopanoid producing cyanobacterium.

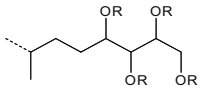
Table 1. The marine cyanobacterial strains surveyed for BHP content are listed and the presence or absence of BHPs is indicated.

Growth Media	Strain name	Order	Genus	Source	BHP (+/-)
SN	WH-8102	Chroococcales	Synechococcus	Waterbury	-
SN	WH-7302	Pleurocapsales	Stanieria	Waterbury	-
SN	WH-8012	Chroococcales	Synechococcus	Waterbury	-
SN	WH-7002	Chroococcales	Synechococcus	Waterbury	-
SN	WH-5701	Chroococcales	Synechococcus	Waterbury	-
SN	WH-7420	Oscillatoriales	Microcoleus	Waterbury	-
SN	WH-8018	Chroococcales	Synechococcus	Waterbury	-
SN	WH-5701	Chroococcales	Synechococcus	Waterbury	-
PRO99	MIT-SB	Prochlorales	Prochlorococcus	Chisolm	-
PRO99	MIT-GP2	Prochlorales	Prochlorococcus	Chisolm	-
SO	WH-8501	Chroococcales	Crocoshpaera	Waterbury	+
SO	IMS-101	Oscillatoriales	Trichodemsium	Waterbury	+
SO	WH-8901	Chroococcales	Cyanothece	Waterbury	+
SO	WH-8903	Chroococcales	Cyanothece	Waterbury	+
SO	WH-8904	Chroococcales	Cyanothece	Waterbury	+
SO	ATCC-51142	Chroococcales	Cyanothece	Waterbury	+

Table 2. Structural diversity and relative abundance of BHPs identified in marine cyanobacterial enrichment cultures and axenic cultures from Table 1. See Figure 1 for structures.

Sample	Type	Media	Ia	Iia	Ic	Ik	If	IVf
Bermuda Tide Pool 01	Enrichment	SO	6%		4%	9%	23%	58%
Bermuda Tide Pool 02	Enrichment	SO	3%			7%	39%	51%
Bermuda Tide Pool 03	Enrichment	SO	8%			3%	27%	62%
Bermuda Spanish Point Mat	Enrichment	SO	17%		16%	1%	66%	
Bermuda Carbonate Biofilm	Enrichment	SO	24%		3%	4%	69%	
Sao Vicente Coralline Algae	Enrichment	SO	73%		16%	9%		
Woodneck Beach Mat	Enrichment	SN	17%	13%		70%		
Woodneck Beach Mat	Enrichment	SO	5%			95%		
Crocosphaera watsonii	Isolate	SO	1%				99%	
Trichodesmium erythraeum	Isolate	SO					19%	81%
Cyanothece sp.	Isolate	SO					100%	





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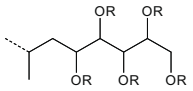

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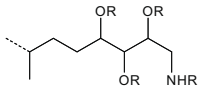

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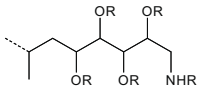

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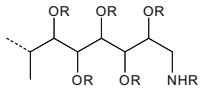

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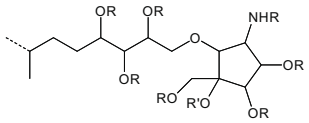

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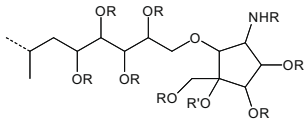

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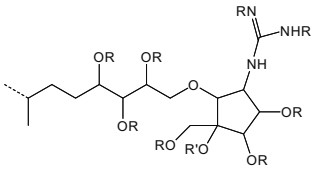

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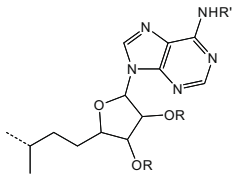

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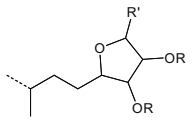

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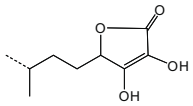

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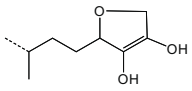

l: R = H
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Figure 1. Structures of BHP that are referred to in the text.

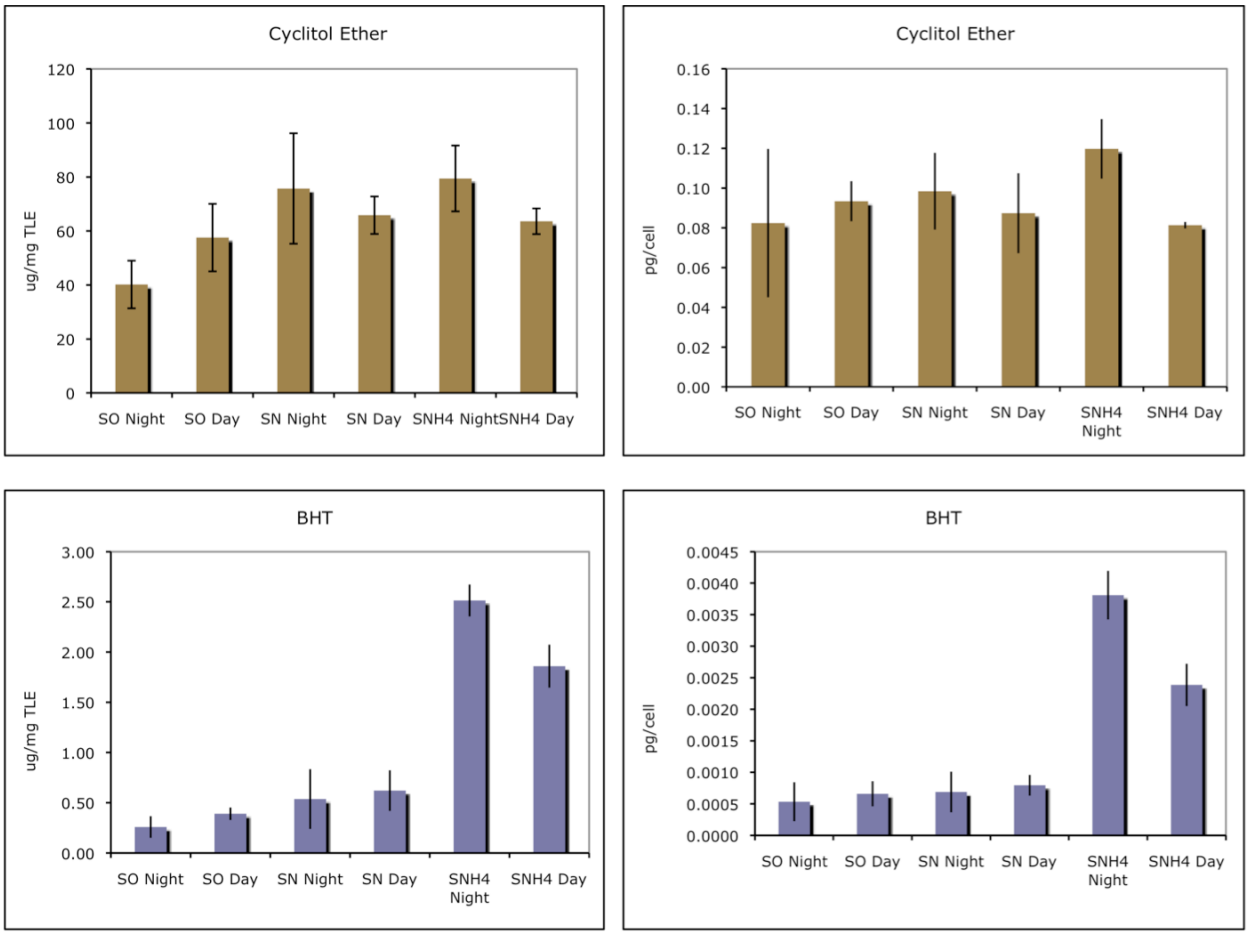

Figure 2. Cellular abundance of BHT-CE (If) and BHT (Ia) in *C. watsonii* grown on different media (SO, SN, and SNH_4) and sampled during light and dark cycles. Cellular abundance is reported relative to the mass of the total lipid extract and as normalized to the number of cells in each sample. Error bars represent the standard deviation of cultures that were grown in triplicate under the same conditions and sampled at the same time.

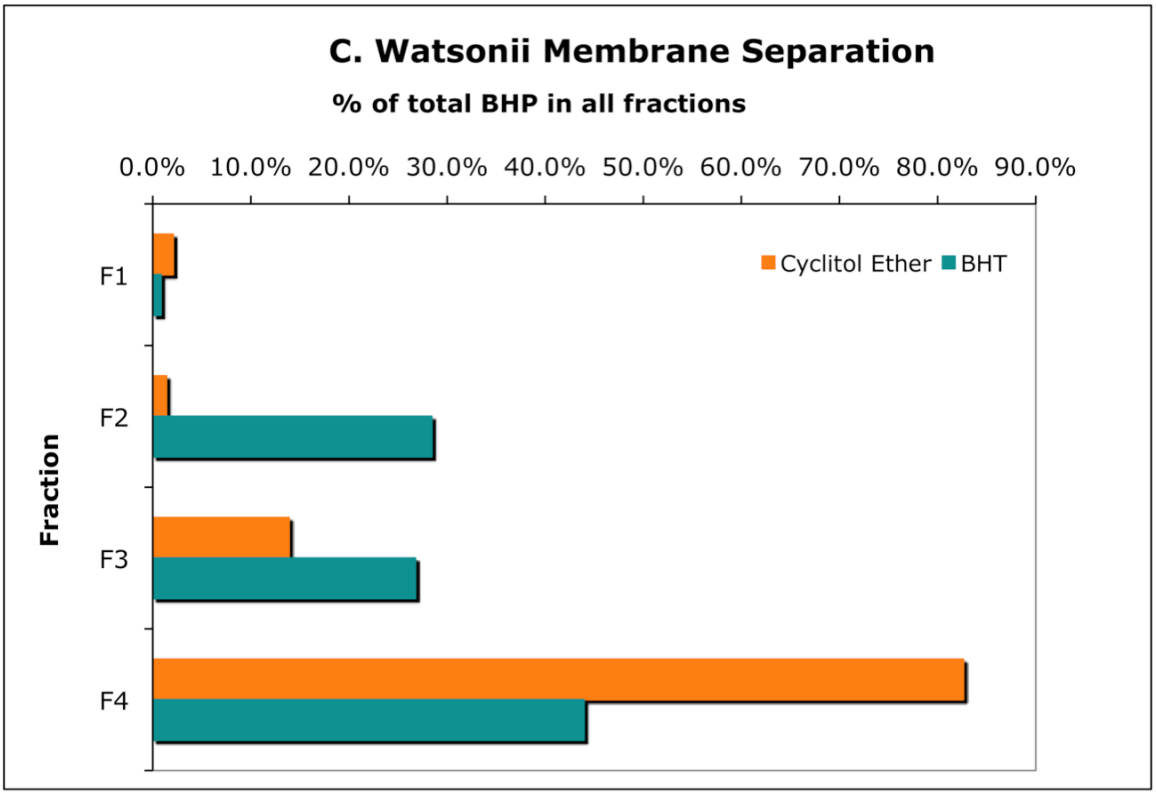

Figure 3. Distribution of cyclitol ether (BHT-CE; If) and BHT (Ia) in four fractions of *C. watsonii* cells that were homogenized and separated by sucrose gradient ultracentrifugation. Fractions F2, F3 and F4 correspond to the cytoplasmic (inner) membrane, thylakoid membrane, and outer membrane, respectively.

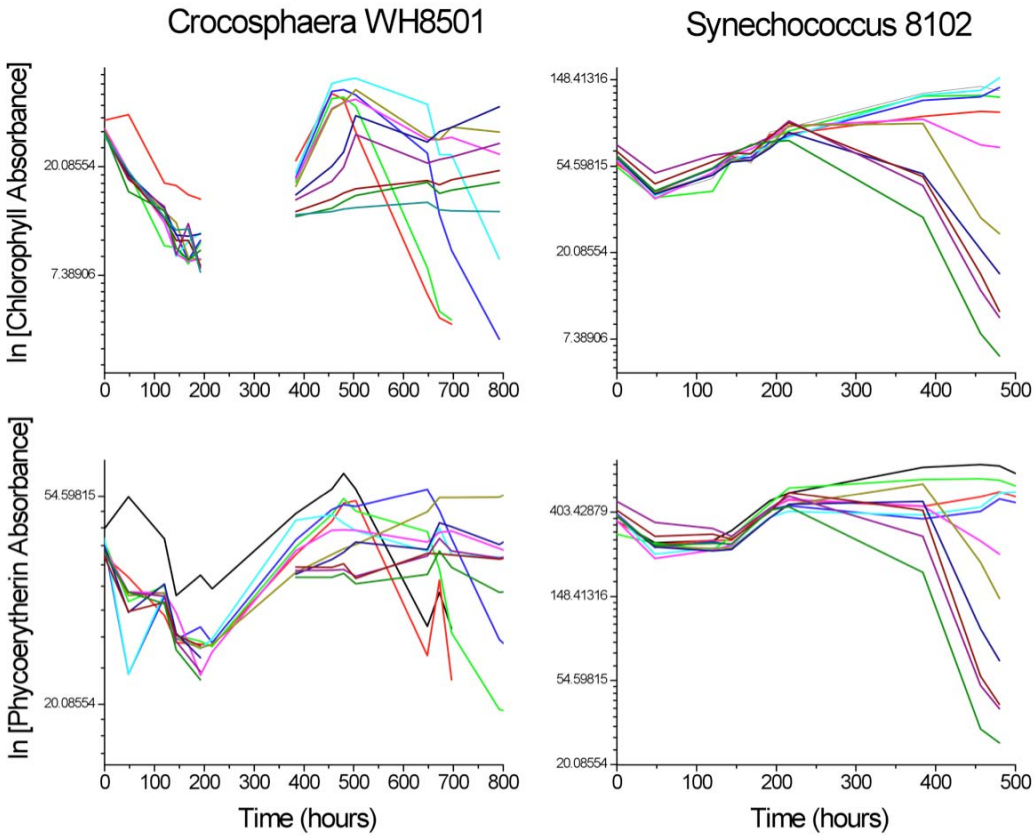

Figure 4. Growth curves for *C. watsonii* and *Synechococcus* WH8102 determined by chlorophyll and Phycoerytherin absorbance over the course of the experiment.

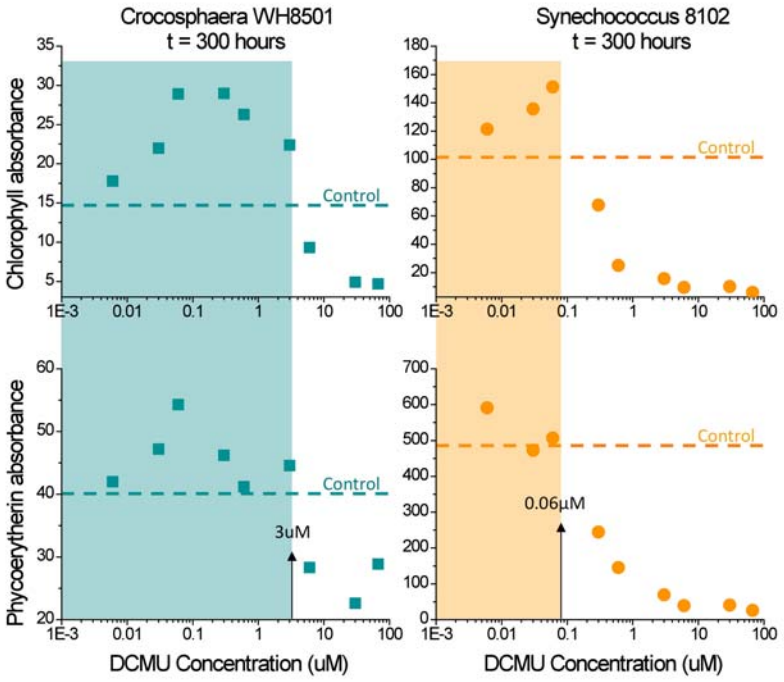

Figure 5. Absorbance of chlorophyll and phycoerytherin measured 300 hours after the addition of DCMU to the batch cultures. Absorbance values for the control batch are indicated by a horizontal dashed line. The initial concentration of DCMU added to each culture is designated by the horizontal axis.

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CHAPTER 5* - HOPANOIDS IN DETERGENT RESISTANT MEMBRANE FRACTIONS OF *CROCOSPHAERA WATSONII*: IMPLICATIONS FOR BACTERIAL LIPID RAFT FORMATION

ABSTRACT

Hopanoids are bacterial membrane lipids with close structural and evolutionary ties to sterols. Extraction of hopanoid containing membranes of *Crocosphaera watsonii* in a non-ionic detergent demonstrates that a hopanoid identified as bacteriohopanetetrol cyclitol ether (BHT-CE) is enriched in a detergent resistant membrane (DRM) fraction. Detergent resistance indicates that hopanoids, like sterols in eukaryotes, are likely associated with putative lipid rafts in bacterial membranes. These results are consistent with previous work suggesting analogous roles of hopanoids and sterols, and may imply that hopanoids are essential to the physiology of putative lipid rafts in hopanoid producing bacteria.

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1. INTRODUCTION

Hopanoids are pentacyclic isoprenoids produced by some bacteria (Rohmer et al., 1984; Pearson and Rusch, 2009) and have been dubbed bacterial "sterol surrogates" (Rohmer et al., 1979; Ourisson et al., 1987). Understanding the biological role of hopanoids is of particular interest to understanding the evolutionary history of bacteria: geologically stable products of hopanoids have been detected as molecular fossils preserved in ancient sedimentary rocks dating back to more than 2.5 billion years before present (Brocks et al. 1999; Rasmussen et al. 2008) and molecular analyses suggest that the last common ancestor of bacteria was a hopanoid producer (Frickey and Kannenberg 2009). The close structural similarities between hopanoids and sterols have fueled speculation that hopanoids in bacterial membranes serve a similar role to that of sterols in eukaryotic membranes (Rohmer et al., 1979; Ourisson et al., 1987; Kannenberg and Poralla, 1999). Studies have focused on membrane rigidification, fluidity and permeability in testing hypotheses for the function of hopanoids (Horbach et al., 1991; Berry et al., 1993; Kleemann et al., 1994; Moreau et al., 1997; Poralla et al., 2000; Doughty et al., 2009; Welander et al., 2009). A key role of sterols in eukaryotic membranes is the formation of lipid rafts. Given their structural

similarities, hopanoids could fulfill a similar role in bacteria, but this hypothesis has remained untested.

The lipid raft hypothesis holds that the cell membrane is not homogenous, but that it consists of coexisting immiscible liquid phases that can self-organize into lipid domains, or lipid rafts (Lingwood and Simons, 2010). Such rafts are thought to serve as platforms that are essential in orchestrating signal transduction, cell polarization and protein trafficking (Rajendran and Simons, 2005). In eukaryotes, sterols play an essential role in the formation of a liquid-ordered (l_o) "raft" phase, which is immiscible with the surrounding phospholipids in a liquid-disordered phase (l_d) (Ipsen et al., 1987; Simons and Vaz, 2004). The acyl chains of phospholipids in the l_o phase have a low conformational freedom owing to the ordering effect of the rigid, semi-planar ring structure of intercalated sterols. Hopanoids possess a similar ring structure, which could have a comparable effect on phospholipid conformational ordering in bacterial membranes. Evidence for lipid rafts in bacteria is beginning to emerge (Matsumoto et al., 2006), but the molecular basis for bacterial lipid rafts is yet to be established.

In eukaryotes, lipids that are associated with a l_o phase have been shown to be particularly insoluble in non-ionic detergents, such as Triton X-100 (TX-100), whereas lipids in the l_d phase are highly soluble (Brown and Rose, 1992). Detergent resistance has been widely applied as a tool for

studying the composition of lipid rafts in cells (Lingwood and Simons, 2007). Density differences between lipids solubilized in detergent and insoluble detergent resistant membrane (DRM) fragments make it possible to separate these fractions on a sucrose gradient by ultracentrifugation. While there is debate over the exact physiological significance of DRM composition, detergent resistance is presently the only well established basis for probing the bulk composition of lipid rafts in living cells (Lingwood and Simons, 2007).

I investigated whether hopanoids in the membranes of *C. watsonii* could be detected in a DRM fraction following TX-100 extraction of homogenized cells. *C. watsonii* is a hopanoid-producing marine cyanobacterium (Talbot et al., 2008) that is widespread in the oceans and is thought to play an important role in the global nitrogen cycle through its ability to meet its cellular nitrogen demand through the fixation of atmospheric N_2 (Dyhrman and Haley, 2006 and references therein). My results indicate that hopanoids may be associated with putative lipid rafts in bacterial membranes and that this hypothesis merits further investigation. If proven, then the emergence of hopanoids in the geologic record could place a minimum age on the evolution of lipid rafts, which was a key innovation in the architecture of the cell membrane.

2. METHODS

Axenic *C. watsonii* (WH8501), isolated by John Waterbury at WHOI, were grown in SN media at 28 °C with a 14:10 hour light-dark cycle (Dyhrman and Haley, 2006). Cells were harvested by centrifugation and the resulting pellet was washed three times in phosphate buffer. Cells were kept on ice throughout the homogenization and extraction procedure. The cells were homogenized by passing through a French pressure cell three times. Homogenized cells were extracted in TX-100 according to methods outlined in Lingwood and Simons (2007).

Detergent extracted cells were separated on a step sucrose gradient by floatation ultracentrifugation as described in Lingwood and Simons (2007) on a Beckman L8M ultracentrifuge with a type 70.1Ti fixed angle rotor at 43,000 rpm for 18 hours. Since TX-100 exhibits a distinct peak in absorbance at 275 nm, the distribution of TX-100 soluble material was determined by measuring the absorbance at 275 nm (A_{275}) in all four fractions (Holloway, 1973) on a Shimadzu UV-1601 spectrophotometer (Fig. 1) in four fractions removed by pipette from the top of the centrifuge tube in the following order: F1 (2.5 ml), F2 (2 ml), F3 (2 ml), F4 (2 ml). F1 was removed as the DRM fraction, as identified by low A_{275} absorbance (Fig. 1). The remaining three fractions were combined as the detergent soluble fraction.

The DRM and detergent soluble fractions were extracted by the method of Bligh and Dyer (1959). The total lipid extracts were acetylated and

analyzed for hopanoids by reversed phase HPLC-APCI/MS^n following methods described previously (Talbot et al., 2003a) on a Thermo Surveyor LC coupled to an LTQ-MS. Structural identification was achieved by comparison of retention time with hopanoids of known structure and identification of characteristic fragmentation patterns reported previously (Talbot et al., 2003a,b).

3. RESULTS AND DISCUSSION

C. watsonii produces BHT-CE as its most abundant hopanoid in addition to bacteriohopanetetrol (BHT), which is present in trace amounts of around 0.1 % of BHT-CE abundance (Talbot et al., 2008). The identity of DRM and detergent soluble factions was confirmed through UV absorbance at 275 nm (A_{275}) (Fig. 1). BHT-CE was detected in the DRM fraction (Fig. 2). A trace amount of the BHT-CE was also detected in the detergent soluble fraction. However, when peak areas are normalized to the volume of sucrose buffer solution in each fraction, BHT-CE is enriched by 10 fold in the DRM fraction (Fig. 2). BHT on the other hand, was detected in the soluble fraction but not in the DRM fraction (not shown). Our observation that BHT and BHT-CE are associated with different fractions supports the assumption that the TX-100 extraction and separation method employed in this study is enriching compositionally distinct cellular components. This observation also suggests that variability in the hopanoid side chain structure may be a

significant factor in determining the intracellular associations that confer detergent resistance to certain hopanoids. The presence and apparent enrichment of BHT-CE – the principal hopanoid in *C. watsonii* – in the DRM fraction shows that this compound is associated with a detergent insoluble component of the membrane of *C. watsonii*, consistent with the association of certain hopanoids with putative lipid raft-like domains.

Hopanoids have in common with sterols a semi-planar polycyclic ring structure that is thought to be essential to the molecular interactions underlying the formation of a liquid-ordered phase in membrane bi-layers containing phospholipids (Simons and Vaz, 2004). Early work on the properties of hopanoids in model membranes indicate that, like sterols, hopanoids have a condensing effect on phospholipid mono-layers (Benz et al., 1983). The observed condensing effect is diagnostic of the increased conformational ordering of the acyl chains, which allows for a closer packing of phospholipids in a liquid-ordered phase. Finally it has been shown that, in the absence of cholesterol, diplopterol can support growth of *Mycoplasma mycoides*. This organism lacks the ability to synthesize sterols, but requires them for growth (Kannenberg and Poralla, 1982). Combined with our results, these observations support the possibility that hopanoids could play a role in the formation of putative lipid rafts in hopanoid producing bacteria.

Since not all bacteria produce hopanoids (Pearson et al., 2007; Rohmer et al., 1984), either their role is not essential to bacterial life, or else bacteria that lack the capacity to synthesize hopanoids possess hopanoid-surrogates that perform analogous roles. It has recently been shown that hopanoids are not essential for the growth of some hopanoid producing bacteria (Welander et al., 2009; Seipke and Loria, 2009), which suggests that the biological role of hopanoids is not essential for survival. But, it is also possible that although hopanoids may not be essential in euxinic cultures under conditions selected for optimal growth, that they might serve a function that is crucial for survival in the environment. These questions will remain enigmatic until the role(s) of hopanoids in bacteria are proven. If hopanoids are shown to be associated with lipid rafts in bacteria, the role of lipid rafts in bacteria will need to be understood in order to elucidate the significance of hopanoids. Furthermore, if lipid rafts are found to occur in non-hopanoid producing bacteria, then other molecular mechanisms will need to be identified to account for raft formation in those organisms.

4 CONCLUSIONS

In the context of these observations, my finding that BHT-CE is enriched in a DRM fraction provides strong evidence in support of the notion that hopanoids could serve as bacterial sterol surrogates in the molecular architecture of putative bacterial lipid rafts. Certainly more work is needed

to broaden these observations to other organisms and hopanoid structures, as it is apparent that not all hopanoids may exhibit detergent resistance. However, my preliminary work provides novel insight on the role of hopanoids and their significance in the geologic record: hopanoids could be biomarkers for putative bacterial lipid rafts and their associated physiological processes and the earliest occurrence of hopanoids in the geologic record may provide a minimum age on the antiquity of lipid rafts in bacteria.

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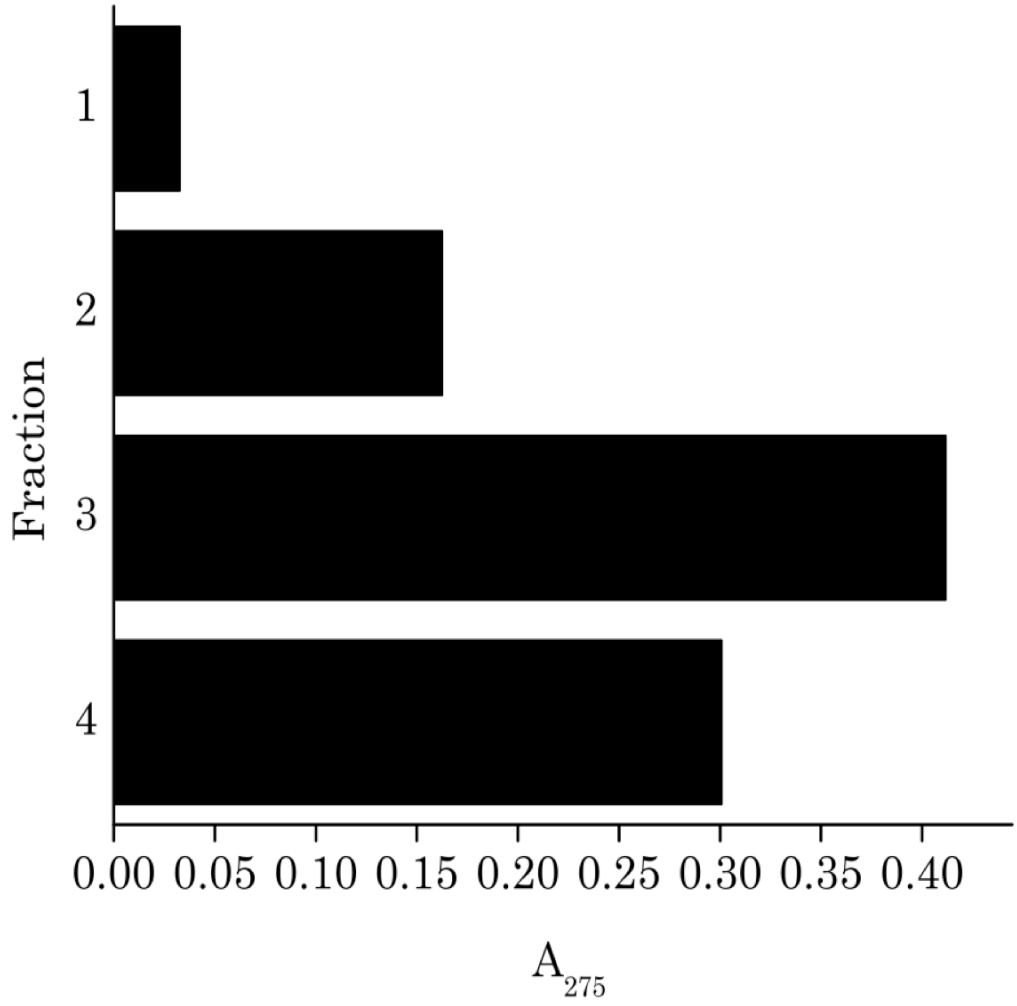

Figure 1: The absorbance at 275 nm (A_{275}) from each sucrose gradient fraction. The intensity A_{275} is proportional to the concentration of TX-100 in each fraction and, therefore, provides a measure of the relative enrichment of detergent soluble material in each fraction. F1 = detergent resistant membrane (DRM) fraction and F2 to F4 = detergent soluble fractions.

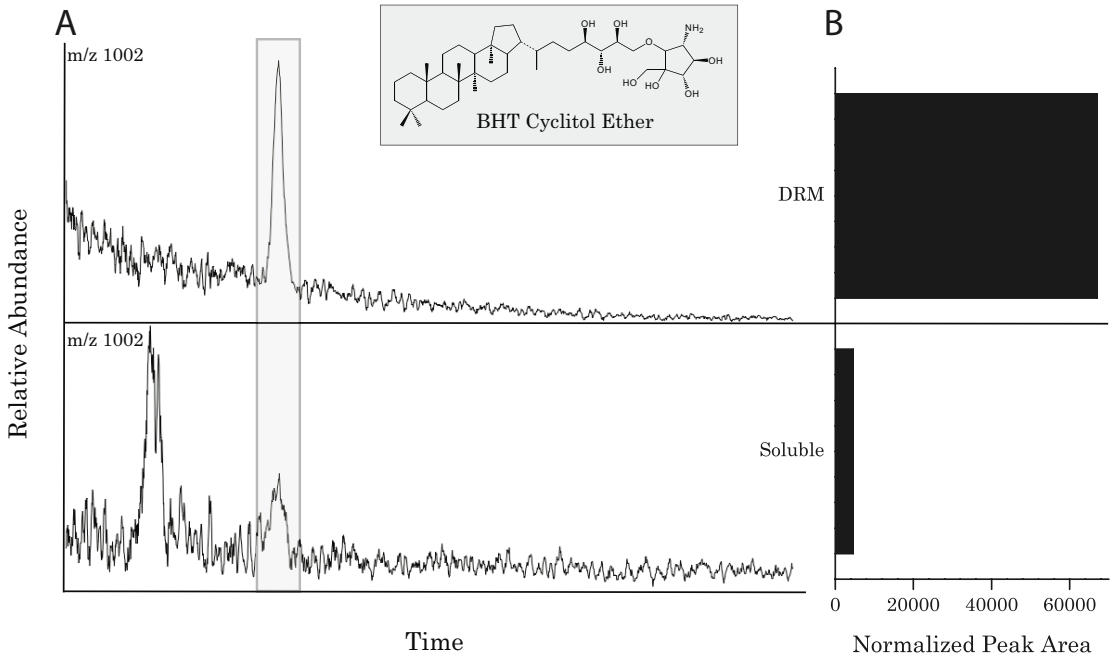

Figure 2: Axis A shows the m/z 1002 partial chromatogram for DRM and soluble fractions, with the BHT-CE peak highlighted. A second peak with a shorter retention time in the soluble fraction does not have a fragmentation pattern that is diagnostic of BHT-CE. Axis B shows peak area normalized to sucrose buffer volume in the two fractions. The structure of BHT-CE is shown.

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CHAPTER 6 - THE DISTRIBUTION AND STRUCTURAL DIVERSITY OF BACTERIOHOPANEPOLYOLS IN MODERN MARINE ENVIRONMENTS

Abstract

Hopanoids have been broadly applied in the marine sedimentary record as taxonomic markers for certain groups of bacteria and their associated biogeochemical processes. However, our ability to rigorously interpret the significance of hopanoids in the geologic record has been greatly limited by a dearth of knowledge surrounding the sources of hopanoids in marine environments. Despite the ubiquity of hopanoids in modern and ancient marine sediments, their provenance in the modern oceans is unknown. In this chapter, we present a survey of bacteriohopanepolyols (BHPs) in a range of marine and proximal marine environments. Our results indicate that BHPs are structurally diverse and ubiquitous within the pelagic zone. We observe pronounced heterogeneity in the spatial and temporal distribution of BHPs indicating great potential for the application of hopanoids as biomarkers for biological processes in the upper ocean and as tracers for organic matter input to sediments. In particular, BHPs appear to be relatively abundant and structurally diverse in low oxygen and oligotrophic environments and in particulate organic matter transported by rivers from terrestrial environments. While the biogeochemical significance of hopanoids in the oceans remains an enigma, their presence and ubiquity in the oceans is now established.

INTRODUCTION

Hopanoids are reputed to be one of the "most abundant natural products on Earth" (Ourisson and Albrecht, 1992) and they are ubiquitous components of modern and ancient marine sediments. They have great potential to inform us about life in the earliest records of Earth history that are preserved in the sedimentary record. Furthermore hopanoids exhibit a range of structural variations, which likely reflect the diversity of their biological sources and functions. The structural diversity of hopanoids therefore suggests that they have the potential to serve as biomarkers for bacterial ecology and biogeochemistry in the environment. However, the structural diversity and distribution of hopanoids in the modern environment is largely unknown, and the oceans in particular remain unexplored. This gap in our knowledge is primarily due to analytical limitations owing to the fact that analysis of hopanoids was traditionally carried out by GC-MS, which requires chemically removing the polar side chain in order to obtain GC-amenable derivatives of the bacteriohopanepolyols (BHPs). Recent advancements in the analysis of intact BHPs by HPLC-MS have made it possible to rapidly screen environmental samples for the full suite of BHP structures that have been characterized in bacterial cultures, and for which mass spectra have been reported.

The principal aim of the work presented in this chapter was to investigate the distribution of hopanoids in marine environments in order to shed light on the biogeochemical or environmental significance of hopanoids preserved in marine sediments. We asked the question: do the hopanoids that are delivered to marine sediments record biological or geochemical processes in the upper ocean? The answer to this question ultimately rests on several key pieces of information concerning the sources, function, and fate of hopanoids in the marine realm. At the outset of the project, a large body of evidence existed supporting the idea that hopanoids could potentially be useful markers for cyanobacterial productivity (Rohmer et al., 1984; Summons et al., 1999; Kuypers et al., 2004), however the abundances and structural diversity of hopanoids in the ocean was unknown, and the potential biological sources enigmatic. Taking advantage of recently developed methods for the analysis of intact bacteriohopanepolyols by HPLC-APCI-MS (Talbot et al., 2001; Talbot et al., 2003; Talbot et al., 2003; Talbot and Farrimond, 2007), we set out to reveal the spatial and temporal distribution of hopanoids in a range of marine environments (Fig. 1).

Our sample selection strategy was aimed at addressing five facets of hopanoid distribution: 1) distributions over a land-sea transition 2) open ocean distribution over a large spatial transect, 3) temporal variability in the upper ocean 4) patterns in vertical distribution, and 5) potential for export to

marine sediments. From this data set we have begun to construct an understanding of how hopanoids might inform us of past conditions in marine environments via their legacy in the sedimentary record.

MATERIALS AND METHODS

Sample collection

Sample collection methodologies for each location are listed in Table 1. Suspended particle matter (SPM) was collected by McLane pump filtration of large water volumes (500-1000 liters). Water was filtered through combusted glass fiber filters (GFFs; 142 mm diameter) with a nominal pore size of 0.7 μm. Samples from the Panama Liquid Jungle Laboratory were filtered by peristaltic pump with GFFs with a nominal pore size of 0.3 μm (Sterlitech #TCLP-5; 142 mm diameter). Filtered material was kept frozen until extraction.

Marine core-top sediment samples were collected using a multicore apparatus, which is designed to gently core the upper meter of seafloor sediment without significantly disturbing loosely packed material (termed "floc") at the sediment water interface. Following shipboard retrieval, multicore sediment was sectioned and kept frozen until extraction. All sediment samples were freeze-dried prior to extraction. Sediment trap samples were collected according to methods described in Wakeham et al., (2002). Depth profiles of salinity, temperature, fluorescence, dissolved oxygen were collected by Niskin/CTD cast.

BHP extraction and analysis

Sample locations, sampling and extraction methodology, and quantification method are listed in Table 1. Samples collected during this study were extracted by the method of Bligh and Dyer as described in Chapter 3. Samples from the Arabian Sea, Cariaco Basin and BATS time series were obtained from Dr. Stuart Wakeham and extracted prior to this study by Soxhlet extraction, described by Wakeham et al., (2002). Quantification was achieved using two different standard compounds. The first samples we analyzed were quantified by comparison with an external standard of acetylated steryl glucoside. Subsequently we began using pregnone diacetate as an internal standard and comparison with standard curve of purified acetylated Bacteriohopanetetrol (BHT). Caution should be taken when comparing the absolute concentrations for samples quantified using BHT and steryl glucoside, as these two standards curves have different response factors and, therefore, will yield different concentration estimates. Methods for BHP extraction and analysis are described in detail in Chapter 3.

Detection Limits

The concentration of total lipid extracts (TLEs) from each sample were all adjusted to around 10 mg/ml to ensure that detection limits did not pose a bias in the number of compounds we could detect. Bacteriohopanetetrol (BHT) was typically the most abundant compound in the samples we analyzed and provided a reference for the lower limit of detection. From the

TLE concentrations we used, the mass of BHT on column was always above 10 ng except in a few instances noted in the results sections below. Given a detection limit of around 0.1 ng on-column, BHPs 100-1000 times less abundant that BHT were within detection limits in most of our samples, except where noted.

ENVIRONMENTAL DATA SETS

I. N.W. African Margin

II. Bermuda Atlantic Time-Series Station

III. Panama – Liquid Jungle Laboratory

IV. Arabian Sea

V. Peru Margin

VI. Cariaco Basin

I. N.W. AFRICA

I-1 Environmental setting

Samples collected off the N.W. African margin during the Changing Holocene Environments of the Eastern Tropical Atlantic (CHEETA) cruise provided an opportunity to observe variations in the abundance and structural diversity of BHPs across a broad geographic range spanning ecological gradients. Because of the differences in potential organic matter inputs, and hydrographic and geochemical conditions of surface waters at stations along the transect (Fig. 3), observations from this sample set may provide insight on some of the environmental controls on BHP abundance and diversity. Stations to the north of the transect were characterized by

relatively cold waters and high nutrient levels, contrasted by southern stations where surface waters were warmer with lower nutrient contents. Furthermore, terrestrial river-transported organic matter inputs are thought to be higher towards the south and aeolian iron- and phosphate-rich dust inputs higher towards the north due to the influence of the dust plumes blowing off the Sahara Desert.

We analyzed suspended particulate matter (SPM) and core top sediments from five stations along the N.W. African margin (Fig. 3). We compare spatial patterns in BHP abundance and diversity within the upper water column and surficial marine sediments across the cruise transect.

I-2 Results

BHPs in Suspended Particulate Matter samples

BHtetrol (Fig 2; Ia; BHT), BHpentol (Fig 2; Ib; BHpentol), BHtetrol cyclitol ether (Fig2; If; BHTCE), and a guanidine-substituted BHT-CE (Fig 2; Ih; G-BHT-CE) were the only compounds that could be detected and verified by their full scan mass spectra (Table 2). A molecular ion with m/z 714 was observed with an appropriate retention time to be considered putatively as BHaminotriol (Fig 2; Ic; aminotriol), however, due to low signal strength no mass spectra were available to confirm structural identity. In some samples a molecular ion with m/z 1060 was identified with a retention time that could

correspond to a BHpentol cyclitol ether (Fig 2; Ig; BHpCE), however the mass spectra had very low intensity signals and were non-conclusive. BHT was detectable in all samples except for the 660-720 m sample at Station 8. BHpentol is typically only detected below the surface 20 meters and is typically most abundant at between 80-200 m. BHT-CE was only detected at station 8 at 10 m (Table 2).

BHPs in Core Top Sediments

A total of thirteen BHPs were identified in core tops sediments from five stations along the N.W. coast of Africa (Table 3). BHT was the most abundant compound ranging between 69-80% of the total BHP content (Table 3A). A second BHT isomer was detected at all stations except for station 13, and it ranged from 2.4-7.3 % relative abundance. Other BHP compounds ranged between 0.2-11% of the total BHP content. The greatest diversity of structures was observed in the two most northern stations (8 and 13). The northern and southern sites differed primarily by the presence of composite BHPs (e.g. BHpCE, BHT-CE, and G-BHT-CE) that were absent or close to detection limits at the southern sites. 2-methylBHT was detected at all stations except for station 22.

Total organic carbon content was higher in the southern stations (22, 26, and 27) than in the northern stations (8 and 13) (Table 2B). BHT

concentration was also higher in the southern stations, both in terms of sediment mass and relative to TOC (Tables 2B and 2C). The relative abundance of adenosylhopane (Fig. 2; Ii; adenosylhopane) was higher in the southern stations as well, whereas composite BHPs showed an overall decrease in relative abundance to the south.

I-3 Discussion

The distribution of BHPs in the water column was patchy with the exception of BHT, which was nearly ubiquitous. BHT, BHT-CE and aminotriol are commonly observed in cultured bacteria and in the environment (Talbot et al., 2003; Talbot et al., 2003; Talbot and Farrimond, 2007; Talbot et al., 2008; Xu et al., 2009). Given their ubiquity, these compounds appear to have little value as biomarkers for specific groups of bacteria or unique source environments. BHpentol has previously been detected in freshwater cyanobacteria and lake sediments (Bisseret et al., 1985; Zhao et al., 1996; Talbot et al., 2003; Talbot et al., 2005; Talbot et al., 2008). We detected BHpentol in samples at all sites except for station 8 and, given its presence in the upper water column, it is possible that it is produced by marine cyanobacteria. However, a marine bacterium has yet to be identified that produces BHpentol. G-BHT-CE has only been identified in non-marine methylobacteria, such as *Methylobacterium organophilum*

(Renoux and Rohmer, 1985). It is, therefore, possible that G-BHT-CE is diagnostic of marine methylobacteria present in the water column.

The patchy distribution of some BHP compounds in the upper water column indicates that the sources of these compounds are not widespread in the pelagic zone and may only thrive under certain conditions. Indeed, there do not appear to be any consistent trends with depth or latitude for any BHP other than BHT. It is likely, therefore, that these compounds may also exhibit a high degree of temporal as well as spatial heterogeneity. In contrast BHT could be detected in all but one sample and was by far the most abundant BHP. This is remarkable considering that only one cultured non-marine cyanobacterium, *Anabena cylindrica*, is known to produce BHT as its sole hopanoid (Talbot et al., 2008). There were no apparent trends in BHP abundance with depth at the five stations we sampled. However, given the low depth resolution of samples, it is not prudent to draw strong conclusions about the vertical profiles of BHPs at this study site.

The diversity of BHPs in the sediments is far greater than can be accounted for by BHPs detected in the upper water column. There are several possibilities that could account for this. The presence of adenosylhopane in the sediment samples suggests that soil-derived BHPs could comprise a significant component of the BHP inventory in these sediments (Cooke et al., 2008; Cooke et al., 2009; Xu et al., 2009); terrestrial BHPs may be delivered

to coastal sediment through riverine transport or aeolian deposition. Along this region of the coast, it has been shown that terrestrial-derived particles are advected away from land over the shelf in a subsurface layer between 200-400m depth in the water column (Karakas et al., 2006). Our samples do not cover this depth range, unfortunately, so it is not presently possible to determine if soil-derived BHPs are abundant in association with such particle layers in the water column. Heterotrophic bacteria growing within the sediments cannot be ruled out as a possible source of BHPs, although it has generally been assumed that they represent a relatively small contribution to sedimentary organic matter (Sinninghe Damste and Schouten, 1997). However, recent findings suggest that microorganisms growing within marine sediments may have a non-trivial impact on sedimentary lipid inventories (Lipp and Hinrichs, 2009). As we discuss in more detail later, the discovery that anaerobic bacteria are capable of synthesizing hopanoids (Fischer et al., 2005; Hartner et al., 2005; Blumenberg et al., 2006) opens the possibility that anaerobic bacteria thriving within sediments could provide a significant source of BHPs to sediments. Additionally, the prominence of N-terminal BHPs, aminotriol and aminotetrol, which are assocatied with methanotrophs and the sulfate reducing bacterium *Desulfovibrio spp.* (Neunlist and Rohmer, 1985; Neunlist and Rohmer, 1985; Neunlist and Rohmer, 1985; Zhou et al., 1991; Cvejic et al., 2000; Talbot et al., 2001;

Blumenberg et al., 2006) may support the possibility of a contribution from anaerobic bacteria. Temporal variability in the diversity of BHPs in the water column could also explain why we do not see more BHP structures in SPM samples which, by their nature, reflect short snapshots of BHP production and export. Finally, if certain BHPs are only present at distinct depths within the water column, the depth resolution of samples could be a limiting factor in our ability to assess the potential structural diversity of BHPs in the pelagic zone. Although this is more likely to be an important factor in pelagic environments characterized by vertical stratification, and not in a well-mixed water column.

Structural diversity is very similar in sediments from all five stations, with some minor variability. This is remarkable considering the large geographic range covered by this transect, and raises the possibility that the majority of BHPs detected in sediments may be derived from a common source environment such as from soil organic matter. However, given the large gradient in soil types and terrestrial environments spanning the latitudes covered along the cruise transect, we would necessarily expect that the BHP composition of terrestrial inputs would be constant at all five stations. The highly conserved structural diversity of BHPs at all five stations could also be explained if a large component of the sedimentary hopanoid inventory is derived from a cosmopolitan group of anaerobic

bacteria thriving within the sediments. There is however variability in the relative abundances of the BHPs, which may indicate that independent processes are responsible for the production and/or export of individual compounds to the sea floor.

The prominence of BHT in the sedimentary BHP inventory is also a striking observation. BHT consistently makes up around 75% of the total BHP inventory, except at station 8 where an abundance of aminotriol (11%) results in lower relative BHT abundance (65%) (Table 2A). In contrast, the absolute quantities of BHP in the sediments changes by over an order of magnitude across this transect (Fig. 4). This suggests a stoichiometric relationship between BHT and other BHP compounds, but it is not clear how such a relationship could be explained.

II. BERMUDA ATLANTIC TIME-SERIES STATION

II-1. Environmental setting

The Bermuda Atlantic Time-Series (BATS) Station is located in the Sargasso Sea at approximately 32°10'N, 64°30'W. The BATS site has been the subject of extensive long-term observations to study the marine biogeochemistry of oligotrophic ocean gyres (Steinberg et al., 2001). Cyanobacteria are a dominant component of the phototrophic population within the photic zone (DuRand et al., 2001), and nitrogen-fixing cyanobacteria can be especially prevalent at certain times of the year (Orcutt et al., 2001). Observing temporal variation and depth distribution of BHPs at BATS in comparison with complementary data on the abundance of microbial populations provides an opportunity to probe the sources of individual BHP structures.

We analyzed the quantitative distribution of BHPs in total lipid extracts from SPM samples collected at 5 meters over a three-year time series from 1993-1996. We also analyzed SPM samples from a depth profile collected on a BATS cruise in November, 2008. Together, these sample sets provide the first observations of BHPs in an oligotrophic marine pelagic environment. The results indicate that BHPs vary considerably over an

annual cycle, and that BHP diversity and abundance is greatest at depths corresponding to maximum chlorophyll fluorescence within the photic zone.

II-2. Results

2.1 Suspended particulate matter time series

Three BHPs were detected in TLEs from 5 m depth SPM samples collected over a three-year period at BATS. On-column mass of BHT ranged from 7-141 ng with an average of 48 ng on column. Given a detection limit of around 0.1 ng on-column, BHPs 10-1000 times less abundant that BHT were within detection limits. BHT was detected in all of the samples, whereas BHpentol and lactoneBHP (Fig. 2; Ik; lactoneBHP) were only detected in a subset of samples. BHT and BHPentol show a seasonal trend with BHT reaching maximum values in September and BHpentol reaching maximum values in March. LactoneBHP is only detectable in three samples and does not appear to exhibit a seasonal trend. BHT is the most abundant compound ranging from around 0.01-0.5 ng/l , whereas BHpentol and BHPlactone were 10-fold less abundant ranging between 0.001-0.05 ng/l.

Measurements of primary production determined by light & dark ^{14}C bottle labeling experiments and heterotrophic bacterial growth rate determined by radiolabeled thymidine uptake obtained from BATS cruise reports were compared with BHP abundance (Fig. 5). Maximum values in

BHpentol concentration occur during peaks in primary production, although not all peaks in primary production are accompanied by the presence of BHpentol, as the compound is only detectable during the early Spring. BHT abundance appears to lag maxima in heterotrophic bacterial growth rates by about three months.

2.2 Suspended Particulate Matter Depth Profile – November, 2008

A total of 8 BHP compounds were identified in SPM samples taken in November, 2009 (Table 4). BHT, aminotriol and lactoneBHP were the most abundant compounds with BHT concentration ranging from 0.1-1 ng/L. At all depths except 200 and 300 m the lactoneBHP was the most abundant compound. Total BHP content, as well as individual compound abundance was highest at 80 m depth, coinciding with the maximum measured chlorophyll fluorescence values (Fig. 6) occurring just below the pycnocline. A smaller mid-depth maximum was observed at 300 m with a very similar molecular fingerprint to the 80 m sample. The greatest structural diversity was observed at 80 m and 300 m, and the lowest structural diversity observed between 150-200 m. The abundance of composite BHPs was highest at 80 m approaching 20% of total BHP (by peak area).

II-3. Discussion

BHT was present at detectable levels year round, whereas BHpentol and lactoneBHP were only detectable during short (1-2 month) intervals. This result is comparable with spatial patterns observed off the coast of N.W. Africa, where BHT was ubiquitous, whereas other BHP structures exhibited spatial heterogeneity. From these combined observations it appears that BHT is spatially and temporally ubiquitous, whereas other BHPs are produced at discrete points in time and space.

BHT exhibits well-behaved seasonal variability over the three-year sampling period. When BHT variability is compared with heterotrophic bacterial growth rate, as measured by ^{3}H-thymidine uptake, the two records show remarkably similar trends. However they appear to be offset by 3 months, with BHT abundance lagging growth rate. While it is not entirely clear what this relationship indicates, it does seem to imply a link between heterotrophic bacterial activity and BHT abundance. Since maximum BHT abundance occurs after maximum growth rates, while growth rate is decreasing, perhaps this indicates that cellular abundance of BHT is higher during stationary growth and lower during exponential growth (bloom conditions). It has been observed anecdotally that the relative abundance of BHT is higher in cultures of *Rhodopseudomonas palustris* during stationary growth phase than during exponential growth phase (Paula Welander, pers. comm.) however a controlled study is yet to be published. An alternative, but

not necessarily mutually exclusive explanation, is that BHT is a degradation product of composite BHPs and that it accumulates as biomass degrades or as cells excrete BHT following degradation within the membrane. This could account for the accumulation of BHT following maximum growth rates, which would, presumably, also coincide closely with maximum cell density, followed by cell death and decay.

BHpentol also exhibits seasonal patterns, however it is only detected during early Spring. Interestingly, the presence of BHpentol also coincides with peaks in measured primary productivity, suggesting a link to a cyanobacterial source. BHpentol has been identified in non-marine *Nostoc spp*. (Bisseret et al., 1985; Zhao et al., 1996), although they are also found in *Acetobacter spp* (Zundel and Rohmer, 1985). Additionally, no cultured marine bacteria are known to make this compound. While, it is possible that some as yet uncultured marine cyanobacterium produces BHpentol, a non-cyanobacterial source cannot be ruled out either.

BHP diversity in the depth profile sampled in November is remarkably high, demonstrating that oligotrophic pelagic environments can be host to bacteria producing a diverse range of hopanoid structures. The number of unique BHP compounds varies considerably with depth and appears to co-vary with total BHP abundance, raising the concern that samples with lower BHP abundance may also have lower diversity because less abundant

compounds fall below the detection limit. However, this is not likely since we have adjusted total lipid extract concentrations such that compounds that are up to 1000-fold less abundant than BHT should be within our range of detection. Therefore, the co-variation in abundance and diversity that we observe is apparently real and not biased by sample abundance and detection limits.

BHP abundance and diversity exhibit maxima at two depths (80 m and 300 m) within the water column. A shallow maximum corresponds to the depth of maximum chlorophyll fluorescence and dissolved oxygen, which suggests a relationship between BHP abundance and the abundance of photo(auto)trophic organisms. A deeper maximum in abundance and diversity at 300 m is not associated with any hydrographic features (salinity, temperature) or with dissolved oxygen concentrations. There is no clear explanation for the deep maxima in abundance and diversity. However, given the similarity in BHP composition at 300 m and 80 m, it is not unreasonable to propose that the BHPs at 300 m may be derived from the lateral advection of organic matter from surface waters to depth along isobars that outcrop at the sea surface.

Both of the composite BHPs that were detected (BHT-CE and G-BHT-CE) exhibit maximum abundance at the same depth as maximum chlorophyll fluorescence when reported both in terms of absolute concentration (not

shown) and relative to total BHP abundance (Fig. 6). While BHT-CE is not exclusively diagnostic of cyanobacteria, it is a major component of all cultured marine cyanobacteria that have been surveyed for BHPs (Chapter 3; Talbot et al., 2008). The presence of two isomers of BHT-CE suggests that this compound is derived from multiple source organisms, since no single cultured bacterium has yet been shown to produce two isomers of this compound.

III. PANAMA – LIQUID JUNGLE LABORATORY RIVER-OCEAN TRANSECT

III-1. Environmental setting

Soils and other terrestrial environments are known to be rich and diverse sources of BHPs (Miller et al., 2000; Talbot et al., 2003; Talbot and Farrimond, 2007; Cooke et al., 2008; Cooke et al., 2009). A few distinct BHP structures that are associated with terrestrial environments have been observed in riverine and estuarine sediments (Cooke et al., 2009). However, the extent to which BHPs that have less specific environmental associations are transported by rivers from land to sea is not clear. We analyzed SPM samples along a transect from river to ocean along the Pacific coast of Panama in order to elucidate the patterns of BHP diversity and abundance across a land sea gradient, and to determine the potential for riverine POC to contribute to marine sedimentary BHP inventory (Fig. 7). The location we chose was particularly advantageous because of the fact that the narrow continental shelf along the Pacific coast combined with local circulation patterns of surface waters allows blue water from open ocean oligotrophic environments to come in close proximity to the coast. Because of this, the transition from estuarine green water to oligotrophic blue water occurs within 2 miles of the shore and could be easily sampled during operations based out of the Liquid Jungle Laboratory field station.

III-2. Results

BHP concentration relative to filtered volume and TLE mass showed 100-fold decrease from river (Station 1) to ocean (Stations 5 and 6) (Fig. 8 A and B). 10 BHPs were detected in SPM samples from the river and coastal waters (Fig. 8 B). BHT was the most abundant compound in all of the samples ranging from ~120 ng/l at Station 5 (blue water) to at Station 1 (up river) ~2 ng/l. BHT and total BHP abundance decreased steadily from Station 1 to Station 5. All other BHPs were about 10 times less abundant than BHT. Sites 1 – 4 all had identical BHP structural distributions with the exception of aminopentol and aminotetrol (Fig. 2; Id; aminotetrol), which were only present at Site 1. In contrast, BHT was the only BHP that could be identified at Stations 5 and 6. Adenosylhopane, which has previously been proposed as a marker for terrestrial soil-derived organic matter (Cooke et al., 2008; Xu et al., 2009), was detected at all stations except for Stations 5 and 6.

III-3. Discussion

The striking differences in structural diversity and concentration of BHPs between the blue water samples and riverine samples indicate that terrestrial and riverine organic matter can be a rich and diverse source of hopanoids. The concentration of BHPs in riverine SPM is 100 times greater than in blue water SPM at this study site (Fig. 8 A). This observation is the

same whether concentration is reported relative to the volume of water filtered (absolute amount of hopanoid) or relative to the mass of the TLE (relative amount of BHP of total extractable lipids). This suggests that the terrestrial organic carbon delivered to coastal sediments by the river has a higher hopanoid content by mass than marine organic carbon exported to sediments. Assuming this is representative of global patterns of BHP distribution then if particulate organic carbon transported by rivers to coastal sediments were to make up only 1% of marine sediment organic matter, terrestrial BHPs would be as abundant as BHPs associated with exported marine POC. Terrestrially-derived BHPs could, therefore, represent a significant component of the BHP inventory in coastal marine sediments. This possibility is supported by our observation of adenosylhopane in surficial marine sediments off the coast of N.W. Africa.

The similar molecular fingerprint at these five stations and decreasing BHP abundance across the transect from river to ocean implies that BHPs are predominately derived from terrestrial sources being transported from up river into the estuary and out to shallow coastal waters. Adenosylhopane and a putative "adenosylhopane analogue" associated with the molecular ion with m/z 761 (Talbot and Farrimond, 2007) are both widespread and abundant in soils (Cooke et al., 2008; Xu et al., 2009). Both compounds are present in the riverine, estuarine, and coastal green water stations from this study.

Adenosylhopane is the second most abundant BHP (next to BHT) in both river samples and in the estuarine samples, indicating that soil-derived BHPs are a major component of the BHP inventory in SPM from these sites. It is also possible, however, that planktonic bacteria thriving in the river, estuary and coastal waters also contribute to the BHP inventory of SPM. This could potentially explain why aminotetrol and aminopentol are only present at Station 1 (up river), and not at any of the down stream stations. Perhaps aminotetrol and aminopentol are produced by a planktonic bacterial source that is only present at Station 1. However, given that aminotetrol and aminopentol have only been identified in methane oxidizing bacteria (Neunlist and Rohmer, 1985; Neunlist and Rohmer, 1985; Neunlist and Rohmer, 1985; Zhou et al., 1991; Cvejic et al., 2000; Talbot et al., 2001) it may be more likely that they are derived from soil or possibly from resuspended river sediment stirred up by boat traffic in that shallow part of the river.

2-methylBHT is also abundant in the riverine, estuarine and coastal green water samples. 2-methylBHT has been frequently observed in terrestrial environments including soils, lake sediments, and bacterial mats in shallow freshwater environments (Talbot and Farrimond, 2007; Pearson et al., 2009; Xu et al., 2009). Previous observations of BHPs along a terrestrial-marine transect in the Bahamas have shown that 2-methylBHT is found in both terrestrial and marine environments (Pearson et al., 2009). This has

particularly significant implications for 2-methylhopanoids in the geologic record. 2-methylhopanoids detected in marine sediments have typically been interpreted as biomarkers for cyanobacteria because of their frequent occurrence in cultured non-marine cyanobacteria (Summons et al., 1999). However, the association of 2-methylhopanoids with terrestrial environments and apparent absence from marine pelagic environments in present times suggests that the geologic record of 2-methylhopanoids may reflect terrestrial sources and rather than marine cyanobacterial sources. However, we cannot rule out the possibility that 2-methylhopanoids might be present in the vast expanses of the ocean, or seasonal/environmental production peaks that we have yet to explore for the presence of hopanoids.

IV. ARABIAN SEA

IV-1. Environmental setting

The Arabian Sea hosts some of the most extensive and productive upwelling and associated oxygen minimum zones (OMZ) in the world (Bange et al., 2000). Seasonal monsoon winds drive upwelling in the summer and winter, leading to intensified primary productivity and export of organic matter (Honjo et al., 1999). The permanent nature of the OMZ combined with strong seasonal shifts in photic zone productivity make this an ideal environment to test the sources and control on hopanoid production and export in fertile and underlying oxygen-depleted water columns. Temporal variations in the abundance of BHPs originating from the photic zone and associated with blooms in primary productivity should be distinguishable from BHPs derived from subsurface communities. By looking at water column depth profiles of suspended particulate matter (SPM), we can infer the source of BHPs through their relative abundances or presence/absence at various depths.

We investigated the spatial, vertical, and temporal distribution of BHPs in the Western Arabian Sea in order to gain insight on the sources and controls on hopanoid production. Core top sediment, suspended particulate matter from 5 m, 500 m, 1000 m and 1500 m and sinking particles collected

by sediment traps at 500 m were sampled at three stations during the US JGOFS Arabian Sea Process Study between 1994-1995 (Fig. 9).

IV-2. Results

2.1 Suspended particulate matter depth profiles

Six BHP structures were identified in SPM samples from stations MS1, MS3, and MS4 (Fig. 10). BHT was the most abundant compound in all of the samples. On-column mass of BHT ranged from 7-187 ng, with an average on-column mass of 52 ng. With on-column detection limits at around 0.1 ng, BHPs 100-1000 times less abundant than BHT were within detection limits. Surface samples and subsurface samples have distinct BHP molecular fingerprints. In general, all surface samples have identical composition while samples from the subsurface all have similar compositions distinct from those above. Two isomers were detected (BHT I and II) in all subsurface samples, however BHT II was absent from surface samples. BHpentol (III) was absent from all surface samples and present in all subsurface samples. Aminotriol (IV) was present at very low concentration from the surface sample at MS1 but was absent from surface samples from the other two stations, and was present in all but one subsurface sample. Bacteriohopaneribonolactone (BHRL; V) is present in all but two of the subsurface samples from MS4. 32,35-anhydrobacteriohopanetetrol (VI) is present in all but one subsurface

sample at MS4, and is absent from all surface samples. The absence of several compounds from the 500m sample at MS4 may be an artifact of low sample abundance (on-column BHT mass was 7ng), which may place some of the less abundant BHPs below detection limits.

Depth profiles of BHPs from sites MS1 and MS3 are shown in Figure 11. Quantitation of BHPs from the sample at 500m from MS4 was not possible because we do not know how much of the original TLE was in the aliquot that we analyzed. However, at stations MS1 and MS3 all BHPs reach maximum values at 500 m depth, except for lactoneBHP at station MS3 which has roughly similar concentrations at surface and 500m. Concentrations of BHT range from 0.8-122.57 ng/L. BHP concentrations for the surface sample at MS4 were significantly higher than other surface samples. However, when concentration is plotted relative to TLE mass, all surface samples show comparable concentrations.

2.2 Sediment traps – 500m depth

Three compounds were detected in TLEs from 500 m sediment traps at stations MS1, MS3, and MS4 (Fig. 12). BHT-I was the most abundant compound in all of the samples. On-column mass of BHT-I ranged from 1-298 ng, with an average on-column mass of 61 ng. With on-column detection limits at around 0.1 ng, BHPs 10-1000 times less abundant than BHT were

within detection limits. The percent organic carbon of the sediment (%Corg) was comparable at all three sites, ranging from 4-19 % with an average of 10%. Total organic carbon (TOC; %) values are relatively stable at all three sites.

TOC flux shows distinct seasonal patterns in variability. At MS1 and MS3 TOC flux peaks during the southwest monsoon (SWM) and northeast monsoon (NEM) and remains low during intermonsoonal periods. At MS4 TOC flux is not as closely linked with the monsoons, and reaches maximum values during the spring intermonsoonal (SI) period and the SWM, but remains low during the NEM. A similar trend in biomarker export was observed by Wakeham et al., (2002) and it has been suggested that flux in this region is may be more heavily influenced by influx of nutrients from eddies rather than from monsoon-driven upwelling (Honjo et al., 1999).

Maximum BHP flux at all three sites correlates closely with TOC flux. In general, the timing of variations in flux is the same for all three BHP structures. However, the timing of maximum BHP flux differs at each site. All three sites show an increase in flux during the SWM, and this is when maximum BHP flux occurs at MS3. BHP flux also increases during the NEM at MS1 and MS3, but not at MS4. The intermonsoonal periods (SI and FI) are characterized by low BHP flux, except at MS4 where maximum BHP flux occurs during the FI.

BHP concentrations (normalized to OC) are highly variable (by up to a factor of 10) and do not appear to be closely correlated to the seasonality of the monsoons. All three BHPs do appear to show strikingly similar patterns of variability. The temporal variability in concentration varies considerably between stations and there are few coeval features in the trends. Both MS1 and MS3 show a late NEM maximum in BHP concentration. BHPs at all three sites reach elevated concentrations midway through the SI. Most of the maxima in BHP concentration are represented by only one or two time points making it difficult to infer the temporal extent of these events.

The relative abundance of BHPs averaged for all sediment trap samples is shown in Figure 14. Variation in relative abundance was very low, as indicated by the low standard deviation for the entire data set. The relative abundances of BHPs are comparable with those observed in subsurface SPM samples.

2.3 Surficial sediments

Six compounds were identified in core top sediments from stations MS1, MS3, and MS4 (Table 5). BHT I was the most abundant compound in all of the samples ranging from 0.11-3.77 μg/g sediment (Table 2). On-column mass of BHT I ranged from 7-413 ng, with an average on-column mass of 194 ng. With on-column detection limits at around 0.1 ng, BHPs 10-1000 times less

abundant than BHT were within detection limits. The relative abundances of BHPs are shown in Figure 13. The BHP composition is identical to the SPM samples, and the relative distributions are strikingly similar.

IV-3. Discussion

Vertical patterns in BHP distribution in SPM indicate that the highest abundance and structural diversity is observed within the OMZ. Temporal variability in concentration and do not reflect seasonal variability, suggesting that conditions leading to hopanoid production are not dependent on monsoon driven upwelling. This is in line with the observation that hopanoids are most abundant at 500m, well below the photic zone. This suggests that other factors are necessary to account for variability in abundance of hopanoid-producing bacteria at depth in the Arabian Sea. The fact that hopanoids that are being produced at 500m are being exported to sediments also indicates that the source organisms may be particle associated; free-floating cells would not be readily exported to depth.

The diversity and relative abundance of BHPs is strikingly constant at all three sites at depths below 500 meters, and throughout the year (Fig. 14). It is most curious that the BHP abundance and diversity is so highly conserved across such large spatial and temporal expanses. A terrestrial source is unlikely since the flux of BHPs is so closely correlated to POC flux,

which is linked to upwelling induced blooms of primary productivity in this system. The simplest explanation is that a single source organism or microbial consortium that is ubiquitous within the OMZ is producing BHPs I through III in a fixed ratio. It is possible that some or all of BHPs I through III represent degradation products of penta- and tetra-functionalized composite BHPs (e.g. BHT-CE). However, such a relationship between BHT and BHpenol with composite BHPs has yet to be demonstrated. The variations that we observe between SPM, sediment trap, and surficial sediments could be attributed to differences in the extent of degradation in these three sample pools, as well as to slight changes in the stoichiometry of BHPs produced by the source organism(s), which exhibits some degree of temporal variability.

V. PERU MARGIN OXYGEN MINIMUM ZONE

V-1. Environmental setting

The Peru-Chile margin is also host to expansive oxygen minimum zones. Wind-driven upwelling leads to an influx of nutrient rich waters from depth (Friederich and Codispoti, 1987), which in turn fuels high levels of primary productivity. Heterotrophic remineralization of organic matter exported from the productive surface waters consumes oxygen rapidly enough to generate anoxic conditions throughout most of the water column. We investigated the abundance and diversity of BHPs in SPM samples from a depth profile spanning the Peru Margin OMZ.

V-2. Results

Six BHP structures were identified in SPM samples from the upper 250 m of the Peru Margin (Fig. 15). BHT was the most abundant compound ranging between 1-7 ng/L. Other BHP compounds were about 10-fold less abundant than BHT and ranged from 0.1-1 ng/L. Two isomers of BHT were detected, one of which was only detectable within the OMZ, where the two isomers were of comparable abundance. The highest concentration of BHT was at 75 m, within the OMZ. Aminotriol was detected in all samples except for 25m above the oxycline, and was most abundant at 50 m depth just below the oxycline. BHpentol was only detectable at 150 m and 250 m. Two

composite BHPs, cyclitol ether and guanidine substituted cyclitol ether, were also detected within the OMZ, but not in the upper photic zone.

V-3. Discussion

BHP abundance and diversity are greatest within the OMZ. This trend is similar to that observed in the Arabian Sea. This could either indicate that the diversity of hopanoid-producing bacteria is much greater within the OMZ or that the membrane composition of bacteria capable of producing hopanoids requires more abundant and diverse BHPs in low oxygen environments. BHT is the only compound detected in the photic zone, which is largely consistent with our observations from other upper ocean sites. Individual BHPs exhibit different depth profiles suggesting that they are derived from different sources within the water column, or produced in greater abundance in response to changing conditions with depth. Aminotriol reaches maximum abundance just below the oxycline. However, given the low sample resolution, it is possible that maximum aminotriol concentrations occur within the oxycline. BHT-CE emerges just below aminotriol at 75 m and is present at constant abundance to the bottom of the profile, spanning the core of the OMZ. Guanidine substituted BHT-CE emerges at 150 m and increases in abundance down to 250 m. These trends in depth profiles suggest that that individual BHP structures occur over distinct depth ranges indicating that they may each associated with a particular chemical horizon within the OMZ,

possibly linked to the succession of thermodynamically favored electron acceptors. This possibility would need to be confirmed by constructing a higher resolution depth profile to elucidate the finer vertical structure in relative abundance.

It is particularly interesting that we did not detect any 2-methylhopanoids. 2-methylBHT (Fig 2; IIa) was previously reported from sediments taken from the Peru Margin (Farrimond et al., 2000), however our data suggests that they are not derived from the water column. While it is possible that we did not detect 2-methylBHT because it is only present at certain times, it is also possible that 2-methylBHT detected in sediments from the Peru Margin are derived from terrestrial sources or bacteria thriving within the sediment.

VI. CARIACO BASIN

VI-1. Environmental setting

The Cariaco Basin is the world's second largest anoxic basin. The basin is isolated by a shallow sill that prevents deep water from mixing with adjacent deep waters from the Gulf of Mexico. Seasonal heating of the surface waters drives stratification, which in turn prevents exchange between deep and surface waters and results in the deep waters being isolated from equilibration with the atmosphere. Because of this, oxygen is depleted by organic matter remineralization at depths below 200-300 meters. In contrast to the Black Sea, the redoxcline of the Cariaco Basin is well below the photic zone, and therefore there are no photosynthetic organisms associated with low oxygen or euxinic conditions at depth.

We investigated the abundance and distribution of BHPs in SPM samples from a depth profile taken from the Eastern Basin of the Cariaco Basin. BHPs were previously detected in the water column of the Black Sea and shown to be most abundant at the chemocline (Wakeham et al., 2007). Interestingly, 2-methylBHT was detected at and below the Black Sea chemocline and not in the photic zone, suggesting that this compound is associated with anaerobic bacteria, possibly including sulfide-utilizing cyanobacteria (Cohen et al., 1986; Post and Arieli, 1997) in that environment.

Given the discovery of 2-methylBHT in *R. palustris* (Rashby et al., 2007) and the capacity for C-2 methylation in some proteobacteria (Welander et al., 2010), it is possible that it is not always associated with a cyanobacterial or phototrophic source in the Black Sea, although the sources of 2-methylhopanoids remain to be determined in the modern oceans. We compare our BHP results from the Cariaco Basin with those reported from the Black Sea in order to determine if any BHP structures are unique to euxinic marine environments.

VI-2. Results

Depth profiles for the Cariaco Basin are shown in Figure 16. Dissolved oxygen concentrations decreased steadily from surface to around 225m. Sulfide becomes detectable at 250 m and increases steadily to the bottom of the measured profile at 900 m. Suspended particulate organic carbon shows a marked decrease from surface to 205 m and then increases abruptly between 236 – 245 m, coinciding with the transition from anoxic to euxinic conditions.

Four BHP compounds were identified in SPM samples from the Cariaco Basin (Fig. 16). Two isomers of BHT were detected (BHT-I and BHT-II). BHT-I and II and aminotriol were the most abundant compounds ranging from 1-10 ng/L. BHpentol concentration ranged from 0.1-1 ng/L. All four compounds exhibited two maxima between 250-280 m and at 326 m.

BHpentol exhibited a third maxima at 42m. BHT-I BHT-II and Aminotriol reached absolute maximum concentrations below the chemocline, whereas BHpentol reached maximum concentration within the photic zone. BHT-I also exhibited a local concentration maxima within the photic zone. However, BHT-II and aminotriol concentrations remained low above the chemocline.

VI-3. Discussion

The distribution of BHPs within the water column of the Cariaco Basin indicates that hopanoid-producing bacteria can be relatively abundant and diverse within marine pelagic redox transitions zones. There are two discrete depths within the redoxcline where BHPs reach maximum values suggesting that hopanoid-producing bacteria are associated with communities at discrete chemical horizons within the chemocline. Furthermore, at the transition from anoxic to euxinic waters, individual BHPs reach maxima at different depths within the chemocline. Aminotriol exhibits a concentration maxima at 270 m below the maxima for BHT I and BHpentol at 256 m. BHT-II and aminotriol appear to be associated almost exclusively with the chemocline; although BHT-II is detected just above detection limits in the photic zone. In contrast, BHpentol is the most abundant within the photic zone, raising the possibility that it may be associated with phototrophic communities. Differences in relative abundance at different depths may indicate that individual BHPs are being produced by different bacterial populations associated with discrete

environmental niches (e.g. specific redox horizons) within the chemocline. Alternatively, this could also reflect differences in the cellular abundance of different BHP compounds expressed in response to varying physiological requirements at different depths.

Despite the fact that hopanoids are considerably more abundant within the chemocline than within the photic zone, hopanoid producing bacteria are likely not a major component of the total bacterial population in the chemocline. When compared with POC concentrations (~20 μg/l) and total fatty acid concentrations (~0.8 μg/l; Wakeham et al., 2010), BHPs concentrations (0.1-10 ng/l) suggest that hopanoids are associated with a relatively rare subset of bacteria within the chemocline. This result is in agreement with a previous study showing low relative abundance of the squalene-hopene cyclase gene (*sqhC*) relative to total DNA in the Global Ocean Sampling expedition (GOS) metagenome (Pearson and Rusch, 2009).

The BHP structural diversity that we observed in the Cariaco Basin differs in some respects from the Black Sea. For instance, we observe a lower diversity of structures in the Cariaco Basin. The N-terminal BHPs aminotriol, aminotetrol, aminopentol, and an unsaturated aminotriol were abundant in the Black Sea (Wakeham et al., 2007) whereas in the Cariaco Basin aminotriol is the only N-terminal BHP we could detect. However, despite the lower diversity of N-terminal BHPs, they comprise roughly half of

the BHP inventory within the chemocline at both locations, suggesting that N-terminal BHPs play an important role in bacteria growing under euxinic conditions. 2-MethylBHT was also reported within the chemocline of the Black Sea, whereas no 2-methylhopanoids were detected in the Cariaco Basin water column. This observation raises the possibility that 2-methylhopanoids are associated with anoxygenic phototrophs or sulfide utilizing cyanobacteria in modern stratified marine basins; the chemocline of the Black Sea hosts communities of anoxygenic phototrophs, whereas the chemocline of the Cariaco Basin is situated well below the photic zone.

GENERAL DISCUSSION

From this first survey of BHPs in the oceans, it is apparent that hopanoids are ubiquitous. BHT is detected in all but one of the samples we analyzed. However, the distribution of other BHP structures was shown to be spatially and temporally heterogeneous (Fig. 15).

Bacteriohopanetetrol (BHT)

The ubiquity and overwhelming abundance of BHT raises some questions about its source and significance. In cultured bacteria, BHT is typically not the most abundant BHP and there is only one published example of an organism that produces BHT as its sole BHP. It is, therefore, quite remarkable that we observe such a predominance of BHT in the environment often in the absence of other BHPs. One possible explanation is that there is an abundance of marine hopanoid-producing bacteria that only produce BHT. However, another explanation is that BHT is a degradation product of tetrafunctionalized composite BHPs such as BHT-CE. The observation that peak BHT abundance lags heterotrophic bacterial growth rate at BATS (Fig. 5) could suggest that BHT accumulates as bacterial biomass degrades. The sole presence of BHT may therefore represent the presence of degraded composite BHPs and not the existence of bacteria that produce BHT as their sole BHP. Distinguishing between these two

possibilities is critical to understanding the ecology of hopanoid-producing bacteria since it would determine whether hopanoid producing bacteria are in fact ubiquitous in the oceans, or whether they only thrive under certain conditions.

Methylated hopanoids

The 2-methylhopanes have been applied widely as a molecular marker for the contribution of cyanobacteria to organic carbon in oils, source rocks, and sediments (Summons et al., 1999; Farrimond et al., 2004; Kuypers et al., 2004), and the geological records of marine sediments and oils frequently have relatively abundant 2-methylhopanes (Summons and Jahnke, 1990; Summons et al., 1999; Talbot et al., 2003; Farrimond et al., 2004; Knoll et al., 2007). However, the distribution of 2-methylhopanoids in this and other studies suggests that marine cyanobacteria may not always be the primary source of methylated BHPs to marine sediments, at least in present times. 2-methylBHT was detected in riverine SPM and marine sediments but could not be identified conclusively in any marine SPM samples. 2-methylBHT was previously detected just below the chemocline in the Black Sea (Wakeham et al., 2007), which could suggest that it is associated with sulfide tolerant cyanobacteria or with an anaerobic hopanoid-producing bacteria in that environment.

Adenosylhopane and homologous compounds

Adenosylhopane has been proposed to serve as a marker for soil organic matter based on its ubiquity and abundance in soils (Cooke et al., 2008). An uncharacterized BHP with similar spectra to adenosylhopane but with different side chain mass is also frequently associated with soils (Talbot and Farrimond, 2007). We could not detect adenosylhopane or related compounds in any sample from the marine pelagic zone. In contrast, they were found to be abundant in riverine SPM samples from Panama. These observations support the potential application of these compounds as soil markers. We also detect adenosylhopane in marine sediments on the N.W. African margin, indicating that terrestrially derived BHPs may be a significant component of the hopanoid inventory in marine continental margin sediments. The role of adenosylhopane in bacteria is yet to be determined. Emerging evidence indicates that adenosylhopane is the first intermediate in the synthesis of BHPs from diploptene (Bradley et al., 2010). This would suggest that all hopanoid-producing bacteria should contain at least trace amounts of adenosylhopane. It is not clear whether soil bacteria produce adenosylhopane as a membrane component or if they simply accumulate a large and detectable reservoir of adenosylhopane as an intermediate in BHP synthesis. Until this uncertainty is resolved, we cannot rule out the possibility that

adenosylhopane may be present in the marine pelagic zone and abundant under certain conditions.

Composite BHPs

Composite BHPs, include both tetra and penta-functionalized BHT-CE as well as G-BHT-CE. Tetra functionalized BHT-CE is one of the most common BHPs in cultured bacteria, and is present in all of the diazotrophic marine cyanobacterial cultures that have been surveyed. Penta-functionalized BHT-CE has only been identified in fresh water cyanobacteria (Talbot et al., 2008). G-BHT-CE has only been detected in cultured *Methylobacterium* species (Renoux and Rohmer, 1985).

LactoneBHP and anhydroBHP

The sources of the lactoneBHP and anhydroBHP remain unclear. LactoneBHP has been identified in the non-marine gram-negative ammonia oxidizing bacterium *Nitrosomonas europa* (Seemann et al., 1999). However, lactoneBHP has only once been observed previously in an environmental sample (Pearson et al., 2009). High concentrations observed in the environment would suggest that it is accumulating either as a degradation product, or as an intended cellular component of certain hopanoid-producing bacteria. Interestingly, surveys of BHPs from terrestrial environments do not

report the presence of lactoneBHP, indicating that its presence at high concentrations may be a unique phenomenon to marine environments.

AnhydroBHP has no known biological sources. The accumulation of anhydroBHP with increasing depth (corresponding to age of deposition) in marine sediments (Cooke et al., 2008) and higher relative abundance in ancient sediments relative to modern settings (Bednarczyk et al., 2005) indicates that this compound may accumulate as a degradation product of other tetrafunctionalized BHPs. Furthermore, it has been shown that anhydroBHP can be synthesized by acid hydrolysis of BHT (Schaeffer et al., 2008). Given the sporadic occurrence and low abundance of anhydroBHP in the modern environments that we've surveyed, it is reasonable to suppose that this compound is in fact a degradation product. However it cannot be ruled out that it is an intended product of some bacteria in certain environments.

Limitations of the current methodology

In this study we only screen for compounds that have been previously characterized in cultured organisms. Given that less than 1% of the bacteria in the environment are represented by bacteria in culture, it is possible that we are not seeing a vast majority of novel BHPs produced by yet to be cultured organisms (Hugenholtz and Pace, 1996). The observation of multiple

isomers of BHPs that have not previously been detected in cultured bacteria provides some support for this possibility. However, identifying novel structures in environmental samples is challenging since BHPs are typically several orders of magnitude less abundant than the most abundant compounds present in the total lipid extract. Performing analyses with a more sensitive instrument, such as a triple-stage quadrupole mass spectrometer (TSQ-MS), may provide one means of overcoming problems associated with low natural abundance of BHPs in some marine environments. However, given the overwhelming complexity of TLEs, trying to search for novel BHPs present in trace amounts is very much like looking for a needle in a haystack. It may be possible to simplify this problem by developing methods to separate BHPs from the total lipid extract prior to LC-MS analysis.

Detection limits present another possible bias to our interpretations of structural diversity. Are the variations in structural diversity that we see real, or are they associated with an arbitrary cutoff imposed by our detection limits? In almost all of the samples that we have analyzed, we have adjusted the concentrations such that the most abundant BHP (typically BHT) is injected with a mass over 10 ng on column. With detection limits around 0.1 ng on column, this means that we should be able to detect compounds that are a minimum of 1% of the most abundant BHP. This is confirmed by the

fact that we typically don't observe compounds with relative abundances less than 1%. Therefore, regardless of the natural abundance of BHPs in any given environment, the detection limits relative to the most abundant BHP should be consistent in all samples.

The problem of detection limits raises another issue: should we be concerned with the possibility that a vast majority of BHPs are present a abundances far below the current detection limits? Presently, we are capable of detecting compounds in the pg/l concentration range. This is ultimately limited by how much the total lipid extracts can be concentrated without exceeding their solubility. The constraint imposed by solubility could be solved by developing separation methods (as suggested above) to separate BHPs and similar compounds from the bulk of the total lipid extract. However, are compounds that are less abundant even relevant to the questions we are asking? In other words, how far into the rare biosphere do we want or need to probe?

Finally, extraction methodology may present biases. In the present study, samples were extracted by two different methodologies: Bligh and Dyer and Soxhlet extractions (Table 1). While there is no *a priori* reason suspect that the two methods would result in different yields or result in the preferential extraction of certain structures over others, the possibility has not been tested. Another problem, which is more fundamental, is the

differential extractability of certain hopanoid structures due to differing cellular associations. For instance, it has been shown that certain BHPs are "unextractable" by conventional approaches, and may only be extracted when the extracted residue of the sample is directly acetylated (Herrmann et al., 1996). Based on this observation, it was proposed that such "unextractable" BHPs are more closely associated with certain, as yet unknown, cellular components (e.g. proteins or cell wall) through strong non-covalent electrostatic bonds, that are only disrupted through direct acetylation. This raises the possibility that we are missing an entire subset of BHPs that have cellular associations rendering them unextractable to Bligh Dyer or Soxhlet extraction. Ultimately, developing a better understanding of the intracellular distribution and molecular associations of hopanoids in membranes may shed light on the significance of such "unextractable" compounds. However, a more complete study of extraction methodology on a range of sample types (e.g. soil, sediment, cells) is much needed to constrain these uncertainties.

The problem of linking hopanoids to their source in the environment

Ultimately the value of BHPs as biomarkers in the ocean is limited by our lack of knowledge of their source organisms. Phylogenetic surveys of SHC in the environment have revealed the apparent rarity of hopanoid-producing bacteria in the marine realm (Pearson et al., 2007; Pearson et al., 2009; Pearson and Rusch, 2009), which is consistent with our results showing the

low abundance of hopanoids in marine environments. Given that hopanoids are associated with rare organisms that are distantly related to cultured hopanoid-producing bacteria (Pearson and Rusch, 2009), the taxonomic affinities of hopanoids, and their association with distinct biochemical or physiological processes remains enigmatic. Without a culture independent means to link structure to biological source in the environment, our ability to assign biomarker status to BHPs is limited.

Potential value of BHPs as biomarkers in the marine geologic record

The presence of hopanoid-producing bacteria within sediments complicates the interpretation of hopanoids in ancient sediments. It was originally erroneously assumed that only aerobic bacteria produced hopanoids. However, their discovery in obligate anaerobic bacteria, and facultative anaerobes grown under anoxic conditions (Sinninghe Damste et al., 2004; Fischer et al., 2005; Hartner et al., 2005; Blumenberg et al., 2006) raises the distinct possibility that bacteria thriving in sediments could contribute to the hopanoid inventory. Our observation of the presence of a second isomer of BHT (BHT II) in sediments may be evidence of an input from anaerobic bacteria, since we typically only observe BHT II in anoxic or euxinic environments. Furthermore, the relatively high abundance and structural diversity of BHPs that we observe in association with the redoxcline of the Cariaco Basin and in the oxygen minimum zones of the Peru

Margin and Arabian Sea indicate that hopanoid-producing bacteria are relatively prominent in low oxygen environments. Given that the redoxcline is situated within the upper few centimeters of sediment throughout most of the ocean, it is plausible to propose that hopanoid-producing bacteria may be abundant within surficial marine sediments, and a significant potential source of structurally diverse BHPs.

Terrestrial supply of BHPs to marine sediments raises another challenge to interpreting the marine record of BHPs. The number of BHPs that have been detected in soils that have been surveyed (Cooke et al., 2008; Pearson et al., 2009; Xu et al., 2009) is, at a minimum, twice the number of structures that we observe in marine pelagic environments. On the Pacific coast of Panama we observed that the concentrations of BHPs in riverine POC are up to roughly 100 times higher than in marine POC. This suggests that even if terrestrial organic carbon transported to marine sediments were to make up only 1% of the total sedimentary organic carbon, terrestrial BHPs could equal or surpass the quantity of marine BHPs in the sedimentary hopanoid inventory. It is, however, possible that the relative importance of terrestrial sources of BHPs changes with terrestrial source environment (e.g. soil type). For instance, a survey of soils from Northern England shows that total BHP concentration can vary by over an order of magnitude (Cooke et al., 2008), although the principal environmental factors associated with such

variability are not yet clear. BHP abundance in Arctic River sediment – presumably derived primarily from terrestrial inputs - also varies quite substantially; BHP concentration in sediment from the Mackenzie River is nearly and order of magnitude less abundant than in sediment from the Kolyma River (Cooke et al., 2009). A systematic study of BHP abundance and diversity in soils from different latitudes (e.g. tropical, subtropical, and polar) has not yet been published, and given differences in the way concentration is reported by different groups (relative to sediment mass, total organic carbon content, or total lipid extract mass), it is not possible to directly compare all existing published data. However, given the large variability in soil BHP abundance within individual study sites, such as Northern England, and the Arctic Rivers, the average abundance of BHPs in terrestrial organic carbon transported by rivers to the ocean would provide a better understanding of global variability in terrestrial BHP input to marine continental shelf sediments.

Our observations have raised several challenges for applying hopanoids as biomarkers in the marine sedimentary record. In addition to uncertainties about principal environmental sources (e.g. terrestrial, pelagic, sedimentary), the biological sources of hopanoids remain enigmatic; The vast majority of bacteria in the oceans remain uncultured (Hugenholtz and Pace, 1996) and the most abundant hopanoid producing bacteria in the oceans

appear to be distantly related from any that exist in culture (Pearson and Rusch, 2009). Additionally, many of the compounds that we have detected are common to many cultured hopanoid-producing bacteria, limiting our ability to assign diagnostic value to BHPs in marine sediments. With the exception of the BHT II isomer, adenosylhopane, and lactoneBHP, the compounds that we observe do not yet appear to show any unique environmental associations. However, given that this survey represents a limited sampling of the temporal and spatial extent of the oceans, there is still great potential for BHPs with diagnostic environmental or taxonomic associations to be discovered. The significance of structural features that are preserved in the ancient sedimentary record are of particular interest. For instance, the 2-methylhopanoids are present throughout the sedimentary record, and show distinct patterns of variability in association with latitude (Knoll et al., 2007) and time (Brocks et al., 1999; Kuypers et al., 2004). Their overwhelming presence in many cultured cyanobacteria (Summons et al., 1999) provides compelling evidence that they may be diagnostic of cyanobacteria and, therefore, oxygenic photosynthesis in the sedimentary record. However, their absence from marine pelagic environments studied to date, and from cultured marine cyanobacteria raises an interesting paradox. Have modern marine cyanobacteria lost the ability to synthesize 2-methylhopanoids, or have 2-methylhopanoids always been associated with non-marine cyanobacteria in

terrestrial environments? This enigma, may be solved in part by a more thorough study of BHPs in the oceans, paying particular attention to overcoming biases imposed by restricted spatial and temporal sampling resolution. Ultimately, however, the significance of 2-methylhopnaoids, and other hopanoid structures, will be best constrained when we have demonstrated their biological role(s) in bacterial membranes in addition to their phylogenetic distribution among bacteria.

CONCLUSIONS

- In the ocean hopanoids are ubiquitous and can be structurally diverse. However, abundance and structural diversity appears to be much lower in marine pelagic environments than in terrestrial environments.
- Terrestrial organic matter appears to be a rich and structurally diverse source of hopanoids to marine sediments.
- Oligotrophic environments in the upper ocean can be a diverse source of hopanoids including some structures that may be produced by nitrogen-fixing cyanobacteria
- Hopanoids are abundant and structurally diverse in low oxygen environments. An isomer of BHT appears to be almost exclusively associated with these environments and has potential to be developed as a biomarker for low oxygen conditions.

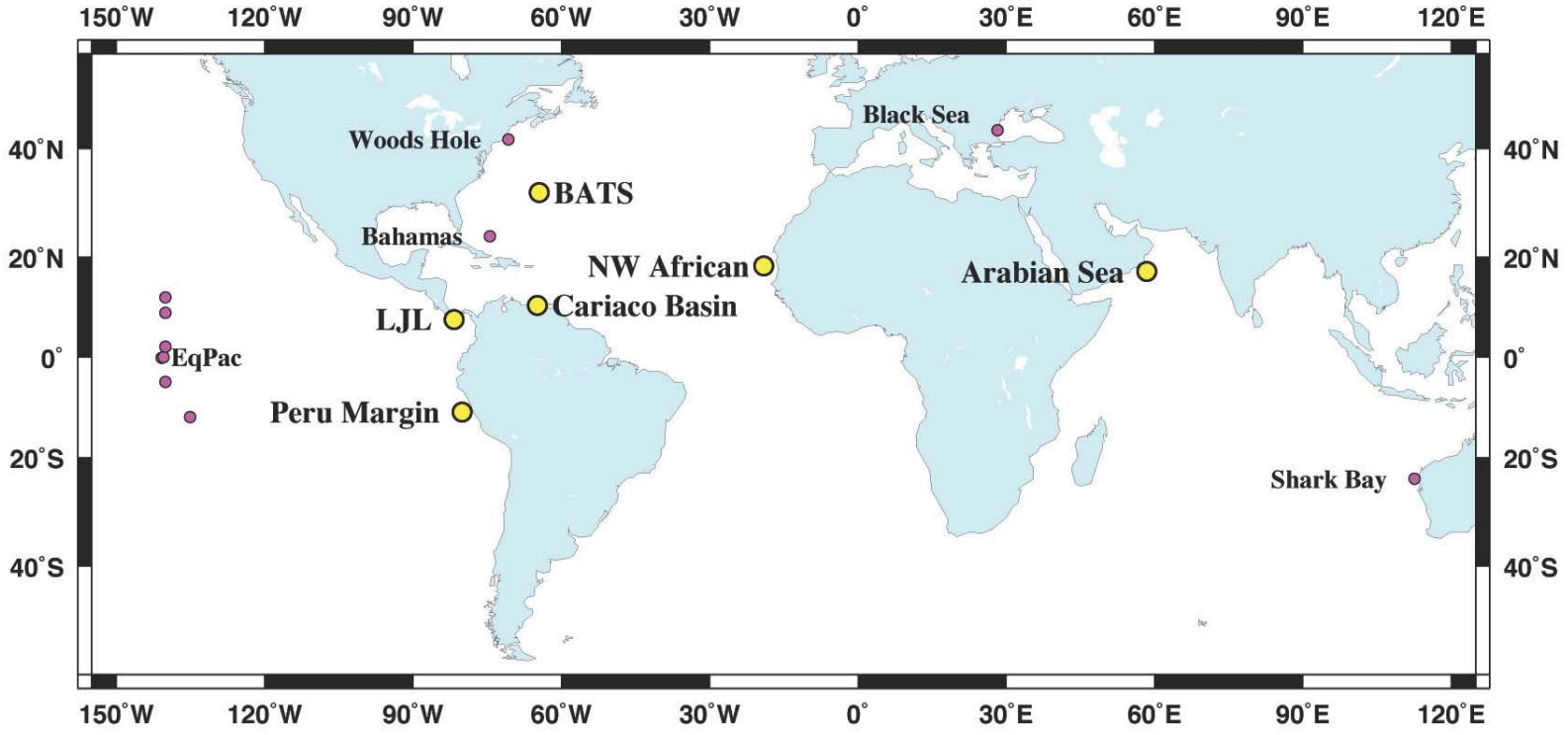

Figure 1: Global map showing the locations of environmental study sites.

Table 1: Sample locations (latitude and longitude) are listed along with relevant sampling and analytical details. Sample types include suspended particulate matter (SPM), sediment, and sinking particles collected by sediment trap.

Location	Latitude	Longitude	Type	Collection Method	Extraction Method	Standard for Quantification
N.W. Africa	18.3° N	19.0° W	SPM	McLane Filtration - 0.7 µm GFF	Bligh and Dyer Extraction	**External standard curve**: Acetylated steryl glucoside
			Sediment	Multi-core		
BATS	32.2° N	64.5° W	SPM - depth profile	McLane Filtration - 0.7 µm GFF	Bligh and Dyer Extraction	
			SPM - time series	McLane Filtration - 0.7 µm GFF	Soxhlet Extraction	
Panama	7.7° N	81.6° W	SPM	Peristaltic Pump - 0.3 µm GFF	Bligh and Dyer Extraction	**Internal standard**: Pregnone diacetate **External standard curve**: aetylated bacteriohopanetetrol
Peru Margin	11.0° S	80.0° W	SPM	McLane Filtration - 0.7 µm GFF	Soxhlet Extraction	
Arabian Sea	17.2° N	58.4° E	SPM	McLane Filtration - 0.7 µm GFF	Soxhlet Extraction	
			Sediment Trap	Indented rotating sphere trap		
			Sediment	Multi-core		
Cariaco Basin	9.8° N	7.5° W	SPM	McLane Filtration - 0.7 m GFF	Soxhlet Extraction	

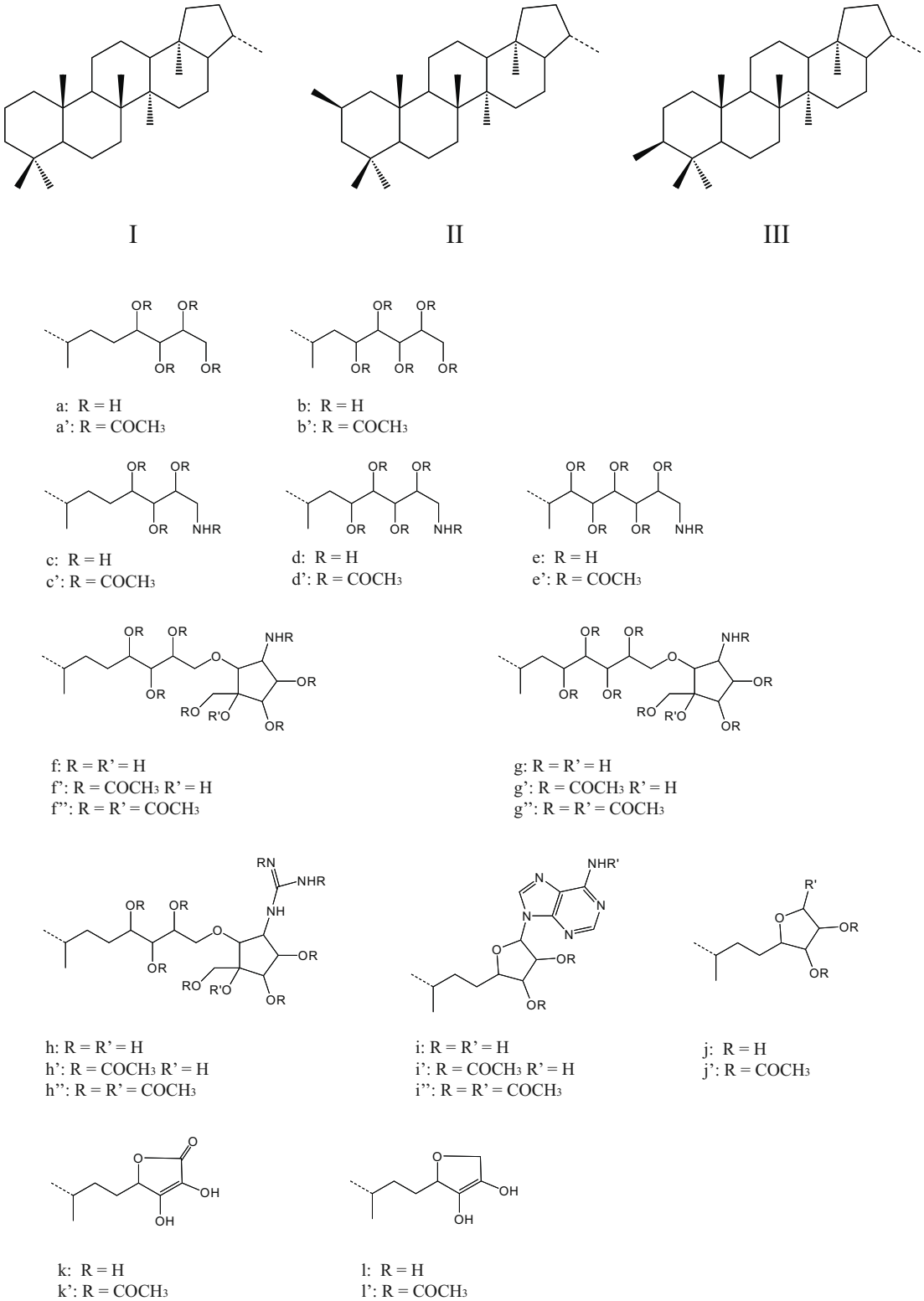

Figure 2: Schematic of the figures referred to in the text.

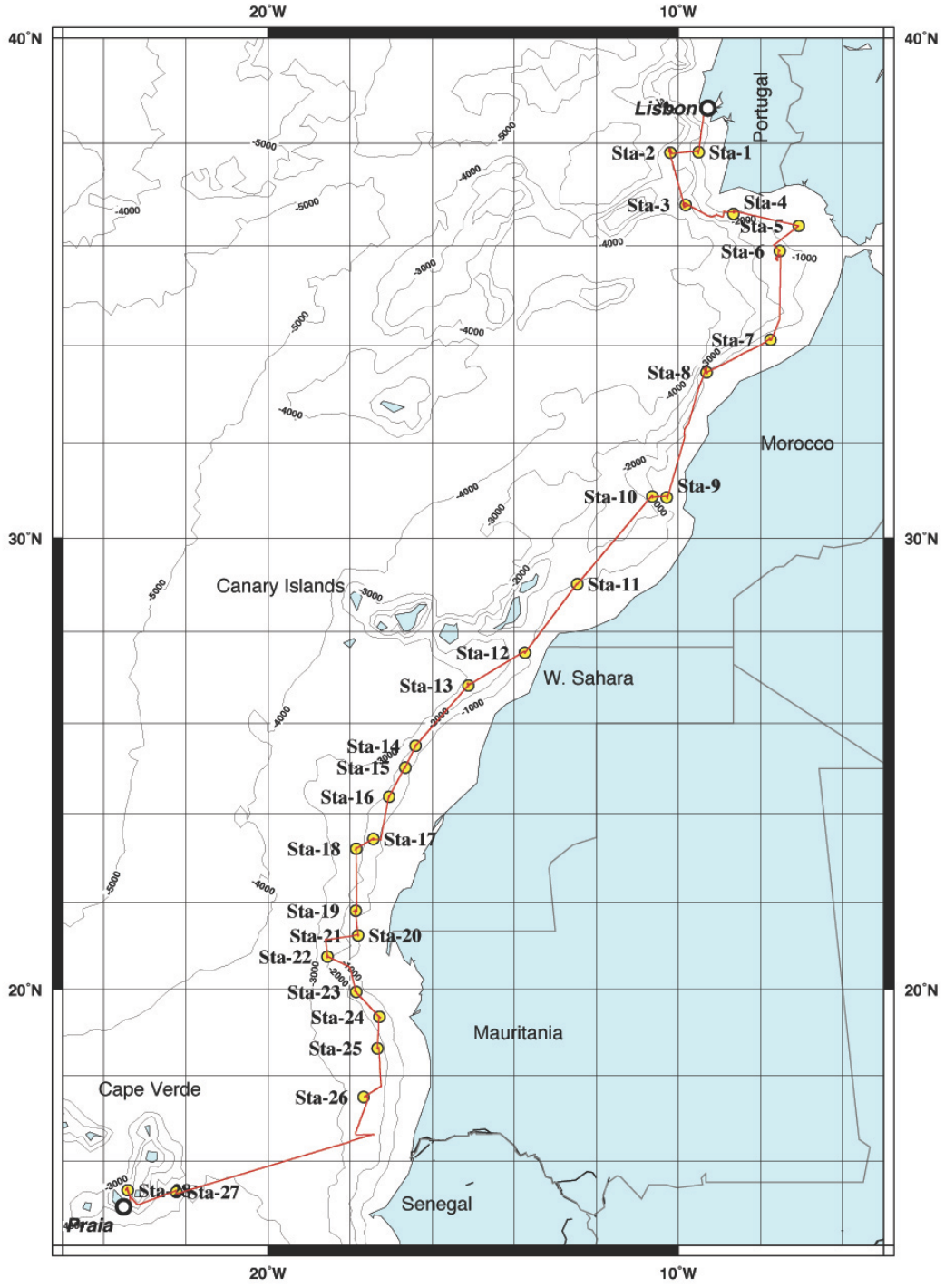

Figure 3: Map showing the locations of sampling stations on the CHEETA cruise. Suspended particulate matter (SPM) was filtered from the upper water column at stations 8, 13, 22, 26, and 27. Surficial sediments were collected by multicore at stations 8, 13, 22, 25, and 28.

Table 2: Relative abundance of BHPs and the concentration of BHT for all SPM samples taken along the CHEETA cruise. Refer to Figure 2 for BHP structures.

Station	Depth	BHP structure			BHT (Ia) concentration ng/l
		Ia	Ib	If	
8	80	100%			0.8
	660-720				0.0
13	10	89%		11%	1.6
	40	100%			1.1
	75	100%			0.1
	200	88%	12%		2.3
22	5	100%			5.3
	12	100%			3.4
	55	87%	13%		4.2
	200	87%	13%		5.1
26	5	100%			3.8
	26	100%			1.7
	80	76%	24%		6.2
27	60	73%	27%		1.5
	200	89%	11%		2.9

Table 3: Relative abundance and concentrations of BHPs detected in sediments from five coring stations corresponding to locations where SPM was sampled. The latitude of each station and relative abundance of BHPs are shown in 3A. Percent organic carbon (%OC) and BHP concentration relative to organic carbon mass are shown in 3B. The mass of each sample and BHP concentrations relative to sediment mass are shown in 3C. Refer to Figure 2 for BHP structures.

A

Station	Latitude	BHP relative abundance (% of total BHP)												
		Ia (I)	Ia (II)	IIa	IIIa	Ib	Ic	Id	Ii	If (I)	If (II)	Ih	Ik (I)	Ik (II)
8	33.53° N	69%	2.4%	0.7%	0.8%	1.7%	11%	1.3%	0.8%	3.1%	2.1%		4.3%	2.2%
13	26.87° N	75%		0.4%	0.6%	2.2%	3.7%			8.1%	1.6%	0.8%	6.3%	0.8%
22	20.74° N	80%	5.5%		0.4%	4.0%	2.5%		1.3%	1.0%			4.2%	1.1%
25	18.65° N	78%	7.3%	0.2%	0.6%	3.4%	2.3%		1.7%	0.9%	0.4%		4.3%	1.1%
28	15.31° N	77%	6.0%	1.0%	1.1%	3.5%	4.1%		3.4%				2.4%	1.6%

B

Station	%OC	BHP concentration ug/g OC												
		Ia (I)	Ia (II)	IIa	IIIa	Ib	Ic	Id	Ii	If (I)	If (II)	Ih	Ik (I)	Ik (II)
8	0.42	439	15	4	5	11	72	8	5	20	13		27	14
13	0.53	353		2	3	11	17			38	7	4	29	4
22	2.36	552	38		2	27	17		9	7			29	8
25	2.35	585	55	1	5	26	17		13	7	3		32	8
28	1.15	901	70	11	12	40	48		40				28	19

C

Station	Sed mass (g)	BHP concentration (ug/g Sediment)												
		Ia (I)	Ia (II)	IIa	IIIa	Ib	Ic	Id	Ii	If (I)	If (II)	Ih	Ik (I)	Ik (II)
8	10.09	1.83	0.06	0.02	0.02	0.05	0.30	0.03	0.02	0.08	0.06		0.11	0.06
13	10.04	1.87		0.01	0.02	0.06	0.09			0.20	0.04	0.02	0.16	0.02
22	10.05	12.99	0.88		0.06	0.64	0.41		0.21	0.17			0.69	0.18
25	10.09	13.74	1.30	0.03	0.11	0.60	0.40		0.30	0.17	0.07		0.76	0.19
28	10.14	10.38	0.81	0.13	0.14	0.47	0.56		0.46				0.32	0.22

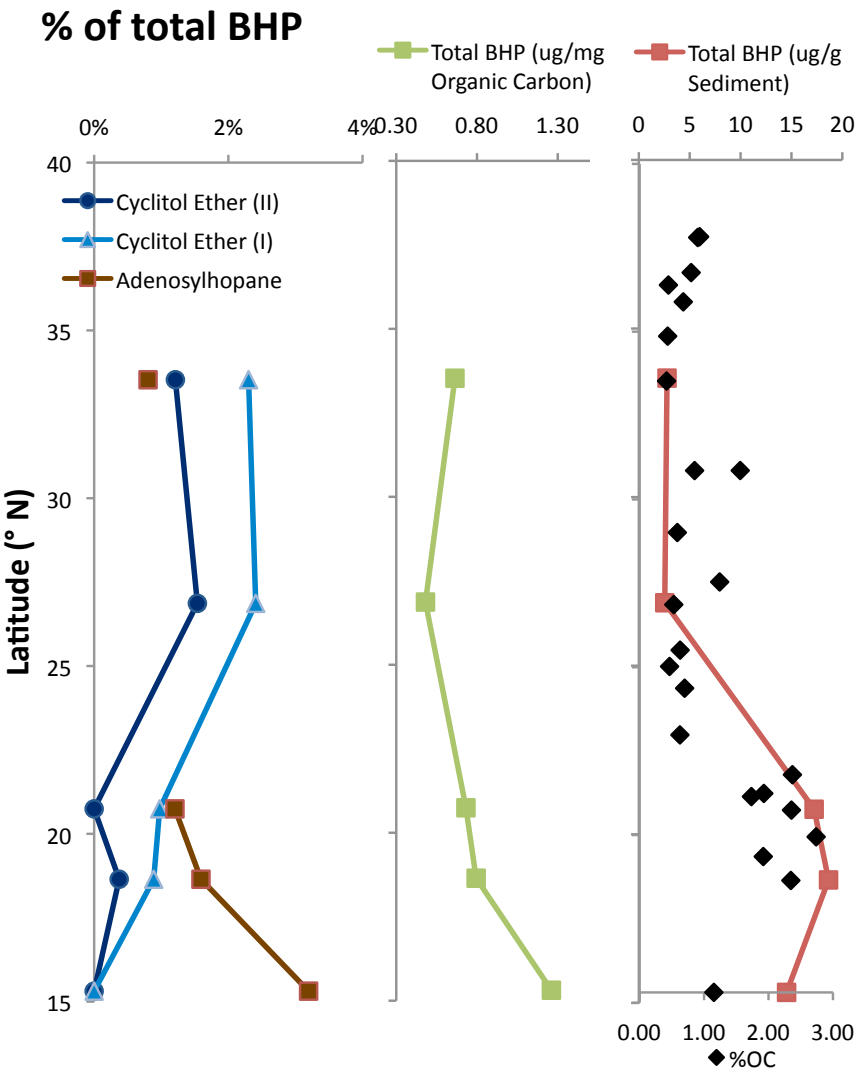

Figure 4: Latitudinal trends in the relative abundance (percent of total BHP) of BHT-CE isomers and adenosylhopane, percent organic carbon (%OC), and BHP concentration relative to sediment mass (ug/g sediment) and organic carbon mass (ug/g OC)

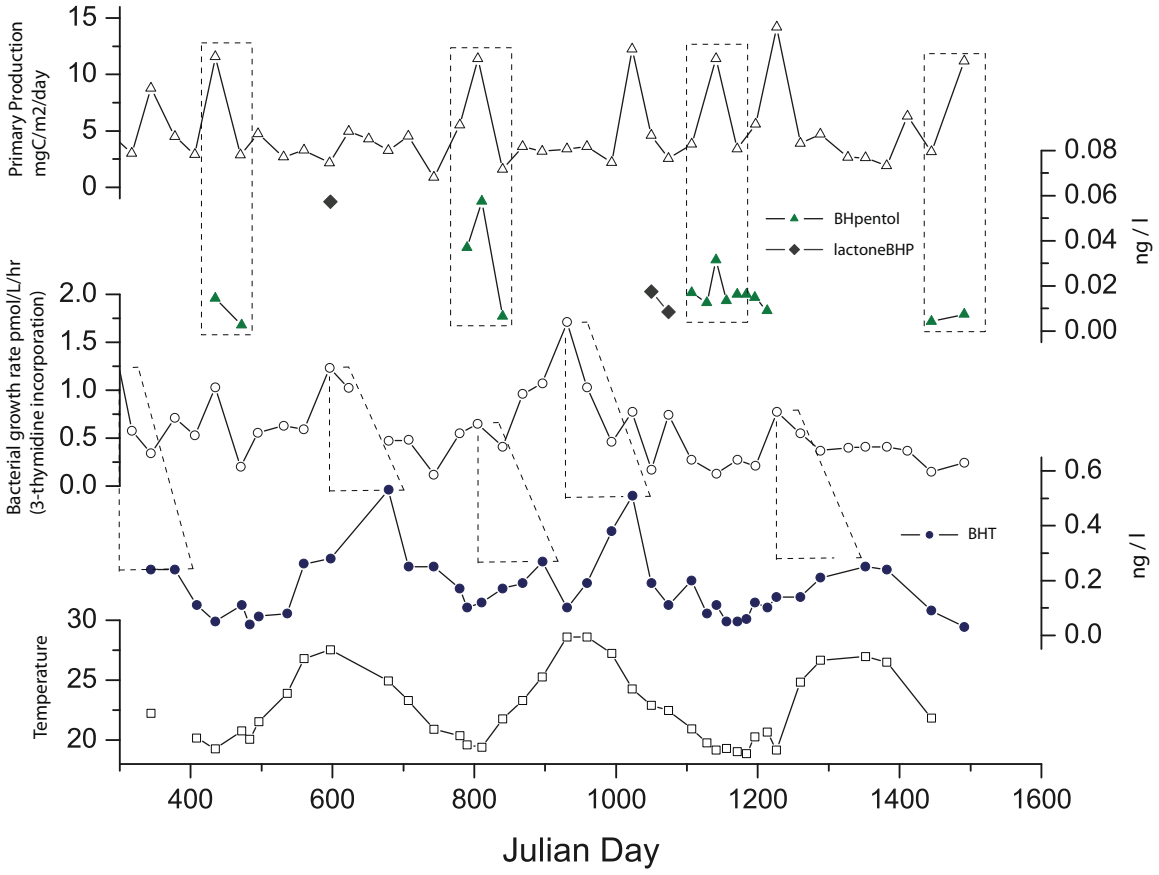

Figure 5: Bermuda Atlantic Time-Series Station data collected over three years (time is shown in days) from the upper 5 meters of the water column. Shown from top to bottom are: primary production estimates from light & dark ^{14}C bottle labeling experiments; Concentrations of BHpentol and lactoneBHP (ng/l) from suspended particulate matter (SPM); Heterotrophic bacterial growth rate determined by ^{3}H-thymidine uptake; concentration of BHT (ng/l); and temperature (°C).

Table 4: BHP abundance data is shown from a depth profile taken at BATS in November, 2008. Shown are the relative abundance of individual BHPs (of total BHP by peak area) and total composite BHPs (sum of compounds If (I), If (II), and Ih), and the number of BHP structures detected at each depth.

Depth	Relative abundance of BHPs (% of total BHP)									% Composite	# of BHPs
	Ia	Ib	Ic	Id	If (I)	If (II)	Ih	Ik	Il		
30	29%	4%	17%		0.2%	1%	4%	45%		5%	7
80	20%		23%	1%	2%	6%	7%	40%	1%	16%	8
140	16%		16%				2%	64%	2%	2%	5
200	55%		23%						23%		3
300	38%		26%	2%	1%	1%	1%	31%	1%	2%	8
500	5%		17%					55%	24%		4

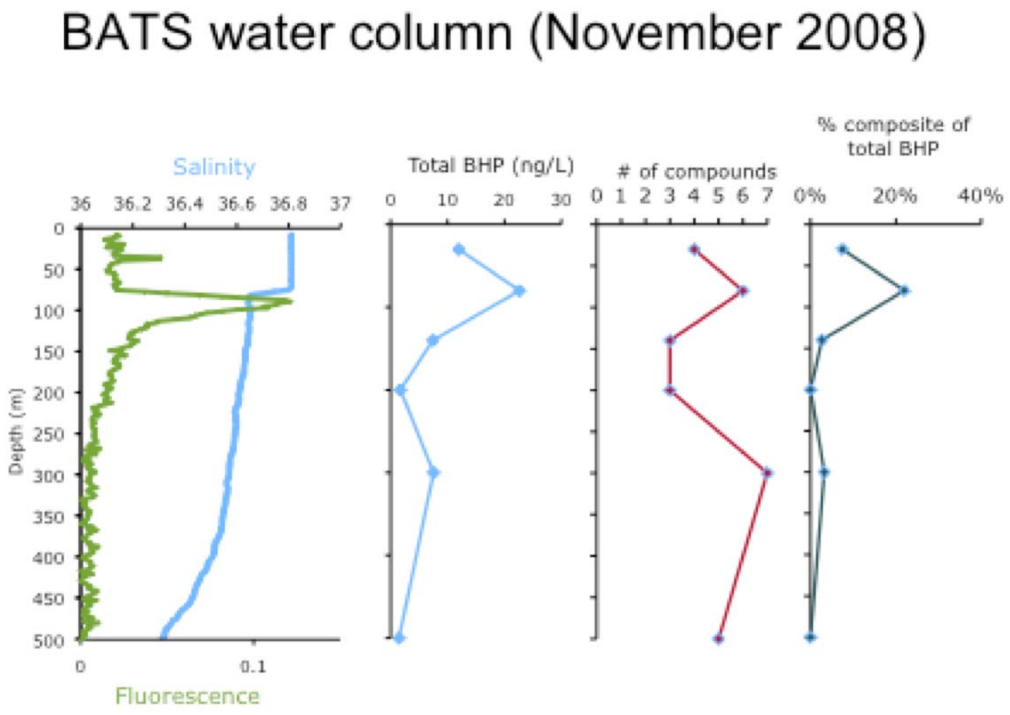

Figure 6: Depth profiles of salinity, fluorescence, total BHP concentration, number of BHP compounds detected at each depth, and the relative abundance of composite BHPs (percent of total BHP). Data collected at BATS in November, 2008.

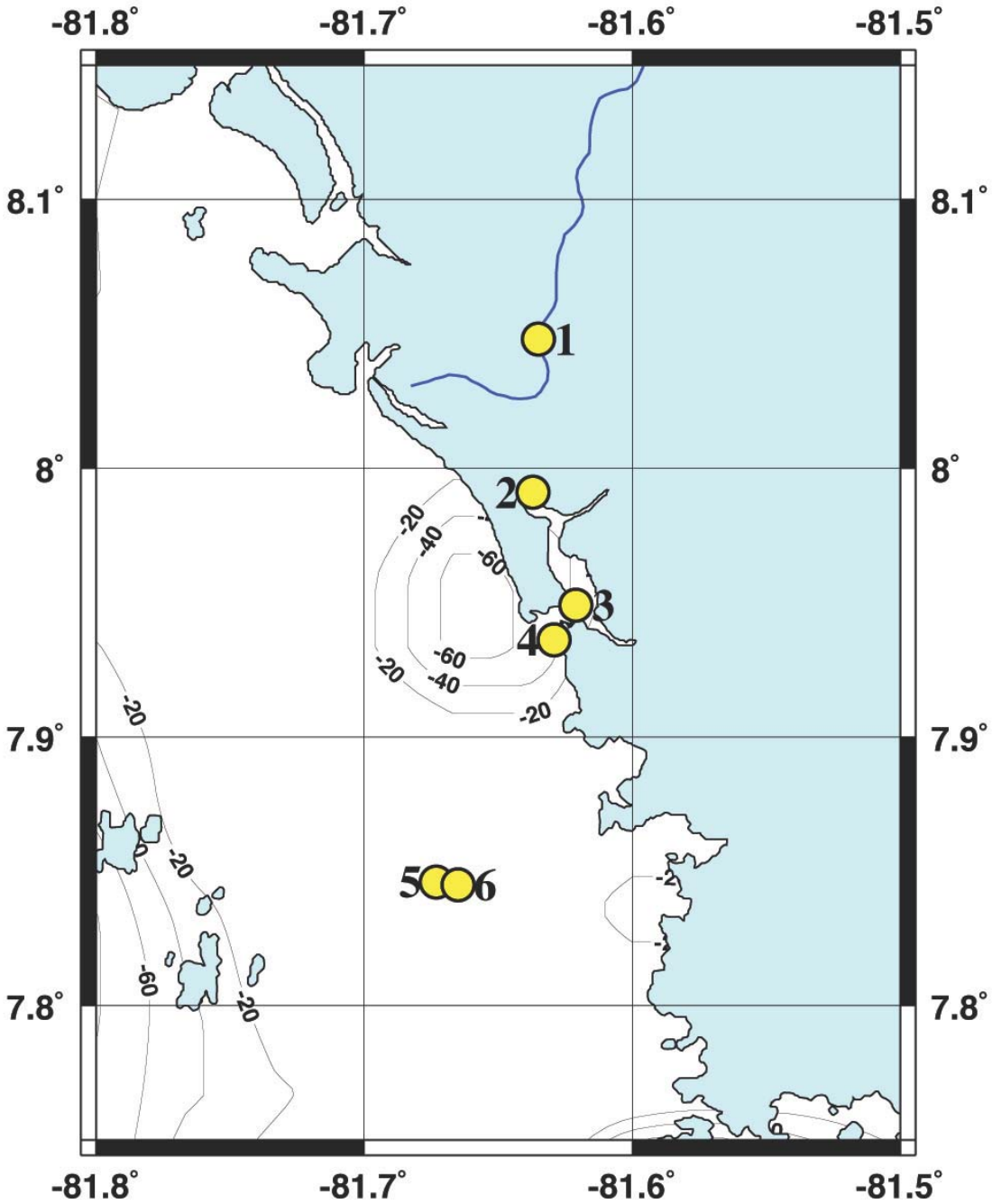

Figure 7: Map showing the locations of samples along a river to ocean transect. Samples were collected from station 1 (up river) to station 6 (blue water) while the tide was rising in January, 2009.

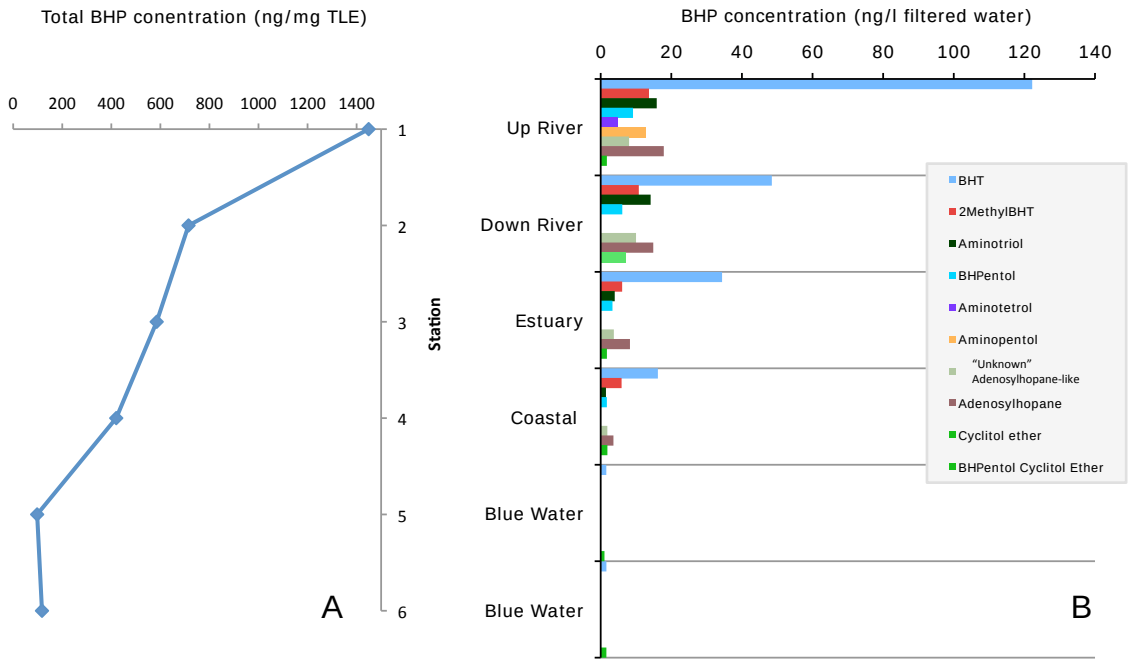

Figure 8: A: total BHP concentration reported relative to total lipid extract (TLE) mass (ng/mg TLE) in suspended particulate matter (SPM) in samples collected along a river to ocean transect along the Pacific coast of Panama, north of the Liquid Jungle Laboratory field station. B: Concentrations of individual BHPs detected in SPM reported relative to volume of water filtered (ng/l filtered water).

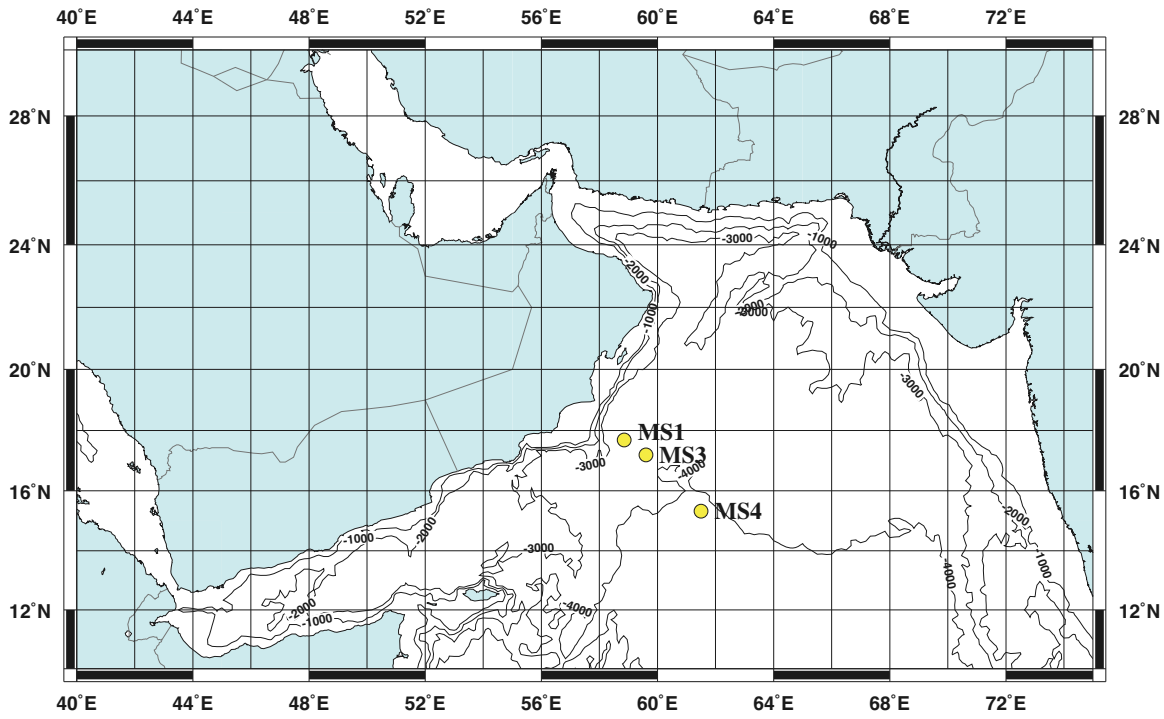

Figure 9: Map showing locations of stations MS1, MS3, and MS4 sampled during the US JGOFS Arabian Sea Process Study between 1994-1995

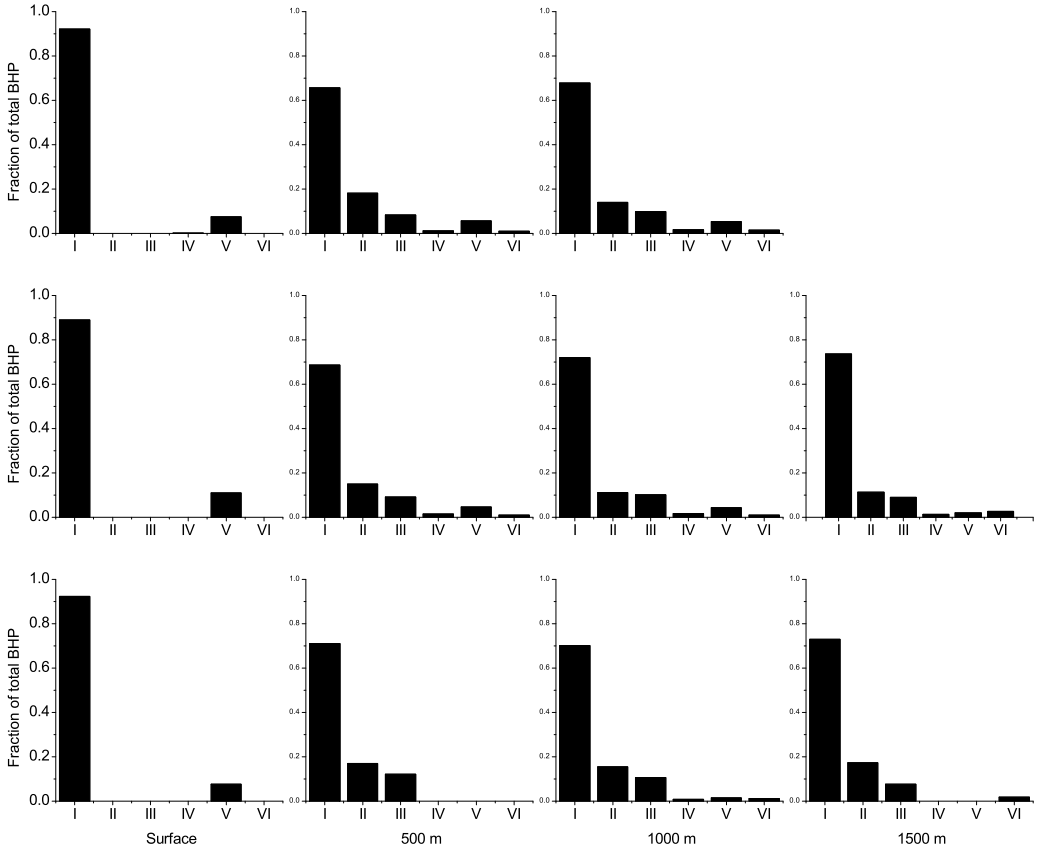

Figure 10. Relative abundance (fraction of total BHP) of BHP structures detected in suspended particulate matter (SPM) samples from stations MS1 (top), MS3 (middle), and MS4 (bottom) at surface (~5 m), 500 m, 1000 m and 1500 m. Samples from 500 m and below are within the oxygen minimum zone at all three sites.

I = BHT I (Ia')
II = BHT II (Ia')
III = BHpentol (Ib')
IV = Aminotriol (Ic')
V = lactoneBHP (Ik')
VI = Il'

See Figure 2 for structures

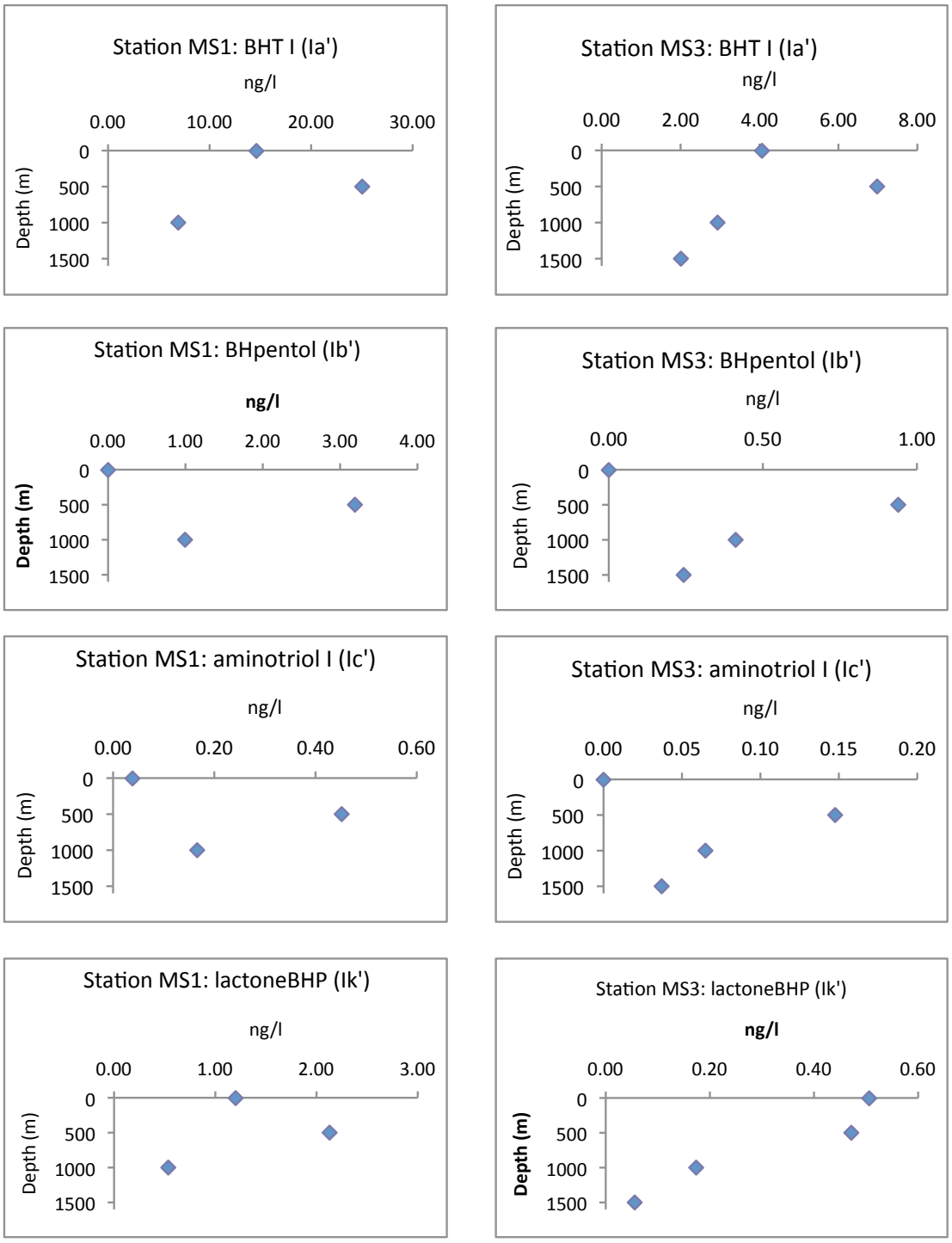

Figure 11: Depth profiles of the concentration of BHPs detected in suspended particulate matter (SPM) samples from stations MS1 and MS3. See Figure 2 for structures.

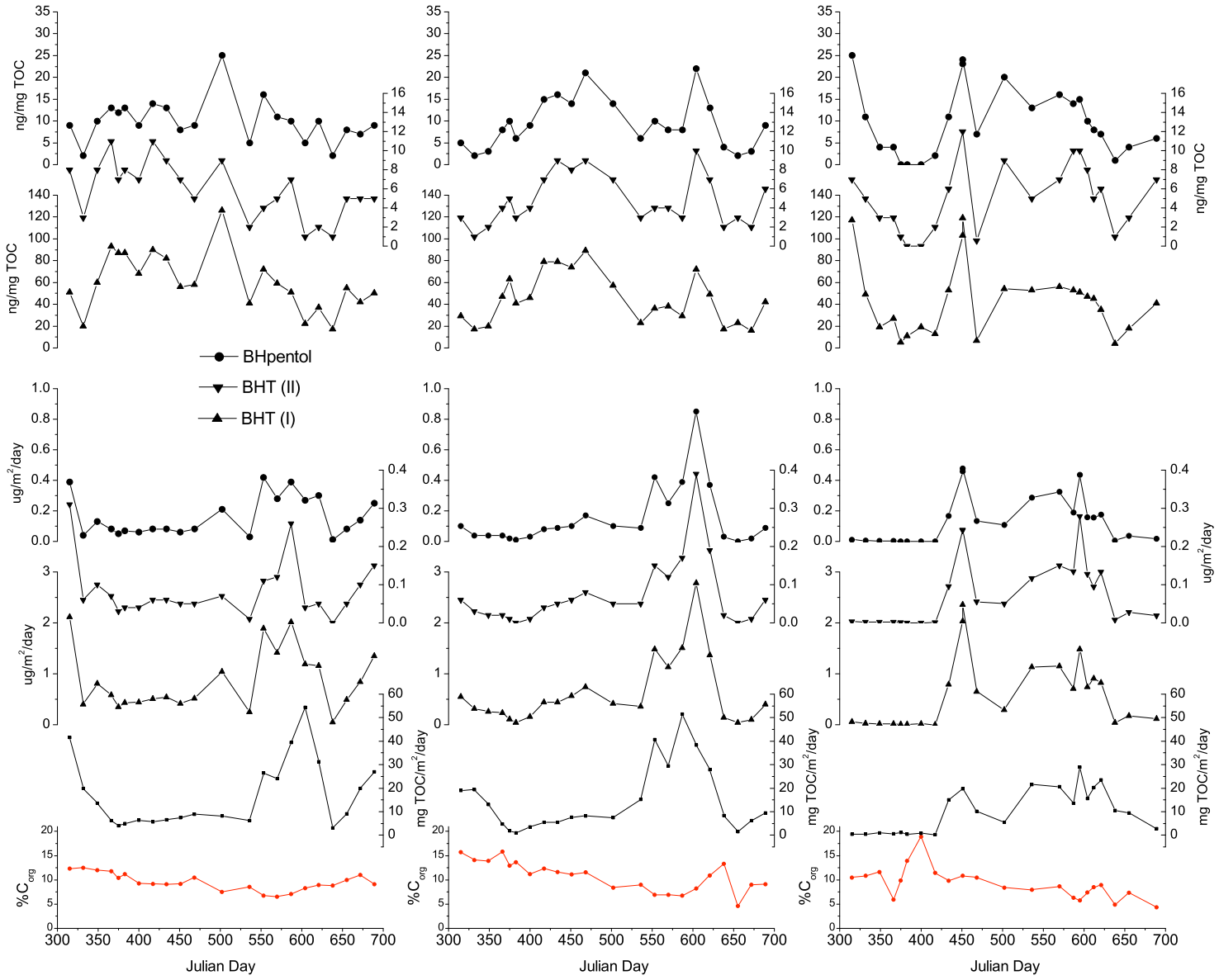
ng/mg TOC
ng/mg TOC
BHpentol
BHT (II)
BHT (I)
ug/m2/day
ug/m2/day
mg TOC/m2/day
%Corg
Julian Day

Figure 12: Time series data from 500 m sediment traps moored at stations MS1 (left), MS3 (middle), and MS4 (right) collected over a one-year period (1994-1995, time shown in Julian day). Shown for each station are (top to bottom): individual BHP concentrations normalized to total organic carbon mass (ng/mg TOC), individual BHP fluxes (ug/m^2/day), total organic carbon (TOC) flux (mg/m^2/day), and percent organic carbon (%OC). A figure legend on the center-left indicates the symbols for the three BHP compounds detected in the sediment trap samples.

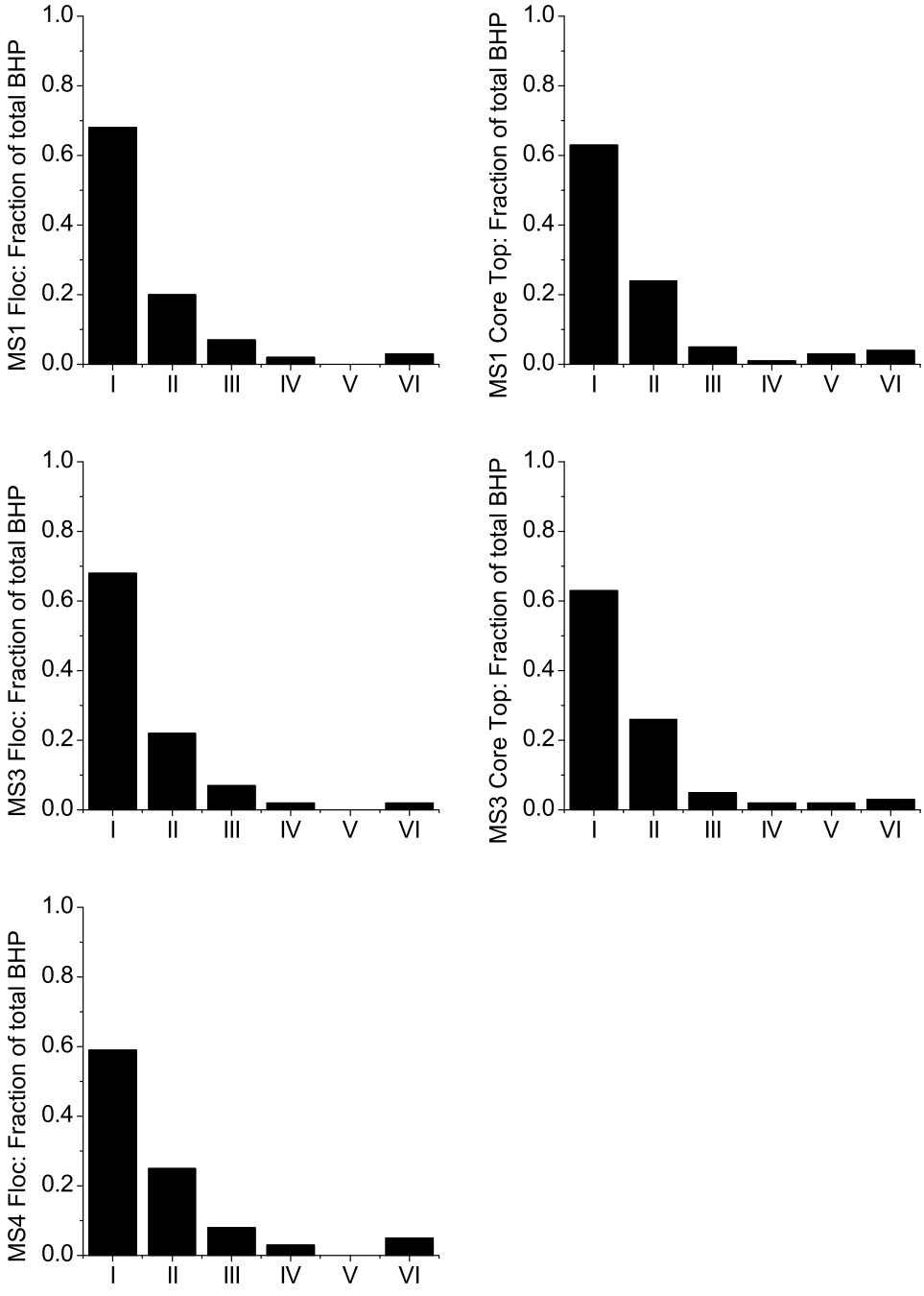
MS1 Floc: Fraction of total BHP
MS1 Core Top: Fraction of total BHP
MS3 Floc: Fraction of total BHP
MS3 Core Top: Fraction of total BHP
MS4 Floc: Fraction of total BHP
1.0
0.8
0.6
0.4
0.2
0.0
I
II
III
IV
V
VI

Figure 13: Relative abundance (fraction of total BHP) of BHP structures detected in core top and floc sediment samples from stations MS1 (top), MS3 (middle), and MS4 (bottom). Floc refers to the unsettled layer of sediment just above the sediment-water interface, and core top refers to the upper 0.5 cm of compacted sediment just below the sediment-water interface.

I = BHT I (Ia')
II = BHT II (Ia')
III = BHpentol (Ib')
IV = Aminotriol (Ic')
V = lactoneBHP (Ik')
VI = Il'
See figure 1 for structures

Table 5: Concentration of BHP compounds detected in core top (0-0.5 cm) and floc (unsettled sediment overlying the core top) from stations MS1, MS3, and MS4. Concentrations for all samples are reported relative to dry sediment mass (µg/g sediment. Additionally concentrations for core top samples from MS1 and MS3 are reported relative to organic carbon mass (ng/mg OC). Refer to Figure 2 for BHP structures.

Station	Depth below sea floor (cm)	ug/g Sediment						ng/mg OC					
		Ia' (BHT I)	Ia' (BHT II)	Ib' (BHpentol)	Ic'	Ik'	Il'	Ia' (BHT I)	Ia' (BHT II)	Ib' (BHpentol)	Ic'	Ik'	Il'
MS1	0-0.5	1.35	0.51	0.10	0.03	0.07	0.08	8.46	3.18	0.64	0.19	0.42	0.50
	FLOC	3.77	1.08	0.42	0.10	0.00	0.17						
MS3	0-0.5	1.09	0.44	0.08	0.03	0.03	0.05	19.89	8.09	1.53	0.48	0.55	0.98
	FLOC	1.55	0.50	0.15	0.03	0.00	0.06						
MS4	FLOC	0.11	0.05	0.01	0.01	0.00	0.01						

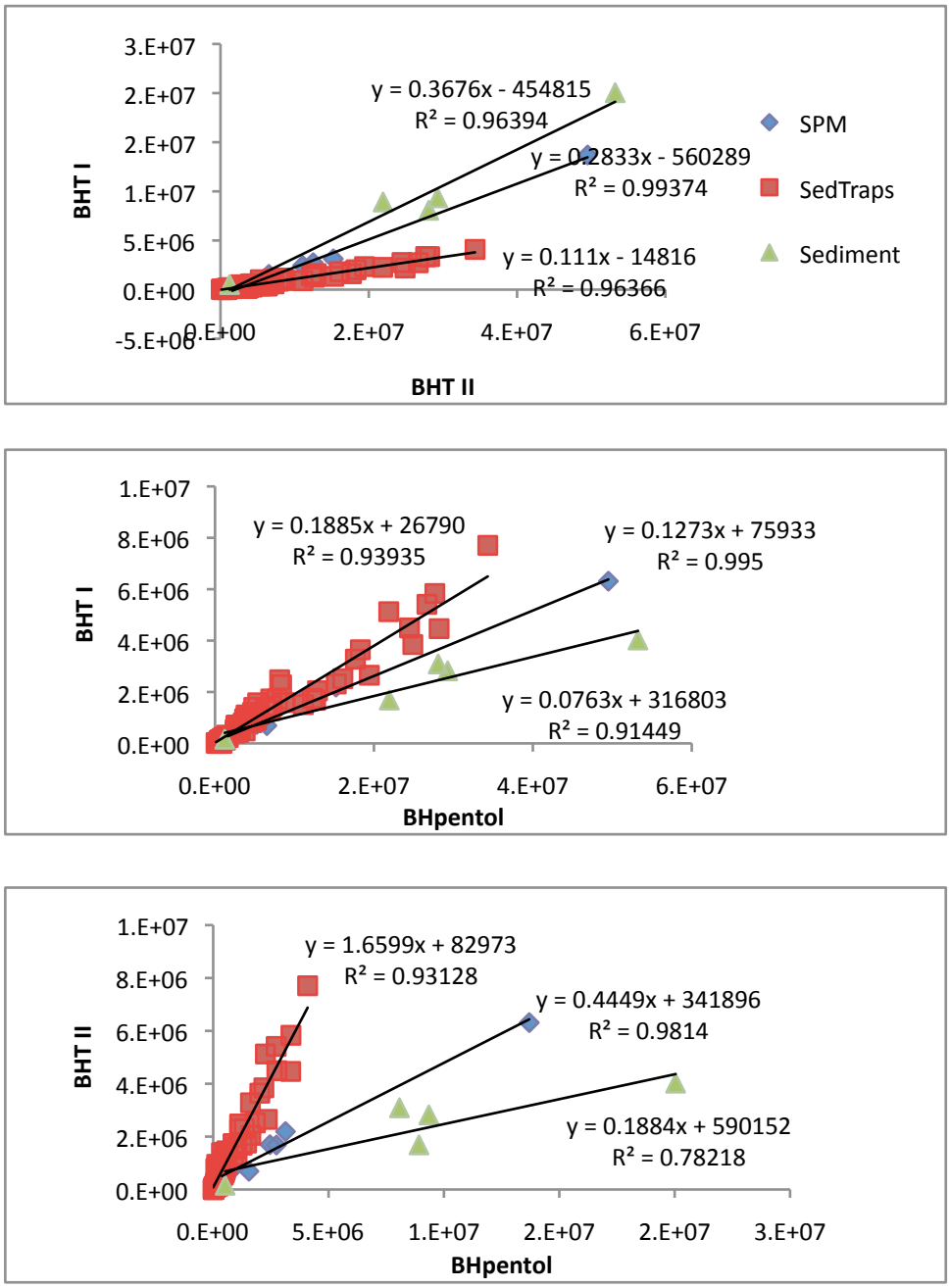

Figure 14: Property-property plots of BHT I vs. BHT II, BHT I vs. BHpentol, and BHT II vs. BHpentol are shown for all suspended particulate matter (SPM; blue diamond), sediment trap (red square), and sediment (olive triangle) samples. The r-squared values and linear equations for trend lines (generated with Microsoft Excel) are shown for each data set on each axis.

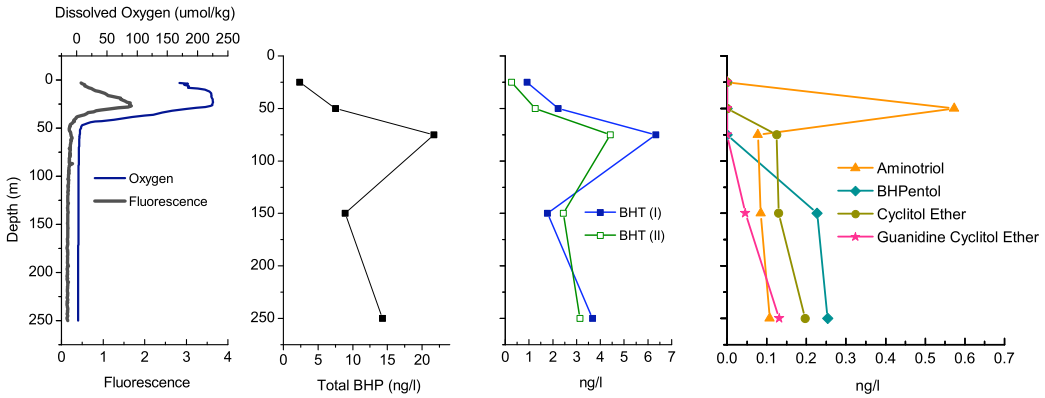

Figure 15: Depth profiles of dissolved oxygen, fluorescence, total BHP, and individual BHPs detected in samples taken from station ISP-1 along the Peru Margin.

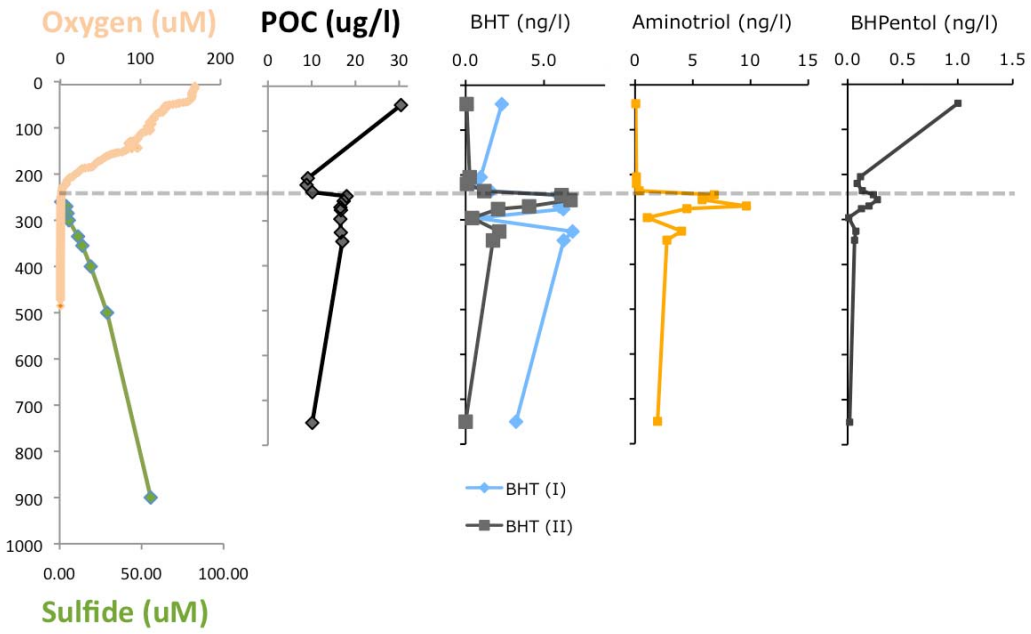

Figure 16: Depth profiles of BHP concentration (relative to volume of water filtered) in suspended particulate matter (SPM), particulate organic carbon, dissolved oxygen, sulfide, and fluorescence from a depth profile taken in the eastern Cariaco Basin.

Table 6: presence/absence of BHPs detected at all sample locations. Samples from each location are pooled into suspended particulate matter (SPM), sediment, or sediment trap samples. SPM samples are further subdivided into photic zone, oxygen minimum zone (OMZ), euxinic or river, estuary, coastal, and offshore.

		Structures from Figure 1													
		Ia'	IIa'	IIIa'	Ib'	Ic'	Id'	Ie'	If'	Ig'	Ih'	Ii'	Ij'	Ik'	Il'
N.W Africa	SPM	O			O				O						
	Sediment	O	O	O	O	O	O					O		O	
BATS	SPM	O			O	O	O		O		O			O	O
Panama	River	O	O		O	O	O	O	O			O	O		
	Estuary	O	O		O	O			O			O	O		
	Coastal	O	O		O	O			O			O	O		
	Offshore	O													
Arabian Sea	SPM photic	O				O								O	
	SPM OMZ	O			O	O								O	O
	Sed Trap	O			O										
	Sediment	O			O	O								O	O
Peru Margin	SPM photic	O													
	SPM OMZ	O			O	O			O		O				
Cariaco Basin	SPM photic	O			O										
	SPM euxinic	O			O	O									

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CHAPTER 7 – DIRECTIONS FOR FUTURE RESEARCH

NITROGEN CYCLING IN SUBTERRANEAN ESTUARIES

In Chapter 2 I demonstrated the presence of anammox bacteria in a subterranean estuary through the detection of ladderanes. This result raises the possibility that anammox may be an important component of the nitrogen cycle subterranean estuarine environments, which could have consequences for the global nitrogen budget. The question we must answer now is: how important is anammox as a pathway for fixed nitrogen removal within subterranean estuaries? This could be addressed through nitrogen isotope labeling experiments within the subterranean estuary. This approach has been applied successfully in other environments to study the rate of anammox and its importance relative to other N-removal pathways (Dalsgaard et al., 2003; Risgaard-Petersen et al., 2003).

HOPANOIDS: SOURCE AND FUNCTION

My survey of hopanoids in marine environments suggests that hopanoids are ubiquitous in the oceans and that they have the potential to inform us of environmental and biological processes in ancient marine environments. However, several challenges to the application of hopanoids as biomarkers in the sedimentary record have emerged from my observations. The environmental sources of hopanoids to marine sediments are not well-

constrained and we presently lack the ability to clearly distinguish between hopanoids derived from terrestrial, marine pelagic, and marine benthic sedimentary sources. In a few instances, certain structures appear to be uniquely associated with specific environments, and the presence and relative abundance of these compounds may provide one means for estimating the contribution of various inputs. For instance, adenosylhopane is ubiquitous and abundant in terrestrial environments has been proposed as a marker for terrestrial organic matter (Cooke et al., 2008; Cooke et al., 2008). In support of that, I found that adenosylhopane appears to be absent from marine pelagic environments (Chapter 6). In addition, the detection of a novel isomer of bacteriohopanetetrol (BHT II) that appears to be associated exclusively with low-oxygen environments holds promise for a novel biomarker for anoxia (Chapter 6). However, many of the structures that I observed do not yet appear to have unique environmental associations, potentially rendering their sources ambiguous in marine sediments. If the carbon and hydrogen isotopic composition of hopanoids from terrestrial, marine pelagic, and marine benthic environments were to be elucidated then isotopic composition of hopanoids in marine sediments may provide one means for distinguishing source.

The biological sources of hopanoids in the oceans are equally enigmatic. From my survey of marine cyanobacteria, it appears that

hopanoids are exclusively associated with nitrogen-fixing marine cyanobacteria (Chapter 4). But this could be an artifact of the small number of marine strains that have been surveyed to date. Furthermore, hopanoid production has not been explored in cultured marine organisms other than cyanobacteria. Indeed, studies of the ocean metagenome indicate that cyanobacterial hopanoid producers may be relatively rare compared with other phylogenetic groups (Pearson and Rusch, 2009). However, given that cultured bacteria likely represent less than 1% of the natural diversity of microorganisms (Hugenholtz and Pace, 1996), it does not appear that we are presently in a position to begin to comprehensively address the phylogenetic diversity of hopanoid production through culture-based studies. This presents a fundamental challenge if we wish to understand the biological sources of individual hopanoid structures in marine environments. Ultimately, what is needed is a culture-independent means to assess the relationship between hopanoid structure and taxonomy. But, how can we link structure to biological source in the environment? Rapid flow cytometric sorting of cells, combined with immunological labeling of genes associated with hopanoid biosynthesis might present one possible means to bridge the gap between source and structure. If hopanoid-producing cells can be selectively labeled, and isolated from natural samples by rapid flow cytometric sorting in large

enough quantities for lipid analyses, then it might be possible to begin to directly link structure to phylogeny in the environment.

Understanding the function of hopanoids may represent the most challenging and, potentially, the most informative facet of hopanoid research. Presumably if we understood the relationship between hopanoid structure and function, we could constrain their potential biological sources and the environments in which they might occur. Existing hypotheses for the role of hopanoids are based on their presumed role in mediating membrane permeability and fluidity. However, we presently lack a clear understanding of how hopanoids behave in membranes at the molecular level; this gap in our understanding has been a major impediment to developing and testing hypotheses surrounding the physiological role of hopanoids in bacteria. The hypothesis that hopanoids form lipid rafts in bacterial membranes (Chapter 5) has not been tested, despite the fact that many striking parallels between sterols and hopanoids have been observed; one of the key properties of sterols in membranes is to induce the formation of liquid-ordered domains, which is thought to be the molecular basis for lipid rafts. The discovery of lipid rafts in bacteria, and the identification of the molecular basis for bacterial rafts would change our understanding of bacterial physiology and the evolution of membrane architecture.

Furthermore, comparative analysis between the membrane physiology of hopanoid-producing bacteria and eukaryotes could provide insight into the evolution of membrane-associated biochemical pathways. One example that is worth investigating is a recent observation surrounding the origin of the Hedgehog pathway, which is an essential component of multicellularity: the Ptc (Patched) protein that is central to the Hedgehog pathway, is closely related (by sequence similarity) to a subfamily of SND transporters in bacteria that very likely target hopanoids (Hausmann et al., 2009). The observation that bacteria and eukaryotes have homologous proteins for the recognition and transport of sterols/hopanoids, and that in eukaryotes this protein is associated with the basis for multicellularity suggests that hopanoids may have a trafficking role in bacteria, similar to that of sterols in eukaryotes. If the molecular basis for multicellularity in eukaryotes was adopted from a bacterial pathway, our understanding of evolution would be altered.

Compared with eukaryotes, the physiology of bacterial membranes has not been well studied. Many of the existing tools and models developed for investigating membrane physiology in eukaryotes have not been applied to bacteria. Studying the biophysics of bacterial lipids in model membranes would provide one avenue for developing an understanding of the static and dynamic properties of bacterial membranes. Ultimately, understanding the

physiology of bacterial lipid biomarkers will further our ability to interpret the geologic record of molecular fossils, and may in some cases allow us to date the antiquity of physiological innovations over the course of Earth history.

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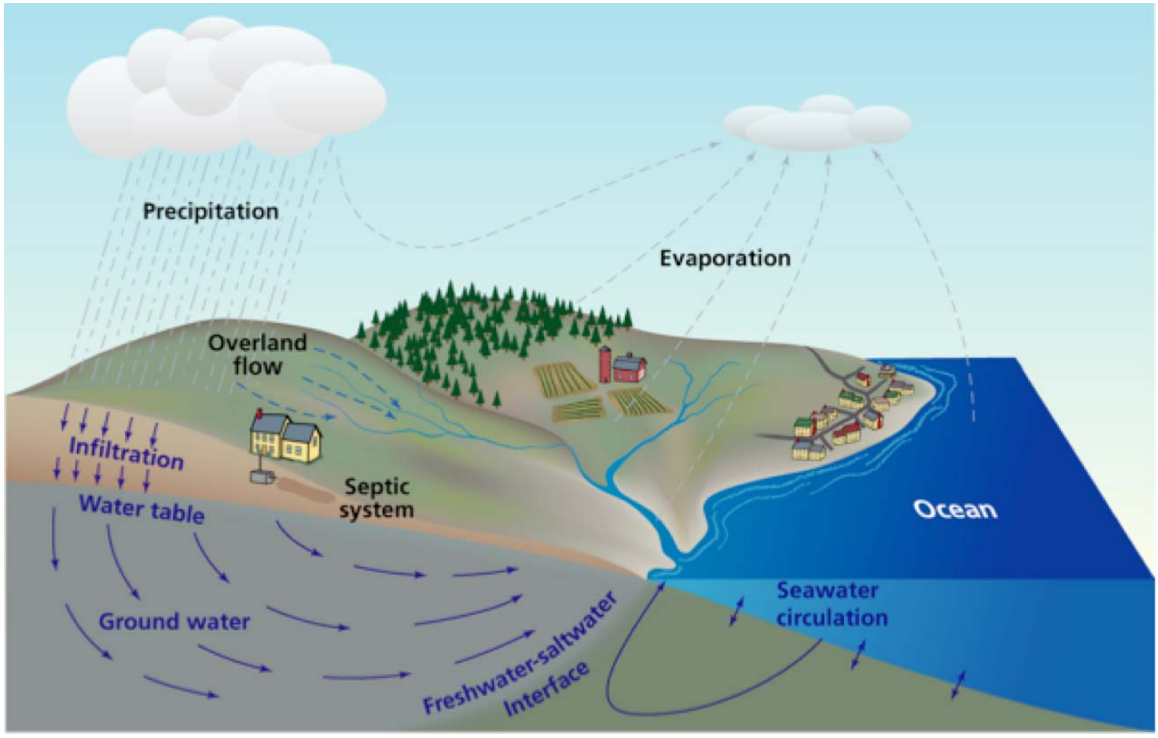
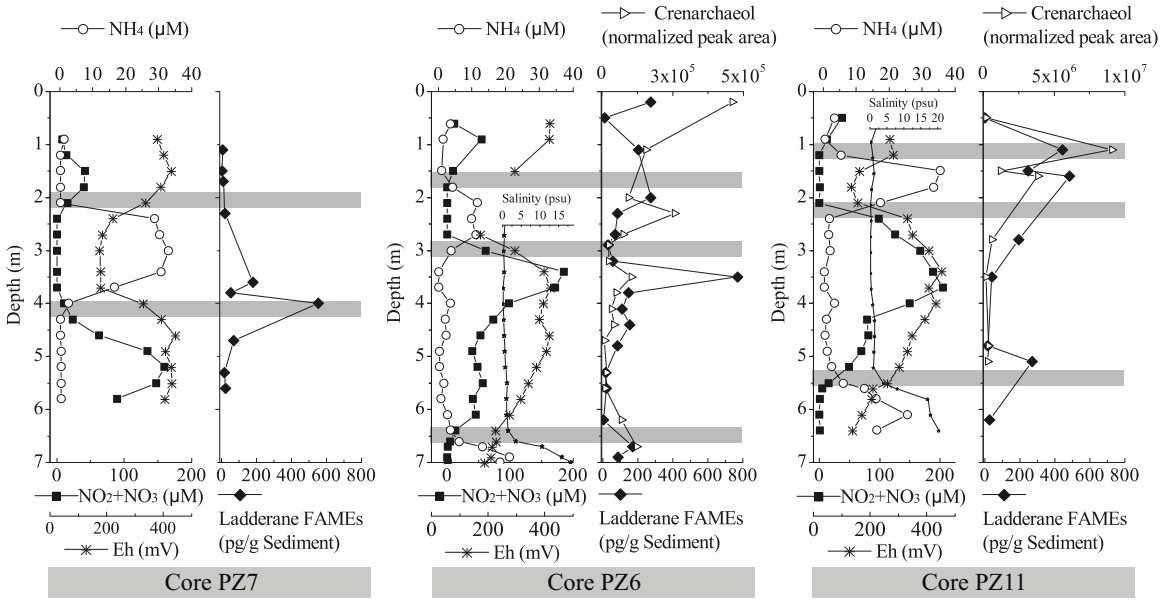
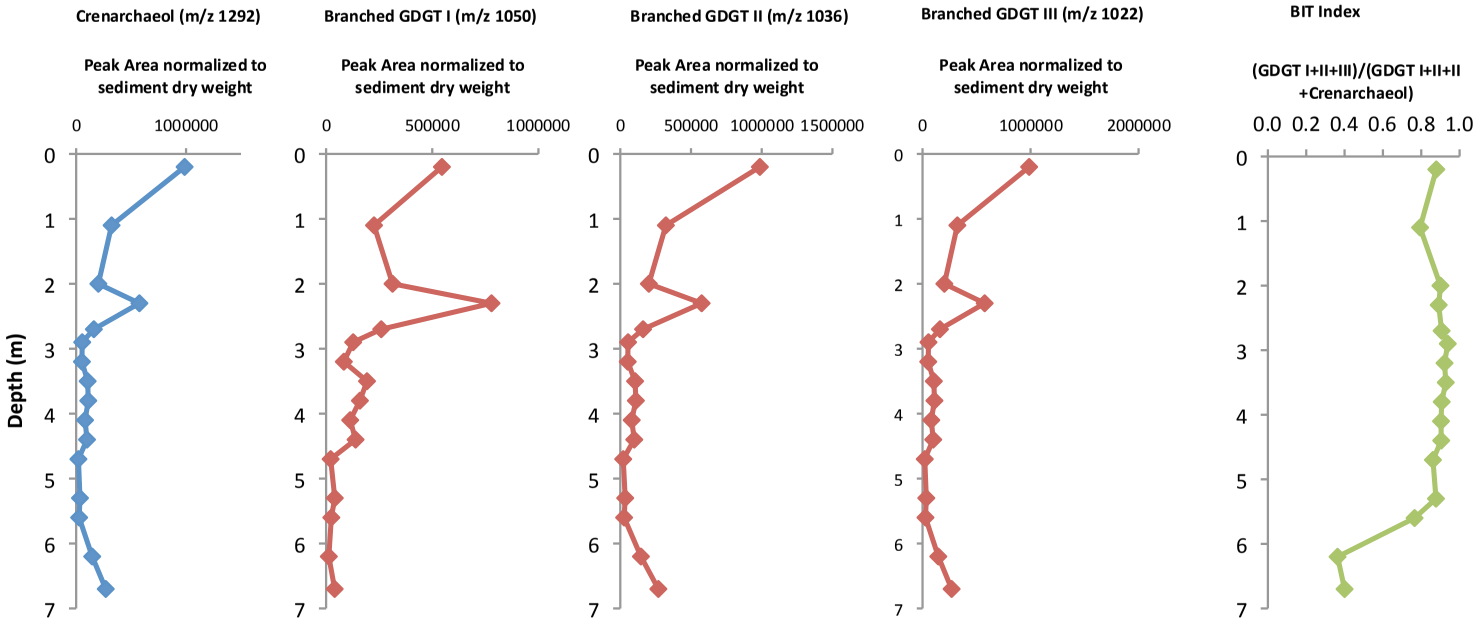
APPENDICES



A2-1: Cartoon depicting the subterranean estuary and its surrounding environment (WHOI Graphics Department).

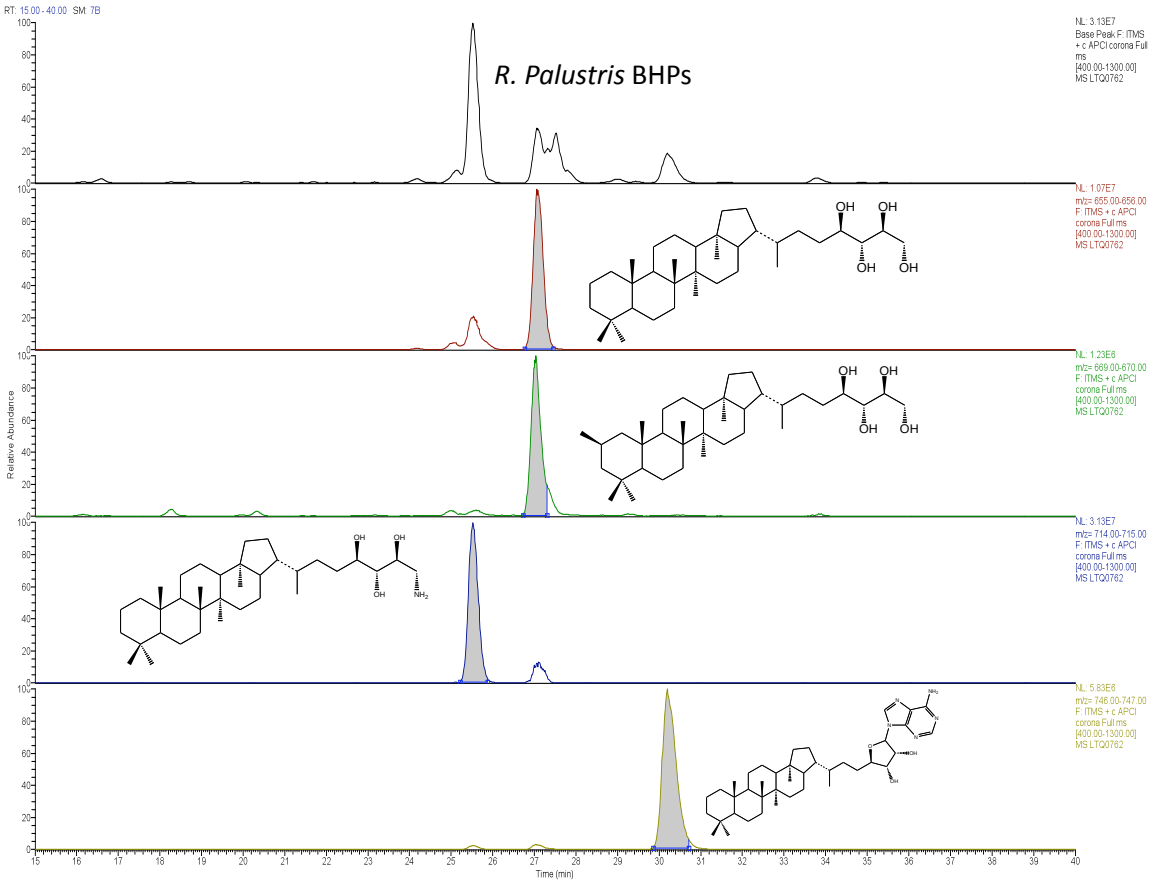


A2-2: Pore water and lipid data from the three cores analyzed in this study (PZ7, PZ6, PZ11). Data for each core station is plotted on two axes; pore water data is shown on the left-hand axis and lipid concentrations are plotted on the right-hand axis. Pore water data includes ammonia, and nitrate+nitrite concentrations, and Eh, which is a measure of redox potential. For sites PZ6 and PZ11 salinity is plotted on a third axis that is inset within the pore water data axis. Ladderane core lipid concentration is reported for all three cores. Crenarchaeol abundance is reported for PZ6 and PZ11 as peak area normalized to sediment mass.

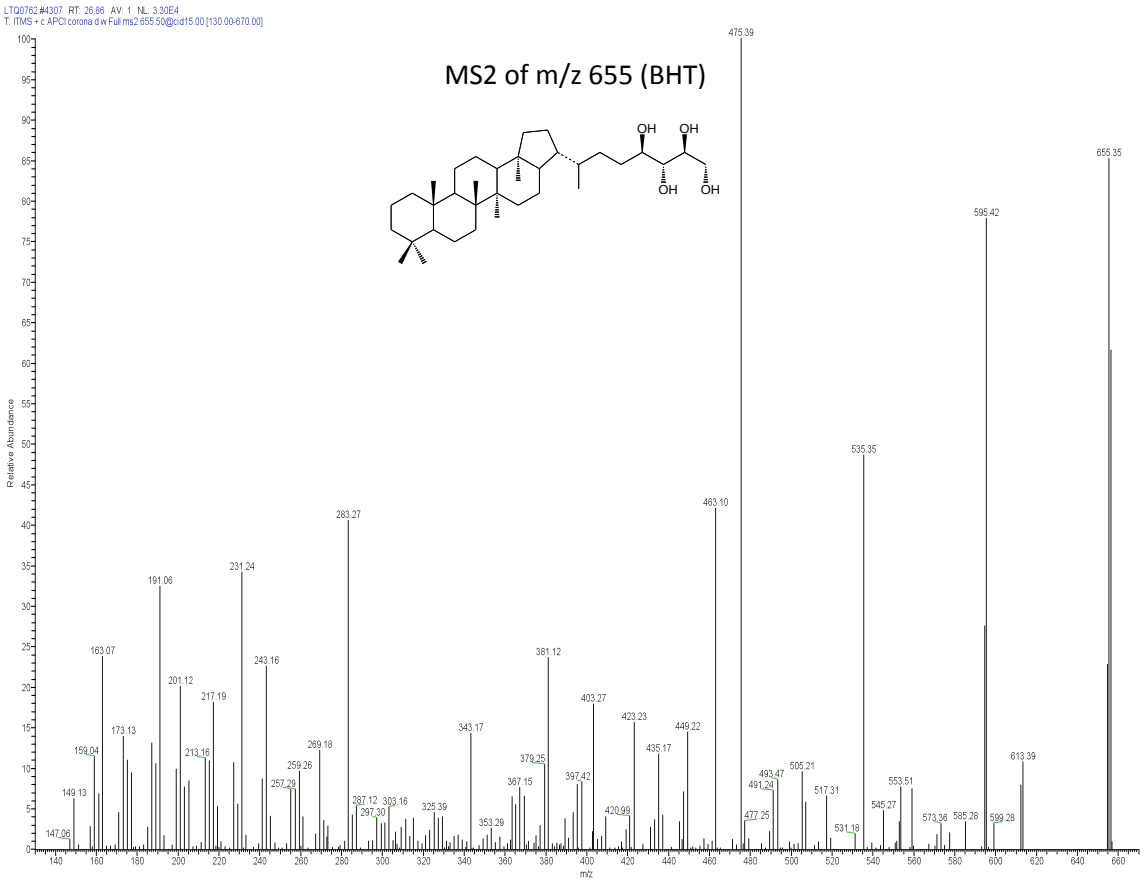


For structures of branched GDGTs see:
Weijers et al. Environmental controls on bacterial tetraether membrane lipid distribution in soils. Geochimica et Cosmochimica Acta (2007)
Damste et al. Newly discovered non-isoprenoid glycerol dialkyl glycerol tetraether lipids in sediments. Chem Commun (2000) (17) pp. 1683-1684
For information on BIT index see:
Hopmans et al. A novel proxy for terrestrial organic matter in sediments based on branched and isoprenoid tetraether lipids. Earth and Planetary Science Letters (2004) vol. 224 (1-2) pp. 107-116

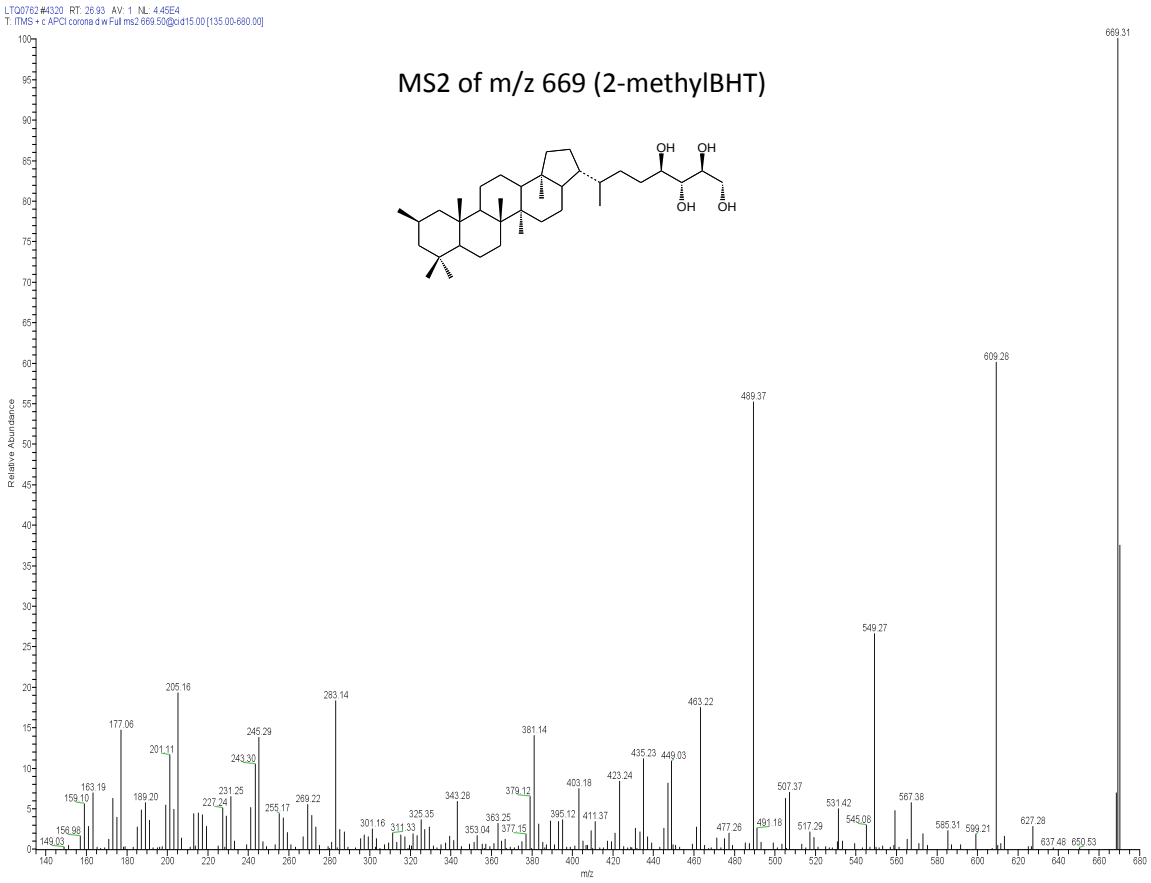
A2-3: Depth profiles of crenarchaeol and branched GDGTs and BIT index at site PZ6.



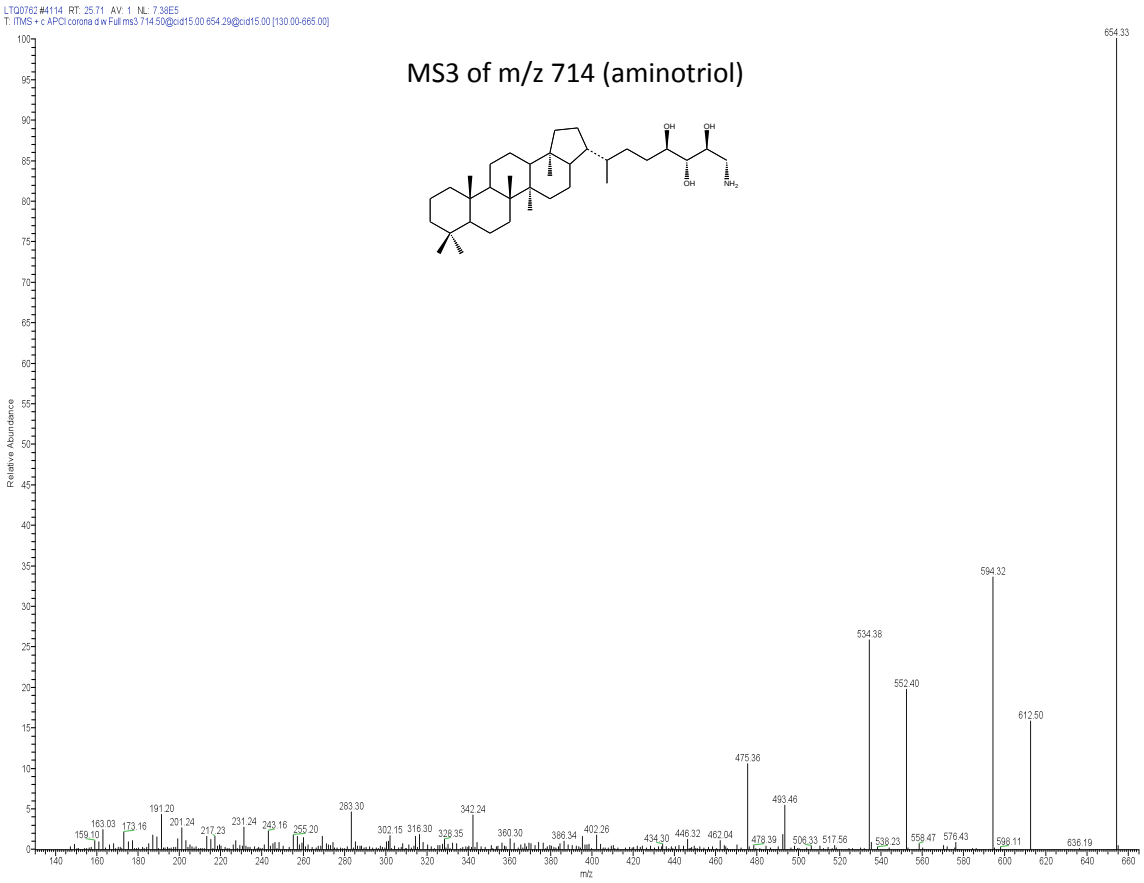
A3-1: Partial chromatograms of total lipid extract from *R. palustris*.



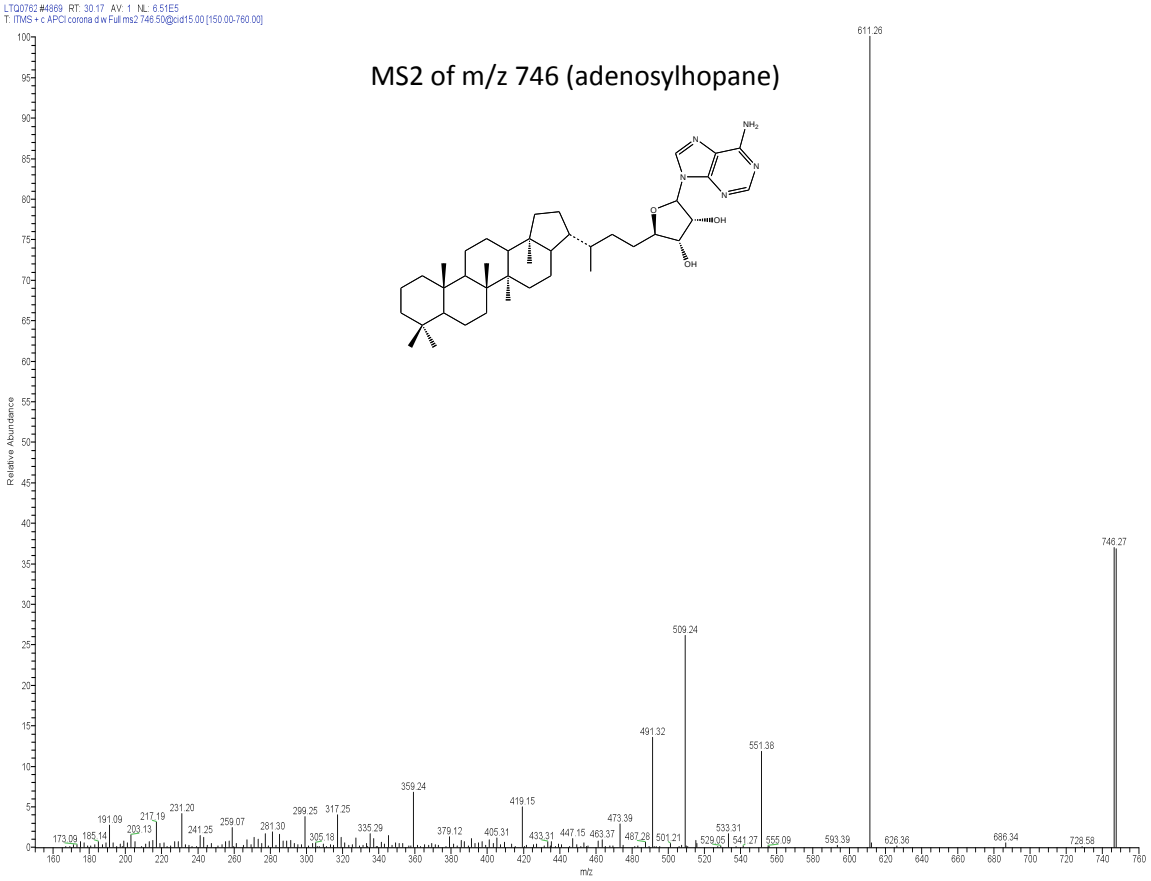
A3-2: Mass spectra of bacteriohopanetetrol (BHT) from *R. palustris*.



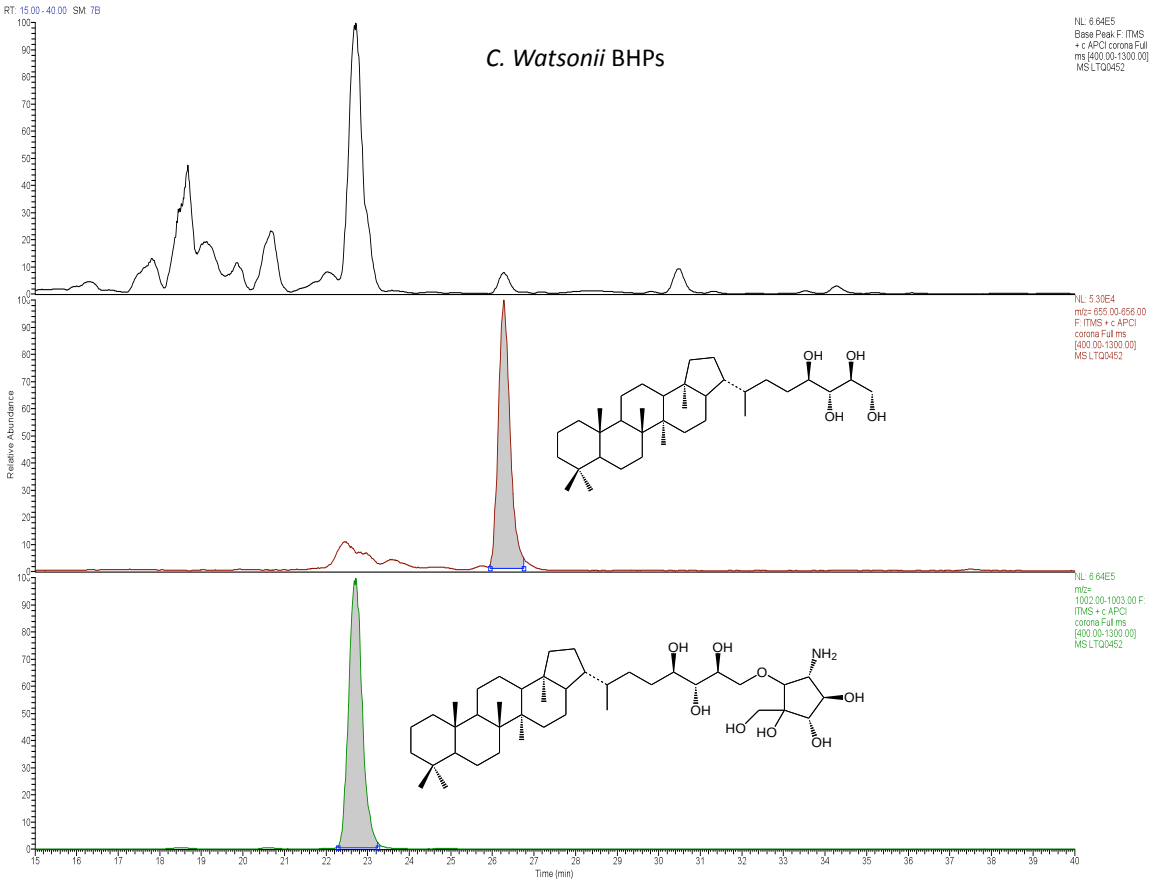
A3-3: Mass spectra of 2-methyl-bacteriohopanetetrol (2Me-BHT) from *R. palustris*.



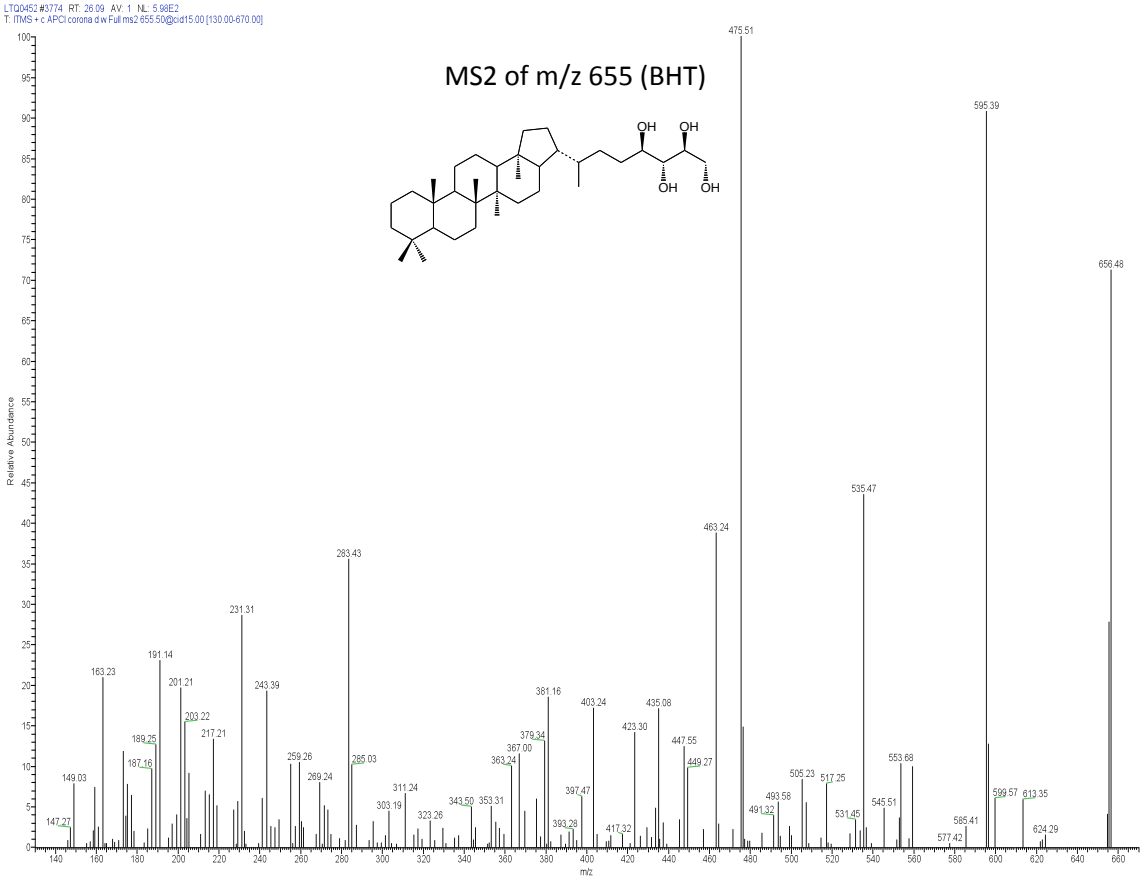
A3-4: Mass spectra of aminotriol from *R. palustris*.



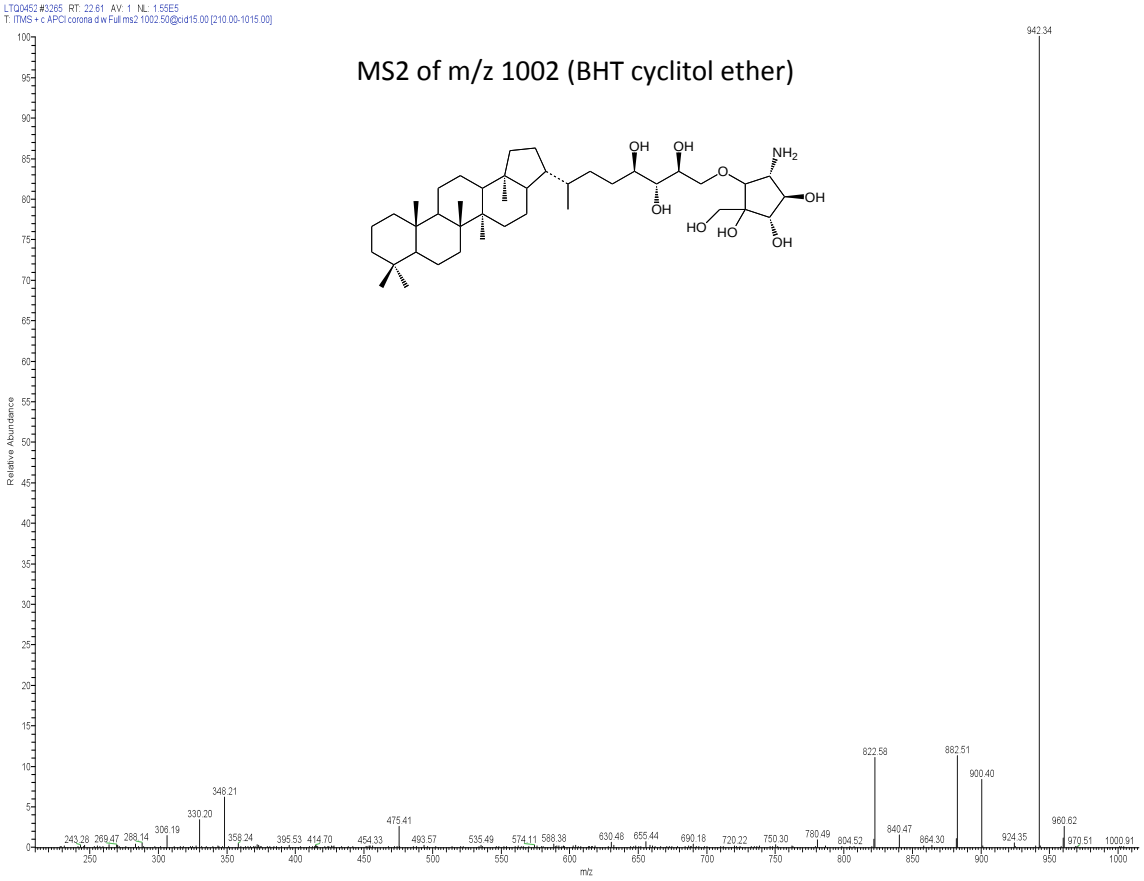
A3-5: Mass spectra of adenosylhopane from *R. palustris*.



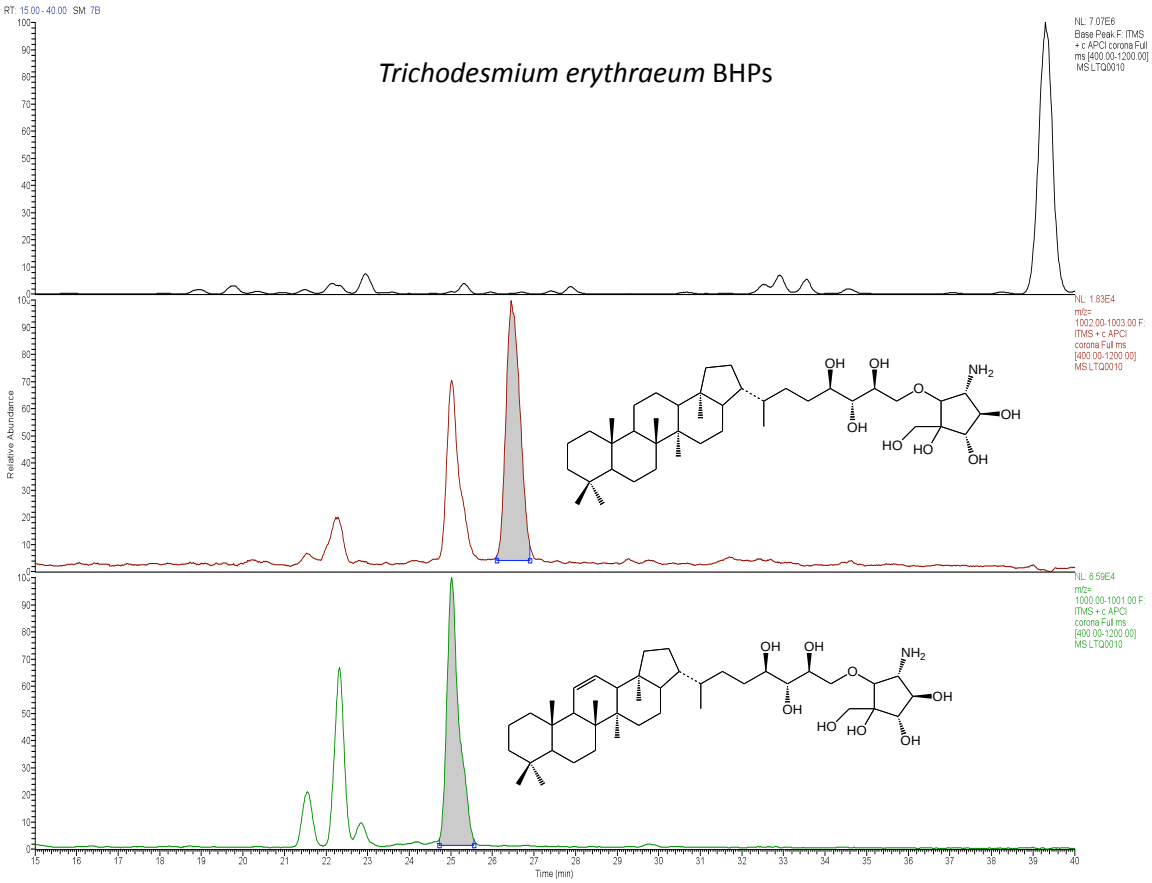
A3-6: Partial chromatograms of total lipid extract from *C. watsonii*.



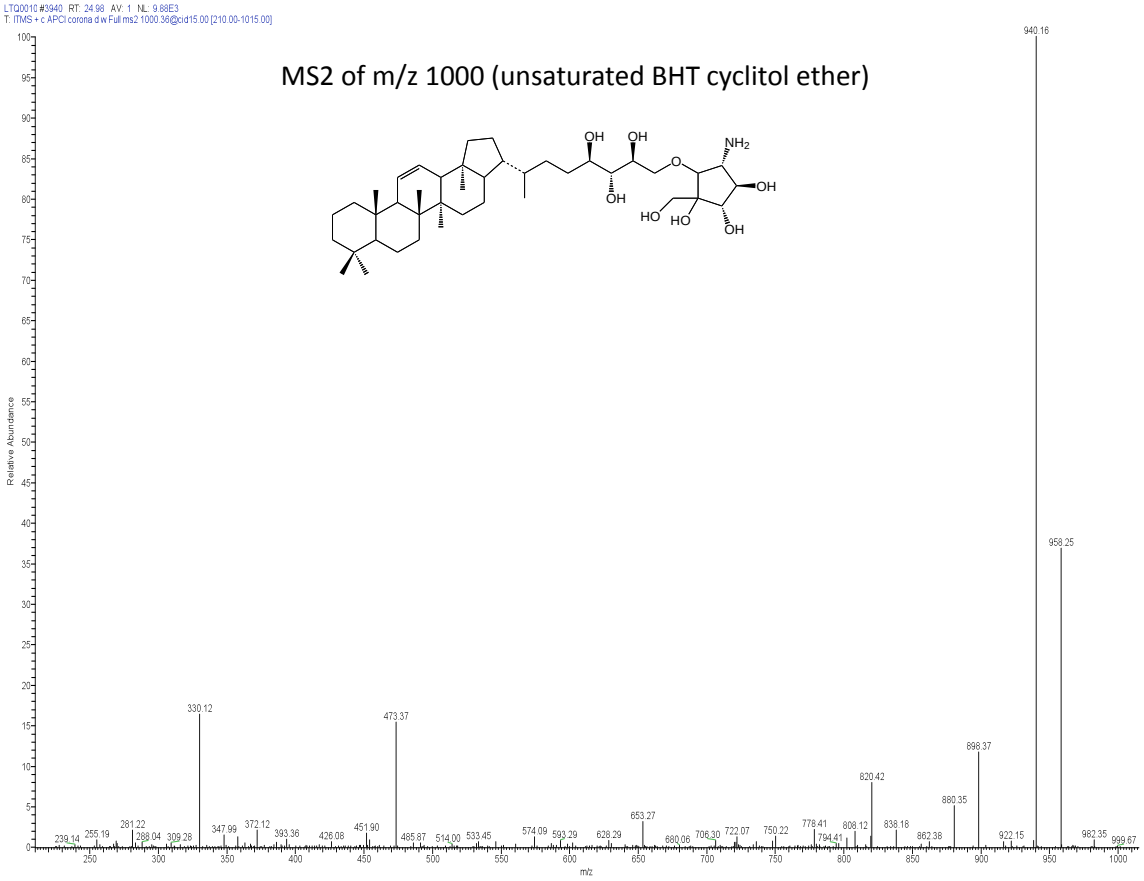
A3-7: Mass spectra of bacteriohopanetetrol (BHT) from *C. watsonii*.



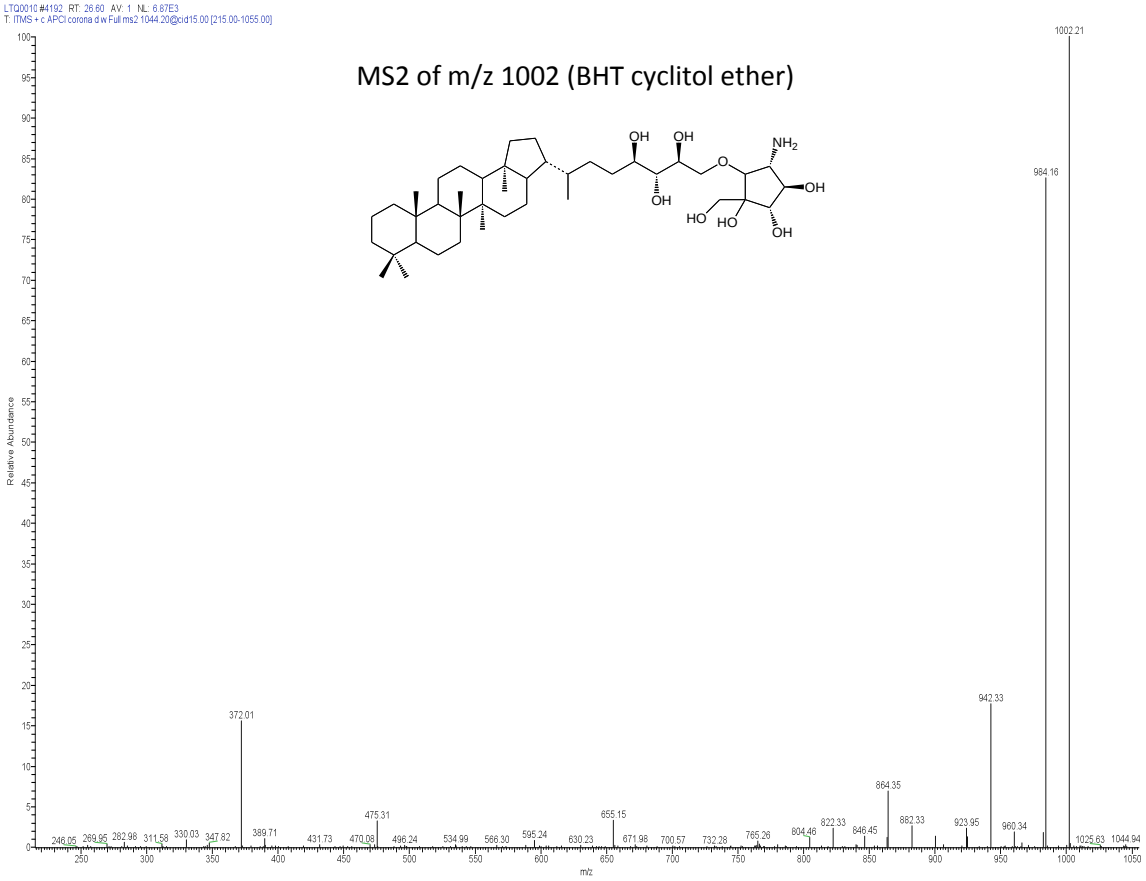
A3-8: Mass spectra of BHT cyclitol ether from *C. watsonii*.



A3-9: Partial chromatograms of total lipid extract from *T. erythraeum*.

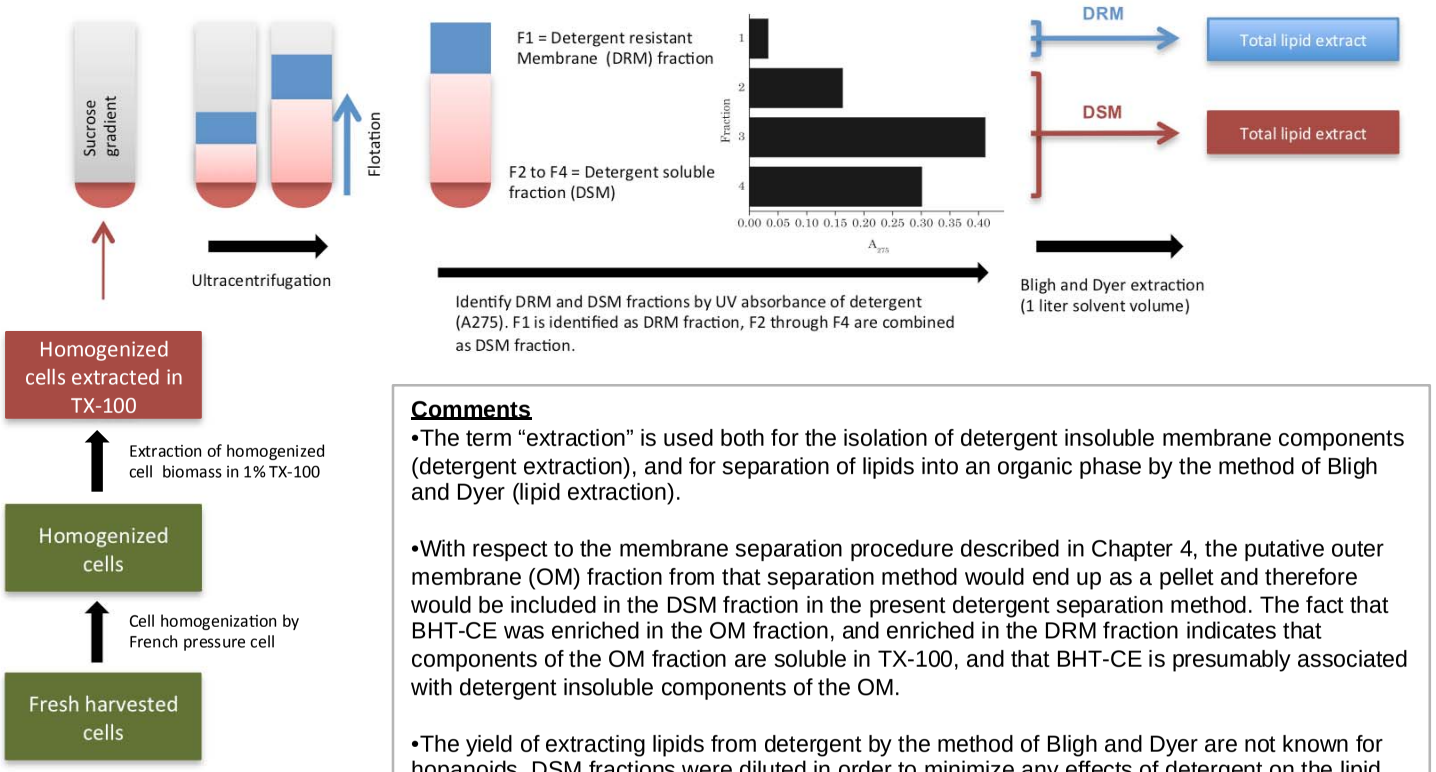


A3-10: Mass spectra of BHT cyclitol ether from *T. erythraeum*.



A3-11: Mass spectra of unsaturated BHT cyclitol ether from *T. erythraeum*.

Chapter 5: Detergent extraction and separation protocol



Comments

•The term "extraction" is used both for the isolation of detergent insoluble membrane components (detergent extraction), and for separation of lipids into an organic phase by the method of Bligh and Dyer (lipid extraction).

•With respect to the membrane separation procedure described in Chapter 4, the putative outer membrane (OM) fraction from that separation method would end up as a pellet and therefore would be included in the DSM fraction in the present detergent separation method. The fact that BHT-CE was enriched in the OM fraction, and enriched in the DRM fraction indicates that components of the OM fraction are soluble in TX-100, and that BHT-CE is presumably associated with detergent insoluble components of the OM.

•The yield of extracting lipids from detergent by the method of Bligh and Dyer are not known for hopanoids. DSM fractions were diluted in order to minimize any effects of detergent on the lipid extraction yields. Total lipid extracts from the DSM fraction were obtained by diluting the detergent fraction with Milli-Q water from 6mL with ~1% TX-100 to 200 mL resulting in a lower detergent concentration (0.03% TX-100). The resulting diluted DSM fraction was then used as the aqueous component of the Bligh and Dyer extraction: 500 ml methanol/250 ml dichloromethane/200ml diluted DSM fraction.

A5-1: Flow chart of the detergent extraction and separation method employed in Chapter 5.


CROSS COUNTRY
The End…
Massachusetts 09
HOPANE
The Spirit of America
BOSTON VOLVO VILLAGE
YANK THESE

A7-1: The End ...