

# Hydrogen-isotopic variability in fatty acids from Yellowstone National Park hot spring microbial communities

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## Abstract

We report the abundances and hydrogen-isotopic compositions (D/H ratios) of fatty acids extracted from hot-spring microbial mats in Yellowstone National Park. The terrestrial hydrothermal environment provides a useful system for studying D/H fractionations because the numerous microbial communities in and around the springs are visually distinct, separable, and less complex than those in many other aquatic environments. D/H fractionations between lipids and water ranged from  $-374\text{‰}$  to  $+41\text{‰}$  and showed systematic variations between different types of microbial communities. Lipids produced by chemoautotrophic hyperthermophilic bacteria, such as icosanoic acid (20:1), generally exhibited the largest and most variable fractionations from water ( $-374\text{‰}$  to  $-165\text{‰}$ ). This was in contrast to lipids characteristic of heterotrophs, such as branched, odd chain-length fatty acids, which had the smallest fractionations ( $-163\text{‰}$  to  $+41\text{‰}$ ). Mats dominated by photoautotrophs exhibited intermediate fractionations similar in magnitude to those expressed by higher plants. These data support the hypothesis that variations in lipid D/H are strongly influenced by central metabolic pathways. Shifts in the isotopic compositions of individual fatty acids across known ecological boundaries show that the isotopic signature of specific metabolisms can be recognized in modern environmental samples, and potentially recorded in ancient ones. Considering all sampled springs, the total range in D/H ratios is similar to that observed in marine sediments, suggesting that the trends observed here are not exclusive to the hydrothermal environment.

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## 1. INTRODUCTION

Recent studies of compound-specific D/H ratios in lipids from marine sediments and particulate organic matter (POM) have documented significant variability, even among molecules with similar biochemical sources of contemporaneous marine origin (Jones et al., 2008; Sachse and Sachs, 2008; Li et al., 2009). Given that the isotopic composition of seawater is nearly invariant, these data suggest the existence of controlling factors independent of water isotopic composition. This is contrary to the prevalent assumption of a nearly constant biological frac-

tionation between lipids and environmental water. Nevertheless, further interpretation of the marine data was hampered by the difficulty of relating individual lipids to specific organisms in complex environmental samples (Li et al., 2009).

Zhang et al. (2009) described culture experiments in which lipid/water fractionations in bacteria appeared to depend on the central metabolic pathway(s) employed by the organism. Moreover, growth on substrates that activate different catabolic pathways lead to dramatically disparate lipid D/H ratios, even in the same organism. Zhang et al. (2009) therefore hypothesized that various enzymes used in central metabolism to reduce  $\text{NADP}^+$  have very different isotope effects, and so produce NADPH (and ultimately lipids) with distinct isotopic compositions. This hypothesis remains to be confirmed in environmental samples.

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The cultured organisms employed by Zhang et al. (2009) such as *Escherichia coli* and *Cupriavidus oxalaticus* are unlikely to be prevalent in most environmental samples. More importantly, the bacteria were grown as axenic cultures in minimal media on single substrates, conditions that bear little resemblance to the complex nutrient and trophic structures present in natural environments. There is thus some uncertainty whether the patterns described by Zhang et al. (2009) will be applicable to microbes in their native habitat. To address that question, we studied the fractionation of hydrogen isotopes in bacterial fatty acids from microbial mats growing in hydrothermal environments of Yellowstone National Park (YNP). We report here the  $\delta D$  values of spring waters and fatty acids in 41 samples collected from 16 springs across YNP.

The terrestrial hydrothermal environment provides a useful system in which to study D/H fractionations in lipids both because of its simplicity and accessibility, and because many of the organisms living there have been intensively studied by others (Hugenholtz et al., 1998; Ward et al., 1998; Jackson, 2001; Fouke, 2003; Spear et al., 2005; Walker et al., 2005; Shock et al., 2010). The numerous microbial communities in and around the springs have limited diversity compared to most soil and/or marine ecosystems, easing the burden of connecting particular lipids to specific parent organisms. This task is further aided by the fact that many representatives of microbial communities featured herein have been isolated in culture (Brock and Freeze, 1969; Brock et al., 1972; Pierson and Castenholz, 1974; Huber et al., 1998) and their lipids have been previously characterized. YNP has the additional benefit of extensive

previous phylogenetic and isotopic studies that provide significant context for our studies (Jahnke et al., 2001, 2004; Zhang et al., 2004; Meyer-Dombard et al., 2005, 2011).

Whereas simplicity is a virtue for identifying the sources of lipids, comparisons across a wide range of physical, chemical, and biological parameters are desirable for surveying the breadth of microbial metabolism. Here again hydrothermal systems provide substantial advantages. Even within a single spring, communities vary systematically with temperature and are frequently visually distinct from one another, allowing for targeted sampling and comparison between different mat types. For example, a classic ecological succession follows temperature and has been studied extensively in outflow channels of Lower Geyser Basin (LGB) type springs such as Octopus Spring (Brock, 1978). The sequence begins with chemolithoautotrophic communities dubbed “pink streamers” living at the highest temperatures (Jahnke et al., 2001), followed by photosynthetic mats ranging from thin yellow biofilms to thick orange and brown mats depending on temperature and water velocity (Castenholz, 1969). Apart from the LGB type springs, extremely variable chemical compositions and energetic potentials exist in the many different geyser basins around the park, giving rise to remarkable microbial diversity (Spear et al., 2005; Shock et al., 2010) and the potential for broad comparisons of isotopic fractionations.

## 2. SAMPLE LOCATIONS AND DESCRIPTIONS

Samples were taken from 16 springs in five hydrothermal areas over the course of two sampling expeditions in

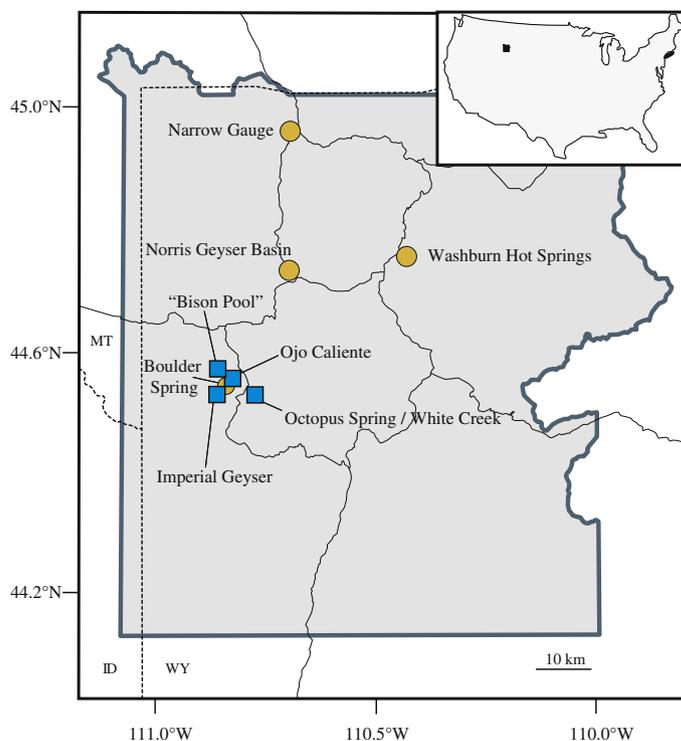


Fig. 1. Location of selected sampling sites. YNP is shown in gray, state boundaries are dashed lines, and roads are thin gray lines. Sample locations are indicated by squares for LGB type springs and by circles for all others.

June of 2008 and 2009 (Fig. 1), and were chosen to cover a range of microbial mat and spring types. The primary focus was to sample classic mat types from LGB runoff channels along with samples of specific mat types from different kinds of springs (Table 1). LGB-type springs included Octopus Spring, ‘Bison Pool’, Ojo Caliente, Imperial Geyser, and four smaller features in the White Creek area. Where possible, we took samples of all three main mat types from these springs including high temperature pink or white streamers, yellow biofilms, and orange photosyn-

thetic mats (Brock and Freeze, 1969; Castenholz, 1969; Brock, 1978). During the 2009 visit, layered mat samples were further dissected into three distinct layers. Samples of the common orange photosynthetic mats were quite variable in morphology and could be broadly divided into two groups, with higher temperature mats being generally thicker with planar stratification and occasionally a green upper surface, whereas lower temperature mats displayed more irregular to coniform laminations. We collected both types of orange mat at Octopus Spring in 2009.

Table 1  
Description of samples.

Spring name <sup>a</sup>	Park reference	Latitude (°N)	Longitude (°W)	Sample number <sup>b</sup>	Type <sup>c</sup>	Temp.	pH
<i>Lower Geyser Basin</i>							
Octopus Spring	LWCGG138	44.53405	110.79784	OS08-1	PS	–	7.9
				OS08-2	YB	–	
				OS08-3	OM-LT	–	
				OS09-1	PS	86.1	
				OS09-2	YB	74.1	
				OS09-3	OM-HT	63.1	
				OS09-4abc	OM-LT	37.9	
‘Brain Pool’	LWCG149	44.53225	110.79654	BP09-1	PS	85±2	8.9
				BP09-2	PS	82.5	
‘Spent Kleenex’	–	44.53247	110.79757	SK09-1	PS	86.9	9.0
‘Fallen Log’	LWCGNNO51	–	–	FL08-1	OM-HT	–	–
				LJ08-1	YB	–	–
‘White Creek’	–	–	–	WC08-1	OM-HT	–	–
<i>Sentinel Meadows</i>							
Bison Pool	LSMG013	44.56953	110.86511	B09-1	PS	81.5	8.4
				B09-2	PS	76.6	
				B09-3	YB	64.5	
				B09-4abc	OM-LT	–	
Ojo Caliente	LR001	–	–	OC09-1	PS	80.2	–
				BS09-1	SR	81.8	9.2
Boulder Spring	–	44.55873	110.84383	BS09-2	SR	78.6	6.9
				IG09-1	YB	69	9.0–9.1
Imperial Geyser	–	44.53167	110.87643	IG09-2abc	OM-HT	64.2	–
				IG09-3	OM-HT	62.5	
				–	–	–	
<i>Norris Geyser Basin</i>							
‘Zygonium mat’	–	44.72885	110.71178	NR08-1	SR	–	2.3
				NR09-1	SR	25.2	
				NR09-2	SR	25.2	
				NR09-3	SR	36.6	
–	–	44.72747	110.71162	–	–	–	3.2
<i>Washburn Hot Spring Group</i>							
‘Boomerang’	WHSNN014	44.75573	110.43007	WB09-1	SR	76.1	3.3
				WB09-2	SR	73.5	
‘DEDS’	–	–	–	–	–	–	–
<i>Mammoth Hot Springs</i>							
Narrow Gauge	MA042	44.96933	110.71044	NG09-1	Carb	46.5	7.9
				NG09-2	Carb	46.5	
				NG09-3	Carb	46.5	
Old Narrow Gauge	MA041	44.96983	110.7103	NG09-4	PS	58.6	6.3
				NG09-5	Carb	34.6	

<sup>a</sup> Spring names are official park names unless indicated with quotation marks.

<sup>b</sup> Samples are named as XX YY-Za, where XX is an abbreviation of the spring name, YY is sampling year, Z is sample number from that location, and a is the mat layer (present only for samples that were dissected). For example OS09-4a refers to Octopus Spring, 2009, location 4, layer a (top).

<sup>c</sup> Mat types are abbreviated as follows: PS – pink streamer, YB – yellow biofilm, OM-LT – orange mat low temperature, OM-HT – orange mat high temperature, SR – sulfur rich, Carb – carbonate hosted orange mat.

In addition to the LGB sample suite, we also collected mats from springs with more varied chemistry. *Zygonium* mat and green biofilms from Norris Geyser basin, and black sediments from Washburn Hot Spring Group, represent sulfur-rich acidic systems. Boulder Spring in the Sentinel Meadow area was neutral to alkaline although high in sulfide and contains unusual black sediments. Mats from Narrow Gauge spring in the Mammoth area are carbonate- rather than silica-depositing springs. Further details and samples names are provided in Table 1 and EA-1.

### 3. METHODS

#### 3.1. Lipid extraction

Samples for lipid analysis were collected using solvent-washed tongs, spoons, or spatulas, and placed directly into pre-combusted glass jars with Teflon cap liners. During the 2009 field season, thick layered photosynthetic mat samples were dissected with a sterile scalpel and dissecting needle using pre-combusted aluminum foil as a work surface. All samples were stored on ice until their arrival at Caltech (<4 days), then were frozen, lyophilized, and stored at  $-20^{\circ}\text{C}$  until lipid extraction. Samples NR09-3 and OS09-2 were thin biofilms on pieces of sinter, and were collected by submerging coated rocks in spring water in sample jars. In the lab, the samples were treated in an ultrasonic bath for 30 min to disaggregate the biofilms. Biomass laden water was then lyophilized to yield concentrated microbial biomass. Temperature and pH of the springs were measured at the time of sampling (Table 1).

Dry biomass samples were first ground to a powder in a solvent-washed mortar and pestle. Samples collected in 2008 were extracted using a modified Bligh–Dyer procedure (Bligh and Dyer, 1959). Samples were first shaken vigorously in a single-phase mixture of dichloromethane (DCM): methanol (MeOH): water (1:2:0.8 v/v). Addition of DCM and  $\text{H}_2\text{O}$  caused the mixture to separate into organic and aqueous phases, from which the organic fraction was collected. Samples collected in 2009 were extracted using a Microwave Accelerated Reaction System (MARS Xpress, CEM Corp.) with 20 ml DCM/MeOH (9:1) at  $100^{\circ}\text{C}$  for 15 min, with stirring. Direct comparison of these two methods on several 2009 samples indicated that both yield similar fatty acid distributions, but with a 4–5 times greater yield from the MARS system.

Total lipid extracts were filtered, dried under a stream of  $\text{N}_2$ , and saponified in 10 ml of aqueous 0.5 M NaOH at  $70^{\circ}\text{C}$  for 4 h. Saponified samples were then extracted three times with methyl tert-butyl ether (MTBE). Elemental sulfur was removed by eluting through acid-washed copper powder ( $-40$  to  $+100$  mesh). After resuspension in hexane, samples were separated into four fractions by solid phase extraction (SPE) on an aminopropyl stationary phase following the method of Sessions (2006). Only data from the fatty acid fraction (F4, eluted in 8 ml of 2% formic acid in DCM) are reported here. Fatty acids were derivatized by heating with  $100\ \mu\text{l}$   $\text{BF}_3/\text{MeOH}$  at  $70^{\circ}\text{C}$  for 10 min to form fatty acid methyl esters (FAMES), and a known

amount of palmitic acid isobutyl ester (PAIBE) was added to each sample as an internal standard. Some samples contained significant amounts of hydroxy-fatty acids that complicated the GC chromatograms. These compounds were separated by column chromatography on 5% deactivated silica gel, eluting the FAMES in three bed-volumes of hexane, and the hydroxy-FAMES in three bed-volumes of acetone.

#### 3.2. Geochemical analyses

Water samples for D/H analysis were collected in pre-combusted glass 2 ml vials with Teflon coated screw caps. Turbid or sediment-laden samples were filtered through 4 and  $0.2\ \mu\text{m}$  syringe filters. Samples were stored on ice in the field and at  $4^{\circ}\text{C}$  until analysis at Caltech using a Los Gatos DLT-100 Liquid Water Isotope Analyzer. Samples were measured in sixfold replicate against two working standards ( $\delta\text{D} = -154.1\text{‰}$  and  $-117.0\text{‰}$ ). Measured isotope ratios were converted to  $\delta\text{D}$  values by comparison with the two standards, and normalized to the SMOW-SLAP scale. Typical precision for these analyses was 1–2‰. All data reduction was performed using Visual Basic code written by us.

Fifteen milliliters of water from each sampling site was collected by syringe, sterile filtered and acidified ( $\text{pH} < 2$ ) for measurement of metals and major cations by inductively coupled plasma atomic emission (ICP-AE) spectroscopy. A second 15 ml aliquot of water was collected and sterile-filtered for analysis of major anions by ion chromatography (IC). Both analyses were performed at the Colorado School of Mines.

Samples for measurement of dissolved  $\text{H}_2$  concentrations were collected via a bubble-stripping method modified from Spear et al. (2005). Water was pumped at approximately 200 ml/min for 20 min through gas-impermeable, Tygon FEP-lined tubing into and through a 1 L gas sampling bulb. Thirty milliliters of air was injected into the gas sampling bulb. Gas samples were collected by syringe and transferred to nitrogen-charged, hydrogen-impermeable glass septum vials and shipped to Microseeps (Pittsburg, PA) for analysis of  $\text{H}_2$  content on an RGA3 reduction gas analyzer (Trace Analytical, Newark, DE).

#### 3.3. Lipid analyses

Lipids were identified and quantified by gas chromatography–mass spectrometry (GC–MS). One microliter of each organic extract was injected into a ThermoFinnigan Trace GC with the effluent split  $\sim 9:1$  between a DSQ mass spectrometer and a flame ionization detector (FID). The sample was injected into a programmable temperature vaporization (PTV) injector operated in splitless mode and heated to  $330^{\circ}\text{C}$  in 24 s. The GC was equipped with a ZB-5 ms GC column (30 m long, 0.25 mm I.D., 0.25  $\mu\text{m}$  film thickness) and was operated with a He carrier gas flow rate of 0.8 ml/min. The oven temperature was held for 1 min at  $100^{\circ}\text{C}$ , ramped at  $20^{\circ}\text{C}/\text{min}$  to  $140^{\circ}\text{C}$ , ramped at  $3.0^{\circ}\text{C}/\text{min}$  to  $250^{\circ}\text{C}$  and held for 1 min, then ramped at  $20^{\circ}\text{C}/\text{min}$  to  $310^{\circ}\text{C}$  and held for 10 min. Compounds were

identified by comparison of mass spectra to the NIST 2004 library and/or by retention time to authentic standards. Concentrations were calculated by comparing integrated FID signals for each peak to the PAIBE internal standard assuming identical response factors for all FAMES. FAMES are reported using the nomenclature “X:Y” where X is carbon number and Y is number of double bonds. The position and stereochemistry of double bonds was not determined. We follow the convention of naming compounds with iso- or anteiso-methyl branches, or cyclopropyl rings, as the total carbon number preceded by *i*-, *a*-, or *cy*-, respectively. Thus ‘*i*-17’ is 15-methylhexadecanoic acid.

D/H ratios of FAMES were measured using a ThermoFinnigan Trace GC coupled to a Delta<sup>plus</sup>XP isotope ratio mass spectrometer (IRMS) via a pyrolysis interface (GC-TC) operated at 1430 °C. External FAME standards were analyzed after every fifth sample. Eight microliters of each sample was injected using a PTV injector operated in splitless mode with solvent venting. A thick-film ZB-5 ms column (30 m long, 0.25 mm I.D., 1.00 µm film) was used for isotope analysis with He carrier gas flow rate at 1.4 ml/min. The GC oven temperature was held at 100 °C for 1 min, ramped at 20 °C/min to 205 °C, ramped at 0.8 °C/min to 220 °C, ramped at 8 °C/min to 320 °C and held for 10 min. Peaks were identified by comparison of retention order and relative height to GC–MS chromatograms. Isotope ratios were calculated using ISODAT NT 2.5 software by comparison to methane reference gas peaks as described previously (Wang and Sessions, 2008) and are reported in the standard  $\delta D$  notation ( $\equiv R_{\text{sample}}/R_{\text{std}} - 1$ ) as permil (‰) variations relative to the VSMOW standard. The root-mean-squared (RMS) error for external FAME standards run both before and between sample runs was 5.47‰ ( $n = 48$ ) for 2008 samples and 5.85‰ ( $n = 156$ ) for 2009 samples. The standard deviation for replicate analyses of unknown analytes averaged 7.5‰ ( $n = 247$ ). Samples were analyzed in triplicate where possible, however low sample abundance prevented this in some samples. The  $H_3$  factor averaged 3.90 ppm/mV (range 3.84–4.02) during analyses of the 2008 samples, and averaged 7.09 (range 6.56–7.46) during analyses of the 2009 samples. Fractionations between lipids and environmental water were calculated as  $\varepsilon_{l-w} = ((\delta D_l + 1)/(\delta D_w + 1) - 1)$  and are reported as permil (‰) variations.

### 3.4. DNA extraction, PCR and DNA sequencing

DNA was extracted from samples using the Powersoil Extraction Kit (MoBio). The manufacturer’s protocol was followed with the 10 min lysis/vortexing step replaced by 1 min bead-beating. qPCR was conducted on a Lightcycler 480 II (Roche) to monitor the plateau point of amplification. A PCR-touchdown annealing temperature strategy (Don, 2010) was employed for 10 cycles to minimize primer dimer formation and the final 15–20 cycles combined the annealing and elongation steps. PCR was stopped for each sample when it appeared the amplification was beginning to

plateau. qPCR volumes were 30 µl total, 9.6 µl of which was DNA template. Reagents and final concentrations were 1× Phusion DNA Polymerase MasterMix with HF Buffer (New England Biolabs), 0.5 µM forward and reverse primer, 8% v/v DMSO, 0.4× Sybr Green I (Invitrogen). Primers incorporated the adapter sequences for pyrosequencing on the GSFLX platform of the Roche 454 Pyrosequencing technology. Additionally, each forward primer had an 8 nt barcode corresponding to an environmental sample that allowed amplicon pools to be sequenced in parallel and binned by sample *in silico* post-sequencing. The small sub-unit (SSU) rRNA gene primers were attached to adapters only (reverse primer) or adapters and barcodes (forward primer) by a 2 nt linker. The sequence for the linker was the two least abundant bases at the adjacent positions to the primer in the Silva SSURef102\_NR alignment and database (Pruesse, 2007) for which the *E. coli* sequence did not have a gap. SSU rRNA gene primers were adapted from 515F (Lane, 1991) and 927R (Jurgens et al., 1997) to account for observed mis-pairings of the primers with archaeal and bacterial sequences from the Silva SSURef102\_NR database. Evaluation of primers was done using custom Python scripts that employed bioinformatics modules from PyCogent (Knight, 2007). The specific SSU rRNA gene primers used were 515f-modified, 5'-GTGYCAGCMGC CGCGGTAA-3', and 927r-modified, 5'-CCGYCAATT CMTTTRAGTTT-3'. Individual sample amplicon volumes were pooled and gel purified (Montage DNA Gel Extraction Kit, Millipore) prior to sequencing. Although, GSFLX adapters were used during PCR amplification, sequencing was done on the later generation Roche 454 Titanium platform.

Sequences shorter than 150 nt and longer than 500 nt were discarded. Additionally, sequences with average quality scores less than 25, errors in the barcode or primer, homopolymer runs greater than 6 nt, and ambiguous base-calls were removed from analyses to improve the quality of the final data as described (Huse, 2007). Initial quality control steps were completed with QIIME (Caporaso, 2010). The flow grams for the remaining sequences were sent through a noise removal algorithm to clean up characteristic pyrosequencing errors (Reeder and Knight, 2010). Following noise removal, the sequences were clustered at 97% identity using UClust (Edgar, 2010) and the most abundant sequence from each cluster was chosen as the cluster representative. Chimeric representative sequence OTUs were identified with ChimeraSlayer and removed from further analyses (Haas et al., 2011). Cluster representatives were classified by recruiting reads to taxonomically annotated near full-length SSU rRNA gene sequences in Silva SSURef102\_NR using BLAST (Altschul, 1990). Prior to classification, sequences in the Silva SSURef102\_NR database with values for the `seq_qual_slv` and `align_qual_slv` metadata fields less than or equal to 50 and the `pintail_slv` field less than or equal to 40 were discarded. UClust, BLAST, noise removal and cluster representative picking were done using Python scripts and wrappers available in the QIIME software package. DNA Sequencing data from this study has been deposited in the Sequence Read Archive (Acc. #: SRA029100).

## 4. RESULTS

### 4.1. Hot spring geochemistry

Hot spring geochemical parameters illustrate the great variability between different types of springs sampled in this study (Table 1 and EA-1). Temperature and pH of source pools varied with location. LGB and Sentinel Meadow springs were near boiling (<95 °C at these elevations) at their sources with neutral to alkaline pH (mean of 8.4), whereas springs in Norris Geyser Basin were characteristically very acidic and cooler than the LGB type springs. The Washburn Hot Spring Group had diverse fluid compositions, but the springs we sampled were sub-boiling (73.5–76.1 °C) and acidic (3.26–4.98). Carbonate springs at Mammoth Hot Springs were more uniform and slightly cooler than the LGB springs and of neutral pH.

Trends in major anion and cation abundance were similar within individual thermal areas. LGB samples were generally characterized by high F, Cl, and Na, and low SO<sub>4</sub> and Fe. Sample BP09-2 had generally lower concentrations of these species as it was taken from the confluence of the hot spring and a creek with presumably mixed meteoric and hydrothermal composition. Norris and Washburn groups were generally similar to each other and were characterized by low F and high SO<sub>4</sub>, B, Ca, Fe, and Mg. Significant differences between the two springs are seen in Cl, K, and Na with Norris being higher than average for the total data set and Washburn lower. Samples from the carbonate hosted Narrow Gauge system were not correlated with any of the other areas, showing very high Ca, Mg, and K, low F, Br, Fe, and moderate values for Cl and Na. Sulfate showed the most striking variability of any

measured species ranging between 15.28 and 2502 mg/L, and was qualitatively correlated to the type of microbial mats present in a spring. Br and NO<sub>3</sub> were universally low throughout the sampled springs. The complete geochemical dataset is presented in Electronic Annex (EA-1).

### 4.2. Patterns of microbial phyla and lipid abundance

We identified 43 distinct fatty acid structures present in 41 samples. Of these, only two (16:0 and 18:0) were found in all samples. The great diversity and patchy distribution of these compounds makes a simultaneous comparison of all samples difficult. In general though, the distribution of fatty acids in a sample varied predictably by mat type, and so for simplicity we discuss the samples in groups based on mat types. The six main groups are denoted as: PS, chemoautotrophic streamers (includes pink, white, and yellow types); YB, yellow biofilms (includes IG09-1 that was a yellow mat); OM-HT, high temperature orange mats; OM-LT, low temperature orange mats; Carb, carbonate hosted orange mats; and SR, sulfur rich samples (includes acidic mats, biofilms, and sediments). While these distinctions are qualitative, they effectively separate lipid-based compositional groups and correspond qualitatively to phylum-level genetic diversity based on 16S rRNA gene sequencing results.

The average phylum-level phylogenetic compositions for each mat-type group are presented in Fig. 2 and are broadly consistent with the results of previous phylogenetic studies (Ward et al., 1998; Meyer-Dombard et al., 2005, 2011; Spear et al., 2005). Individual samples within the groups were similar enough to justify averaging except in the case of the SR group, where it was more appropriate to present

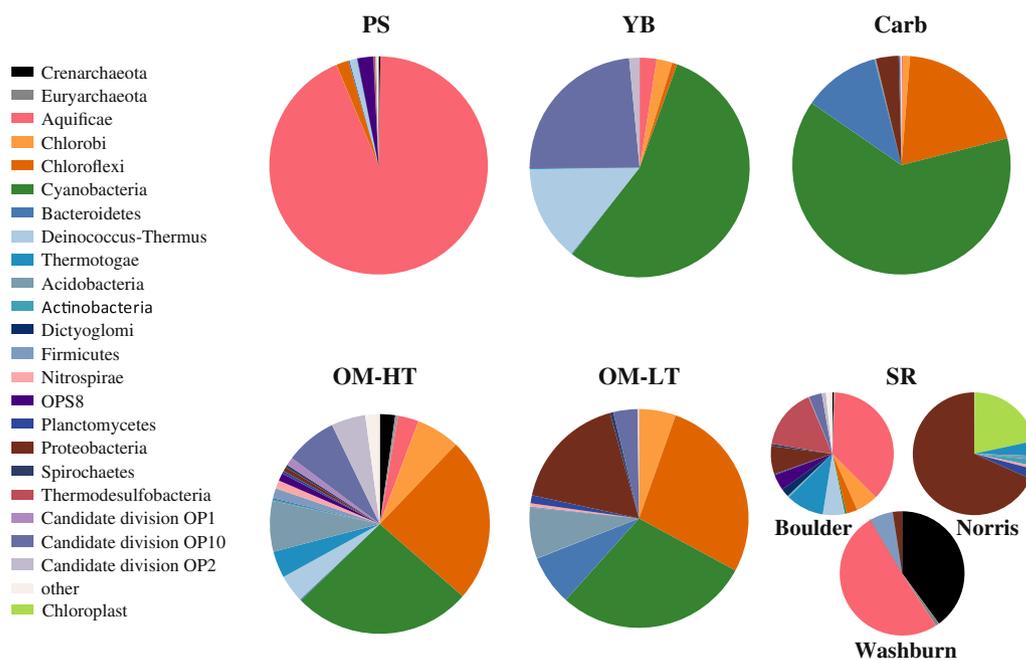


Fig. 2. Phylum-level genetic diversity of mat types based on SSU rRNA gene sequence. Large circles represent average compositions for particular mat types, whereas small circles represent average compositions for individual hydrothermal areas.

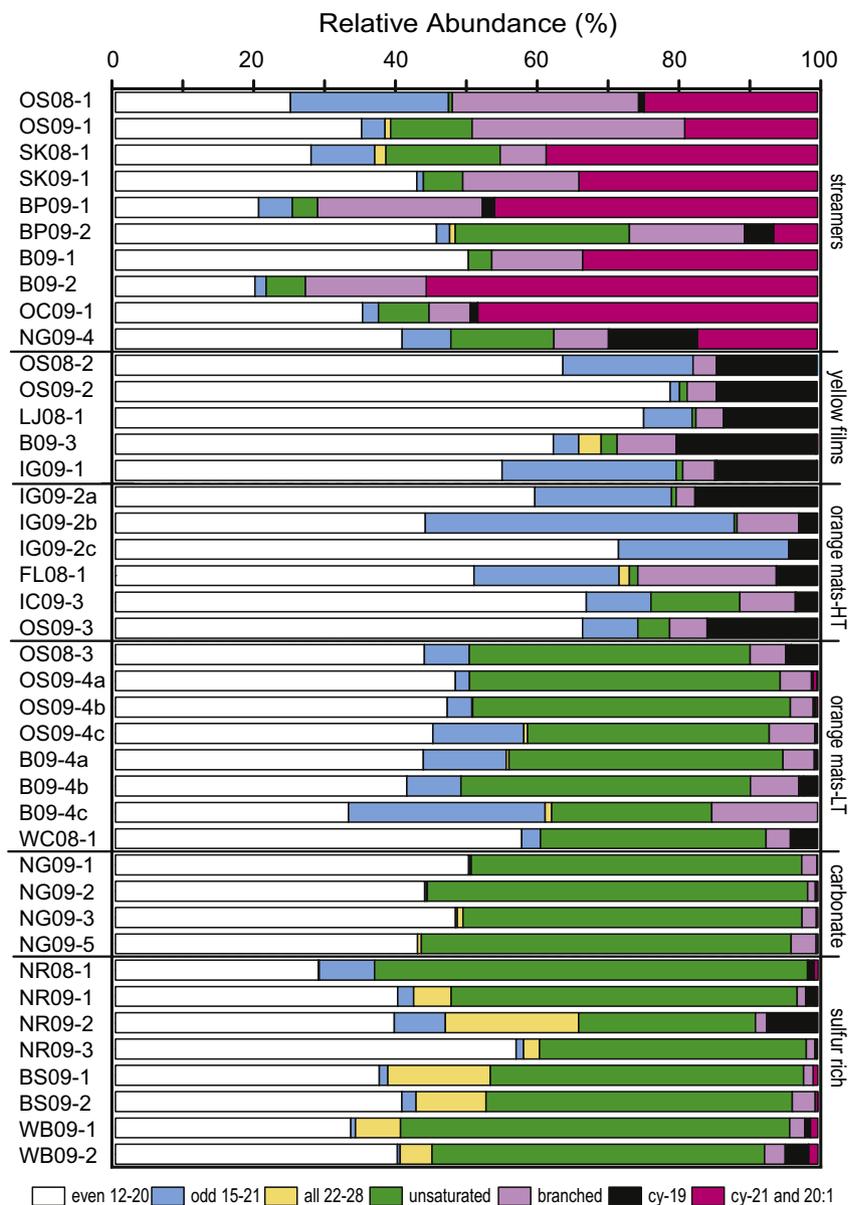


Fig. 3. Fatty acid distributions for each sample. Fatty acids with related structures and/or origins are binned together. Samples are ordered into characteristic groups based on mat type as described in the text, and as indicated on the right side of the graph.

the hydrothermal areas separately. The most striking compositional distinction is the dominance of the Aquificae in PS samples. They are also present, but at much lower levels, in both YB and OM-HT mats. The phototrophic mats (YB, OM-HT, OM-LT, and Carb) show an expected high proportion of photosynthesizing organisms, each comprising greater than half the population. There is a relative lack of Chloroflexi in YB mats compared to the large populations present in the other mat types, perhaps representing an upper temperature bound for the Chloroflexi as YB mats are the hottest photosynthetic mat samples. While the phototrophic populations of OM-HT and OM-LT mats are quite similar at the phylum level, the other (non-phototrophic) halves of these communities are quite different from one another. The heterotrophic population in OM-

HT mats is extremely diverse with the largest components being *Thermus*, *Thermotoga*, *Acidobacteria*, and Candidate division OP10. This contrasts with OM-LT type mats that instead have significant contributions from Bacteroidetes, *Acidobacteria*, and *Proteobacteria*. This is similar to bacterial clone libraries in Meyer-Dombard et al. (2011) which show significant variability in the non-photosynthetic portion of biofilms between various downstream LGB samples. The Carb mats have low numbers of heterotrophs relative to phototrophs and relative to the other mat types.

SR type samples were extremely diverse, precluding their representation as a single diversity wheel. Boulder spring samples contain significant contributions of *Aquificae*, *Thermodesulfobacteria*, *Proteobacteria*, and various heterotrophs. Washburn samples were dominantly split

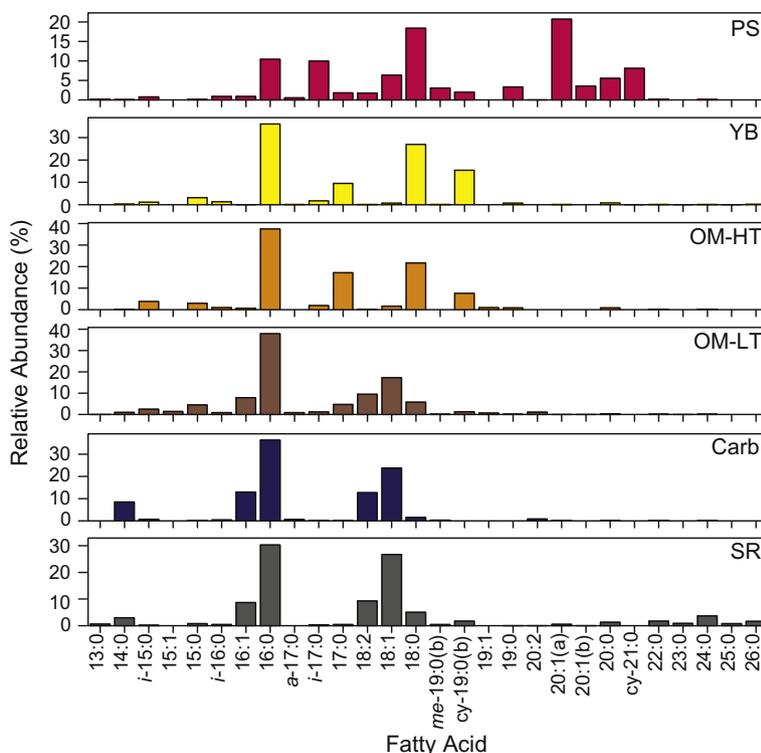


Fig. 4. Average relative abundances of individual FA in each mat type. Compounds with very low relative abundance ( $<0.6\%$ ) are not shown.

between Aquificae and Crenarchaeota with minor contributions from Firmicutes and Proteobacteria. The Norris diversity wheel includes only NR09-2 and NR09-3 because NR09-1 (Zygonium mat) failed to amplify. These samples were both dominated by Proteobacteria but NR09-3 shows a significant (42.9%) proportion of chloroplast sequences that we attribute to diatoms based on microscopic inspection. Previous studies have shown that Zygonium mats contain significant proportions of eukaryotic algae from the genera *Zygonium* and *Cyanidium* (Rothschild, 2001; Walker et al., 2005).

The relative abundances of fatty acids (FA) in each sample are summarized in Figs. 3 and 4, illustrating the similarity between individual samples in a mat type and clear compositional distinctions between the different mat types. The streamer (PS) communities were the most variable, but are also distinct from all other groups. They were characterized by a large proportion of 20:1 and cy-21 FA, along with significant amounts of branched compounds. Samples BP09-2 and NG09-4 showed visual evidence of pigmented microbes encrusting streamers and were distinguished by lower relative abundances of 20:1 and cy-21 and much larger amounts of cy-19. YB mats contained  $>70\%$  straight-chain FA (both even and odd chain lengths) and cy-19, with minor abundances of branched and unsaturated compounds in some samples. OM-HT samples were similar to YB samples but with higher proportions of odd-chain FA and variable contributions of unsaturated and branched compounds. OM-LT, Carb, and SR mats were very different from those above but quite similar to each other. These groups were comprised primarily of equal proportions of even-chain and unsaturated FA. The OM-LT mats were

differentiated from the others by a slightly larger contribution of odd-chain and branched FA, whereas the SR samples showed a unique contribution of long-chain FA ( $C_{22}$ – $C_{28}$ ).

Specific locations that were sampled in both 2008 and 2009 showed significant variability over time. This is particularly true in the higher temperature pairs OS08-1/OS09-1, SK08-1/SK09-1, and OS08-2/OS09-2. For these three pairs, the relative proportion of odd-chain FA decreased from 2008 to 2009, but there was no systematic trend in corresponding increases. Samples of low temperature orange mat from Octopus Spring were roughly consistent between years (OS08-3/OS09-4abc). Subsections (abc) of dissected mat samples had similar lipid profiles despite appearing visually distinct.

### 4.3. Isotopic compositions of lipids and water

Values of  $\delta D$  for the different spring waters were fairly consistent and averaged  $-132 \pm 5\%$  for the 2008 samples and  $-138 \pm 8.5\%$  for 2009 samples (EA-1). Most of the 2009 samples were near  $-140\%$ , but samples WB09-1 and WB09-2 were more D-enriched at  $-117\%$ . These values are consistent with measured cold surface water and snow from the YNP area which ranged from  $-115\%$  to  $-153\%$  and  $-88\%$  to  $-178\%$ , respectively (Kharaka et al., 2002). In comparison, lipid  $\delta D$  values spanned a  $>400\%$  range, providing a vivid demonstration of the highly variable biotic fractionations that occur.

D/H fractionations between fatty acids and source spring waters (quantified as  $\epsilon_{l/w}$ ) observed in this study ranged from  $-374\%$  to  $+41\%$  (Fig. 5). In addition, these

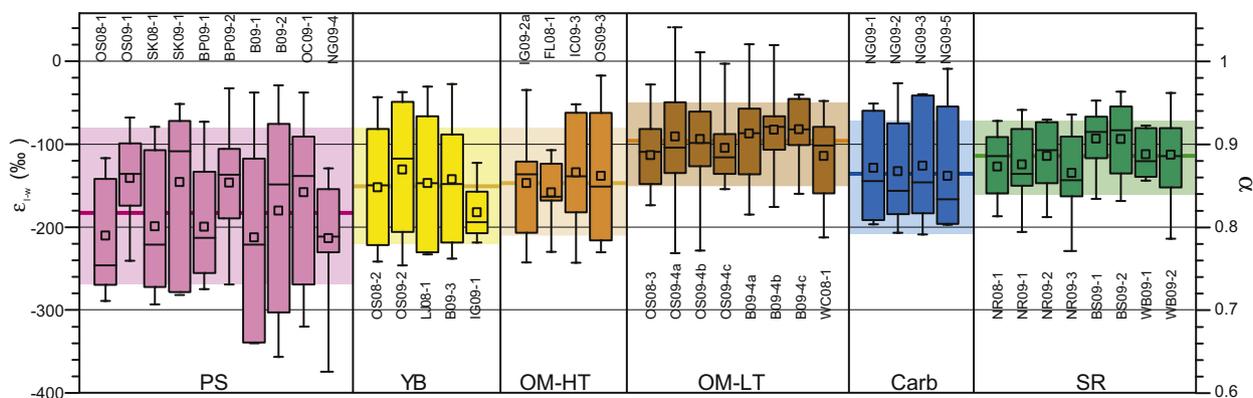


Fig. 5. The range of isotopic fractionations in each sample (individual bars) and for mat types (separate colors). For each sample the boxes contain 50% of the data with the upper and lower quartile shown by the whiskers. The mean and median of the data points are indicated by the square and line, respectively. The mean values ( $\pm 2\sigma$ ) for each group are shown in the background lines and shading. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

fractionations varied systematically by mat type, with streamer samples exhibiting larger and more variable fractionations ( $-191 \pm 100\text{‰}$ ) than the other types, and extending to extremely negative values (i.e., strong D-depletions of lipids). Fractionations in other mat types were more consistent, and decreased in the order YB ( $-151 \pm 71\text{‰}$ ), OM-HT ( $-147 \pm 65\text{‰}$ ), Carb ( $-137 \pm 69\text{‰}$ ), SR ( $-116 \pm 44\text{‰}$ ), and OM-LT ( $-97 \pm 53\text{‰}$ ). Lipids from OM-LT mats were the least D-depleted on average, and in some cases the fractionations were positive, indicating that lipids were D-enriched relative to water. Interestingly, orange mats from the carbonate system had  $\delta\text{D}$  values more similar to those from OM-HT mats in siliceous springs, even though lipid abundance and 16S diversity suggest they were more similar to OM-LT type mats. Duplicate samples from the same spring in multiple years show variable consistency. PS mat pairs from Octopus Spring shifted significantly toward smaller fractionations in 2009 compared to 2008, whereas YB and OM-LT samples were relatively consistent.

## 5. DISCUSSION

The striking covariance between mat type, FA abundance, microbial community composition, and lipid D/H fractionations highlights the influence of individual species on isotopic fractionation. We first examine the characteristic fractionations of specific microbes as deduced from biomarker FAs. We then relate these trends to the larger dataset to examine which organisms control the isotopic variability that is apparent in these systems.

### 5.1. Streamer communities

Bacteria in the order *Aquificales* produce a unique distribution of fatty acids that can help differentiate the contributions of these organisms to environmental samples. *Aquificales* isolates from Octopus Spring make abundant 20:1, cy-21, and 18:0 FA with minor amounts of 16:1, 16:0, 18:1, cy-19, 20:0 and 22:1 (Fig. 5a; Jahnke et al.,

2001). The 20:1 and cy-21 FA are unusual and have not been reported in high abundance in organisms beyond these chemoautotrophic bacteria. These same lipids also dominate pink streamer samples from Octopus Spring (Fig. 6c and d), though they were not the only lipids present.

Another important member of the highest-temperature ecosystems of YNP are filamentous bacteria from the genus *Thermus*. The best known is *Thermus aquaticus*, first isolated from springs in the White Creek, Boulder Spring, and Sentinel Meadow areas (Brock and Freeze, 1969). Lipids of *Thermus* species are also unusual for Gram-negative bacteria and consist primarily of iso- and anteiso-branched FA (Fig. 5). When grown at its optimum growth temperature, *T. aquaticus* lipids are dominated by *i*-15, *i*-16, 16:0, and *i*-17 with smaller amounts of *a*-15, 16:1, and *a*-17 (Nordstrom and Laakso, 1992). The relative proportions of these lipids are variable both with strain color and with growth temperature (Nordstrom, 1993). The presence of abundant *i*-19 – which has not been previously reported in *Thermus* – in our samples might be attributed either to a broader spectrum of lipids produced by *Thermus*, or to an alternative microbial source such as the uncultured OPS8 found in our sequencing libraries. The genus *Thermotoga* has also been reported as a major constituent of the Bison Pool streamer communities (Meyer-Dombard et al., 2005). Although there is no published lipid information for *Thermotoga*, a similar lipid profile seems reasonable based on its phylogenetic and physiological similarity to *Thermus* (Huber et al., 1998).

Based on these published lipid profiles, the fatty acids in PS type mats can be explained as resulting mainly from a combination of *Aquificales* and *Thermus*-like organisms (Fig. 6). The abundance of 20:1 FA is strongly correlated with the abundance of *Aquificales* 16S sequences across all spring types ( $r = 0.76$ ), further supporting that attribution. Of course, contributions from other uncharacterized organisms cannot be entirely ruled out. Some streamer samples contained small amounts of odd-chain and cy-19 FA, tentatively attributed to *Chloroflexus*- and *Chlorobium*-like organisms (see below). This is supported by the small

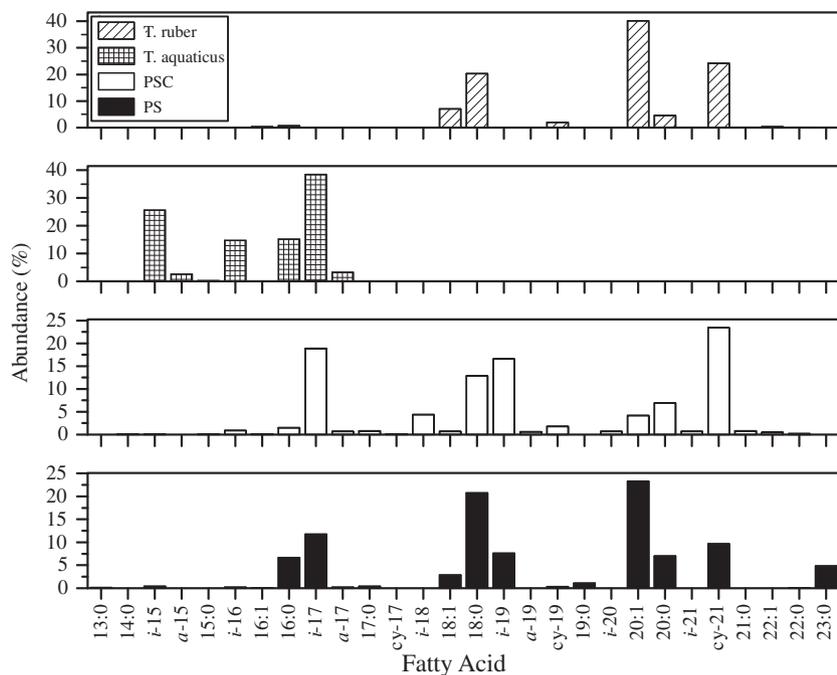


Fig. 6. Relative abundances of FA produced by *Thermocrinus ruber* (A) (Jahnke et al., 2001), *Thermus aquaticus* grown at 70 °C (B) (Nordstrom, 1993), and pink streamer communities examined by Jahnke et al. (2001) (C) and this study (D). The distribution of fatty acids in the environmental samples can largely be explained as a combination of the upper two diagrams.

amount of Chloroflexi DNA in the streamer samples (Fig. 2). These lipid contributions are thus in accord with previous phylogenetic studies on the composition of pink streamer communities (Meyer-Dombard et al., 2005), and with our own sequencing data.

The average D/H fractionations between fatty acids and source water for PS type mats are shown in Fig. 7a, together with their likely source organisms. Data for *i*-19 FA was not available because it partially co-eluted with the internal standard. Fractionations span a very large

range and exhibit an apparently systematic increase with chain length. Lipids that are diagnostic of the chemoautotrophic *Aquificales* (20:0, 20:1, *cy*-21) exhibit very strong D-depletions, whereas those attributable to heterotrophic *Thermus* (*i*-15, *i*-16, *i*-17) are D-enriched. Fatty acids of intermediate chain length, particularly 18:0, 18:X, and *cy*-19, have isotopic compositions between the two end members. It is unclear whether they derive solely from *Aquificales*, from other (possibly photosynthetic) bacteria, or perhaps have mixed sources. The latter is quite likely

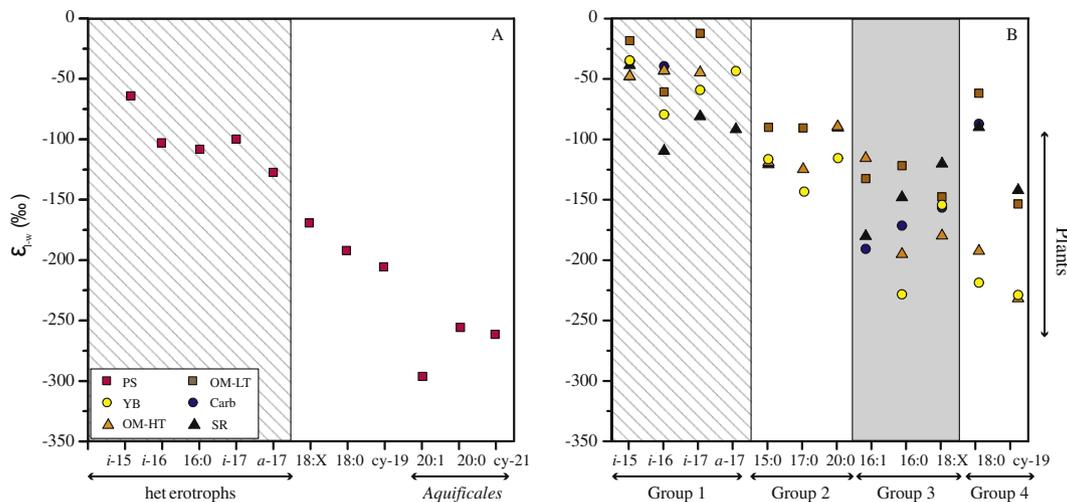


Fig. 7. Average D/H fractionations between fatty acids and spring waters in PS (A) and YB, OM-HT, OM-LT, Carb, and SR (B) type mats. The typical range of fractionations in higher plants is shown to the right for reference. Compounds are ordered along the x-axis to group FAs from apparently similar sources, and the specific groups (1–4) are discussed in the text.

for the common 18:0 and 18:X fatty acids. It is thus possible that the trend of decreasing  $\delta D$  value with FA chain length does not represent a biosynthetic feature per se, but rather reflects the varying contributions of different organisms. On the other hand, this trend is one of the most persistent features of our dataset, and so could conceivably be related to lipid biosynthesis. It is in the opposite direction of the correlation observed by Chikaraishi et al. (2004) for FA in marine macroalgae.

These trends in fatty acid  $\delta D$  values are broadly consistent with the conclusions of Zhang et al. (2009) about the influence of metabolism on D/H fractionations. At the extreme temperatures where the PS communities thrive, the base of the food chain is supported by the chemoautotrophic *Aquificales*. While these organisms have been previously shown to grow on  $H_2$ , elemental S, or formate, their actual growth substrate in this environment has not been determined. Regardless, any of these substrates would be predicted to yield large D-depletions in lipids (Valentine et al., 2004; Zhang et al., 2009), consistent with our data. Similarly, the relative D-enrichment observed in biomarker lipids from heterotrophic *Thermus* in these samples is also in accord with the work on cultured isolates, where aerobic heterotrophy consistently produced the most D-enriched fatty acids (Zhang et al., 2009).

## 5.2. Photosynthetic mats

Photosynthetic mat communities are significantly more diverse than the chemolithoautotrophic PS type mats making precise identification of lipid sources more difficult. In general, the phototrophic mats of LGB type runoff channels are composed of cyanobacteria, anoxygenic phototrophs (*Chloroflexus* and *Chlorobi*), and various heterotrophs. Our 16S rRNA gene sequence data support these distinctions with 21–82% of sequences belonging to cyanobacteria, 20–27% (except YB which is low) belonging to Chloroflexi, and much of the remainder attributable to heterotrophic bacteria. For clarity we separate the discussion of the lipids from these samples into four groups. Group 1 consists of D-enriched, methyl-branched FA characteristic of heterotrophic bacteria. Group 2 comprises 15:0, 17:0, and 20:0 FA with fractionations clustering tightly around  $-100\%$  to  $-125\%$ , suggesting a common origin. Group 3 includes 16:0, 16:1, 18:X and shows a significantly larger range in isotopic composition, with  $\epsilon$  values ranging from  $-125\%$  to  $-225\%$ . The fourth group comprises only cy-19 and 18:0 but is remarkable because these compounds appear to have two very different sources in various mat types.

Long-chain ( $C_{22}$ – $C_{28}$ ) FAs were also present in relative abundance in SR type springs, though were rare in other samples. Possible sources for these compounds include windblown leaf waxes, fungi, and unusual bacterial products. We regard an *in situ* source as more plausible given the lack of mechanism for concentrating windblown contributions in these samples relative to all others. The abundances of these compounds show strong covariation with *Thermodesulfobacteria* and proteobacterial 16S rRNA gene sequence abundance, and so might originate in those

groups. However, due to uncertainty about their origins, we do not consider these compounds further.

### 5.2.1. Group 1: Heterotrophs

The methyl-branched FA in our dataset form a coherent group based on  $\delta D$  values (Fig. 7B) as well as relative abundance between mat types (Fig. 3). These compounds are D-enriched compared both to other FA in the same samples, and to the same FA in PS type samples (Fig. 7A). The average  $\delta D$  values for these FA are similar between the different mat-type groups, although there is a slight apparent enrichment of OM-LT samples over YB and SR samples. Among photosynthetic mats, these lipids are most abundant in the OM-HT and OM-LT mat types although they are still present at low abundance in Carb and SR mats.

As discussed above, thermophilic aerobic heterotrophs such as *Thermus* are known to produce large abundances of branched fatty acids, and so are the presumed main sources of these lipids. However, both the isotopic variability of the branched FA and the greater phylogenetic diversity of the OM-HT and OM-LT samples suggest additional heterotrophic sources in these samples. Based on the phylogenetic data, one possible source of these lipids are the Acidobacteria. *Acidobacterium capsulatum* was shown to produce large amounts (>50% of total fatty acid) of *i*-15:0 (Kishimoto et al., 1991). This is supported by the observation that in PS samples, *i*-17:0 is more abundant than *i*-15:0, whereas in samples from photosynthetic mats *i*-15:0 is more abundant. The cultured strains of *Thermus* produce both FA but with more *i*-17:0 than *i*-15:0. On the other hand, most of the Acidobacterial 16S rRNA gene sequences from OM-HT and OM-LT are most closely related to the photoheterotrophic *Candidatus Chloracidobacteria thermophilum* (Bryant et al., 2007). With no published fatty acid production profiles for this newly isolated organism, we are unable to determine if it is responsible for the *i*-15:0 FA. Our 16S data also reveal large numbers of *Deinococcus-Thermus* in YB and to a lesser extent OM-HT mats. Carb samples did not contain large amounts of either phyla and instead the heterotrophic communities are largely Thermotogae and Proteobacteria.

The relative D enrichments and significant isotopic variability of the branched FAs are consistent with their derivation from a mixed community of heterotrophs (Zhang et al., 2009). The D enrichment is particularly strong in OM-HT and OM-LT type mats where typical fractionations are around  $-50\%$ , whereas YB and SR type mats are typically somewhat less D-enriched (except for *i*-15:0 FA).

### 5.2.2. Group 2: Chloroflexus

The second group of lipids includes 15:0, 17:0, and 20:0 FA and exhibits relatively little isotopic variance with mean  $\delta D$  values near  $-100\%$ . Three lines of evidence suggest that *Chloroflexus*-like organisms are the primary source for 15:0 and 17:0 FA. First, cultured representatives of the genus *Chloroflexus* are known to produce both 17:0 and 19:0 (along with other fatty acids described below; Kenyon and Gray, 1974), whereas odd-chain FA are not commonly found in cyanobacteria. The production of 15:0 FA by

Chloroflexi has not been previously described, but seems likely based on the presence of 17:0 and 19:0 FA. Second, odd-chain FA are most abundant in the OM-HT and OM-LT mats, where organisms from the phylum Chloroflexi account for roughly one-quarter of the 16S rRNA gene sequences (Fig. 2), making them one of the two most abundant organisms in this mat type. Moreover, the abundances of 15:0 and 17:0 FA are well correlated with each other ( $r = 0.63$ ) and with the abundances of Chloroflexi 16S rRNA gene sequences ( $r = 0.74$  and  $0.50$ , respectively) in our samples. Third, the  $\delta D$  values of the Group 2 lipids are similar to each other but different from those of other lipids (such as 16:1 and 18:X) that can likely be attributed at least in part to cyanobacteria (see below), the other abundant group of organisms in these mats.

Based on this evidence, we suggest that 15:0 and 17:0 FA are dominantly produced by green non-sulfur bacteria from the phylum Chloroflexi. The origin(s) of 20:0 FA are less clear. *Chloroflexus* is known to produce 20:0 as a minor fatty acid (Kenyon and Gray, 1974), but the abundances of 20:0 FA and Chloroflexi 16S rRNA gene sequences are inversely correlated ( $r = -0.40$ ), suggesting that some other source is probable. Nevertheless, the isotopic compositions of 20:0 are virtually identical to those of 15:0 and 17:0.

One of the more interesting aspects of these lipids is that – for a given mat type – the putative Chloroflexi lipids appear to be D-enriched by 25–50‰ relative to those produced wholly or partially by cyanobacteria (see Group 3 below). This trend is also supported by the systematic enrichment of OM-LT lipids over those in Carb and YB, given that OM-LT mats are rich in *Chloroflexus* compared to the other two. Zhang et al. (2009) observed a relative D-enrichment of the lipids of *Rhodospseudomonas palustris* during photoheterotrophic vs photoautotrophic growth. This is potentially significant given that culture-based studies reveal *Chloroflexus* commonly employs photoheterotrophic metabolism under anaerobic conditions (Pierson and Castenholz, 1974), as well as previous C-isotopic work suggesting a photoheterotrophic metabolism for *Chloroflexus* in these mat communities (van der Meer et al., 2007). However, the signal may be complicated by increased inputs of heterotroph lipids to OM-LT that may also serve to enrich the average isotopic composition.

### 5.2.3. Group 3: Mixed phototrophs

Unsaturated C<sub>16</sub> and C<sub>18</sub> FA make up approximately half of the fatty acids from OM-LT, Carb, and SR mat types, but <5% of the fatty acids in OM-HT mats. This is the dominant compositional difference between OM-LT and OM-HT mat types (Figs. 3 and 4). These lipids are grouped with 16:0 FA based on isotopic composition, though their patterns of abundance are only slightly correlated ( $r = 0.42$  for 16:0 vs 16:1 FA,  $0.17$  vs 18:1 FA, and  $0.34$  vs 18:2 FA). This group likely represents contributions from a number of sources, with the Chloroflexi and cyanobacteria being the most abundant. Cultured *Chloroflexus* species are known to produce significant quantities of 16:0, 18:0, and 18:1 FA, with minor quantities of other fatty acids including 16:1, 18:2, and 20:1 (Kenyon and Gray, 1974). Cultured thermophilic *Synechococcus* strains have

been shown to produce mostly 16:0, 16:1, and 18:1 fatty acids (Kenyon, 1972). This distribution is also consistent with the sole report of fatty acid distributions for the filamentous cyanobacterium *Phormidium* (Jahnke et al., 2004). This genus has been implicated in forming the coniform orange mat morphologies in low temperature mats similar to samples OS08-3 and OS09-4 (Jahnke et al., 2004). Although the C<sub>16</sub> and C<sub>18</sub> fatty acids potentially have very diverse sources, comparison between lipid abundance and sequence data shows cyanobacterial abundance to be well correlated ( $r = 0.67$ ) with 16:0 FA abundance, moderately correlated with 16:1 and 18:2 FA ( $r = 0.38$  and  $0.35$ , respectively), and uncorrelated ( $r = 0.01$ ) with 18:1 FA. Thus, cyanobacteria are likely a prominent – but not sole – source for these lipids.

The relatively low abundance of unsaturated fatty acids in YB and OM-HT samples is hard to reconcile with the large numbers of cyanobacterial sequences in these mats. While homeoviscous adaptation to growth at high temperature is one possible explanation for these composition trends, it is not supported by the isotopic data. However, cyanobacterial diversity at the genus level indicates distinctly different populations in YB and OM-HT mats compared to all other photosynthetic mat types (Fig. 8). We suggest that this diversity is contributing to the differences in production of unsaturated FA by cyanobacteria in different mat types.

The isotopic compositions of Group 3 lipids exhibit two main characteristics. First, they are highly variable, typically spanning a ~100‰ range of  $\delta D$  values. We attribute this primarily to the fact that they represent mixtures of products from multiple and different, although probably still photosynthetic, sources in each spring, and that these multiple sources apparently express somewhat different fractionations. Second, the isotopic compositions of these fatty acids are completely overlapping with the range commonly observed in plants and algae (Fig. 7). The mean value for this group in our study (–151‰) is very similar to the mean for C<sub>16</sub> and C<sub>18</sub> fatty acids in Santa Barbara Basin sediments (Li et al., 2009) where they are believed to derive from marine algae. Thus we infer that the fractionations exhibited by photoautotrophic thermophiles are not inherently different from those in higher plants and algae despite their relatively extreme environmental setting.

### 5.2.4. Group 4: 18:0 and cy-19

Lipid Group 4 is comprised of 18:0 and cy-19 FA. These compounds are united by their unusual, bimodal distribution of D/H fractionations where the generally low-temperature Carb, OM-LT, and SR samples yield relatively small fractionations (more D-enriched lipids) compared to the high-temperature PS, OM-HT, and YB samples that exhibit larger fractionations (more D-depleted lipids).

While 18:0 FA is present in relatively high abundance throughout most samples, and has many possible sources, cy-19 FA is abundant only in YB and OM-HT samples where it contributes between 3% and 20% of total FA abundance. Despite its correlation with the high-temperature mat samples, cy-19 is not yet known in the published literature to be produced by *Synechococcus* or *Chloroflexus*.

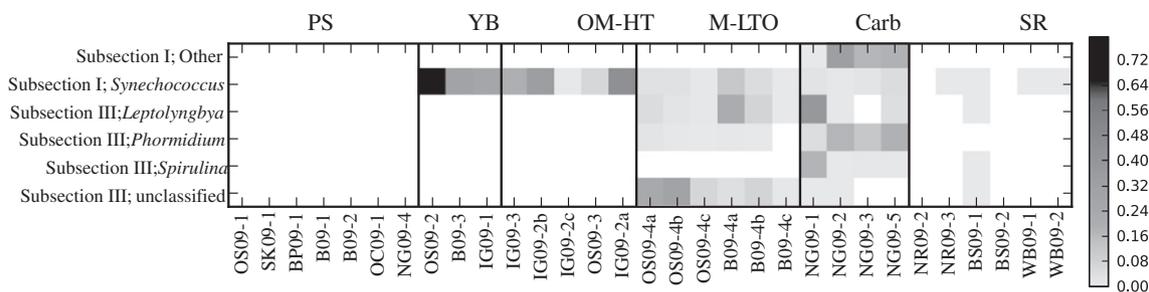


Fig. 8. Distribution of 16S rRNA gene sequences for selected phyla and subdivisions of the cyanobacteria in different mat types. Plotted values are the fractional abundance of sequences in that group relative to all sequences in the sample.

While many bacteria are capable of producing FA containing cyclopropyl rings (Grogan and Cronan, 1997), most are not found in the hydrothermal environment. An exception is the thermophilic green-sulfur bacterium *Chlorobium tepidum* (Ward et al., 1998). Our sequencing libraries confirm the presence of Chlorobi in many of the samples that contain cy-19, however correlation between the abundance of Chlorobi sequences and cy-19 FA is low ( $r = 0.11$ ). *Thermocrinus ruber* (an Aquificales) is also known to produce small amounts of cy-19 FA, and is also uncorrelated with cy-19 abundance ( $r = -0.19$ ). Surprisingly, the abundance of Candidate division OP10 was well correlated to cy-19 ( $r = 0.56$ ), and could account for the high concentrations found in the YB and OM-HT. Additional potential sources of these lipids are the alphaproteobacterial genera *Acidiphilium* and *Acidomonas* that have been shown to produce cy-19 up to 20% of total fatty acid abundance (Kishimoto et al., 1991).

The magnitude of D/H fractionations exhibited in both 18:0 and cy-19 FA are near  $-200\text{‰}$  in high temperature mats (PS, YB and OM-HT), marking them as likely photoautotroph or perhaps even chemoautotroph products (i.e., compare to Group 3 and *Aquificales* in Fig. 7). This is interesting given that neither OP10 nor the mentioned alphaproteobacteria are known to be photo- or chemo-autotrophs. However, in the case of OP10 only a single isolate has so far been described, and is a thermophilic aerobic heterotroph (Stott, 2008). These observations lead to three competing hypotheses regarding the origins of 18:0 and cy-19 in high-temperature mats: (1) they are produced exclusively by Aquificales in all high-temperature mat types, but their  $\delta\text{D}$  values are inexplicably different from the other FA attributed to this group. This explanation is not consistent with the higher abundance of cy-19 in YB and OM-HT mat types. (2) They are produced by previously undescribed species of OP10 or alphaproteobacteria with photoautotrophic or chemoautotrophic metabolism and hence large D/H fractionations. A potential problem with this explanation is that the putative source organisms are not readily apparent in 16S rRNA gene sequences from PS type mats. (3) They represent a mixture of sources, including *Aquificales*, Chlorobi, OP10, and alphaproteobacteria, and their average isotopic compositions coincidentally arrive at roughly the same values in all mat types. The latter hypothesis is most likely for 18:0 FA, given that  $\epsilon_{1/w}$  values for individual samples span a fairly wide range ( $-116\text{‰}$  to  $-247\text{‰}$ ).

However, for cy-19 the range of fractionations is much smaller ( $-195\text{‰}$  to  $-242\text{‰}$  for the PS, YB, and OM-HT mat types), and a single source (or less diverse consortium) in all three mat types seems more likely. Our current data do not allow us to resolve this question. Nevertheless, our ability to predict that organisms in Candidate phylum OP10 might be capable of photo- or chemoautotrophy highlights the potential utility of hydrogen-isotopic data.

In OM-LT, Carb, and SR type mats, the 18:0 and cy-19 FA both have significantly more D-enriched compositions. For 18:0 the fractionations reach  $-50\text{‰}$ , suggesting that this lipid must derive almost entirely from heterotrophs in those mat types. This is somewhat surprising given that phototrophs still make up  $>50\%$  sequence abundance in Carb and OM-LT samples. For cy-19, the fractionations are closer to  $-150\text{‰}$ , suggesting either a phototrophic source (similar to 16:0 and 18:X FA) or a mixture of both D-depleted (similar to those in YB and OM-HT mats) and D-enriched (presumably heterotroph) lipids.

### 5.3. Applications of lipid D/H ratios

The preceding discussion focuses on the metabolic basis for large differences in D/H fractionations between different microbes. But regardless of mechanism, the patterns of lipid  $\delta\text{D}$  observed in YNP springs are robust and – in many cases – diagnostic of particular mat types. They may thus serve as useful ‘fingerprints’ for assessing the composition and function of ancient hydrothermal systems via organic biomarkers.

To facilitate such an analysis, we compared the abundances and hydrogen isotopic compositions of several common fatty acids as a function of mat type (Fig. 9). Fatty acids with double bonds or cyclopropyl rings were excluded because of their relatively poor preservation potential. Fig. 9 indicates several characteristics that could be usefully employed in an ancient system. For example, streamer communities are characterized by great abundance and strong D-depletion of long-chain (especially  $\text{C}_{20}$ ) fatty acids. Yellow biofilm-type mats are distinguished by abundant, strongly D-depleted 16:0 and 18:0 FA, whereas orange LGB-type mats are typified by abundant, D-enriched short-chain branched and odd-numbered FA (e.g., *i*-15:0). High- vs low-temperature orange mats seem to be differentiated by the  $\delta\text{D}$  value of 16:0, with lower-temperature mats having a more D-enriched composition. We tentatively

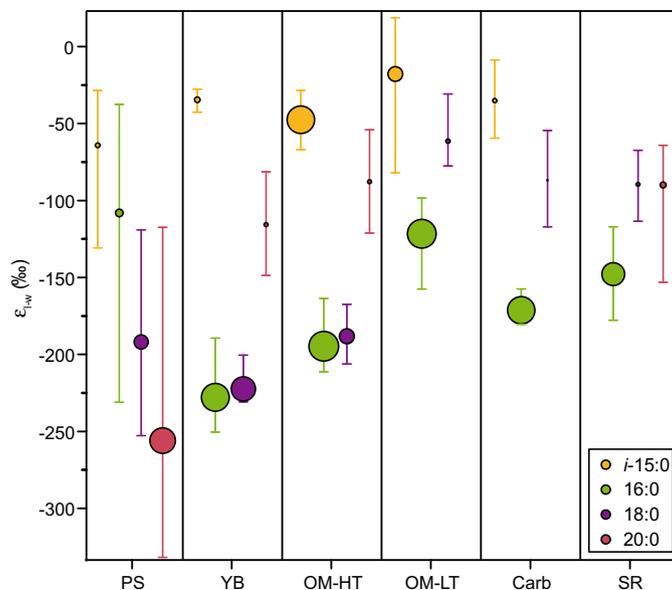


Fig. 9. Mean isotopic compositions for *i*-15:0, 16:0, 18:0, and 20:0 FAs in each mat type. The size of each symbol is proportionate to lipid abundance. The size of *i*-15 and 20:0 symbols have been exaggerated by factors of 8 and 6, respectively. Bars represent the total range of  $\delta D$  values.

interpret this signal as reflecting a more dominant heterotrophic source for 16:0 in the lower-temperature mats.

The utility of these kinds of ‘fingerprints’ will, of course, depend on whether the signals observed in YNP are ubiquitous or more regional in character. They do at least seem to be consistent across the greater Yellowstone region and in a recent report of fatty acid  $\delta D$  from Naruko Hot spring in northeastern Japan (Naraoka et al., 2010). The fidelity of H-isotopic compositions in quite warm environments over long times will also have to be investigated, as hydrogen exchange may be fairly rapid at the ambient temperatures of these systems. Nevertheless, given the large and (apparently) systematic variations in lipid D/H across YNP, there is significant potential for the use of these data as fingerprints for ancient hydrothermal environments.

A second potential use for lipid  $\delta D$  values in these hydrothermal systems is the attribution of lipids with unknown origins to particular microbes. For example, the cy-19 FA can be tentatively attributed to OP10 in YB and OM-HT type mats based on strong D-depletions, but must have other origins in Carb and OM-LT mats. Because of the large variance, lipid D/H ratios can also be quite useful for quantitative apportionment of multiple sources. For example, palmitic acid (16:0) is extremely common and can have a wide range of bacterial (and eukaryotic) sources. Based on comparisons of  $\delta D$  values in PS and OM-LT type mats, the palmitic acid appears to derive mainly from heterotrophs (e.g., *Thermus*), whereas in YB and OM-HT type mats it is almost exclusively an autotrophic product (cyanobacteria and/or *Chloroflexus*).

#### 5.4. Relevance to other environments

While this study focuses on a terrestrial hydrothermal environment, the trends observed here are likely applicable

to more moderate environments as well. Previous studies of both marine and lacustrine samples have observed very large ranges in lipid  $\delta D$  values. These included a range of  $-32\text{‰}$  to  $-348\text{‰}$  for *n*-alkyl lipids in marine sediments (Li et al., 2009),  $-73\text{‰}$  to  $-237\text{‰}$  for fatty acids extracted from marine particulate organic matter (Jones et al., 2008), and  $-166\text{‰}$  to  $-255\text{‰}$  for fatty acids from lacustrine sediments (Chikaraishi and Naraoka, 2005). The bulk of the fractionations observed in the current study fall within these ranges, although the total range is somewhat larger at  $+41\text{‰}$  to  $-374\text{‰}$ . Additionally, a trend of D-enriched heterotroph lipids relative to those of photosynthetic algae has been observed in the marine environment (Jones et al., 2008; Li et al., 2009). Similar to our findings, Jones et al. (2008) observed an inexplicable D enrichment in *i*-15:0 and 15:0 fatty acids compared to all others. Based on the results presented here, we suggest that these lipids might be produced by marine heterotrophs where the less D-enriched lipids might come from phototrophs or another metabolism with characteristically moderate isotopic fractionations. While the extreme values observed in this study are exceptional, we suggest that the hydrothermal environment may not be unusual in the production of extreme isotopic values, but rather in our ability to separate communities so that these values can be observed.

We have demonstrated that differences in the sources of common lipids can have a significant effect on their H-isotopic composition. While many of the organisms discussed in this study are specific to the hydrothermal realm, the metabolisms they practice are much more cosmopolitan. Here, subtle shifts in microbial communities were significant enough to influence the bulk signal. We suggest that over sufficient timescales, hydrogen isotopic variability of lipids from marine and lacustrine sediments may be influenced both by the water  $\delta D$  and by variations in microbial community.

## 6. CONCLUSIONS

Fatty acids from a diverse suite of microbial communities living in terrestrial hydrothermal environments show systematic variations in H-isotopic composition that appear linked to the presence of specific types of bacteria. Lipids derived from chemoautotrophic bacteria (mainly *Aquificales*) showed strong D-depletions, down to  $-374\%$  relative to growth water. In comparison, lipids derived from heterotrophic bacteria were relatively D-enriched, with fractionations relative to growth water typically only  $-50\%$  to  $-100\%$ . Fatty acids from photosynthetic bacteria exhibited intermediate fractionations similar to those found in higher plants. Lipids from *Chloroflexi* were slightly D-enriched relative to those of cyanobacteria, perhaps reflecting photoheterotrophic metabolism in the former organism. These data, based on well-characterized environmental samples, both confirm and extend the observations of Zhang et al. (2009) that the utilization of different central metabolic pathways by bacteria is recorded in the H-isotopic composition of lipids.

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## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.gca.2011.05.038](https://doi.org/10.1016/j.gca.2011.05.038).

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