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Diel metabolomics analysis of a hot spring chlorophototrophic microbial mat leads to new hypotheses of community member metabolisms

Young-Mo Kim, Shane Nowack, Millie T. Olsen, Eric D. Becraft, Jason M. Wood, Vera Thiel, Isaac Klapper, Michael Kühl, James K. Fredrickson, Donald A. Bryant, David M. Ward and Thomas O. Metz

Dynamic environmental factors such as light, nutrients, salt, and temperature continuously affect chlorophototrophic microbial mats, requiring adaptive and acclimative responses to stabilize composition and function. Quantitative metabolomics analysis can provide insights into metabolite dynamics for understanding community response to such changing environmental conditions. In this study, we quantified volatile organic acids, polar metabolites (amino acids, glycolytic and citric acid cycle intermediates, nucleobases, nucleosides, and sugars), wax esters, and polyhydroxyalkanoates, resulting in the identification of 104 metabolites and related molecules in thermal chlorophototrophic microbial mat cores collected over a diel cycle in Mushroom Spring, Yellowstone National Park. A limited number of predominant taxa inhabit this community and their functional potentials have been previously identified through metagenomic and metatranscriptomic analyses and in situ metabolisms, and metabolic interactions among these taxa have been hypothesized. Our metabolomics results confirmed the diel cycling of photorespiration (e.g., glycolate) and fermentation (e.g., acetate, propionate, and lactate) products, the carbon storage polymers polyhydroxyalkanoates, and dissolved gasses (e.g., H₂ and CO₂) in the waters overlying the mat, which were hypothesized to occur in major mat chlorophototrophic community members. In addition, we have formulated the following new hypotheses: (1) the morning hours are a time of biosynthesis of amino acids, DNA, and RNA; (2) photo-inhibited cells may also produce lactate via fermentation as an alternate metabolism; (3) glycolate and lactate are exchanged among Synechococcus and Roseiflexus spp.; and (4) fluctuations in many metabolite pools (e.g., wax esters) at different times of day result from species found at different depths within the mat responding to temporal differences in their niches.

Keywords: gas chromatography-mass spectrometry, metabolomics, microbial mats, polyhydroxyalkanoates, Roseiflexus, Synechococcus, wax esters
Introduction

Microbial communities inhabiting extreme environments in Yellowstone National Park (YNP) have been investigated for more than half a century (Brock, 1972, 1998). In particular, chlorophototrophic (i.e., chlorophyll-based phototrophs) microbial mat communities present in the effluent channels of Octopus Spring and Mushroom Spring within the Lower Geyser Basin have been intensively studied (Brock, 1978; Ward et al., 2012). As a result of metagenomic (Klatt et al., 2011) and metatranscriptomic (Liu et al., 2011, 2012; Klatt et al., 2013) analyses, an objective and more complete understanding of the major taxa inhabiting the upper 2 mm of the 60–65°C regions of the Mushroom Spring mat, in terms of their contribution to the gene pool and their functional potentials, has emerged (Table 1). Cyanobacteria from the genus Synechococcus are the predominant primary producers driving metabolism in these communities via oxygenic photosynthesis (Klatt et al., 2011; Liu et al., 2011). Synechococcus spp. fix CO₂ and synthesize, and possibly excrete, metabolites that are then consumed by (photo)-heterotrophic members of the community, including several Chloroflexi, especially Roseiflexus spp. (Table 1), which were formerly thought to be exclusively photoheterotrophs. However, genomics, metagenomics, and metatranscriptomics analyses have revealed that Roseiflexus spp. also have the genetic potential to fix CO₂ (Klatt et al., 2007; Van Der Meer et al., 2010). Collectively, cyanobacteria and Roseiflexus spp. account for the majority of the biomass of the upper 0–2 mm portion of the mat community (Table 1), and thus they should have the greatest influence on the metabolites in this portion of the mat. Two additional Chloroflexi, Chloroflexus spp. and a novel, apparently phototrophic, Anaerolineae-like taxon, and two aerobic/anaerobic, anoxygenic photoheterotrophs, Chloracidobacterium thermophilum (Bryant et al., 2007; Garcia Costas et al., 2012) and “Candidatus Thermochlorobacter aerophilum” (Liu et al., 2012), also occur in the upper photic layer of the mat. Non-chlorophyllous, heterotrophic bacteria have been detected in the upper mat community, but they are much less abundant (Liu et al., 2011), and are unlikely to strongly influence mat metabolites. Heterotrophs, together with the photoheterotrophic and photomixotrophic community members, can be considered potential consumers of metabolites produced by cyanobacteria and possibly other mat inhabitants.

Studies performed by Konopka (1992) and Nold and Ward (1996) showed that CO₂-fixing chlorophototrophic community members undergo diel metabolic switching. Recently, metatranscriptomics analyses have provided a comprehensive view of diel transcription patterns in predominant mat taxa (Liu et al., 2011, 2012; Klatt et al., 2013), and have led to new hypotheses about Synechococcus spp. and Roseiflexus spp. metabolisms. For instance, Synechococcus spp. express genes involved in photosynthesis diurnally and have the genetic potential to produce glycogen, which they accumulate during the day (Van Der Meer et al., 2007). Extremely high irradiance during the day leads to O₂ supersaturation combined with CO₂ depletion (as indicated by elevated pH), causing production and possible accumulation of toxic levels of glycogen, a common product of photorespiration ( Bateson and Ward, 1988). Synechococcus spp. also have the genetic potential to conduct fermentation with production of lactate, acetate, ethanol and formate (Bhaya et al., 2007). When photosynthesis declines in the evening, O₂ uptake by aerobically respiring community members exceeds O₂ production and the mat becomes anoxic, except within the upper ~150 µm. Fermentation genes, as well as genes involved in N₂ fixation, are expressed at this time, consistent with measured N₂ fixation driven by fermentative metabolism at night and by light in the early morning (Steunou et al., 2006, 2008).

Diurnal transcription patterns of the genes involved in CO₂ fixation suggested that Roseiflexus spp. can conduct photomixotrophic metabolism, in which they combine CO₂ fixation with assimilation of low-molecular weight organic compounds, possibly produced by Synechococcus spp. (Klatt et al., 2013). Other transcription patterns suggested that Roseiflexus spp. construct and decompose intracellular polymers, including glycogen, polyhydroxyalkanoates (PHAs) and possibly wax esters (genomic and metagenomic analyses show that Synechococcus spp. lack the ability to synthesize PHAs (Bhaya et al., 2007; Klatt et al., 2011). Because external reductants such as H₂ and H₂S are not present in the oxic mid-day photic layers of the mat, it was further hypothesized that utilization of these intracellular storage polymers may provide reductants and organic intermediates for photomixotrophic CO₂ incorporation during the day. As suggested by Bauld and Brock (1973), organic compounds produced by CO₂-fixing community members might be cross-fed to (photo)-heterotrophic or mixotrophic mat community members. Little is known about metabolite exchange in the mat, although it has been shown that acetate, butyrate, ethanol, glycolate, lactate, and propionate are photoassimilated into filamentous community members (Anderson et al., 1987; Bateson and Ward, 1988).

Metabolomics has been successfully applied to characterize the metabolic responses of diverse organisms, both qualitatively and quantitatively, under various growth conditions (Koek et al., 2011). These measurements are increasingly used to study microbial communities (Mosier et al., 2013; Xie et al., 2013). In the current study, a combination of untargeted and targeted metabolomics analyses was performed to quantify five groups of metabolites. Volatile organic acids, polar metabolites, wax esters, and PHAs were measured in the mat, while selected dissolved gases and inorganic ions were quantified in the overflowing water. Measurements of acetate, propionate, and glycolate in the mat, as well as H₂, CO₂, and CH₄ in the water, were performed to test hypotheses regarding the production of these products during different parts of the diel cycle. Similarly, targeted measurements of wax esters and PHAs were performed to characterize these molecules as intracellular carbon and energy storage polymers that should undergo diel cycling if photomixotrophy occurs as hypothesized in Roseiflexus spp. (Klatt et al., 2013).

Finally, untargeted metabolomics measurements were performed to identify and quantify polar metabolites extracted from the mat samples (intracellular) and interstitial fluids (extracellular) to identify additional metabolites that are changing during the diel cycle and that may be available for possible metabolic exchange among mat community members, respectively. In addition to
TABLE 1 | Major members of Mushroom Spring mat photosynthetic layer and their metabolisms.

| Organism type     | % of rRNA<sup>a</sup> | Mode of primary carbon assimilation in culture<sup>b</sup> | Hypothesized mode of primary carbon assimilation in mat<sup>c</sup> | Potential carbonaceous substrates consumed based on evidence of<sup>d</sup> | Potential metabolites inferred from genomic/metagenomic analyses | Storage polymers inferred from genomic/metagenomic analyses |
|-------------------|------------------------|----------------------------------------------------------|-------------------------------------------------|-----------------------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
|                    |                        |                                                          | Transporter genes                | Pathway genes           | Use by representative isolates<sup>e</sup>                       | From photosynthesis | From fermentation |                           |                        |                                     |
| Synchococcus spp. | 27.0                   | autotrophy                                              | autotrophy                        | CO₂, urea, carbohydrates, amino acids | CO₂/bicarbonate, urea, carbohydrates | CO₂/bicarbonate, glycolate, glyoxyylate, oxalate, glycerate, formate, glycine, serine<sup>f</sup> | lactate, formate, acetate, ethanol | glycanogen, cyanophycin |
| Roseiflexus spp.  | 33.4                   | heterotrophy                                            | mixotrophy                        | lactate, fructose, citrate, ornithine, amino acids, peptides, putrescine, spermidine, glucosamine, amino glycolate, manniol, trehalose, acetylmuramate, diacetyl chitobiose, melibiose, dimethoxytoluene | CO₂/bicarbonate, glycolate, lactate, citrate, fructose, amino acids/peptides, ornithine, putrescine, spermidine, glucosamine | fructose, glucose, acetate, pyruvate, lactate, propionate, succinate, malate<sup>f</sup> | lactate, acetate, propionate | glycanogen, PHAs, wax esters |
| Chloracidobacterium spp. | 10.0                  | heterotrophy                                            | heterotrophy                       | oligopeptides, spermidine, putrescine, glutamate, ribose, branched chain amino acids, mannose | mannose, amino acids, glutamate | 2-oxo-glutarate, amino acids<sup>h</sup> | lactate, alcohols | glycanogen |
| Candidatus Thermochlorobacter spp. | 6.0                   | heterotrophy                                            | heterotrophy                       | oligopeptides, sugars, oxalate, formate, maltose, acetate, methionine, bicarbonate, glutamate, hydroxymethyl-pyrimidine | amino acids, carbohydrates, formate, acetate, methionine, glutamate, hydroxymethyl-pyrimidine | lactate, acetate, CO₂ | glycanogen |
| Chroflexus spp.   | 2.5                    | autotrophy and heterotrophy<sup>i</sup>                 | mixotrophy                        | oligopeptides, branched chain amino acids, glutamate, maltose, hydroxymethyl-pyrimidine, ribose, spermidine, putrescine, acetylglicosamine, glycerol-3-phosphate, rhamnose, arabinose | CO₂, acetate, glycolate, glyoxyylate, lactate, glucose, fructose, sucrose, mannose, lactose, galactose, maltose, maltodextrin, trehalose | CO₂ | lactate | glycanogen, PHAs, wax esters |

(Continued)
evaluating the above hypothesized metabolisms, these data were collectively used to formulate new hypotheses of community metabolisms and metabolite exchange.

**Materials and Methods**

**Chemicals and Materials**

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. A mixture of fatty acid methyl esters (FAMEs; C8–C28) dissolved in hexane was prepared for use as a retention index standard. PHA polymers were purchased from Sigma-Aldrich or were provided as a gift by Prof. Alexander Steinbüchel at University of Münster, Germany. Deionized and purified water was used to prepare buffer and standard solutions (Milli-Q System Advantage A10, Merck Millipore, Billerica, MA). All solvents and chemicals were obtained in the highest purity available.

**Sample Collection**

**Mat Samples**

For whole-mat (i.e., intracellular and extracellular metabolites combined) analyses of volatile organic acids, polar metabolites, wax esters, and PHAs, core samples were collected in from a microbial mat in the effluent channel of Mushroom Spring in the Lower Geyser Basin (YNP, WY) where the temperature of water in the sampling area varied from 58 to 62°C during the diel cycle. A cork-borer with a 8 mm inner diameter was used to collect the same volume of mat sample, and a razor blade was used to separate the top 5 mm of each mat core such that the analyses were focused on the top green phototrophic layer and the red-orange undermat layers in the zone that contain most of the biological activity (Ward et al., 1987) (Figure 1). The samples were transferred to microcentrifuge tubes and immediately frozen in a Dewar containing liquid nitrogen. Mat samples were collected in triplicate at 14 time points between 13:30 h on September 21, 2012 and 11:00 h the following day.

For analyses of extracellular metabolites, mat core samples were collected at 03:00, 09:00, 13:00, and 19:00 h (n = 6, each) during the same diel cycle. Once collected, three core samples from each time point were immediately frozen as described above for use as unrinsed controls, while the remaining three samples were transferred to 15 mL Falcon tubes containing 1 mL of spring water that had been filtered through a 0.2-µm filter. Since the 68°C source pool of Mushroom Spring is lined with microbial mat, in order to avoid metabolites that might have diffused from the mat to overflowing water, we used water from the source pool (92°C) of chemically similar Octopus Spring (Papke et al., 2003), which is well upstream of photosynthetic mats (maximum range of 72–74°C). This water did not contain significant levels of any of the organic compounds detected in this study. The re-suspended mat cores were then quickly disrupted onsite by vigorous shaking, and the biomass and rinse water were then immediately separated using a centrifuge (16,025 × g for 5 min). The supernatant was transferred to a clean microcentrifuge tube and the rinsed biomass and the rinse water samples were immediately frozen with liquid nitrogen. All samples were stored at −80°C until further processing. This process did not
result in release of metabolites identified in analyses of biomass, suggesting that it did not cause leakage of constituents from intact cells.

Water Samples
Duplicate water samples were collected at 03:00, 07:00, 09:00, 11:00, 13:00, 15:00, 17:00, 19:00, and 23:00 h, during the same diel cycle. The temperature at the collection site was approximately 60°C in the main effluent channel. Channel water was filtered through 0.4 µm HTTP Isopore™ polycarbonate membrane filters, collected in 160-ml serum bottles, and then after several exchanges of the serum-bottle volume, sealed with butyl-acetate stoppers (without head-space).

Metabolite Extraction
A single metabolite extraction protocol was used for the analysis of the various classes of metabolites described herein. Frozen mats were thawed at room temperature and 100 µL each of Nanopure™ water and zirconia-silica beads (0.1 mm size; Biospec Products; Bartlesville, OK) were added, respectively, to the samples and vigorously vortexed for 2 min. This bead-beating process was repeated after the samples were maintained at room temperature for 5 min. A mixture of chloroform/methanol (400 µL; 2:1, v/v) spiked with 20 µg of 13C-labeled acetate (Sigma-Aldrich catalog number 282022-250) was added to each disrupted mat sample, and the mixtures were repeatedly vortexed to ensure thorough mixing. The samples were centrifuged at 15,000 × g for 5 min at 4°C to separate aqueous and organic layers from precipitated proteins.

For analysis of acetate and propionate, aliquots (50 µL) of the aqueous layer from each extract were transferred to glass vials equipped with glass inserts for direct GC-MS analysis without chemical derivatization. Because acetate and propionate are volatile molecules, all samples were immediately analyzed after extraction.

For untargeted analysis of polar metabolites, aliquots (150 µL) of the remaining aqueous layer from each sample extract were transferred to glass vials and completely dried in vacuo. The dried extracts were stored at -20°C until chemical derivatization.

For analysis of wax esters, aliquots (200 µL) of the organic layer from each extract were analyzed directly using GC-MS without chemical derivatization.

For analysis of PHAs, the remaining organic layer from each extract was combined with the corresponding protein pellet, and the combined extract and pellet were completely dried in vacuo. The samples were hydrolyzed using a modification of the method reported by Lageveen et al. (1988). Briefly, the dried pellets were dissolved in methanol containing 15% H2SO4 (v/v) and incubated at 100°C for 15 h. The resulting PHA monomers were extracted with chloroform and analyzed by GC-MS.

Metabolomics Analyses
An Agilent 7890A gas chromatograph coupled with a single quadrupole 5975C mass spectrometer (Agilent Technologies, Inc.) was used for all analyses. Samples were analyzed in duplicate by optimized GC-MS methods, which varied according to the classes of molecular targets as described below.

Acetate and propionate were quantified in a targeted fashion using 13C-labeled acetate as an internal standard. Briefly, mixtures of unlabeled acetate and propionate at different concentrations were combined with constant amounts of 13C-labeled acetate in order to construct calibration curves. 13C-labeled acetate was then spiked into microbial mat lysates prior to extraction of metabolites, and the measured ratios of unlabeled acetate and propionate to labeled internal standard were used to accurately quantify the target molecules. A polar column (HP-FFAP; 30 m × 0.250 mm × 0.250 µm; Agilent Technologies, Santa Clara) was used. The temperature of the GC inlet was maintained at 200°C, and samples (1 µL) were injected in splitless mode with a helium gas flow rate of 1.0 mL min⁻¹. A temperature gradient from 40 to 200°C over 20 min was used, and data were collected over the mass range 20–300 m/z. To reduce any carry over arising from the direct injection of the aqueous layers (a mixture of methanol and water) from the metabolite extraction procedure, pure methanol blanks were analyzed between each sample.

For untargeted analysis of polar metabolites, extracted metabolites in the dried aqueous layers were chemically derivatized to trimethylsilyl esters as previously described (Kim et al., 2013). Metabolite extracts were dried in vacuo again to remove any residual moisture. To protect carbonyl groups and reduce the number of tautomeric isomers, methoxyamine (20 µL of a 30 mg mL⁻¹ stock in pyridine) was added to each sample, followed by incubation at 37°C with shaking for 90 min. To derivatize hydroxyl and amine groups to trimethylsilylated (TMS)
forms, N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) (80 µL) was added to each vial, followed by incubation at 37°C with shaking for 30 min. The samples were allowed to cool to room temperature and analyzed on the same day. A HP-5MS column (30 m × 0.25 mm × 0.25 µm; Agilent Technologies) was used for untargeted analyses. Samples (1 µL) were injected in splitless mode, and the helium gas flow rate was determined by the Agilent Retention Time Locking function based on analysis of deuterated myristic acid (Agilent Technologies, Santa Clara, CA). The injection port temperature was held at 250°C throughout the analysis. The GC oven was held at 60°C for 1 min after injection, and the temperature was then increased to 325°C by 10°C/min, followed by a 5 min hold at 325°C. Data were collected over the mass range 50–550 m/z. A mixture of FAMEs (C8–C28) was analyzed together with the samples for retention index alignment purposes during subsequent data analysis.

For analysis of wax esters, aliquots of the organic layer from the metabolite extracts were directly injected into the GC-MS. For analysis of PHA monomers, the acid-hydrolyzed samples were analyzed. Wax esters and PHA monomers were chromatographically separated using the same HP-5MS column as described above. Samples (1 µL) were injected in splitless mode. The GC oven was held at 60°C for 5 (wax esters) or 10 (PHA monomers) min after injection, and the temperature was then increased to 325°C by 10°C/min, followed by a 1 (PHA monomers) or 5 (wax esters) min hold at 325°C. The helium gas flow rate was 1.0 mL/min and the injection port temperature was held at 250°C throughout the analysis. Data were collected over the mass range 50–600 (PHA monomers) or 50–700 (wax esters) m/z.

All GC-MS raw data will be made available via the MetaboLights metabolomics data repository (http://www.ebi.ac.uk/metabolights/) under study identifier MTBLS187.

Metabolomics Data Analysis

The relative amounts of acetate and propionate in the mat samples were quantified by isotope dilution mass spectrometry. Standard curves for acetate and propionate were constructed as described above, and the integrated peak areas of acetate, propionate, and 13C-acetate in mat samples were determined using the corresponding extracted ion chromatograms (EICs; acetate, m/z 60; propionate, m/z 74; and 13C-acetate, m/z 62). The peak areas of endogenous acetate and propionate were divided by that of 13C-acetate to obtain ratios of unlabeled/labeled target molecules.

GC-MS raw data files from untargeted analyses of polar metabolites were processed using MetaboliteDetector (Hiller et al., 2009). Retention indices (RI) of detected metabolites were calculated based on the analysis of the FAME standard mixture, followed by their chromatographic alignment across all analyses after deconvolution. Metabolites were then identified by matching GC-MS features (characterized by measured retention indices and mass spectra) to an augmented version of the Agilent Fiehn Metabolomics Retention Time Locked (RTL) Library (Kind et al., 2009), which contains spectra and validated retention indices for over 700 metabolites. All metabolite identifications were manually validated to reduce deconvolution errors during automated data-processing and to eliminate false identifications. The NIST 08 GC-MS library was also used to cross-validate the spectral matching scores obtained using the Agilent library. A heat-map analysis was also carried out after z-score transformation of the obtained signal intensities and with K-means clustering (K = 5, Distance metric: Euclidean) using DanteR (Taverner et al., 2012).

For wax ester analysis, the 10 most abundant species from >30 detected and quantified in the microbial mat samples were selected based on a previous report (Dobson et al., 1988), and their abundances were determined by the EIC method described above. For PHA analysis, the monomers were also quantified using the EIC method described above. A common representative fragment ion (m/z 103) was used for quantifying both 3-hydroxybutyrate and 3-hydroxyvalerate.

Dissolved Gas Analysis

Dissolved gases (CO₂, H₂, and CH₄) were determined using closed head-space GC as described (Inskeep et al., 2005).

Solar Irradiance Analysis

The incident downwelling irradiance was logged throughout the field campaign with a LI-1400 light meter equipped with a LI-192 quantum irradiance sensor (LI-COR, Lincoln, NE).

Results

Triplicate samples were taken from a 60°C region of Mushroom Spring mat at approximately 2-h intervals over a diel cycle (Figure 1). The top 5 mm was removed for solvent extraction and separate analyses of polar and volatile aqueous metabolites, PHAs and wax esters.

Polar Metabolites in the Mat

Untargeted metabolomics analyses were performed to identify fluctuations in polar metabolites. This analysis resulted in identification of 58 metabolites that were reproducibly detected in the 42 samples over the diel cycle. The time-course abundance patterns of these 58 metabolites are shown individually in Supplemental Figure S1. K-means clustering was used to categorize these patterns of temporal changes in relative abundances, resulting in five clusters of metabolites (Table 2) that each contained metabolites sharing similar patterns of abundance fluctuation over the diel cycle (Figure 2).

Metabolites detected in Cluster A accumulated in the predawn and early morning and included 3-hydroxybutyrate and 3-hydroxyvalerate, the monomeric units of PHA. These two compounds showed similar patterns of relative abundance over the diel cycle, as well as to the monomers liberated from acid hydrolysis of intact PHA polymers (see below). Many of the metabolites of Cluster A were lowest in relative abundance in the afternoon and began to increase at 03:00 h, peaking by 09:00 to 11:00 h. Adenine, ornithine (indistinguishable from arginine during GC-MS analysis), dihydroxyacetone phosphate, α-hydroxyglutaric acid, sophorose and phosphoinositol showed similar diel profiles.

Cluster B contains metabolites that showed highest abundances in late morning. These included the majority of the
TABLE 2 | List of categorized metabolites showing diel cycling patterns.

| Cluster | Patterna | Metabolites |
|---------|----------|-------------|
| A (8)   | Increase in early morning (03:00–11:00) | adenine, dihydroxyacetone phosphate, 3-hydroxybutyric acid, a-hydroxoglutaric acid, 3-hydroxovaleric acid, L-ornithine, phosphonoisitol*, sophorose |
| B (28)  | Increase in late morning (07:00–11:00) | L-asparagine, L-cysteine, fumaric acid, D-glucose, D-glucose-6-phosphate, L-glutamic acid, L-glutamine, glycine, hypoxanthine, inosine, lumazine, L-lysine, D-malic acid, maltose, maltontriote, methylcitric acid*, nicotinic acid, L-phenylalanine, phosphate ion, L-pyroglutamic acid, ribose, L-serine, succinic acid, L-threonine, thymine, L-tyrosine, uracil, L-valine |
| C (9)   | Increase in afternoon (11:00–15:30) | carbonate ion, citric acid, glyceric acid, glycolic acid, L-homoserine, oxalic acid, 2-oxo-glutaric acid, ribose, L-serine, succinic acid, L-threonine, thymine, L-tyrosine, uracil, L-valine |
| D (11)  | Increase in late afternoon/early evening (14:30–22:00) | adenosine, benzoic acid, glycerol-3-phosphate, L-(+)-lactic acid, D-(-)-melezitose, 1-methyl nicotinamide, 3-phosphoglyceric acid, pyrophosphate, D-(-)-trehalose, urea, xylopyranose |
| E (2)   | Increase at night (19:00–22:00) | fructose, sucrose |

aClusters are the same as those shown in Figure 2. Numbers in parentheses correspond to the numbers of metabolites comprising the cluster.

bMetabolites identified by the NIST spectral library only.

amino acids identified in the mat, precursors for the synthesis of nucleic acids, such as hypoxanthine, inosine, phosphoric acid, ribose, thymine, and uracil, as well as intermediates in glycolysis (e.g., glucose and glucose-6-phosphate) and the citric acid cycle (e.g., fumaric, malic, and succinic acids). The abundance profile of maltose, an α-1,4 disaccharide of glucose, paralleled that of glucose, whereas maltontriote, an α-1,4 trisaccharide of glucose, initially increased in abundance, gradually declined and then remained low with minor oscillations throughout the afternoon. A number of metabolites (e.g., asparagine, glycine, malic acid, phenylalanine, succinic acid, threonine, tyrosine, and valine) showed maximal abundances at 11:00 h, followed by an abrupt decrease near mid-day, which was then followed by a secondary maximum around 14:00–15:00 h.

Metabolites assigned to Cluster C showed highest abundance in the early afternoon, a time that correlates to peak photosynthetic activity over the diel cycle (see Discussion). Organic acids such as citric, glycergic, oxalic, 2-oxo-glutaric acid (α-ketoglutaric acid), and pyruvic acids were detected in highest abundance during the period of 12:00 to 16:00 h. In contrast to the proteinogenic amino acid serine, the abundance of homoserine was highest from 11:00 to 14:00 h, during which there was an abrupt decrease at mid-day.

Cluster D metabolites accumulated in the late afternoon. Among these, the amounts of lactate and urea dramatically increased from 12:30 to 15:30 h, then gradually decreased until mid-night. In contrast, benzoic acid, glycerol-3-phosphate and trehalose, an α,α-1,1 disaccharide of glucose, showed peak abundance in the early evening (17:00 h).

Only two metabolites, fructose and sucrose, were assigned to cluster E; these accumulated around 19:00 to 22:00 h, decreased at 23:00 h, and then exhibited a relatively low but constant abundance from midnight to noon.

Volatile Fatty Acids in the Mat
To quantify acetate and propionate in the mat accurately, 13C-labeled acetate was spiked into samples as an internal standard before metabolite extraction, and the ratios of unlabeled acetate and labeled acetate was spiked into samples as an internal standard...
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FIGURE 2 | Heat map view of z-scored polar metabolite abundances over a diel cycle. A total of 58 metabolites were reproducibly detected \((n = 3)\) and confidently identified in the mat samples. The scale bar indicates the z-score transformed average intensity values of metabolites. K-means clustering was performed to categorize the metabolites based on their diel abundance patterns. Cluster A—increase in early morning; Cluster B—increase in late morning; Cluster C—increase in afternoon; Cluster D—increase in late afternoon/early evening; Cluster E—increase at night.

changed throughout the diel cycle in a complex pattern. In general, the wax ester abundances showed decreases from mid-night to mid-day, except for increases in morning and afternoon, followed by an increase again in the evening. Interestingly, \(i,n\) forms of \(C_{31}, C_{32},\) and \(C_{35}\) wax esters increased before \(n,n\) forms.

**Metabolite Partitioning in the Mat**

To evaluate the potential for metabolite exchange among members of the community, we analyzed additional mat core samples that were collected during four time points over the diel cycle and measured metabolites that were excreted or were otherwise extracellular. For this experiment, warm, filtered hot spring water was used to rinse the mat samples on site to avoid release of metabolites due to osmotic shock. Glycolate and lactate were the only metabolites confidently identified in the rinse waters (data not shown). We compared the levels of these metabolites between the rinsed and control mats (Figures 5A,B) at 4 time points during the diel cycle. The highest level of glycolate in control and rinsed mat samples occurred at 13:00 h. Otherwise, the level of glycolate was relatively the same at 03:00, 09:00, and 19:00 h. In contrast, the abundance of lactate in the rinsed mat samples was equal across the four time points. The levels of lactate in the control mat were much higher than in the rinsed mat and increased from 03:00 to 19:00 h. Figure 5C shows data for glycolate and lactate over the full diel in unrinsed mat samples. The diel trends for glycolate in Figure 5A (control mat) and Figure 5C (unrinsed samples from the full diel sampling) clearly track each other. Similarly, the data for lactate in control mat from the rinsing experiment (Figure 5B) shows a rise in lactate abundance beginning at 13:00 h, which matches the time of the rise in lactate in the unrinsed samples from the full diel sample collection (Figure 5C). However, while the lactate abundance continues to rise to 19:00 h in the control mat from the rinsing experiment, it has begun to decline by 15:30 h in the unrinsed samples from the full diel experiment.

**Gases in the Overflowing Water**

The amounts of three gaseous molecules in the water overflowing the 60°C mat—\(\text{CO}_2\), \(\text{H}_2\), and \(\text{CH}_4\)—were also measured over the diel cycle (Figure 6). While the levels of hydrogen and carbon dioxide were lower during the day, methane abundance fluctuated, with maxima at 07:00, 12:30, and 23:00 h.
Discussion

The application of systems biology approaches is expanding from lab-cultured samples to complex environmental communities. In this way, integrated studies are becoming more common for understanding biological systems through the combination of data from metagenomics, metatranscriptomics, metaproteomics, and metametabolomics analyses. Interpreting data from metabolomics analyses of a complex microbial community is challenging because many taxa may contribute to metabolite pools and because they may do so at different times during a diel cycle. Furthermore, metabolite concentrations represent pools that are influenced by production and consumption, as well as by diffusion, and all three factors are closely coupled in aquatic microbial mats. Thus, metabolite fluctuations with time likely represent periods of net production/accumulation or consumption/diffusion. Nevertheless, the data obtained in this study supported existing hypothesized metabolisms of major taxa in the mat and led to new hypotheses based on novel observations, as discussed below.

Integration of Metabolomics and Gene Expression Data: Support of Existing Hypotheses on *Synechococcus* spp. and *Roseiflexus* spp. Metabolisms within the Mat Community

In this section, we interpret certain metabolomics results in the context of hypotheses generated from previous diel metatranscriptomics studies (Liu et al., 2011, 2012; Klatt et al., 2013). Although the metatranscriptomics results are from a different year (September 2009), the long-term stability of the mat community, its composition and structure (Ramsing et al., 2000; Ferris et al., 2003; Ward et al., 2006; Becraft et al., 2011; Meldredz et al., 2011), processes conducted during diel cycles by phototrophic community members based on O$_2$ concentration profiles, and expression of *Synechococcus* photosynthesis and N$_2$ fixation genes (Ramsing et al., 2000; Ward et al., 2006; Steunou et al., 2008; Jensen et al., 2011; Liu et al., 2011, 2012) as measured between 1996 and the present, make comparisons of data collected at comparable temperature sites and times of the year valid. Indeed, comparison of solar irradiance and glycolate levels over a diel cycle in mat samples collected in 2011 showed very similar abundance profiles as the data presented here (Supplemental Figure S3).

**Synechococcus** spp.

Based on diel changes in glycogen (Van Der Meer et al., 2007) and metatranscriptomics analyses (Liu et al., 2012), we...
hypothesized that Synechococcus spp. shift from daytime photosynthesis and the production of glycogen to nighttime glycogen fermentation (Van Der Meer et al., 2007). Consistent with this hypothesis, fermentation products that mat Synechococcus populations have the genetic potential to produce (e.g., acetate and lactate) accumulated during the afternoon and night (Figures 3, 5C).

Mid-day extremes of light and O₂ concentration, as well as CO₂ depletion (suggested by a rise in pH, which shifts the carbonate equilibrium) have been shown to lead to photorespiratory production of glycolate (Bateson and Ward, 1988). Thus, we hypothesized that Synechococcus spp. in the mat experience photorespiration during periods of high light irradiance. Supporting this hypothesis, CO₂ in the water flowing over the mat decreased during the day (Figure 6), and glycolate accumulated between ∼12:00 and ∼16:00 h (Figure 5C). Production of glycolate at peak solar irradiance correlated with the expression of Synechococcus spp. genes encoding photosynthesis machinery (Liu et al., 2012).

Additionally, nighttime and early morning N₂ fixation by Synechococcus has been demonstrated (Steunou et al., 2006, 2008), and because mat Synechococcus lack an uptake hydrogenase, we hypothesized that H₂ accumulation should temporally follow N₂ fixation. Diel patterns of H₂ concentration in the water above the mat (Figure 6) are consistent with this prediction.

Roseiflexus spp.
Noting the diel cycling of transcript abundances encoding enzymes associated with the 3-hydroxypropionate pathway and the production and consumption of polymers known to be produced by Roseiflexus spp., Klatt et al. (2013) hypothesized that Roseiflexus spp. shift from a photomixotrophic metabolism leading to glycogen synthesis during the day to nighttime fermentation of glycogen, coupled with nighttime synthesis of PHA and/or wax esters, whose breakdown during the day could in turn provide the necessary metabolites for photomixotrophy. Consistent with this hypothesis, diel glycogen cycling was previously demonstrated by Van Der Meer et al. (2007). Also consistent with the hypothesis, levels of CO₂ in the water overflowing the mat and of intracellular fermentation products known to be used by Roseiflexus [e.g., acetate, propionate, and lactate; based

**FIGURE 5** | Measured levels of glycolate (A) and lactate (B) from the rinsed and control (un-rinsed) mats. The difference between the two conditions is regarded as a portion biologically available by excretion and diffusion in the mats, which can be taken up by other heterotrophic bacteria in the communities. Glycolate and lactate profiles in the unrinsed mat over the full diel cycle (C) are shown as a reference. The glycolate and lactate abundances were z-score transformed (i.e., normalized), and the value s plotted are mean ± standard error (n = 3). Solar irradiance is shown in solid gray.

**FIGURE 6** | Gaseous metabolites over the diel cycle. Solar irradiance (solid gray) and CO₂, H₂, and CH₄ levels in water overflowing the mat are shown. Metabolite values plotted are mean ± standard error (n = 3).
on genomic (Van Der Meer et al., 2010; Bryant et al., 2012) and metagenomic (Klatt et al., 2011) analyses and on laboratory growth experiments (Hanada et al., 2002) are lower during the day.

In addition, PHAs, measured by their constituent monomers (e.g., 3-HB and 3-HV) after acid hydrolysis of the polymers, were relatively higher at night and in the early morning, followed by a decrease during the day (Figure 4A). The accumulation of 3-HB and 3-HV as free monomers in the morning (Figure 2, Supplemental Figure S1), together with methyl-citrate, an intermediate in the oxidation of propionate (which could be derived from 3-HV), provides evidence that PHAs are being degraded in the early morning. These observations are consistent with previous metatranscriptomics data on expression of Roseiflexus spp. PHA biosynthesis genes, and our previous hypothesis that these molecules might be used for mixotrophic metabolism by filamentous anoxygenic phototrophic bacteria (Klatt et al., 2013).

Wax esters generally cycled in a manner consistent with the expression patterns of Roseiflexus genes associated with their production and degradation, supporting their hypothesized involvement in photomixotrophy. However, these compounds fluctuated in a complex manner, possibly reflecting differences due to the timing of metabolisms of different Roseiflexus species (see below).

**Novel Observations Leading to New Hypotheses**

In this section, we highlight novel observations of metabolism in the Mushroom Spring microbial mat community with respect to metabolites identified or measured for the first time, as well as to the time of day at which certain metabolites showed peaks in accumulation. These observations were then used as the basis upon which new hypotheses have been formulated.

**Detection and Accumulation of Previously Unreported Metabolites**

The accumulation of CH₄ in the mat at mid-day was unexpected (Figure 6), since methanogenesis is an anaerobic process that should only occur in the anoxic nighttime mat (Ward, 1978; Sandbeck and Ward, 1981). However, genomic and metagenomic analyses indicate that Synechococcus spp. have the potential to metabolize phosphonate (Gomez-Garcia et al., 2011), which can also lead to methane production. We therefore hypothesize that the mid-day peak in methane concentration is a result of Synechococcus spp. metabolism of phosphonates.

Metabolites in cluster B accumulated specifically in the morning and in general reached their highest levels at 11:00 h. The metabolites present in this cluster (most amino acids, hypoxanthine, inosine, phosphoric acid, ribose, thymine, and uracil) imply that amino and nucleic acid biosynthesis occur maximally during the early morning period. Interestingly, all of these nitrogen-rich compounds reached peak levels shortly after the maximal period of N₂ fixation by Synechococcus spp., which occurred between 06:00 and 10:00 h in the morning (Steunou et al., 2008). This period also corresponded to the time when total mRNA levels increased sharply in members of the major phototrophic taxa that occur in the mats Liu et al., 2011, 2012; Klatt et al., 2013). While not unexpected, these collective observations lead to the hypothesis that the morning hours represent a time when RNA, DNA, and protein biosynthesis rates are maximal for major taxa in the mat.

At midday (11:00 to 12:00 h) there is an abrupt decline in all metabolites of cluster B, when metabolites of cluster C, including glycolate, oxalate, carbonate, citrate, and phosphonopyruvate, accumulated (Figure 2). The accumulation of glycolate (as discussed above), glycerate, and oxalate is likely due to photorespiration by Synechococcus spp. (Bateson and Ward, 1988; Bauwe et al., 2010). Interestingly, the abundance of carbonate ion also increased at this time, consistent with extreme CO₂ consumption and elevated pH during peak periods of photosynthesis shifting the equilibrium of dissolved inorganic carbon (Revsbech and Ward, 1984; Jensen et al., 2011). Also of interest is the observation that peak production of glycolate coincides with the abrupt decrease in levels of certain metabolites (asparagine, glycine, malic acid, phenylalanine, succinic acid, threonine, tyrosine, and valine) in cluster B, suggesting a decrease in activity in these metabolic pathways possibly due to photoinhibition. At the same time as the abrupt decrease in abundances of cluster B metabolites and just after the peak in glycolate abundance (~12:00 h), the levels of lactate in the mat begin to increase, with maximal abundance at ~15:00 h and correlating with a second peak in glycolate abundance (Figure 5C). We hypothesize that Synechococcus spp. may be a source of the peak in lactate abundance at this time via fermentation either as an alternative metabolism for photoinhibited cells closest to the mat surface, or because cells deeper in the mat experience a shorter period of peak solar irradiance (Becraft et al., this issue; Olsen et al., this issue), or both.

**Metabolite Exchange**

Metabolic interactions among community members are key features stabilizing the composition and function of microbial communities. In a chlorophototrophic microbial community, organic compounds produced and excreted by CO₂-fixing taxa could be used as nutrients by (photo-)heterotrophic or mixotrophic mat community members. Indeed, diurnal transcription patterns of the genes involved in CO₂ fixation have suggested that Roseiflexus spp. in the Mushroom Spring mat community can conduct photomixotrophic metabolism, presumably using organic compounds produced and excreted by other community members. In this section, we discuss the potential for metabolic exchange between Synechococcus and Roseiflexus spp.

Two metabolites, the photorespiration product glycolate and the fermentation product lactate, were identified in the extracellular fractions of the rinsing experiment and were therefore available as nutrients for members of the mat community. Glycolate was most abundant in the mat during the early afternoon (Figures 2, 5C, and Supplemental Figure S1). At 13:00 h, the amount of glycolate associated with mat biomass was much lower (25–30%) in the rinsed compared to the unrisned control samples, suggesting that glycolate is excreted into the extracellular milieu (Figure 5A). At other time points examined, the amounts of glycolate were similar in rinsed or unrisned samples, suggesting a balanced consumption and production or
that photorespiration is less active at lower irradiance levels. Although, Klatt et al. (2013) did not observe significant changes in transcription patterns in Roseiflexus spp. during the same time period as the peak in mat glycolate abundance, these organisms are still the most likely consumers of glycolate because glycolate derived from glycolate by oxidation can readily be assimilated by the 3-hydroxypropionate bi-cycle (Klatt et al., 2007).

In contrast, a very sharp and large increase (~60-fold above the minimum) in transcript abundance for lactate permease in Roseiflexus sp. at approximately 18:00 h has been observed (Bryant et al., unpublished data), just after the afternoon increase in lactate abundance in unrinced vs. rinsed mat samples in our experiment (Figure 5B). This observation suggests that Roseiflexus sp. might utilize a significant proportion of the lactate produced. Indeed, lactate levels declined in the early evening hours after the spike in lactate permease transcripts occurred. As with glycolate, the lower levels of lactate during the night may indicate an efficient balance between production and consumption. Based on these observations, we hypothesize that glycolate, and possibly lactate (as discussed above), are mostly produced and excreted by the cyanobacteria (i.e., Synechococcus spp.) during the early afternoon and are available to other mat inhabitants, particularly Roseiflexus spp., as a carbon and energy source.

It is interesting that only glycolate and lactate were identified in the rinse water. We have considered several possible explanations for this observation. It is possible that other extracellular metabolites (e.g., volatile fatty acids, ethanol) may have been lost during the in vacuo drying of the rinse water samples, as previous analyses have shown that these compounds accumulate in the aqueous fraction during dark, anaerobic incubation of mat samples (Anderson et al., 1987). Alternatively, our sampling of extracellular metabolites, which occurred at 03:00, 09:00, 13:00, and 19:00 h, may not have occurred during the peak times of metabolite excretion. A third possibility is that certain metabolites are rapidly scavenged from the extracellular milieu as soon as they are excreted. The last possibility is that there were no other metabolites that were excreted.

**Depth- or Temporally-Resolved Metabolisms**

As mentioned above, a complex pattern of wax ester abundances was observed, with peak abundances in the predawn, morning, and afternoon periods, and differential timing of i,n- and n,n-forms of the same wax esters. Such complexity might arise because of contributions from multiple taxa capable of wax ester synthesis with different diel timing. As shown in Table 3, although Roseiflexus wax esters are a better match to wax esters found in the mat, Chloroflexus also makes n,n forms of C_{31}, C_{32}, and C_{35} wax esters, and the different abundances of these forms might relate to differential timing of wax ester synthesis in members of these two genera. Such could also be the case for different Roseiflexus species. Zeng et al. (1992) showed that the ratio of i,n- to n,n-forms of C_{31–35} wax esters increased nearly 5-fold in mat layers 4–5 mm below the surface of the highly similar Octopus Spring mat, raising the question of whether different species of Roseiflexus, with different vertical distributions, experience different light regimes and have different timing of wax ester synthesis and degradation. Taxon-related and/or depth-related differences in metabolisms may be generally important, because similar small-scale fluctuations were observed in PHA, glycolate, and fermentation products. Furthermore, a number of metabolites (e.g., asparagine, glycine, malic acid, phenylalanine, succinic acid, threonine, tyrosine, and valine) showed maxima in abundances at 11:00 h, followed by an abrupt decrease near mid-day, which was then followed by a secondary maximum around 14:00–15:00 h. Metabolomics analyses were conducted on the top 5 mm region of the mat, whereas the transcription results of Klatt et al. (2013) were from the top 2 mm region. Different taxa (and/or different species within these taxa) inhabit different vertical regions of the mat (Ramsing et al., 2000; Becraft et al., 2011), and we hypothesize that they exhibit maximal metabolic rates for specific processes at different times during the diel cycle.

**Table 3** Wax ester content of mat and Chloroflexus and timing of abundance changes in the mat.

| Wax ester | Roseiflexus | Chloroflexus | mat | Evening rise start | Night peak | Morning peak | Afternoon rise start | Afternoon peak |
|-----------|-------------|--------------|-----|-------------------|-----------|-------------|---------------------|---------------|
| C_{31} n,n | +           | ++           | 19:00 | 01:00 | 05:00 | 14:30 | 15:30 |
| C_{31} i,n | +           | +            | 17:00 | 22:00 | 03:00 | 11:30 | 14:30 |
| C_{32} n,n | ++          | +            | ++    | 19:00 | 01:00 | 05:00, 09:00 | 14:30 | 15:30 |
| C_{32} i,n | + ++        | + ++         | 19:00 | 01:00 | 05:00, 09:00 | 12:30 | 15:30 |
| C_{33} n,n | +           | +            | ++    | 19:00 | 01:00 | 05:00, 09:00 | 14:30 | 15:30 |
| C_{33} i,n | +           | ++           | 19:00 | 01:00 | 05:00, 09:00 | 14:30 | 15:30 |
| C_{34} n,n | + ++        | ++           | 19:00 | 01:00 | 05:00, 09:00 | 13:30 | 15:30 |
| C_{34} i,n | + ++        | +            | 19:00 | 01:00 | 05:00, 09:00 | 13:30 | 15:30 |
| C_{35} n,n | +           | ++           | 19:00 | 01:00 | 05:00, 09:00 | 15:00 | 17:00 |
| C_{35} i,n | +           | +            | 19:00 | 01:00 | 05:00, 09:00 | 13:00 | 17:00 |

aRelative abundances of wax esters in Roseiflexus spp., Chloroflexus spp., and the Mushroom Spring microbial mat are indicated by “+” for low abundance, “++” for moderate abundance, and “+++” for high abundance. Data for Roseiflexus spp. and Chloroflexus spp. are from Van Der Meer et al. (2010).
Indeed, evidence that *Synechococcus* species with different depth distributions (Becraft et al., this issue), light adaptations (Nowack et al., this issue) and gene expression timing (Olsen et al., this issue), strongly supports this hypothesis.

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**Supplementary Material**

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00209/abstract

**References**

Anderson, K. L., Tayne, T. A., and Ward, D. M. (1987). Formation and fate of fermentation products in hot spring cyanobacterial mats. *Appl. Environ. Microbiol.* 53, 2343–2352.

Baijona, M. M., and Ward, D. M. (1988). Photoexcretion and fate of glycolate in a hot spring cyanobacterial mat. *Appl. Environ. Microbiol.* 54, 1738–1743.

Baud, J., and Brock, T. (1973). Ecological studies of *Chloroflexus*, a gliding photosynthetic bacterium. *Arch. Microbiol.* 92, 267–284. doi: 10.1007/BF00409281

Bauwe, H., Hagemann, M., and Fernie, A. R. (2010). Photoscpiration: partners, players and origin. *Trends Plant Sci.* 15, 330–336. doi: 10.1016/j.tplants.2010.03.006

Becraft, E. D., Cohan, F. M., Kühl, M., Jensen, S. I., and Ward, D. M. (2011). Fine-Scale distribution patterns of *Synechococcus* ecological diversity in microbial mats of Mushroom Spring, Yellowstone National Park. *Appl. Environ. Microbiol.* 77, 7689–7697. doi: 10.1128/AEM.05927-11

Bhaya, D., Grossman, A. R., Steunou, A.-S., Khuri, N., Cohan, F. M., Hamamura, N., et al. (2007). Population level functional diversity in a microbial community revealed by comparative genomic and metagenomic analyses. *ISME J.* 1, 703–713. doi: 10.1038/isjem.2007.46

Brock, T. D. (1972). “One hundred years of algal research in Yellowstone National Park,” in Proceedings of the International Symposium on Taxonomy and Biology of Blue-Green Algae, ed T. V. Desikachary (Madras), 393–405.

Brock, T. D. (1978). *Thermophilic Microorganisms and Life at High Temperatures*. New York, NY: Springer-Verlag, doi: 10.1007/978-1-4612-6284-8

Brock, T. D. (1998). Early days in Yellowstone microbiology. *ASM News* 64, 137–140.

Bryant, D. A., Costas, A. M. G., Maresco, J. A., Chew, A. G. M., Klatt, C. G., Bate- son, M. M., et al. (2007). *Candidatus Chloracidobacterium thermophilum*: an aerobic photosynthetic acidobacterium. *Science* 317, 523–526. doi: 10.1126/science.1134326

Bryant, D. A., Liu, Z., Li, T., Zhao, F., Garcia Costas, A. M., Klatt, C. G., et al. (2012). “Comparative and functional genomics of anoxicogenic green bacteria from the taxa *Chlorobi*, *Chloroflexi*, and *Acidobacteria*,” in *Advances in Photosynthesis and Respiration: Functional Genomics and Evolution of Photosynthetic Systems*, eds R. L. Burnap and W. Vermaas (Dordrecht: Springer), 47–102.

Dobson, G., Ward, D. M., Robinson, N., and Eglinton, G. (1988). Biogeochemistry of hot spring environments: extractable lipids of a cyanobacterial mat. *Chem. Geol.* 68, 155–179. doi: 10.1016/0009-2541(88)90093-9

Ferris, M. J., Kühl, M., Wieland, A., and Ward, D. M. (2003). Cyanobacterial ecotypes in different optical microenvironments of a 68°C hot spring mat community revealed by 16S–23S rRNA internal transcribed spacer region variation.

App. Environ. Microbiol. 69, 2893–2898. doi: 10.1128/AEM.69.5.2893-2898.2003

Garcia Costas, A. M., Tsukatani, Y., Rijpstra, W. I. C., Schouten, S., Wielander, P. V., Summons, R. E., et al. (2012). Identification of the bacteriochlorophyll, carotenoids, quinones, lipids, and hopanoids of *Candidatus Chloracidobacterium thermophilum*. *J. Bacteriol.* 194, 1158–1168. doi: 10.1128/JB.06421-11

Gomez-Garcia, M. R., Davison, M., Blain-Hartnagel, M., Grossman, A. R., and Bhaya, D. (2011). Alternative pathways for phosphate metabolism in thermophilic cyanobacteria from microbial mats. *ISME J.* 5, 141–149. doi: 10.1038/isjem.2010.96

Hanada, S., Takai, S., Matsuura, K., and Nakamura, K. (2002). *Roseflaxus castenholzi* gen. nov., sp. nov., a thermophilic, filamentous, photosynthetic bacterium that lacks chlorosomes. *Int. J. Syst. Evol. Microbiol.* 52, 187–193.

Hillen, K., Hangebroek, J., Jäger, C., Spura, J., Schreiber, K., and Schomburg, D. (2009). Met aboliteDetector: comprehensive analysis tool for targeted and non-targeted GC/MS based metabolome analysis. *Anal. Chem.* 81, 3429–3439. doi: 10.1021/ac802689k

Inskeep, W. P., Ackerman, G. G., Taylor, W. P., Kouzabal, M., Korf, S., and Macur, R. E. (2005). On the energetics of chemolithotrophy in nonequilibrium systems: case studies of geothermal springs in Yellowstone National Park. *Geobiology* 3, 297–317. doi: 10.1111/j.1462-4669.2006.00059.x

Jensen, S. I., Steunou, A.-S., Bhaya, D., Kuhl, M., and Grossman, A. R. (2011). *In situ* dynamics of *O*₂, *pH* and cyanobacterial transcripts associated with CCM, photosynthesis and detoxification of ROS. *ISME J.* 5, 317–328. doi: 10.1038/ismej.2010.131

Kim, Y.-M., Schmidt, B. J., Kidwai, A. S., Jones, M. B., Kaiser, B. L. D., Brewer, H. M., et al. (2013). *Salmonella* modulates metabolism during growth under conditions that induce expression of virulence genes. *Mol. Biosyst.* 9, 1522–1534. doi: 10.1039/c3mb25598k

Kind, T., Wohlgemuth, G., Lee, D. Y., Lu, Y., Palazoglu, M., Shahbaz, S., et al. (2009). FiehnLib: mass spectral and retention index libraries for metabolomics based quadrupole and time-of-flight gas chromatography/mass spectrometry. *Anal. Chem.* 81, 10038–10048. doi: 10.1021/ac9019522

Klett, C. G., Bryant, D. A., and Ward, D. M. (2007). Comparative genomics provides evidence for the 3-hydroxypropionate autotrophic pathway in filamentous anoxicogenic phototrophic bacteria and in hot spring microbial mats. *Environ. Microbiol.* 9, 2067–2078. doi: 10.1111/j.1462-2920.2007.01323.x

Klett, C. G., Liu, Z., Ludwig, M., Kuhl, M., Jensen, S. I., Bryant, D. A., et al. (2013). Temporal metatranscriptomic patterning in phototrophic *Chloroflexi* inhabiting a microbial mat in a geothermal spring. *ISME J.* 7, 1775–1789. doi: 10.1038/isjem.2013.52
Klatt, C. G., Wood, J. M., Rusch, D. B., Bateson, M. M., Hamamura, N., Heidelberg, J. F., et al. (2011). Community ecology of hot spring cyanobacterial mats: predominant populations and their functional potential. *ISME J.* 5, 1262–1278. doi: 10.1038/ismej.2011.73

Koek, M., Jelena, R., Greet, J., Tas, A., and Hankemeier, T. (2011). Quantitative metabolomics based on gas chromatography mass spectrometry: status and perspectives. *Metabolomics* 7, 307–328. doi: 10.1007/s11306-010-0254-3

Konopka, A. (1992). Accumulation and utilization of polysaccharide by hot-spring phototrophs during a light-dark transition. *FEMS Microbiol. Lett.* 102, 27–32. doi: 10.1111/j.1574-6968.1992.tb05792.x

Lageveen, R. G., Huisman, G. W., Preusting, H., Ketelaar, P., Egnink, G., and Witholt, B. (1988). Formation of polymers by *Pseudomonas oleovorans*: effect of substrates on formation and composition of poly-(R)-3-hydroxyalkanoates and poly-(R)-3-hydroxyalkenones. *Appl. Environ. Microbiol.* 54, 2924–2929.

Liu, Z., Klatt, C. G., Ludwig, M., Rusch, D. B., Jensen, S. J., Kuhl, M., et al. (2012). *Candidatus Thermochlorobacter aerophilum*: an aerobic chlorophototrophic member of the phylum Chlorobi defined by metagenomics and metatranscriptomics. *ISME J.* 6, 1869–1882. doi: 10.1038/ismej.2012.24

Liu, Z., Klatt, C. G., Wood, J. M., Rusch, D. B., Ludwig, M., Wittekindt, N., et al. (2011). Metatranscriptomic analyses of chlorophototrophs of a hot-spring microbial mat. *ISME J.* 5, 1279–1290. doi: 10.1038/ismej.2011.37

Madigan, M. T., and Brock, T. D. (1975). Photosynthetic sulfide oxidation by *Chlorobium aurantiacum*, a filamentous, photosynthetic, gliding bacterium. *J. Bacteriol.* 122, 782–784.

Melendez, M. C., Lange, R. K., Cohan, F. M., and, Ward, D. M. (2011). Influence of molecular resolution on sequence-based discovery of ecological diversity among *Synechococcus* populations in an alkaline siliceous hot spring microbial mat. *Appl. Environ. Microbiol.* 77, 1359–1367. doi: 10.1128/AEM.02032-10

Mosier, A. C., Justice, N. B., Bowen, B. P., Baran, R., Thomas, B. C., Northen, T. R., et al. (2013). Metabolites associated with adaptation of microorganisms to an acidophilic, metal-rich environment identified by stable-isotope-enabled metabolomics. *MBio* 4, e00484–e00412. doi: 10.1128/mBio.00484-12

Nold, S. C., and Ward, D. M. (1996). Photosynthetic partitioning and fermentation in hot spring microbial mat communities. *Appl. Environ. Microbiol.* 62, 4598–4607.

Papke, R. T., Ramsing, N. B., Bateson, M. E., and, Ward, D. M. (2003). Geographical isolation in hot spring cyanobacteria. *Environ. Microbiol.* 5, 650–659. doi: 10.1046/j.1462-2920.2003.00460.x

Ramsing, N. B., Ferris, M. J., and, Ward, D. M. (2000). Highly ordered vertical structure of *Synechococcus* populations within the one-millimeter-thick photic zone of a hot spring cyanobacterial mat. *Appl. Environ. Microbiol.* 66, 1038–1049. doi: 10.1128/AEM.66.3.1038-1049.2000

Revsbech, N. P., and Ward, D. M. (1984). Microelectrode studies of interstitial water chemistry and photosynthetic activity in a hot spring microbial mat. *Appl. Environ. Microbiol.* 48, 270–275.

Sandbeck, K. A., and Ward, D. M. (1981). Fate of immediate methane precursors in low-sulfate, hot-spring algal-bacterial mats. *Appl. Environ. Microbiol.* 41, 775–782.

Steunou, A.-S., Bhaya, D., Bateson, M. E., Melendez, M. C., Ward, D. M., Brecht, E., et al. (2006). *In situ* analysis of nitrogen fixation and metabolic switching in unicellular thermophilic cyanobacteria inhabiting hot spring microbial mats. *Proc. Natl. Acad. Sci. U.S.A.* 103, 2398–2403. doi: 10.1073/pnas.0507513103

Steunou, A.-S., Jensen, S. L., Brecht, E., Becraft, E. D., Bateson, M. E., Kilian, O., et al. (2008). Regulation of nit gene expression and the energetics of N2 fixation during the diel cycle in a hot spring microbial mat. *ISME J.* 2, 364–378. doi: 10.1038/ismej.2007.117

Tank, M., and Bryant, D. A. (2015). Nutrient requirements and growth physiology of the phototrophic Acidobacterium, *Chloracidobacterium thermophilum*. *Front. Microbiol.* 6:226. doi: 10.3389/fmicb.2015.00226

Taverner, T., Karpievitch, Y. V., Polpitiya, A. D., Brown, J. N., Dabney, A. R., Anderson, G. A., et al. (2012). DantelR: an extensible R-based tool for quantitative analysis of -omics data. *Bioinformatics* 28, 2404–2406. doi: 10.1093/bioinformatics/bts449

Van Der Meer, M. T., Schouten, S., Damste, J. S., and, Ward, D. M. (2007). Impact of carbon metabolism on 13C signatures of cyanobacteria and green nonsulfur-like bacteria inhabiting a microbial mat from an alkaline siliceous hot spring in Yellowstone National Park (USA). *Environ. Microbiol.* 9, 482–491. doi: 10.1111/j.1462-2920.2006.01165.x

Van Der Meer, M. T. J., Klatt, C. G., Wood, J., Bryant, D. A., Bateson, M. M., Lammerts, L., et al. (2010). Cultivation and genomic, nutritional, and lipid biomarker characterization of *Roseiflexus* strains closely related to predominant in situ populations inhabiting Yellowstone hot spring microbial mats. *J. Bacteriol.* 192, 3033–3042. doi: 10.1128/JB.01610-09

Ward, D. M. (1978). Thermophilic methanogenesis in a hot-spring algal-bacterial mat (71 to 30 degrees C). *Appl. Environ. Microbiol.* 35, 1019–1026.

Ward, D. M., Bateson, M. E., Ferris, M. J., Kuhl, M., Wieland, A., Koep pel, A., et al. (2006). Cyanobacterial ecotypes in the microbial mat community of Mushroom Spring (Yellowstone National Park, Wyoming) as species-like units linking microbial community composition, structure and function. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 361, 1997–2008. doi: 10.1098/rstb.2006.1919

Ward, D. M., Castenholz, R. W., and, Miller, S. R. (2012). *Cyanobacte ria in Geothermal Habitats,* in *Ecology of Cyanobacteria II,* ed B. A. Whittington (Rotterdam: Springer), 39–63. doi: 10.1007/978-94-007-3855-3_3

Ward, D., Taney, T., Anderson, K., and, Bateson, M. E. (1987). *Community Structure and Interactions Among Community Members in Hot Spring Cyanobacterial Mats,* in *Synopsis of the Society for General Microbiology* (Cambridge).

Xie, G., Zhang, S., Zheng, X., and Jia, W. (2013). Metabolomics approaches for characterizing metabolic interactions between host and its commensal microbes. *Electrophoresis* 34, 2787–2798. doi: 10.1002/elps.201300017

Zeng, Y. B., Ward, D. M., Brassei, S. C., and Eglington, G. (1992). Biogeochemistry of hot-spring environments. 3. Apolar and polar lipids in the biologically-active layers of a cyanobacterial mat. *Chem. Geol.* 95, 347–360. doi: 10.1016/0009-2541(92)00021-V

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