Impact of temperature fluctuations on hot spring cyanobacteria

Overview

Photoautotrophic cyanobacteria evolved over 2.45 billion years ago; producing the oxygen that eventually resulted in the great oxygenation event approximately 2.3 billion years ago (Shih et al., 2017; Tomitani et al., 2006). Continuing to be major primary producers today, cyanobacteria inhabit diverse ecosystems ranging from caves and desert soil crusts to oceans and hot springs (Behrendt et al., 2020; Billi, 2019; Hamilton, 2019). Hot springs have been present for ~3.5 billion years on Earth and are thought to have existed on ancient Mars (Djokic et al., 2017). Cyanobacteria have been found in hot springs at temperatures up to ~73ºC, the upper temperature limit of photosynthesis. The cyanobacteria living at or near ~73ºC inform the upper temperature limit of biological oxygen production on Earth and potentially on other worlds.

Cyanobacterial growth at ~73°C has only been identified in the *Synechococcus* A/B clade found in hot spring outflows in and near Yellowstone National Park (YNP) (Allewalt et al., 2006; Becraft et al., 2011, 2015, 2020; Brock et al., 1967; Ferris and Ward, 1997; Miller et al., 1998, 2009; Ward et al., 1998, 2006). While thermophilic cyanobacteria are found globally, there are no regions outside of YNP and the surrounding geothermal areas where phototrophs persist at ~73°C (Papke et al., 2003). The *Synechococcus* A/B clade is highly diverse, consisting of multiple ecotypes (ecologically distinct populations) that have different thermal niches (Miller et al., 1998; Pierson and Castenholz, 1971; Steunou et al., 2006; Ward et al., 2012). The primary drivers of ecological distinction between *Synechococcus* ecotypes are geographic distance and variation in environmental temperature, light intensity, and pH. Thermotolerance ranges are a major factor in distinguishing ecotypes (Miller at al., 2009; Ward et al., 2006; Ramsing et al., 2000).

The large role of temperature in ecotype differentiation may be due to the negative impact

that heat stress has on phototrophic machinery. High temperatures inactivate photosystems, aggregate proteins and light harvesting complexes, photobleach photosynthetic pigments, and denature RuBisCo and RuBisCo activase (Cimdims et al., 2014; Hamilton, 2019; Mathur and Jajoo, 2014; Salvucci et al., 2001). While studies have identified cyanobacterial heat shock responses that help mitigate the detrimental impacts of heat stress on photosynthesis, these studies have been limited to temperatures <50°C (Asadulghani et al., 2003; Blondin et al., 1993; Cimdims et al., 2014; Nakamoto et al, 2000; Rajaram et al., 2014; Sakthivel et al, 2009; Suzuki et al., 2006).

Frequent exposure to high temperatures (~60-70°C) approaching the upper temperature limit of photosynthesis occurs in hot spring outflows known as geysing outflows. The periodic temperature fluctuations present in these outflows are due to the eruptions of a geyser at the outflow source. Each eruption generates a burst of flow that travels down the outflow channel. Changes in in flow rate are followed by changes in water temperature at sites along the outflow.

Geysing and nongeysing (stable) hot spring outflows together provide a unique opportunity to address the knowledge gaps of (1) the impact of inconsistent site temperature on cyanobacterial diversity and (2) the physiological responses of thermophilic cyanobacteria to heat stress. The overarching question of this proposal is: what is the impact of fluctuating temperature on *Synechococcus* ecotype diversity and physiology in YNP hot spring outflows?

The following specific aims will be used to address this question:

1. Evaluate differentiation in the heat stress responses of ecotypes in stable outflows.
2. Elucidate ecotype diversity in geysing outflows in comparison to stable outflows.
3. Examine extent of heat stress in ecotypes in geysing outflows.

Background and Rationale

Cyanobacteria in Hot Springs

Figure 1: A YNP hot springs outflow. The black dashed line marks the visible phototrophic temperature fringe. In this image, phototrophs are present in orange microbial mats.

In hot springs, there is a visible phototrophic fringe at ~73ºC (see Figure 1), where the temperature becomes too hot to support phototrophic growth and phototrophs cease to be part of the microbial community. While thermophilic hot spring cyanobacteria have evolved from less thermotolerant ancestors multiple times (Miller and Castenholz, 2000; Swingley et al, 2008), cyanobacteria have not evolved to persist at temperatures above ~73ºC.

The upper temperature limit of photosynthesis is likely due to physiological constraints of phototrophy itself. Phototrophic light and electron harvesting components are sensitive to heat stress, as are key enzymes such as RuBisCo and RuBisCO activase (Cimdims et al., 2014; Hamilton, 2019; Mathur and Jajoo, 2014; Salvucci et al., 2001). In addition, damaging reactive oxygen species (ROS) and toxic Calvin intermediates form more rapidly at higher temperatures (Allakhverdiev et al., 2008; Li, 2016). While these thermally induced constraints on phototrophy have been well explored, the mechanisms of increased thermotolerance by phototrophs living at phototrophic fringe sites have not been established. Ecotypes of the *Synechococcus* A/B clade are the only phototrophs to persist at ~73ºC. Due to the significant impact of temperature on photosynthesis and in *Synechococcus* ecotype niche partitioning, ecotypes found at different temperatures are expected to have differentiated heat stress responses.

Genomic and Thermal Niche Differentiation in *Synechococcus* Ecotypes

The major *Synechococcus* A/B clade ecotypes are designed with the identifiers of A', A, and B'; they are associated with approximate respective temperature ranges (Figure 2) of 55°C-

73°C, 40°C-70°C, and <40°C-65°C (Allewalt et al, 2006; Becraft et al, 2011; Ferris and Ward, 1997; Miller at al., 2009; Ward et al., 2006). These ecotypes reveal adaptations required for high temperature photosynthesis and elucidate the role of niche separation in genetic differentiation.

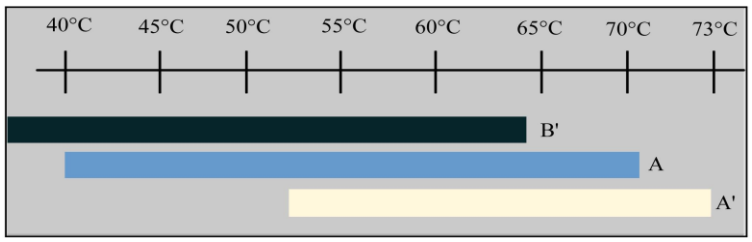

Figure 2: Distribution of major ecotypes (A', A and B') along the thermal gradient of a typical stable hot springs outflow.

The only ecotype found at the phototrophic fringe is A', which has a < 20°C temperature range. The other two major ecotypes, A and B', have a ≥ 30°C range, supporting the hypothesis proposed by Miller and Castenholz (2000) that there is a tradeoff in thermal range for the specialized capability of growth at the phototrophic fringe (~73ºC).

Ecotype specific genetic differentiation is closely tied to functional differentiation (Becraft et al., 2020). Genetic diversity between ecotypes is seen in genes related to photosynthesis, including RuBisCO (Ribulose-1,5-bisphosphate carboxylase/oxygenase) (Miller et al., 2013), and photosynthetic reaction center protein PsaA (Becraft et al., 2011), and in genes relating to phosphate metabolism (Gomez-Garcia et al., 2010) and nitrogen fixation (Steunou et al., 2006). Ecotypes also have differentiated in diel transcriptomic patterns of nitrogen fixation and photosynthesis (Olsen et al., 2015; Steunou et al., 2006). Ecotypes found at phototrophic fringe sites (A'-like ecotypes) have distinctly differentiated from the less thermophilic A-like and B'-like ecotypes in RuBisCO and nitrogen fixation genes (Miller et al., 2013; Kees et al., unpublished).

Ecotypes found at the phototrophic fringe sites may also exhibit diversification from less thermophilic ecotypes in genes related to heat stress response. For instance, genetic differentiation in A'-like fringe ecotypes has been identified in both an uncharacterized protein in the sigma-70 regulatory protein family and in *Cyt* c_M (Kees et al., unpublished), an electron carrier protein

suggested to participate in regulatory functions during multiple stress conditions (Bialek et al., 2016; Hiraide et al., 2015). This work will determine the heat stress responses of *Synechococcus* ecotypes and evaluate the extent of heat stress response differentiation across the diversity of *Synechococcus* ecotypes to assess the impact of heat stress on ecotype diversification.

Stable and Geysing Hot Spring Outflows

Hot springs are found globally in most volcanic regions (Bryan, 2008). The Hamilton lab has expanded on existing work on hot spring phototrophy by examining phototrophic presence in YNP hot springs, including previously unstudied springs with a range of geochemistries (Bennett et al., 2020; Hamilton et al., 2019; Havig and Hamilton, 2019). This proposal will further expand the scope of research to include geysing outflows.

Most hot spring outflows found globally are stable outflows; over half of the world's geysing outflows are located within YNP (Bryan, 2008). Geysing outflows have periodic bursts of flow following the pattern of a geysing cycle, while stable outflows maintain a steady rate of flow. The geysing cycle is due to underground activity within a complex "plumbing system" of narrow tubes that connect regions of high porosity sand and gravel (Bryan, 2008). Heated water builds up in the "plumbing system," beneath the geyser, creating a hot, pressurized environment. As water accumulates, it begins to overflow onto the surface, and the pressure below ground drops, resulting in a drop of the boiling temperature of the water and creating a flash boiling event: more and more water in the system boils, escaping in an eruption. This continues until either the water is depleted or the temperature in the "plumbing system" is below the boiling point (Bryan, 2008). On the surface, the geysing cycle is seen as a pattern beginning with an onset of flow, followed by a period of steady flow, and a period without flow until the next eruption begins. Geysing cycles vary both in consistency and length (Herwitz and Manga, 2017), with most being inconsistent.

Periodic temperature fluctuations of up to 40°C have been observed at sites with phototrophic microbial mats at two geysing outflows in the Lower Geyser Basin region of YNP — Flat Cone and an unnamed feature we colloquially named ‘The Jolly Jelly ’(YNP Thermal Feature Inventory ID LFMNN010) (Hamilton and Havig, 2022). Figure 3 demonstrates temperature variation at phototrophic fringe sites with *Synechococcus* ecotypes present in Jolly Jelly, Flat Cone, and a stable outflow colloquially named Bison Pool (Hamilton and Havig, 2022).

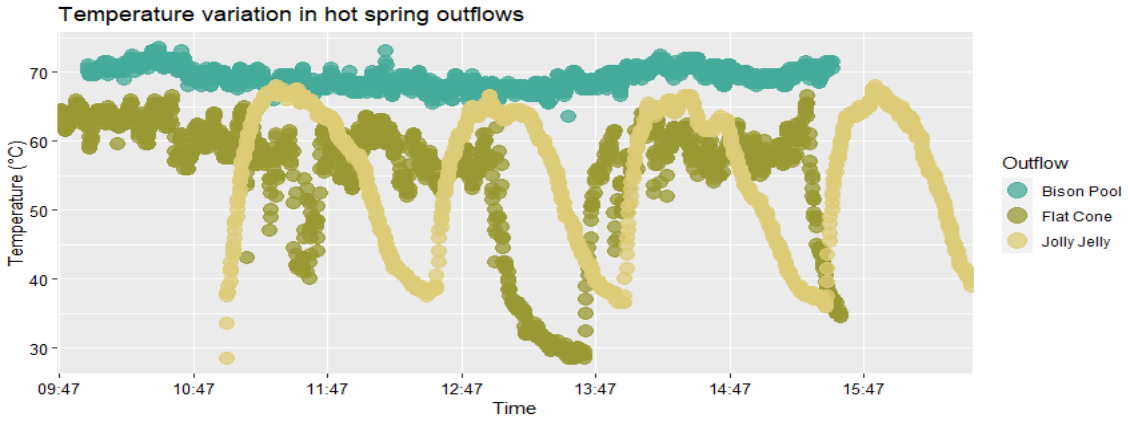

Figure 3: Temperature over time in three outflows of different types in YNP. Blue-green points represent a stable outflow, Bison Pool. Green and tan represent two geysing outflows, Flat Cone and Jolly Jelly. Jolly Jelly has more consistent geysing periods.

We have found that median site temperature may drive *Synechococcus* ecotype diversity in Jolly Jelly and Flat Cone (Hamilton and Havig, 2022), but we have not yet identified the thermal range breadths of geysing ecotypes, nor have we identified genetic diversity specific to ecotypes in geysing outflows. In addition to median site temperature, it is likely that other temperature parameters such as mean, minimum and maximum site temperature (Table 1) play a role in ecotype diversity.

Table 1	Mean	Median	Max	Min
Bison Pool OF	69.2°C	69°C	73.5°C	63.5°C
Flat Cone OF	54.9°C	58°C	66.5°C	28.5°C
Jolly Jelly OF	55.2°C	59°C	68°C	28.5°C

Table 1: Mean, median, maximum, and minimum site temperatures for the outflows (OFs) in Figure 3.

Geochemical data from Jolly Jelly and Flat Cone indicate that their respective dissolved sulfide

concentrations are 4-11 ug/L and 17-36 ug/L, dissolved silica concentrations are 3.31-4.69 mM and 5.26-5.74 mM, and dissolved iron concentrations are, for both, <0.04 mg/L. The pH range of phototrophic sites is pH 8.4-9.1 at Jolly Jelly and pH 8.5-8.8 at Flat Cone. While geochemical differences between sites at time of sampling will be taken into account when assessing diversity, determining the impact of geysing cycles on geochemistry is outside of the scope of this proposal. This work will focus on the interplay between temperature and *Synechococcus* ecotypes.

Specific Aims and Research Plan

Aim 1: Evaluate differentiation in the heat stress responses of ecotypes in stable outflows.

Introduction: Heat shock proteins and related proteins contribute to heat stress resistance and recovery in cyanobacteria from lower temperature (<50°C) environments (Blondin et al., 1993; Rajaram et al., 2014; Suzuki et al., 2006). Heat stress responses in ecotypes within stable outflows will be evaluated by assessing changes in transcript presence and abundance following transplant to higher temperatures. Previous work has demonstrated cyanobacterial heat stress transcript expression to peak in 15-45 minutes following heat shock (Blondin et al., 1993; Suzuki et al., 2006). Ecotype transcriptomes will be surveyed to establish the extent of differentiation in heat shock protein expression patterns following a 30-minute heat shock.

Hypothesis: Heat stress responses differ between the A'-like ecotypes found at the phototrophic fringe and the A-like and B'-like ecotypes found throughout a lower, broader temperature range.

Research Methods: Transplant experiments will occur at six sites as outlined in Figure 4. Samples will be collected and flash-frozen in triplicate for each treatment group (pre-transplant samples, in-place bottle-effect control samples, transplanted dark control samples, and transplanted experimental group samples) for six sites (Table 2). RNA will be extracted from samples using the Qiagen RNeasy Power Biofilm Kit, and submitted to the University of Minnesota Genomics

Center (UMGC) to undergo Illumina sequencing to generate paired end, 2 x 150 bp reads.

Table 2

Site #	Source temperature		Transplant Temperature (Experimental group and dark control group)	Expected Abundant Ecotypes
1	55°C	→	65°C	B' and A
2	57°C		65°C	B', A and A'
3	60°C		70°C	B', A and A'
4	62°C		72°C	B', A and A'
5	65°C		70°C	A and A'
6	65°C		72°C	A and A'

Table 2: Source temperatures, transplant temperatures and targeted ecotypes for the transplant experiments.

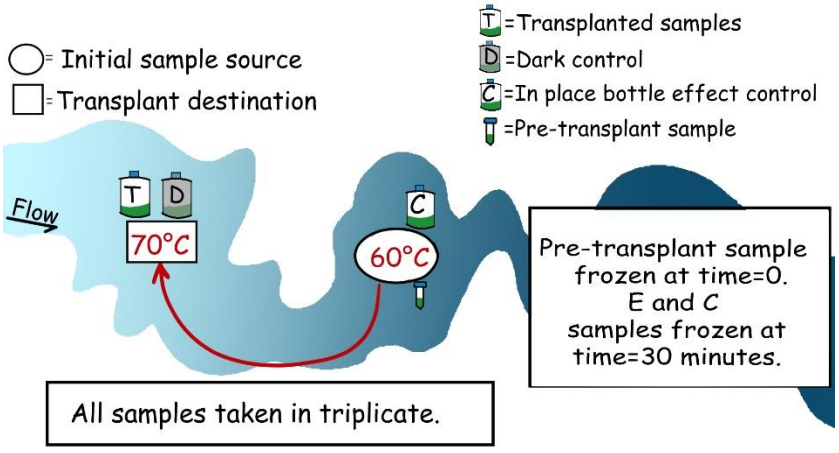

Figure 4: Phototrophic mat material will be collected at each of six sample sites (only one pictured for simplicity) into three pre-transplant samples and six glass bottles. Three bottles will be in-place bottle-effect controls, remaining at the sample site.

Data Analysis Methods: Sequences will be trimmed and cleaned using Trimmomatic (Bolger et al., 2014) and undergo removal of rRNA using SortMeRNA (Kopylova et al., 2012). To generate an annotated reference transcriptome, (mRNA) sequences will be assembled using Megahit (Li et al., 2016), with coding sequences predicted using Prodigal (Hyatt et al., 2010) and annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al., 2008). The Hamilton lab has *Synechococcus* A/B clade metagenome assembled genomes (MAGs) from these hot springs that can be used as references in assembly. mRNA sequences will be mapped to the references and quantified using Salmon (Patro et al., 2017). Genes differentiated between

ecotypes, including the photosystem gene *psaA*, will be used to identify the ecotypes of assembled transcriptomes (Becraft et al., 2011; Miller et al., 2013; Melendrez et al., 2011). The transcriptome data outputted from Salmon will be normalized using a variance stabilizing transformation. Log_2 fold changes in transcript abundances between the pre-transplant samples and the transplanted samples will be calculated by using negative binomial regression. This will be followed by a Wald test to test the significance of log_2 fold transcript abundance changes between the pre-transplant samples and the transplanted samples. These three functions of variance stabilizing transformation, negative binomial regression and Wald test will be run using R package DESeq2 (Love et al., 2014). A PERMANOVA will be used to test that the pre-transplant control samples and the in-place bottle effect control samples have statistically equivalent gene expression patterns.

The log_2 fold change between ecotype transcriptomes in pre-transplant samples and transplanted samples will be visualized as heatmaps to identify differentially expressed transcripts with a log_2 fold change >2, using the R package pheatmap (Kolde, 2018). Additional heatmaps of the differentially expressed transcripts will be constructed to contrast heat stress associated expression patterns between ecotypes, and a PERMANOVA will be used to test whether the expression patterns are statistically different.

Expected Outcomes: Ecotypes with broader temperature ranges are expected to have a less extreme response to heat stress, evidenced by lower transcript abundances of heat stress related proteins. Genes that are differentially transcribed following heat stress and genetically differentiated between ecotypes likely play a role in the diversification of *Synechococcus* A/B clade heat stress responses. Transcripts differentially expressed in this aim will be monitored in enrichment cultures in the experiment outlined in aim 3 of this proposal.

Challenges: Normal diel transcriptomic patterns should not create significant differences

between the pre-transplant control samples and the in-place bottle effect control samples within the time frame of the experiment. However, if these control groups are different, then only the in-place bottle effect control samples will be compared to the transplanted samples. If no ecotype specific heat stress transcriptome patterns are observed, this would intriguingly reveal that heat stress responses may not be ecotype specific and may not play a major role in ecotype thermal niche partitioning. Additionally, while having more replicates would result in more robust statistical analyses, it is not possible to increase the number beyond triplicates due to constraints introduced by the narrow width of the hot spring outflows and the biomass limitations for sample collection set by our sampling permit.

Furthermore, as heat and light stress are inexorably linked in phototrophs (Allakhverdiev et al., 2008; Asadulghani et al., 2003; Tóth et al., 2011), it is difficult to confidently assess patterns to heat stress alone. All transplant experiments will take place at the midday to keep light consistent. While dark controls will assist in distinguishing between light and heat effects, light stress associated transcripts generated in this experiment will be considered as indirectly due to heat stress.

Aim 2: Evaluate ecotype distribution and diversity in geysing systems.

Introduction: Amplicon sequence variants (ASVs) of the 16S rRNA gene have previously been used to identify ecotypes (Allewalt et al, 2006; Ferris and Ward, 1997; Ward et al., 2006). As ecotypes often have relatively high 16S similarity and greater difference elsewhere in the genome, the use of ASVs rather than operational taxonomic units (OTUs) provides the ability to distinguish ecotypes more finely with this gene. Genes that evolve more rapidly than the 16S rRNA gene have more significant variation between ecotypes, providing greater molecular resolution in demonstrating ecotype diversity when used for phylogenetic reconstructions (Becraft et al.,2011,

2015, 2020; Melendrez et al., 2011). These genes include *psaA*, which encodes part of the reaction center core of Photosystem I. This aim will use both 16S rRNA and *psaA* sequences taken from sites along geysing outflows to evaluate distribution of ecotypes in geysing outflows and estimate both ecotype temperature ranges and what drives the temperature ranges. Additionally, this aim will use 16S rRNA sequences to compare ecotype presence and abundance between geysing and stable outflows, as the Hamilton lab has previously collected 16S data from stable outflows.

Hypotheses: (1) Geysing outflows have lower *Synechococcus* ecotype diversity than stable outflows. (2) Ecotype diversity within geysing outflows is primarily driven by median site temperature.

Research Methods: Triplicate samples will be taken from the phototrophic layer of the microbial mat at multiple sites along each of two distinct geysing outflows, and flash frozen immediately after collection. The sampling site temperatures (52°C, 57°C, 62°C, 67°C, and 72°C) were selected to cover a wide thermal range, targeting multiple ecotypes. DNA will be extracted using a DNeasy PowerSoil Total DNA Kit. Resulting DNA will be submitted to the UMGC for PCR amplification of the 16S rRNA gene as well as the *psaA* gene. PCR amplification of *psaA* will be done using *psaA* primers specifically developed for use with the Synechococcus A/B clade (Becraft et al., 2015). PCR will be followed by Illumina sequencing at the UMGC.

Data Analysis Methods: 16S sequences will be cleaned, trimmed, aligned, and binned into ASVs using Mothur (Schloss et al., 2009). Statistical significance of 16S ASV community compositional differences between stable and geysing outflows will be assessed via PERMANOVA, and within-group dispersion will be tested with the betadisper test of dispersion.

Prior to analysis, *psaA* sequences will be cleaned, trimmed, and aligned in Mothur (Schloss et al., 2009). To generate a custom reference file for the alignment of *psaA* sequences, reference

psaA sequences will be trimmed to start and end with the primer pair in Mothur (Schloss et al., 2009) and aligned using Clustal Omega (Sievers and Higgens, 2018). The reference *psaA* sequences for alignment will come from both existing reference *psaA* sequences from Hamilton lab metagenomes and publicly available *psaA* sequences (Becraft et al., 2011; Bhaya et al., 2007). Reference *psaA* sequences for *Synechococcus* OS-A and OS-B (Bhaya et al., 2007) will be used to classify sequences into the two major clades.

Ecotype diversity will be estimated using both 16S and *psaA* sequence data. This will be done via visualization of the Faith's phylogenetic diversity, Shannon's diversity and Chao1 species richness indices. The Wilcoxon rank-sum test will be used to assess statistical significance of differences in 16S diversity index values between outflows.

The 16S and *psaA* sequences will be used to construct phylogenetic trees in MEGA using maximum likelihood sequence reconstruction (Sudhir et al., 2018) with the statistical support of branching patterns determined via bootstrapping. Temperature range data of sample sites where each ecotype was found will be overlaid on the trees to visualize the relationship between related ecotypes and thermal range. This will reveal the temperature niches occupied by distinct and diverse ecotypes in geysing outflows. The impact of different thermal parameters (median, mean, maximum, minimum and range of site temperature) on diversity will be assessed using CAP plots and generalized linear models with a Poisson distribution to fit sequence count data.

Expected Outcomes: Geysing outflows likely have lower diversity than stable outflows, as temperature fluctuations along the outflow may have a homogenizing effect on ecotypes present, selecting for fewer ecotypes with broad thermal niches. Jolly Jelly's temperature fluctuations are more frequent; this greater geysing cycle consistency will result in higher diversity in Jolly Jelly than in Flat Cone. There will be greater similarity in community compositional between the two

geysing outflows than between geysing and stable outflows. Novel ecotypes may be identified at geysing sites, revealing greater *Synechococcus* A/B clade diversity.

Challenges: Two is not an adequate sample size to represent the breadth of variation in geysing outflows. However, these outflows are highly distinct from each other, allowing for better representation of geysing outflows broadly. While I will be able to suggest hypotheses about thermotolerance and thermal range breadth in geysing outflows, future work surveying more outflows will be needed to support generalized conclusions about ecotype distributions and diversity in geysing outflows.

Aim 3: Determine extent of heat stress in ecotypes in geysing outflows.

Introduction: In eukaryotic phototrophs, heat stress increases the production of Reactive Oxygen Species (ROS), negatively impacting photosynthesis (Allakhverdiev et al., 2008). Little is known about the interplay between heat stress and ROS in cyanobacteria. Transcriptomes of heat stressed ecotypes in stable outflows (data collection described in aim 1) will be compared to transcriptomes of ecotypes at the phototrophic fringe sites of two geysing outflows to evaluate presence or absence of heat stress responses at these sites. Due to the cyclical nature of heat stress in geysing outflows, it is expected that ecotypes present will exhibit a novel transcriptomic pattern, continuously expressing transcripts associated with mitigating heat stress.

In-lab experiments will be used to further elucidate the impact of temperature fluctuations on *Synechococcus* physiology. Enrichment cultures of *Synechococcus* ecotypes will be exposed to controlled temperature fluctuations while being monitored for changes in ROS production, photosynthetic rates (quantified by carbon fixation rates), and transcriptomes.

Hypothesis 3: Ecotypes from geysing outflows continuously express heat stress responses at low levels to mitigate the impact of cyclical exposure to high temperatures both *in vivo* and in lab.

Research Methods: (3a) Triplicate samples will be collected from phototrophic fringes sites at two geysing outflows along with site-associated thermal data, and flash frozen. RNA extraction and sequencing will follow the protocol described in aim 1.

Data Analysis Methods: (3a) RNA will be prepared for analysis following the methods described in aim 1. Annotated heatmaps of transcript abundances will be used to assess the presence of *Synechococcus* heat stress related transcripts. DESeq2 (Love et al., 2014) will be used to assess differential expression and potential statistical significance of transcriptomic differences between geysing outflows and transplanted samples in stable outflows.

Research Methods: (3b) Samples from geysing outflow Jolly Jelly will be enriched by inoculating 50uL of pH-adjusted BG11 media (Rippka et al., 1979) with environmental sample. Prior to the experiment, 16S rRNA sequencing will be used to confirm ecotype identities in the enrichments. Jolly Jelly was chosen for the enrichment source over Flat Cone as its predictable cycle is reproducible in-lab. In addition to these enriched cultures, there will be a positive control group enriched from a stable outflow, a negative control group containing media with no cells and a control group of geysing enrichment kept in the dark to account for light stress effects. The experimental group and three control groups (stable outflow enrichment control, cell free control, and dark geysing enrichment control) will be inoculated with ^{13}C in the form of $NaH^{13}CO_3$ prior to undergoing 280 minutes of simulated geysing cycles in an incubator, following the temporal temperature shifts as seen in site-associated thermal data from Jolly Jelly (Table 3). During this period, subsamples will be taken every 20 minutes to evaluate ROS abundance, carbon fixation rate and transcriptomes at multiple temperature points, to establish whether physiological thermal stress is occurring (Figure 5).

Sample timepoints (minutes)	0, 100, 200	20,120, 220	40, 140, 240	60, 160, 260	80, 180, 280
Temperature	63°C	68°C	63°C	50°C	38°C

Table 3. Timepoints and temperatures for subsamples to be collected. Timepoints are minutes after the experiment start time.

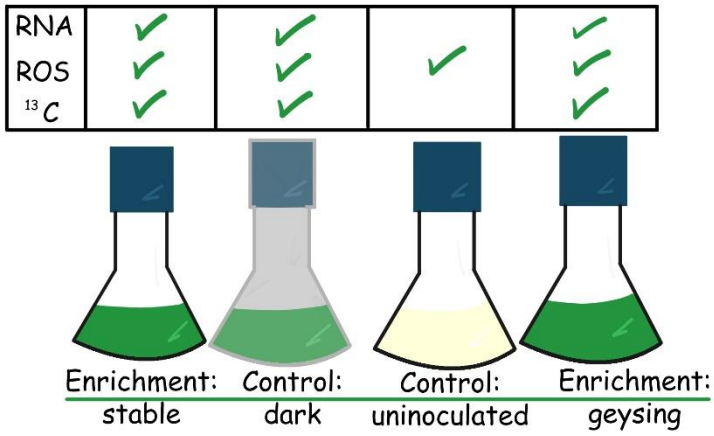

Figure 5: Overview of subsampling schematic, with each checkmark indicating a subsample for each timepoint of the simulated geysing experiment.

Measuring carbon fixation rates: The first set of subsamples (5-mL) will be frozen at time of collection. This set of subsamples will later be thawed, rinsed, dried, homogenized and weighed in preparation for ^{13}C stable isotope measurement as described in Bennett et al, 2020. Following sample preparation, samples will be submitted to the UC Davis Stable Isotope Facility, where a Costech Instruments Elemental Analyzer (EA) periphery connected to a Thermo Scientific Delta V Advantage Isotope Ratio Mass Spectrometer (IR-MS) will be used to assess the amount of ^{13}C incorporated into biomass. Carbon uptake/carbon fixation rate at each timepoint will be calculated as total micrograms of C taken up divided by the grams of organic C per gram of sample, divided by the time since the previous sample was collected (Bennett et al., 2020). Carbon fixation rates will be normalized by dry biomass weight. *Measuring ROS levels:* The second subsample (5-mL) will be vortexed to break up cyanobacterial filaments, and OD700 will be used as a measure of biomass for later normalization (normalization by dividing the ROS measure by OD700). After

measurement of OD700, ROS quantification will be done following protocol outlined in Rajneesh et al (2017). In summary, the subsample will be inoculated with a dye, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), acquired from ThermoFisher. DCFH-DA enters cells and fluoresces upon reaction with any ROS. Quantification of ROS will be done via measuring DCFH-DA fluorescence using fluorescence spectrometry with an excitation wavelength of 485 nm and an emission band between 500 and 600 nm (Rajneesh et al., 2017). As ROS are highly reactive, they will be quantified rapidly following collection. *Acquiring transcriptomes:* The third subsample (5mL) will be flash frozen for RNA extraction, sequencing, and processing as described in aim 1.

Data Analysis Methods: (3b) One-way repeated measures MANOVA will be used to determine statistical significance of changes in the two dependent variables (carbon fixation rates and ROS abundance) throughout the simulated geysing cycle between the experimental and control groups. Timepoint will be used as the predictor rather than temperature, as each temperature will be experienced at multiple timepoints. For transcriptome analyses, as in aim 1, annotated heatmaps of transcript abundances will be used to assess the presence of *Synechococcus* heat stress related transcripts throughout the geysing cycle. DESeq2 (Love et al., 2014) will be used to assess differential expression and potential statistical significance of transcriptomic differences throughout the progression of the simulated geysing cycle.

Expected outcomes: Ecotypes in geysing outflows likely continuously express heat stress associated transcripts to support growth throughout temperature fluctuations. Thus, the proteins that moderate heat stress damage would be constantly present in the system as opposed to being newly generated each time the temperature rises. This would be evidenced by presence of heat stress related transcripts in samples taken from geysing outflows and by a lack of statistically

significant changes in the ecotypes enriched from geysing outflows as the simulated geysing cycles progress. Continuously expressing stress responses will result in the geysing ecotypes maintaining steady carbon fixation rates and mitigating accumulation of toxic ROS throughout the experiment. The control ecotypes from stable outflows will have a buildup of heat stress related transcripts, decreased carbon fixation rates and increased ROS production due to their greater inhibition by the recurring high temperatures. The dark control will be used to assess the extent of stress related transcription that is due to heat stress alone versus the combination of heat and light stress together.

Challenges: Heat stress in-lab may not be representative of heat stress *in vivo* due to differences between culture conditions and environmental conditions. Heat and light stress both increase ROS production. As temperature increases, the intensity of light stress will increase. The extent of ROS production from heat stress cannot be distinguished from ROS produced by light stress in this experiment. As in aim 1, light stress will be considered analogous to indirect heat stress and will be accounted for by using a dark control.

Conclusions

The proposed work will contribute to an increased understanding of the evolution of the ecologically distinct populations within the photoautotrophic thermophilic cyanobacterium *Synechococcus* sp. in YNP hot springs, by assessing the impact of flunctuating temperature on *Synechococcus* ecotype diversity and physiology and determining the drivers of ecotype diversity in geysing outflows. In addition, it will evaluate the extent of heat stress response differentiation between the major groups of *Synechococcus* ecotypes within stable outflows and assess presence of heat stress related transcript in ecotypes within geysing outflows. The capability of mitigating the impact of high temperature has significant implications for the ubiquity of photoautotrophy.

Photoautotrophy originated on early Earth and played an important role in shaping modern Earth via significant contributions to primary productivity throughout the history of the Earth and oxygenation of the Earth's atmosphere. Photoautotrophic cyanobacteria contribute to elemental nutrient (C, N, O) cycling and have been implicated as the origin of plant chloroplasts. As light is an energy source expected to reach the surface of many celestial bodies, it is possible that phototrophic organisms might reside on other worlds. Therefore, understanding the interactions between phototrophic organisms and environmental factors that constrain phototrophy, such as high temperature, is key for the future of both community ecology and astrobiology.

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