

Going with the flow:

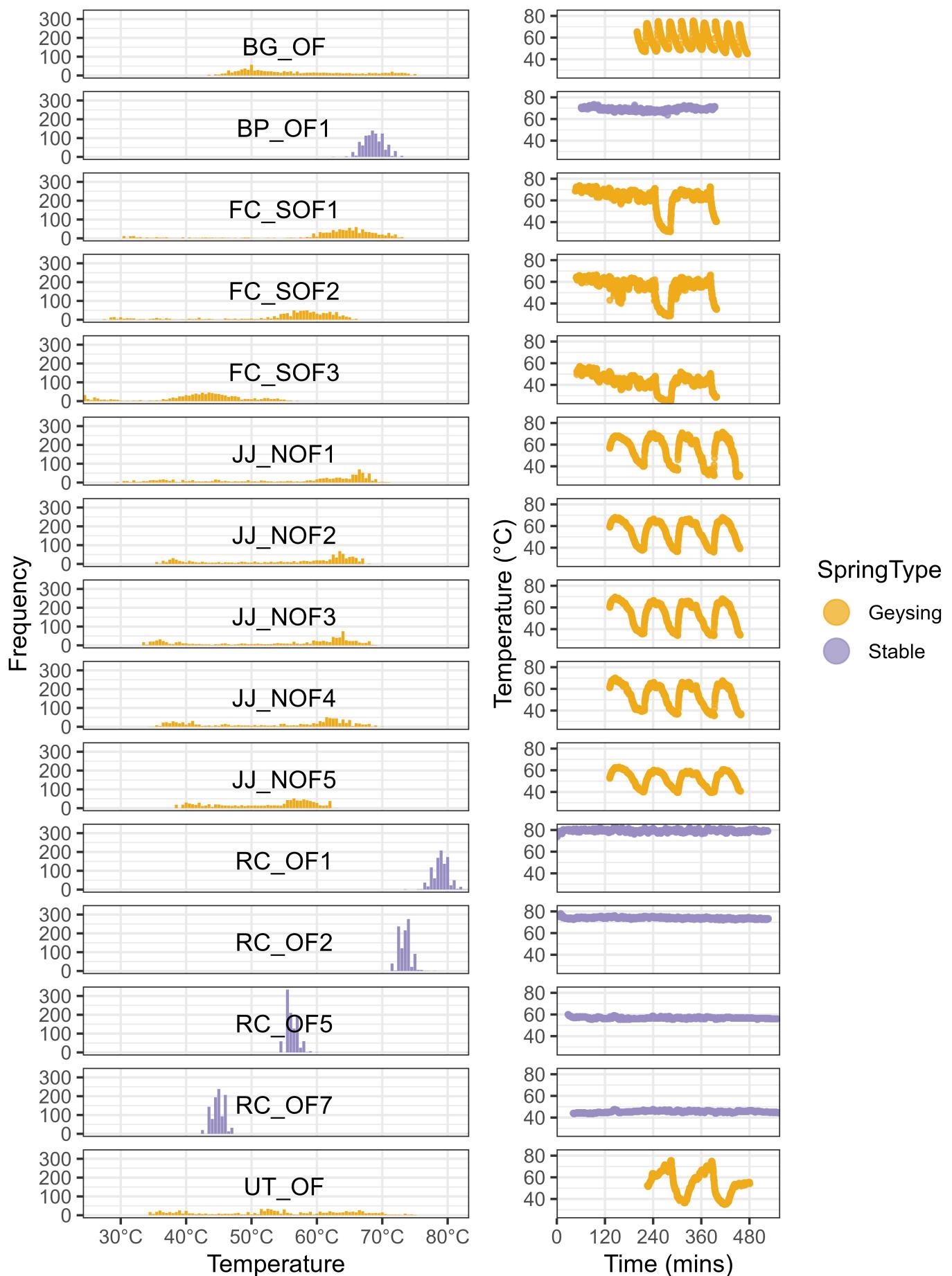
Cyanobacterial Diversity in Geysing and Nongeysing Springs

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Brief Introduction

Over half of the world's geysers are located within Yellowstone National Park (YNP) (Bryan, 2008). Geysers experience geysing cycles- periodic eruptions that result in bursts of water flowing along the outflow channel. A 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. While YNP hot spring microbial communities have been the subject of studies for decades, the impact of geysing cycles on hot spring microbial communities remains an understudied area.



Cyanobacteria are a highly relevant microbial phylum; significant to Earth's oxygen and carbon cycles. The *Synechococcus* A/B clade of photoautotrophic cyanobacteria is prevalent and highly diverse in Yellowstone National Park hot springs at temperatures up to ~73°C (Allewalt et al., 2006; Becroft et al., 2011, 2015, 2020; Brock et al., 1967; Ferris and Ward, 1997; Miller et al., 1998, 2009; Ward et al., 1998, 2006). No phototrophs exist at temperatures higher than ~73°C in any sites globally. At this temperature, there is a visible phototrophic fringe; a transition in the community where phototrophs are no longer present. Much of the work with *Synechococcus* A/B has been done in nongeysing/stable hot springs, which have a consistant rate of flow and stable site conditions. Diversity in the *Synechococcus* A/B clade is in the form of ecotypes; ecologically relevant genotypes with different temperature ranges in stable springs. *Synechococcus* A (Syn-A) and *Synechococcus* B (Syn-B) are the two major ecotype clades, and the Syn-A subclade, *Synechococcus* A', is a third.

Synechococcus A/B is evolutionarily interesting as an early branching photoautotroph (having branched right after the primitive thylakoid-less *Gloeobacter*). It is ecologically interesting because of its diversification into thermal niches in stable hot springs– and its ability to surpass the niches that are so established in stable hot springs, if temperature conditions are instead inconsistent. If fluctuations in temperature do not limit *Synechococcus* diversity, this could expand our knowledge as to the conditions that life can tolerate. Cyanobacterial proliferation despite the environmental challenge drives home the unexpected ability of photosynthesis tolerating such extreme conditions.

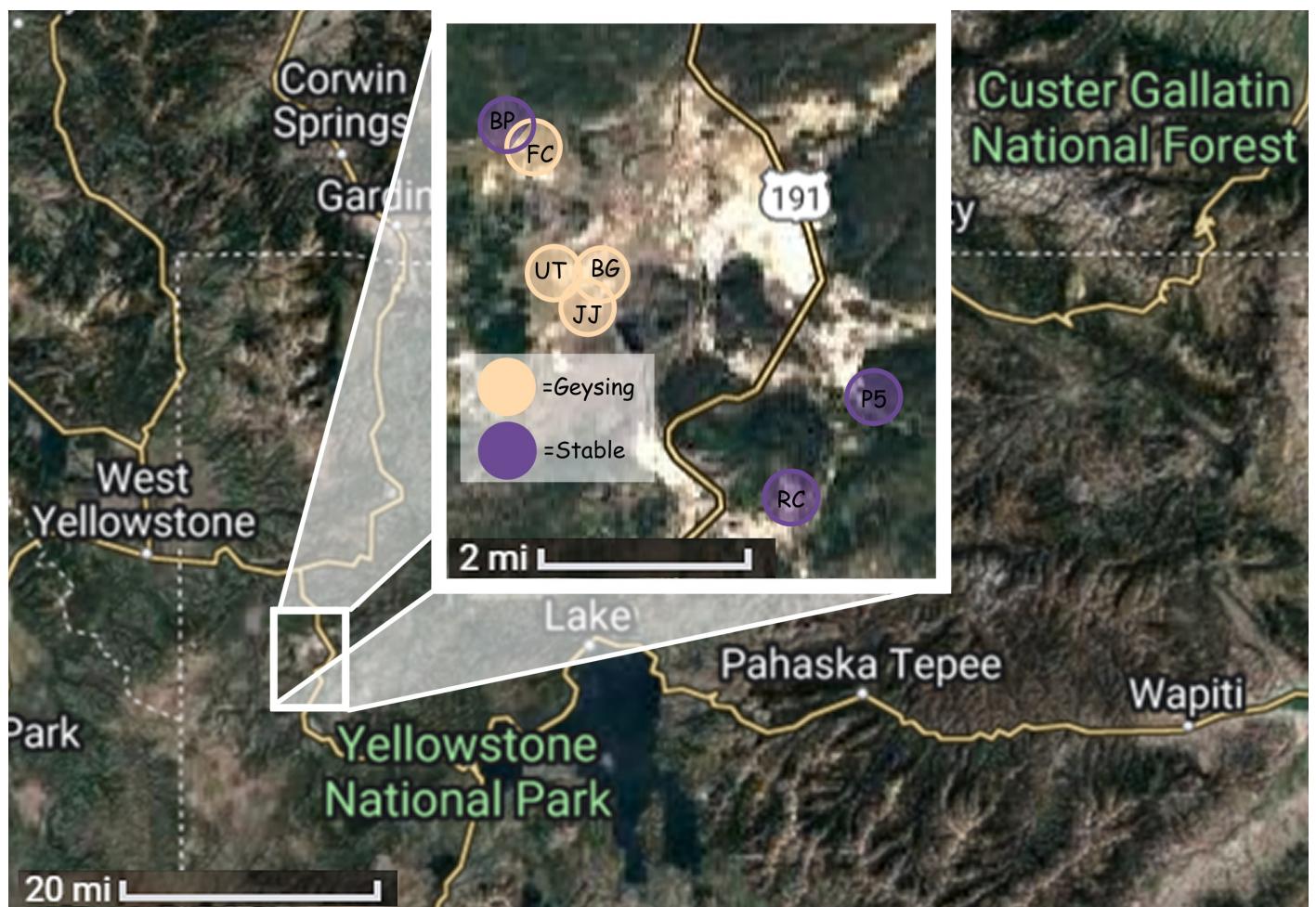
Syn-A', Syn-A, and Syn-B are associated with approximate respective temperature ranges of 55°C-73°C, 40°C-70°C, and <40°C-65°C (Allewalt et al., 2006; Becroft et al., 2011; Ferris and Ward, 1997; Miller et al., 2009; Ward et al., 2006). Only Syn-A', with a 20°C temperature range, is found at the phototrophic fringe. Syn-A and Syn-B have a ≥ 30°C range. These *Synechococcus* have recently been confirmed present in geysing springs (Hamilton and Havig, 2022). In contradiction to 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 (~73°C), in geysing outflows, we see all ecotypes are capable of tolerating the wide temperature ranges of a geysing site. This may be due to the short period of time the site spends at any given temperature. There is still much to be elucidated about how the unique temperature conditions in geysing springs impact both *Synechococcus* and the community at large.

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 et al., 2009; Ward et al., 2006; Ramsing et al., 2000), likely due to the large impact of temperature on cellular function, particularly, photosynthetic components. Phototrophic light and electron harvesting components are sensitive to heat stress, as are key enzymes such as RuBisCo and RuBisCo activase (Cimdins et al., 2014; Hamilton, 2019; Mathur and Jajoo, 2014; Salvucci et al., 2001). While the thermally induced constraints on phototrophy have been well explored (Blondin et al., 1993; Rajaram et al., 2014; Suzuki et al., 2006), the mechanisms of increased thermotolerance by phototrophs living at phototrophic fringe sites have not been established. *Synechococcus* A'/A/B are the only phototrophs to persist at or near the upper temperature limit of photosynthesis, ~73°C. Due to the impact of temperature on photosynthesis, *Synechococcus* is likely to continuously express heat stress related proteins throughout the geysing cycle. This work focuses on the interplay between temperature and the microbial community, particularly *Synechococcus* ecotypes, and aims to begin an avenue to research to assess the impact of fluctuating temperature on *Synechococcus* diversity and physiology.

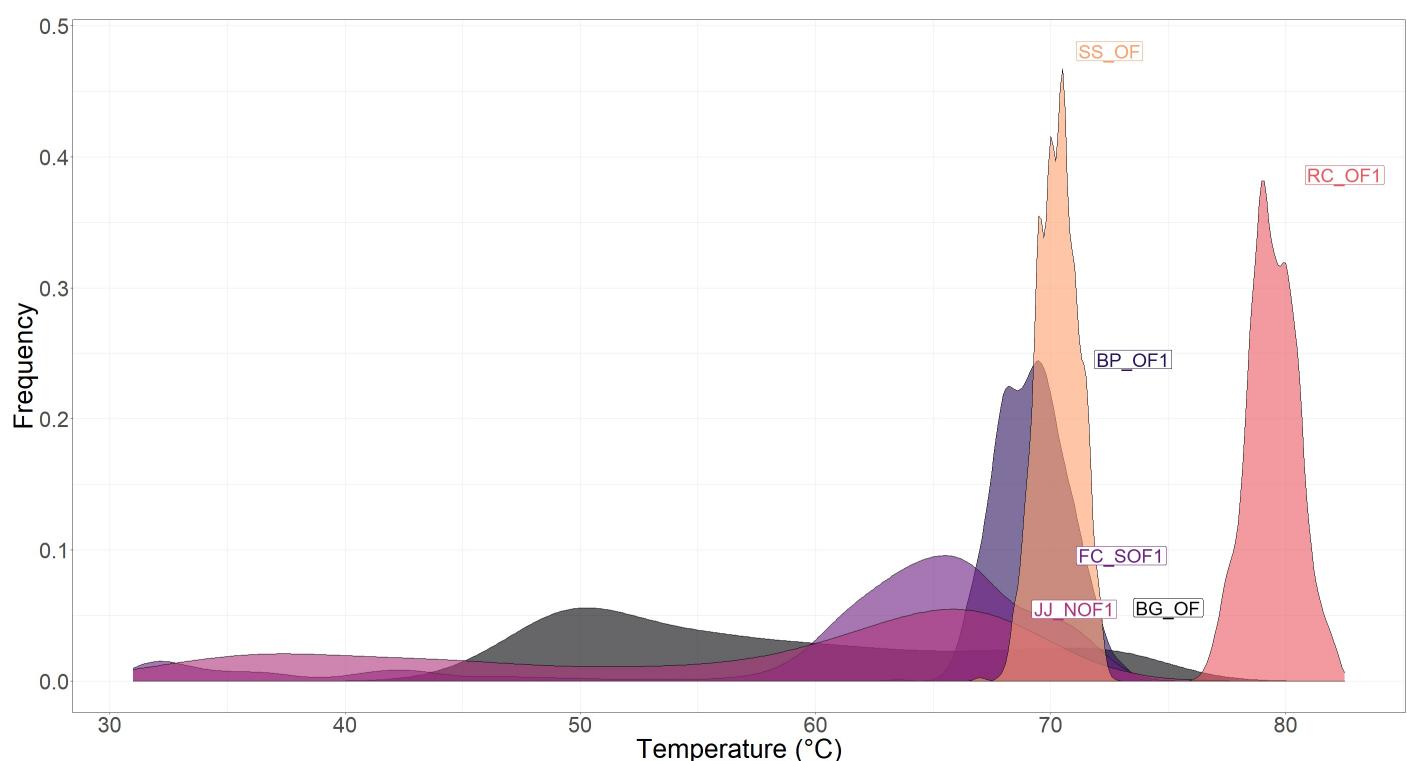
Both observed diversity (richness) and Shannon diversity (richness and evenness) were used to investigate the ecological diversity of geysing and nongeysing springs, in both the full microbial community, and the organism of interest- *Synechococcus* A/B. While *Synechococcus* diversity was found to be similar between spring types, *Synechococcus* relative abundance was higher in geysing sites. Metatranscriptomics were used to assess what microbial functions were differentially expressed between stable and geysing hot springs. Many *Synechococcus* genes are more highly expressed in geysing sites, including genes involved in photosynthesis and the nitrogen cycle. This pattern appears to be stronger for *Synechococcus* A clade (Syn-A) than for *Synechococcus* B clade (Syn-B). Phototrophy is not inhibited by fluctuating temps; *Synechococcus* alpha and beta diversity overlap between spring types. This is significant when considering the role that temperature is known to play on the diversification of phototrophic cyanobacteria. The decreased number of taxa overall (eg, lower community level observed diversity) in geysing sites, in combination with consistent observed cyanobacterial diversity in both spring types, results in a higher relative abundance of cyanobacteria in geysing sites than in stable sites. Further affirmation that photoautotrophy is not impacted by temperature fluctuation is indicated by the lack of change in photosynthetic gene expression throughout a geysing cycle. Comparison of key photosynthetic genes' expression throughout temp fluctuations reveals that phototrophy, along with other microbial metabolisms, likely remains consistent throughout a geysing cycle.

Methods:

Sample collection: Triplicate microbial mat samples were taken from several distinct geysing and nongeysing outflows at phototrophic fringe sites and one or more cooler downstream sites, in 2021-2022. These biomass samples were flash frozen immediately after collection. Sampling site temperatures were selected to cover a wide thermal range. DNA was extracted from samples that were taken from 4 geysing springs– Blue Grotto (BLG), Flat Cone (FC), Jolly Jelly (JJ), and Undercut Terror (UT) – and 2 nongeysing springs– Bison Pool (BP) and Par 5 (PR). RNA was extracted from samples that were taken from 2 geysing springs– FC, JJ – and 3 nongeysing springs – BP, PR and Rabbit Creek (RC). Sample collection was facilitated by Yellowstone National Park under research permit YELL-2021-SCI-7020.



Site map indicating sampling locations. Geysing sites: Flat Cone (FC), Undercut Terror (UT), Blue Grotto (BG), and Jolly Jelly (JJ). Stable/nongeysing sites: Bison Pool (BP), Rabbit Creek (RC), Par 5 (P5).



Nucleic acid extraction and sequencing: (**DNA**) DNA was extracted using a DNeasy PowerBiofilm Total DNA Kit and submitted to the University of Minnesota Genomics Center (UMGC) for library preparation and sequencing of the 16S rRNA gene. Illumina sequencing was conducted at UMGC for DNA sequences using 16S primers with Nextera adapter sequences. (**RNA**) RNA was extracted using the Qiagen RNeasy Power Biofilm Kit, and submitted to the University of Minnesota Genomics Center (UMGC) to undergo ribosomal reduction using a RiboZero kit and Illumina sequencing to generate paired end reads.

Data Analysis Methods; data cleaning and processing: (**DNA**) DNA sequence data was cleaned, trimmed, aligned, and binned into ASVs using dada2 (Callahan et al., 2016). No cyanobacteria were detected in 2/3 replicates of one of the BP samples, therefore, those replicates were excluded from downstream analysis. A custom reference file was created for clade-level taxonomy classification using OH28, OS-A and OS-B as references for A', A and B (Becraft et al., 2011; Bhaya et al., 2007). ASVs from this study, along with sequences from previous work in the Hamilton lab and additional reference sequences, were included in phylogenetic reconstructions to place ASVs from this study in a broader phylogenetic context. (**RNA**) Raw RNA reads were trimmed to remove adapters and poor quality sequences using Trimmomatic (Bolger et al., 2014). Remaining rRNA was filtered out using SortMeRNA. Short reads were assembled into contigs, and a de novo metatranscriptomic assembly was constructed, using Trinity prior to quantification of mRNA counts using Salmon. The resulting counts matrix was annotated following the use of Trinotate taxonomy and functional annotation. Trinotate includes wrappers for transdecoder, which identifies likely coding regions in transcript data (Haas, BJ. <https://github.com/TransDecoder/TransDecoder> (<https://github.com/TransDecoder/TransDecoder>)), and signalP, which predicts signal peptides (Teufel et al., 2022). Following the operations of transdecoder and signalP, HMMER (Finn et al., 2011) and BLAST (Altschul et al., 1990) are used to assign Uniprot annotations. These annotations populated a Trinotate SQLite database, which was used to annotate the transcript and isoform counts matrices generated by Trinity assembly. The annotated transcript level ("gene" level) counts matrix was utilized in downstream analyses.

Data Analysis Methods; data analysis and statistics: (**Site data**) Temperature frequency plots, and a site map were created to provide environmental context to sequence data. (**DNA**) Sequences were aligned using MUSCLE (Edgar, 2004) within MEGA (Tamura et al., 2021), and trees were visualized in MEGA using maximum likelihood sequence reconstruction with the statistical support of branching patterns determined via bootstrapping. Trees were saved in newick format to be joined to the ASV abundance and taxonomy data for downstream analysis using R (R Core Team, 2019). R package ggplot2 (Wickham, 2016) was used for data visualization. R packages used in alpha diversity analyses include Phyloseq (McMurdie & Holmes, 2013) and the tidyverse (Wickham et al., 2019). Phyloseq's estimate_richness function was used to obtain Shannon diversity and observed diversity values for the full dataset and the dataset following filtering only for phyla Cyanobacteria and Chloroflexi. Wilcoxon rank-sum test was done on diversity versus spring type for both the full and Synechococcus-only datasets, with a bonferroni correction done on the resulting p-values. Bray-Curtis dissimilarity matrices of log-transformed data were the basis for construction of PCA plots. ADONIS was conducted on centered log-ratio (CLR) transformed count data, followed by a betadisper test of dispersion, to determine whether community compositions were significantly different. ASV counts were transformed into relative abundances by sample prior to the comparison of the prevalence of "abundant" ($\geq 2\%$ relative abundance in at least one site). (**RNA**) Reads were filtered to remove low abundance reads; those kept were >10 counts per million (CPM) in 2+ samples. Note, results should be taken as tentative/in need of further validation, due to the low numbers of replicates (1-2). In the volcano plots that show the results of differential expression analysis, an abundance of transcripts had high log2Fold change between stable and geysing flows, yet had high p-values. This pattern is associated with sample size and high variability. Analyses confirm that there is high variability in the nongeysing/stable samples. Both Pearson's correlation coefficient and a plot of the expression of the top 500 DE genes in the dataset indicate that Par 5 and one of the two RC sites cluster apart from the rest of the samples.

MSI slurm scripts used:

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R scripts used:

RNA analyses

DESeq.R - Preparing metatranscriptomic dataset for DE analysis, beginning with the Salmon results file and ending with differential expression results, awaiting visualization. Biological Metadata.R - Creating a reference spreadsheet of all transcript IDs and their UniProt annotations.

16S analyses

DADA2_16S.R - In this script, DADA was used to clean 16S sequences, demarcate ASVs and create an ASV abundance table, and create a taxonomy table for the ASVs. 16S_analysis.R - All downstream analyses in 16S data, including intermediate steps that produced objects used in the data visualization scripts. Figs for Q3 were also made in this script.

psaA analyses

DADA2wPsaA - In this script, DADA was used to clean psaA sequences, demarcate ASVs and create an ASV abundance table, and create a taxonomy table for the ASVs. DADA2phyloseqPsaA- All downstream analyses in psaA data, including intermediate steps that produced objects used in the data visualization scripts.

File pathway to create all figures in this document

Brief Introduction: Site map figure was made using Google maps, and the GPS coordinates on site metadata spreadsheets. Temp Plots.png and Fringe Site Temp Freq.png were made in Temperature Plots.R. Question 1: DADA2_16S.R -> 16S_analysis.R -> Diversity Figures.R Question 2: DADA2_16S.R -> 16S_analysis.R -> Relative Abundance of Genera.R Question 3: (first plot) DADA2_16S.R -> 16S_analysis.R + Tree Made with MEGA -> TreeWithTempRanges.R (second plot) DADA2_16S.R -> 16S_analysis.R + Tree Made with MEGA -> Abundance bubble plot (line 254), Dumbbell plot (line 353), Phylo tree (line 552), the three plots were aligned in photo editing program Clip Studio. Question 4: DADA2wPsaA.R -> DADA2phyloseqPsaA.R -> PsaA Phyloseq object. DADA2_16S.R -> 16S_analysis.R -> Syn only Phyloseq object. PsaA Phyloseq object + Syn only Phyloseq object -> Syn psaA vs 16S.R Question 5: Used PsaA sequences from DADA2phyloseqPsaA.R, and also extracted which transcript IDs were associated with PsaA seqs in "Retrieve PsaA IDs.R" -> Tree Made with MEGA + Aligned PsaA Sequences -> Making PsaA Tree and PID plot.R Question 6: DESeq.R -> Stable vs geysing DE heatmap.R Question 7: DESeq.R -> Stable vs geysing DE heatmap.R Question 8: DESeq.R -> Boxplots For Log CPM of Genes of Interest.R Question 9: DESeq.R -> DE gene expression bt geysing ON and OFF.R

Results

Question 1: Does diversity differ between stable and geysing springs?

Results: Alpha diversity differs between springs types when looking at the overall community, with diversity (both Shannon and Observed) being higher in nongeysing springs. Distinctions between spring types via bray-curtis similarity are difficult to parse in this PCA plot, indicating a degree of homogeneity between springs. However, diversity indexes underscore that *Synechococcus* is not among the taxa inhibited by fluctuating temps. *Synechococcus* richness and evenness are consistent across springs. In addition, there is little-to-no change in phototrophic gene expression across geysing timepoints (Question 9).

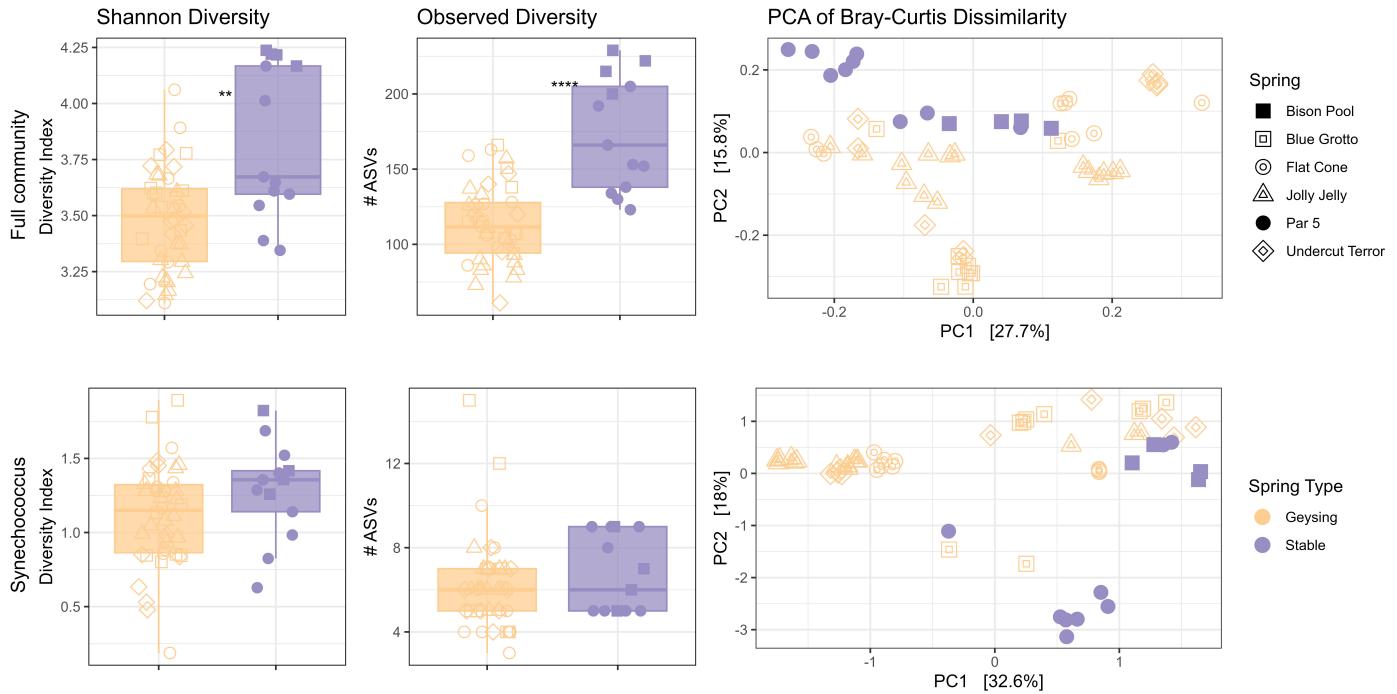
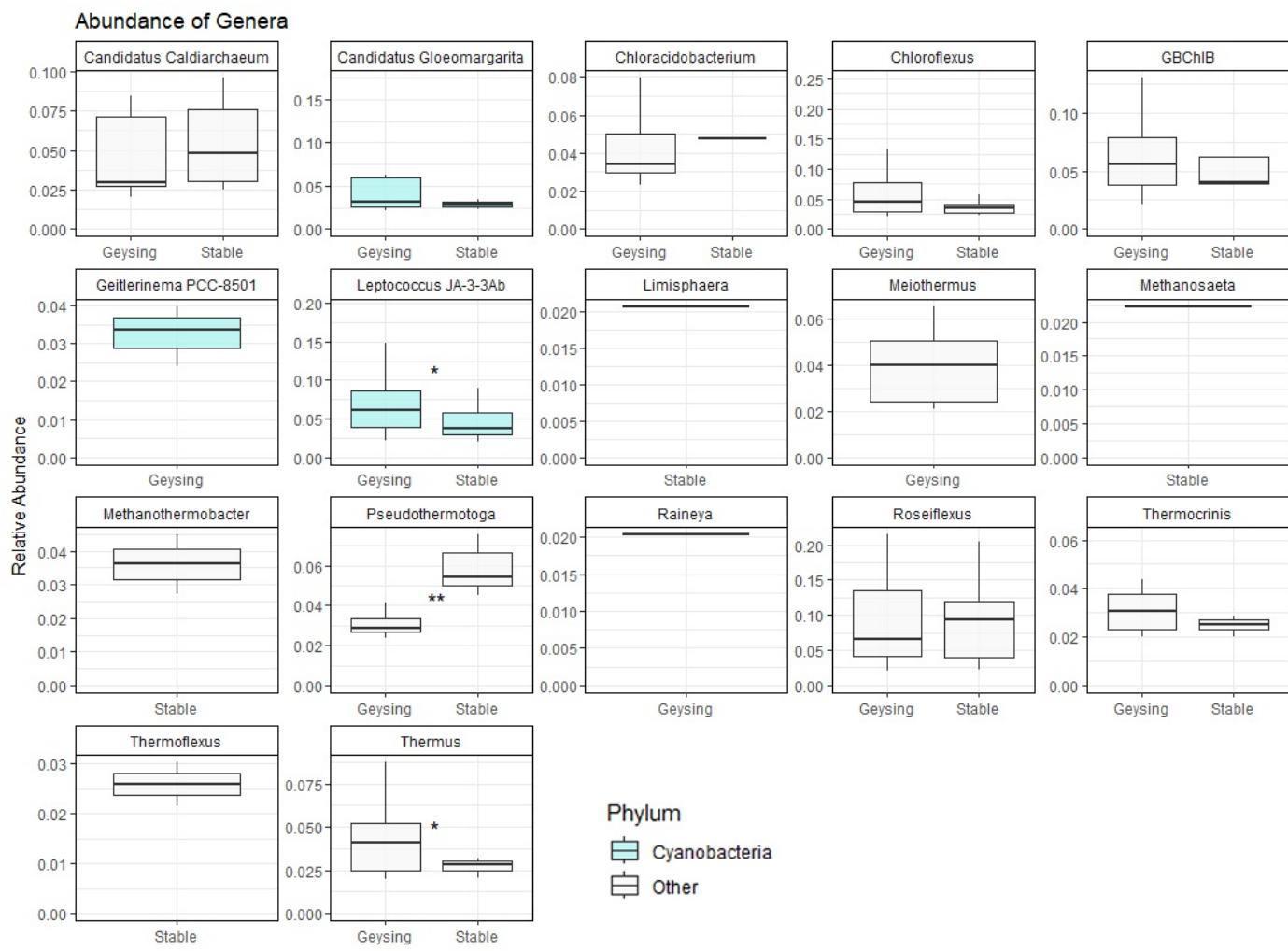


Figure 1: Alpha and Beta Diversity

Question 2: If not Cyanobacteria, which taxa are differentially abundant?

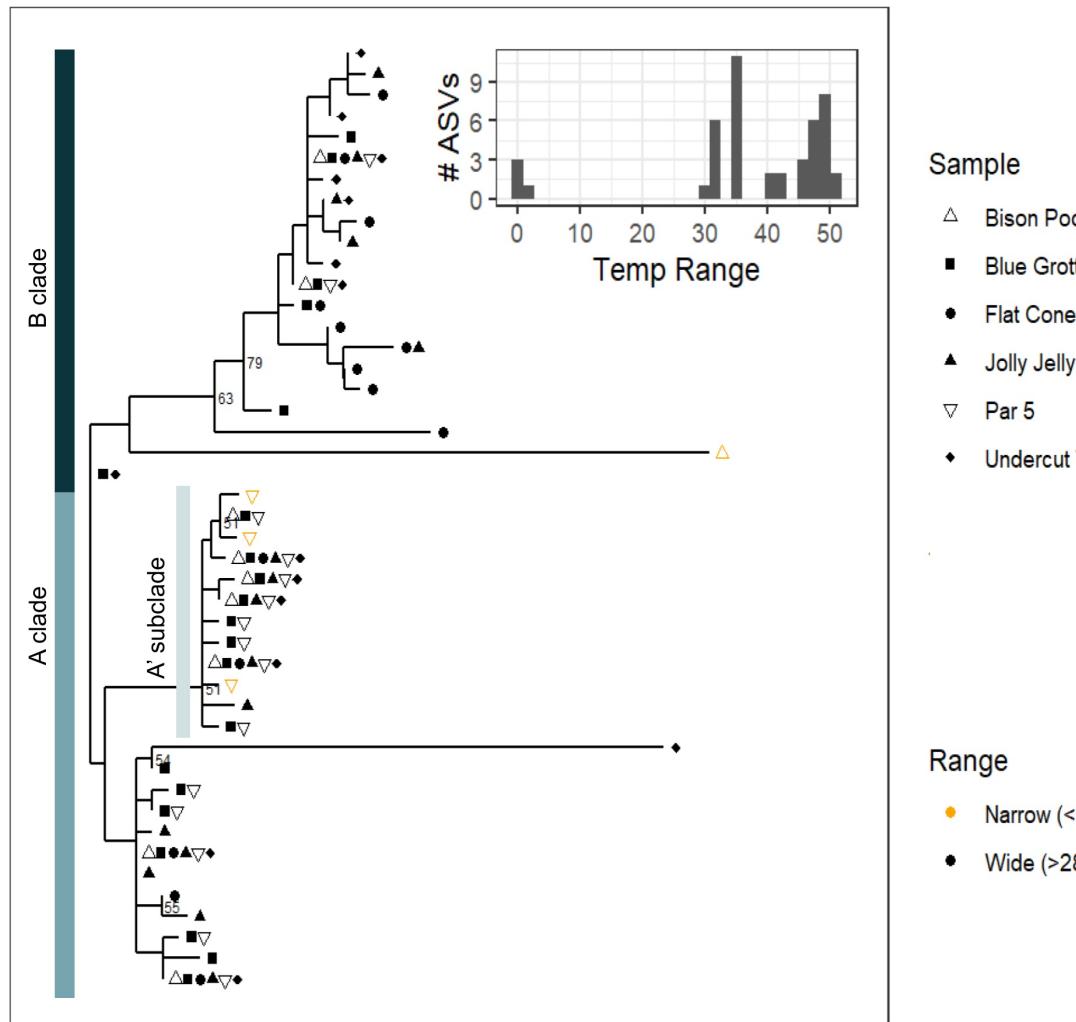
Results: While cyanobacterial **diversity** is not different, there are significantly more more cyanobacteria in terms of relative abundance. Eg, there are proportionally more cyano in geysing springs, though the actual number of cyanobacterial OTUs is similar. This is because the geysing sites had fewer OTUs on the community level/lower community level richness. Most likely, a large part of this is due to nongeysing/stable springs having more “unique” taxa, eg, taxa only found in nongeysing springs. In terms of the number of genera found at >2% relative abundance in only one spring type: 9 taxa are found only in nongeysing sites, and 2 genera are only found in geysing sites. One of those two genera, *Geitlerinema PCC-8501*, is a cyanobacterium. Perhaps geysing springs select for more generalistic organisms? Of the 16 taxa found at 2% relative abundance in both spring types, *Synechococcus* and *Thermus* are higher proportion of the community in geysing springs, while *Pseudothermatoga* is of higher abundance in stable springs. These results suggest that, overall, diversity differences between spring types are of a presence/absence nature.



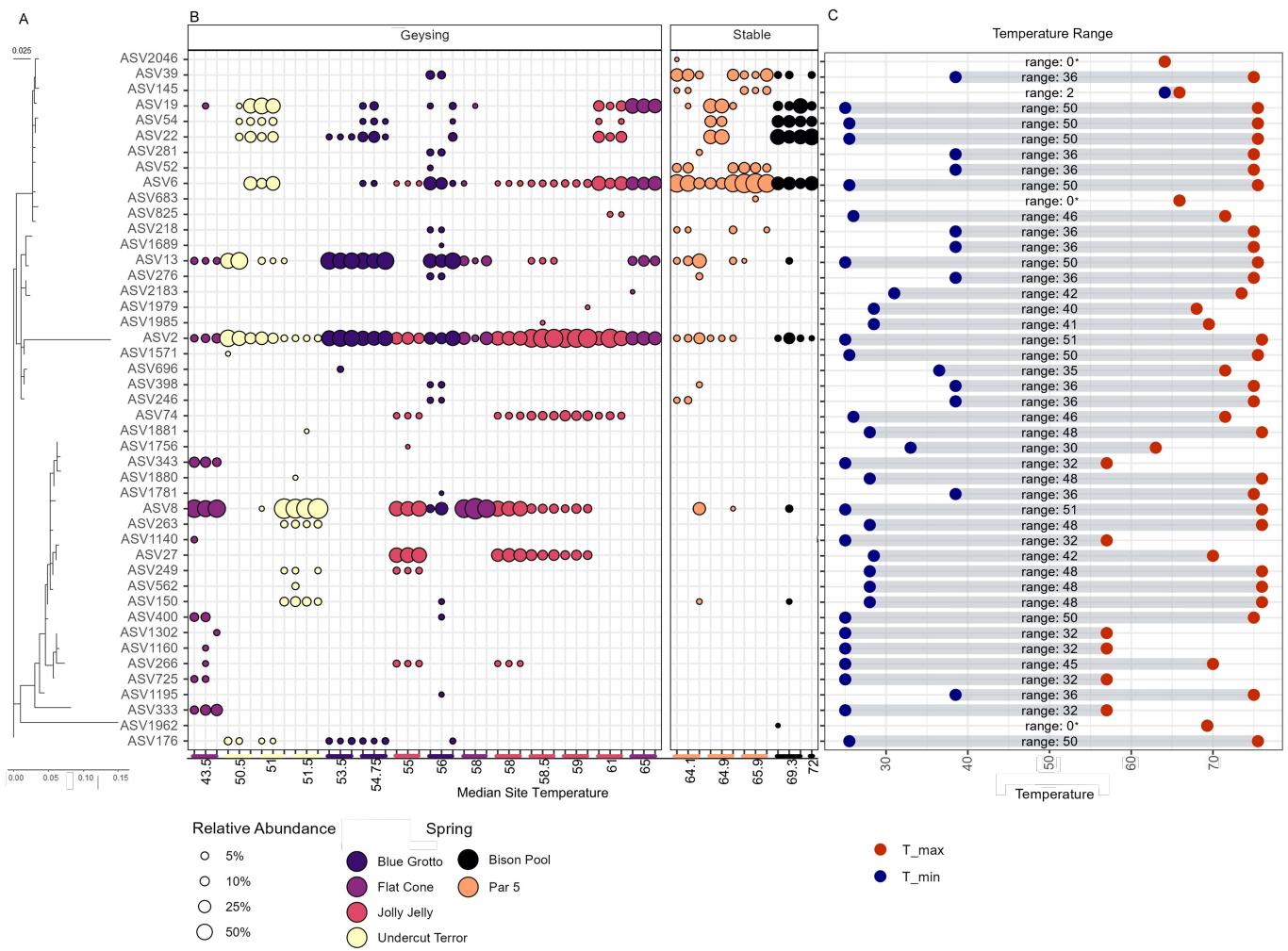
Relative abundance of genera (>2% relative abundance)

Question 3: Ecotype subclade A' is proposed to have diverged from A and B clade in certain genes while specializing to a high, narrow temperature range. Are they present in geysing springs, and, if so, how is their management of fluctuating temperatures?

Results: Ecotype A' is present in geysing springs. The below figure demonstrates a lack off relationship between 16S phylogeny and an ability to tolerate wide temperature ranges in geysing sites (with a temperature range >28C between Tmin and Tmax in the dataset). The relative lack of B clade diversity in the nongeysing sites here is likely due to site temperatures sampled; these higher temperatures sampled favored A clade ecotypes. *Synechococcus* A/B clade ecotypes are not compelled to follow the temperature ranges defined for them in nongeysing springs; sites that experience wide temperature range that includes the upper temperature limit for photosynthesis are home to all 3 ecotypes. While the presence of ecotypes downstream may be partially due to being washed downstream, this is not the case for upstream sites, and it is likely that all 3 ecotypes are actively growing in geysing springs. Future work may reveal unique mechanisms for survival that are ubiquitous in all geysing ecotypes and not present in their nongeysing counterparts.



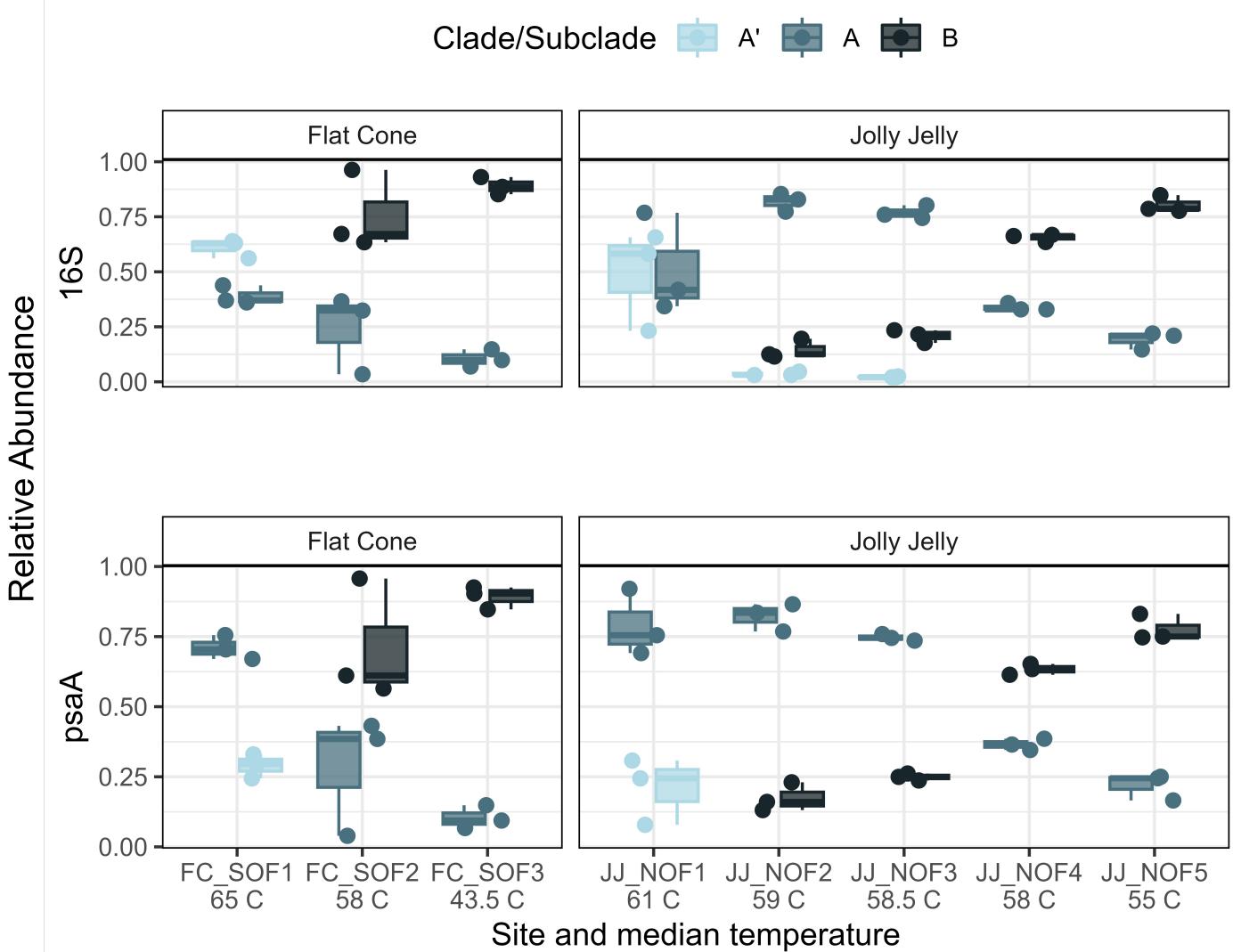
From YNP_Sum21_stable_and_geysing_16S/Triplete16Sanalysis/TreeWithTempRanges.R



16S Results: most ASVs are found across a wide range of temperatures. This figure presents a more nuanced view of the results that are summarized in the previous figure.

Question 4: PsaA is used for diversity analysis in previous work. Do psaA results here by JJ and FC correspond to the 16S results?

Results: Yes, in most instances. However—there are more A-like psaA sequences in the phototrophic fringe sites of FC and JJ than 16S would suggest, and fewer A'-like psaA sequences. This may indicate that some of the organisms in fringe sites have an A-like 16S sequence and an A'-like psaA sequence, which is reasonable given the rate of evolution in the two genes, but presents a confounding variable for studies which rely on only one amplicon (eg 16S or psaA) sequence as a metric for diversity.

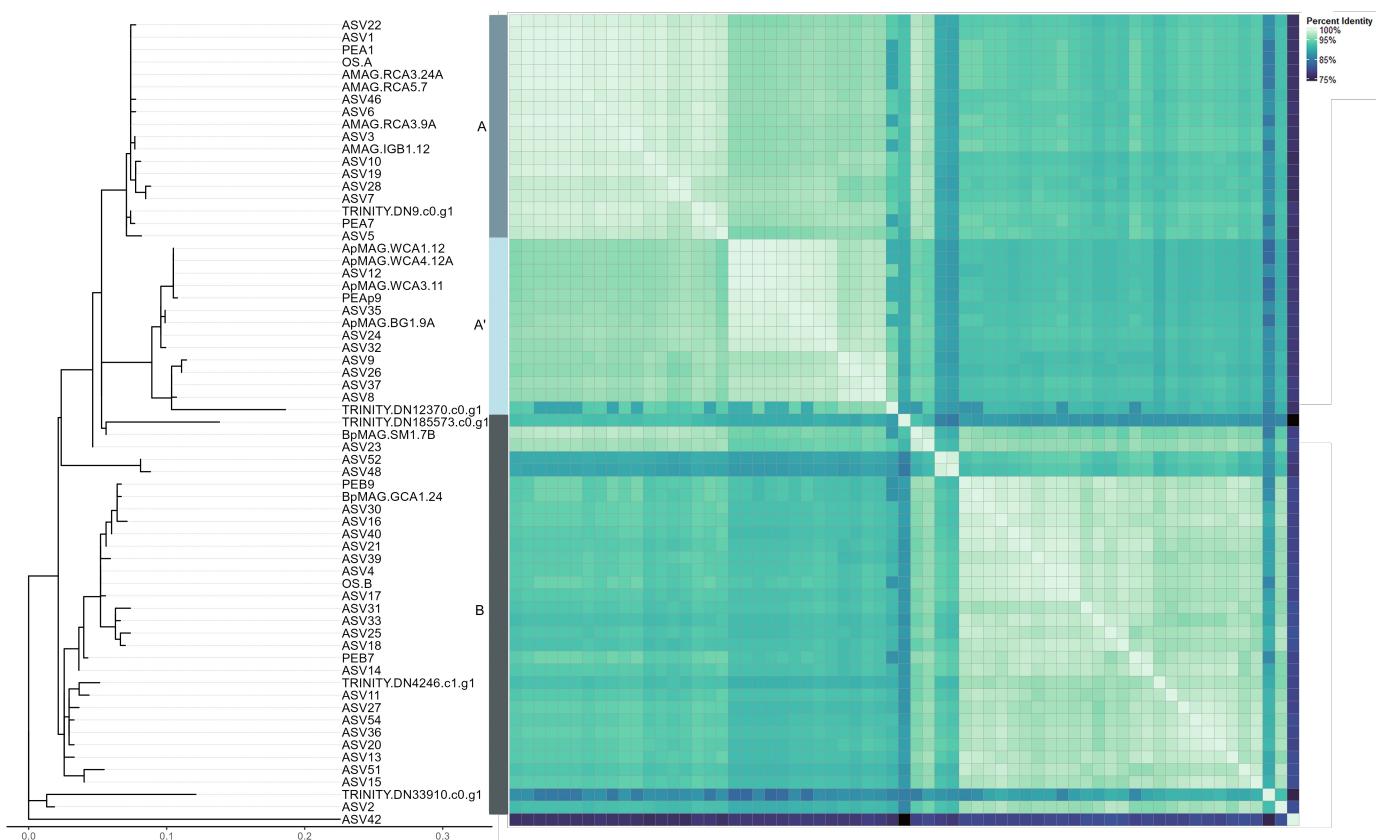


Relative abundance of Synechococcus 16S and psaA variants

Question 5: Are the PsaA seen in amplicon seq representative of the population? Eg, are they identical to the PsaA found in the metatranscriptome?

Results: For the most part, ASVs are clearly divided into A/A'/B, and most RNA sequences follow this pattern. Notably, there are fewer RNA PsaA sequences than DNA PsaA sequences. This may be due to the grouping of highly similar PsaA transcripts; the method the Trinity assembly uses to determine which “isoform-level” results should be grouped into different “genes” for “gene-level” results is a bit of a black box. It is recommended to do analysis on the “gene” level rather than the “isoform” level, thus, that is what was done in the analyses with the RNA data here.

Sample names beginning with: ASV = from the psaA amplicon dataset TRINIT.DN= RNA dataset A/Ap/BMAG= MAGs from previous Hamilton lab study OS.A/OS.B= reference sequences for Syn A & B



Made in "psaA and PID plot.R"

Question 6: What is differentially expressed between stable and geysing springs- are the functional differences between stable and geysing springs?

Result: Interestingly, there seems to be a strong divide in the behavior of *Synechococcus* and the response of most other organisms. Out of the top 5000 differentially expressed genes (out of ~12000 differentially expressed genes), most of the *Synechococcus* genes are more highly expressed in geysing sites, while more of the non-Syn genes are more highly expressed in stable sites.

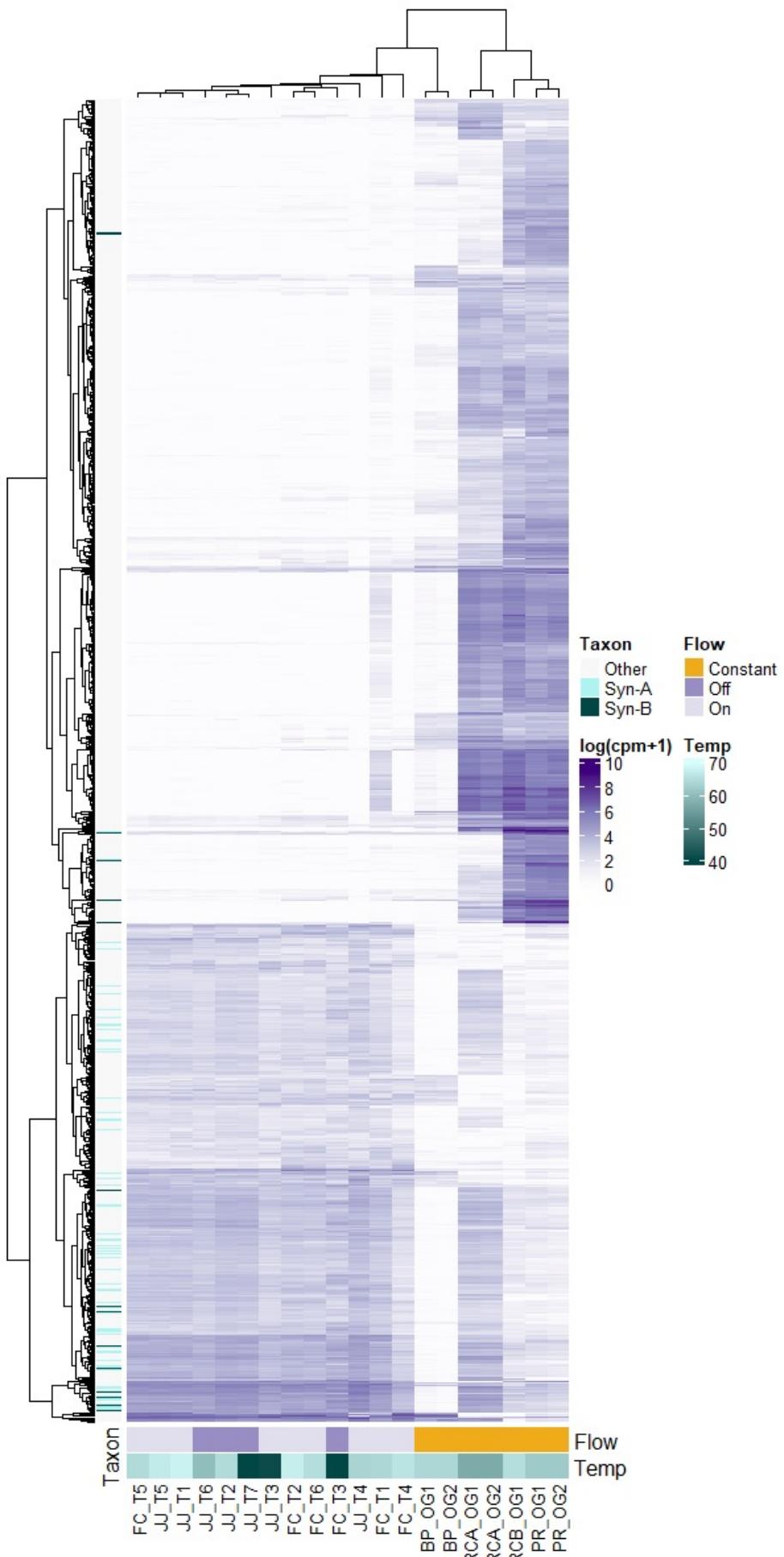
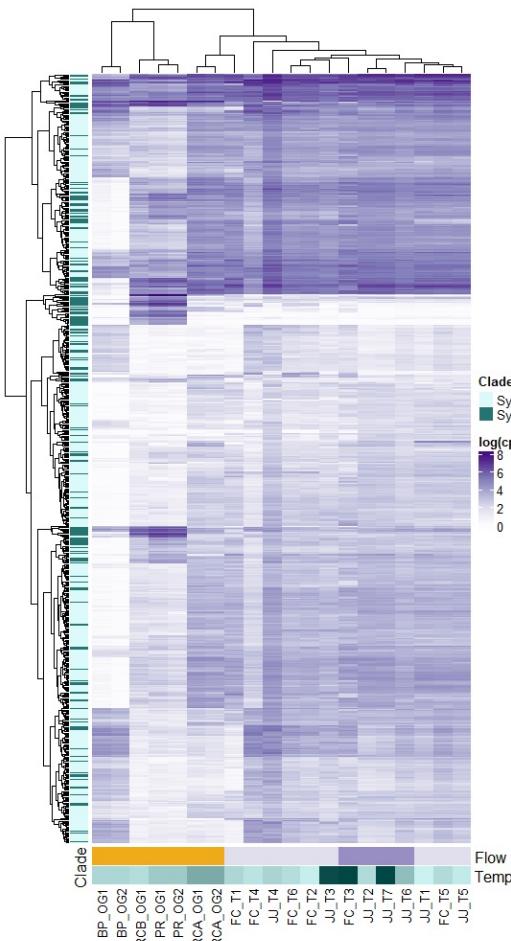


Figure created in: "Stable vs geysing DE"

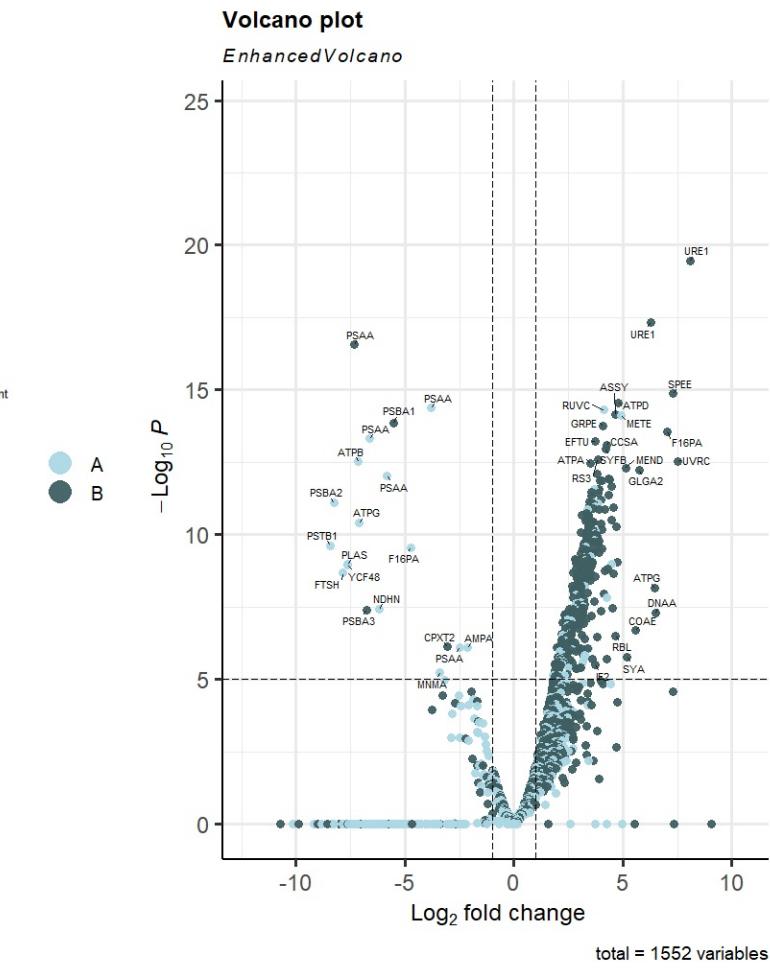
heatmap.R"

Question 7: Which cyanobacterial genes are differentially expressed?

Result: As in with the full community, we don't see a strong division between geysing-on and geysing-off. However, there is a little more distinction in on vs off in this *Synechococcus* only dataset than in the full community. 671 of the 1552 genes belonging to Syn in the dataset are represented here. (~10% of all differentially expressed genes are *Synechococcus*, and about 43% of *Synechococcus* genes are differentially expressed.) Syn-A and Syn-B appear to be experiencing different patterns. Most of the *Synechococcus* genes that are more highly expressed in geysing sites are Syn-A, while the Syn genes that are more highly expressed in stable springs are a mixture of Syn-A and Syn-B genes. Within the heatmap, we observe what seems to be groupings of genes with a similar expression pattern. Within each grouping, there will generally be a subgroup containing most of the Syn-B genes associated with that group.



Synechococcus Heat Map



Synechococcus Volcano Plot

Figures created in: "Stable vs geysing DE heatmap.R"

Question 8: Are cyanobacterial heat stress related transcripts differentially expressed between spring types and/or within a geysing cycle?

Result: Within a geysing cycle, differential expression analysis revealed no change in transcriptomic expression between conditions of water flow “on,” when the site was actively geysing, and water flow “off,” in between eruptions (results in question 9). The first 4 columns of this figure provide a more nuanced view, demonstrating the consistency of gene expression across the timepoints of Flat Cone (columns 1 and 2) and Jolly Jelly (columns 3 and 4). In Flat Cone, only timepoint 3 is in an “off” state. In Jolly Jelly, timepoints 2, 6 and 7 are in an “off” state. A handful of genes exhibit significantly higher expression in one timepoint in one spring: CH601 for both Syn-A and Syn-B in timepoint 5 of Flat Cone, Syn-A psaA in timepoint 1 in Flat Cone, and atpB is in timepoint 4 in Jolly Jelly. However, the veracity of the results of site-to-site comparisons is weaker than comparing all “on” samples to all “off” samples, as there is only one replicate per sampling time point, per spring, for these geysing samples.

These genes are not differentially expressed throughout the time period of a geysing cycle, supporting a hypothesis that *Synechococcus* are either not experiencing heat stress OR that they mitigate their heat stress in a non-transcriptional way.

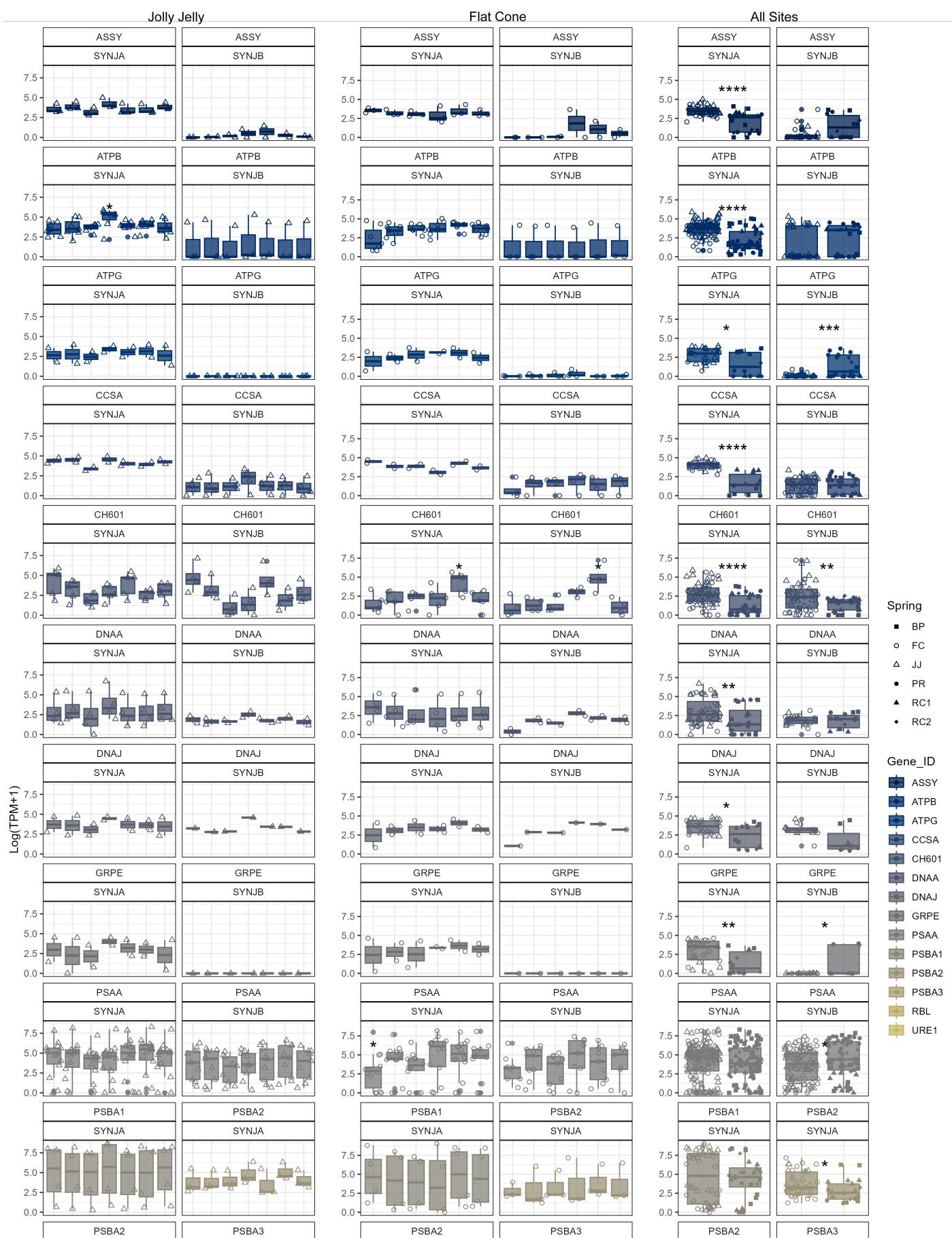
Indeed, as there are very little change in gene expression between conditions of water on and water off, potentially the rapid rate of temperature change favors an alternative to a transcriptional heat stress related response, such as post-translational modifications. Further research will be needed to clarify how *Synechococcus* and the rest of the community withstand the conditions off geysing springs.

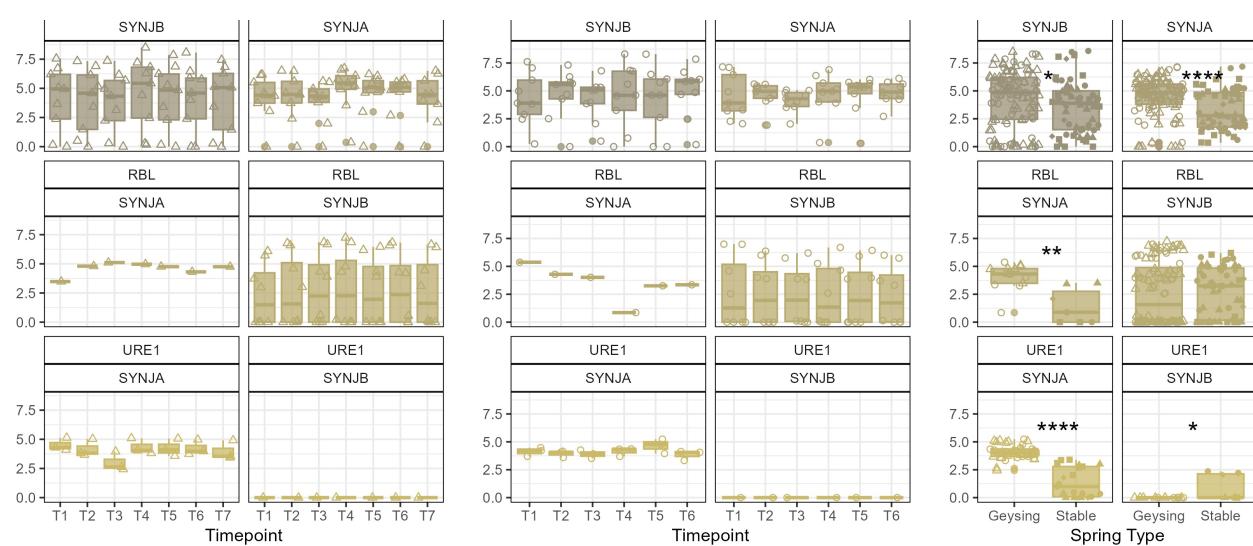
All information in the table is from the UniProt site.

Information about genes displayed in Q8 figure

Gene_ID	Gene	Details
PSBA1	Photosystem II protein D1, form 1	D1/D2 bind the primary electron donor for Photosystem II
PSBA2	Photosystem II protein D1, form 2	
PSBA3	Photosystem II protein D1, form 3	
PSAA	Photosystem I component	psaA is part of the heterodimer complex that binds the primary electron donor of Photosystem I
CCSA	Cytochrome c biogenesis protein	Required to attach heme to cytochrome c6 and cytochrome f
RBL	Ribulose bisphosphate carboxylase, large subunit	Subunit of RuBisCo, a key Calvin cycle protein
ATPG	ATP synthase gamma chain	Thought to regulate ATPase activity
DNAA	Chlorophyll a/b binding protein	Chlorophyll a/b binding protein

DNAA	Chromosomal replication initiator protein	Binds to the origin of replication during the initiation of DNA replication
GRPE	Protein folding chaperone	Prevents the aggregation of stress-denatured proteins; works in association with DnaKJ
DNAJ	Protein involved in heat shock response and protein folding	Prevents the aggregation of stress-denatured proteins; works in association with DnaK and GrpE
CH601(GROEL)	Protein folding chaperone	Essential in assisting protein folding; works with GroES
URE1 (UREC)	Urease subunit alpha	Nitrogen metabolism
ASSY	Argininosuccinate synthase	Biosynthesis of the amino acid arginine

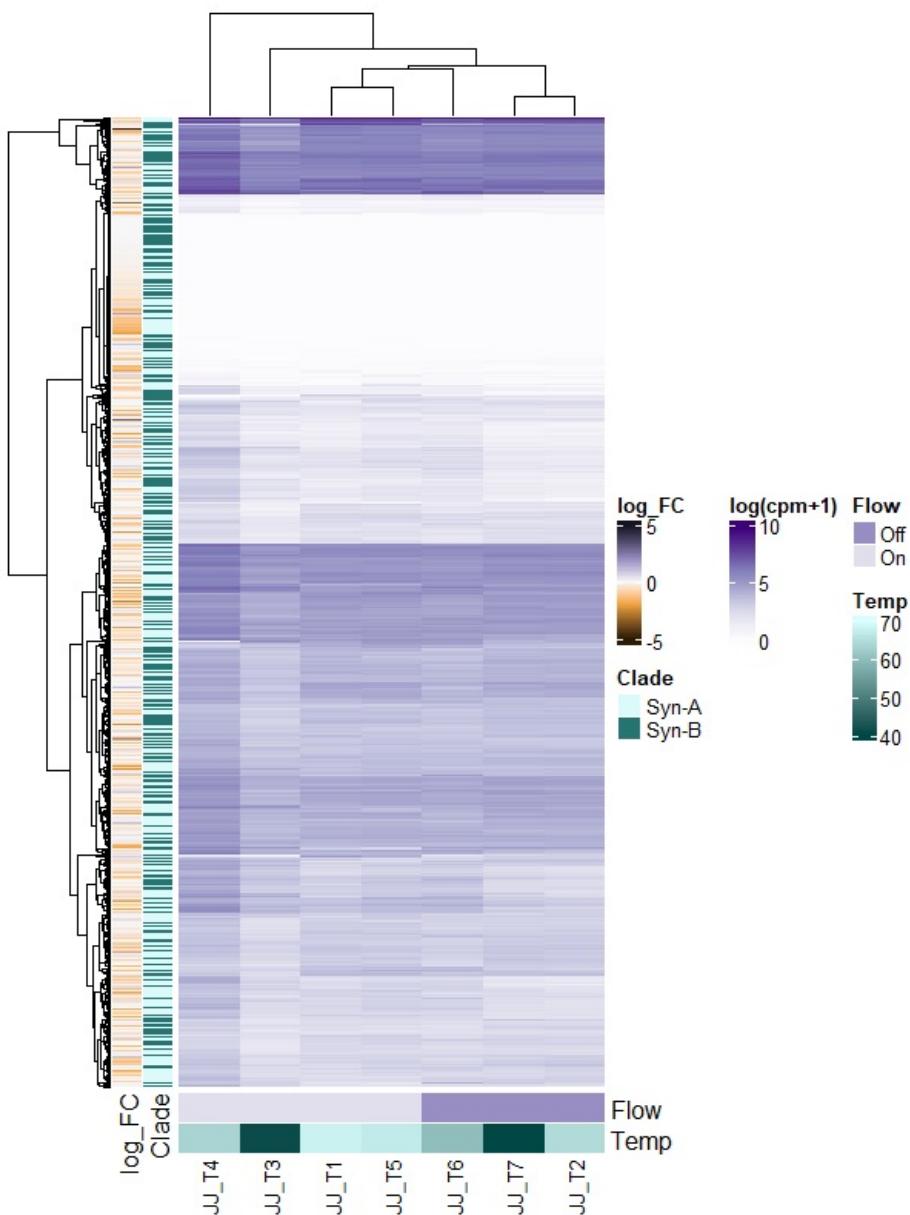




Differentially expressed *Synechococcus* genes

Question 9: What is differentially expressed throughout the geysing cycle/ between conditions of water on and water off?

Result: Jolly Jelly had 4 flow “on” and 3 flow “off” samples from the same site; enabling direct comparison to the “on” and “off” states of the metatranscriptome of a geysing site. This was not possible for Flat Cone, as for it there was only 1 sample in the flow “off” state. Given the limited number of community-wide differentially expressed genes between conditions of “on” and “off” conditions, it is possible that the rapid rate of temperature change favors alternative mechanisms, such as post-translational modifications, to cope with heat stress. Alternatively, these results could suggest that the organisms in these springs are adapted to fluctuating conditions and can maintain stable gene expression despite changes in their environment. Although no genes were differentially expressed between conditions, a heatmap was created to visualize the distribution of gene expression to assess any patterns which may be visible to the eye.



Synechococcus Heatmap, Jolly Jelly

Synechococcus does not mount a transcriptional-level response to the rapid temperature fluctuations in geysing sites. Transcription remains relatively unaffected by the changing environment of a geysing system, with no genes being differentially expressed at a statistically significant level between conditions of flow "on" versus flow "off." However, there is some moderate clustering of "on" sites and "off" sites in the below figure, which demonstrates rates of *Synechococcus* gene expression in the Jolly Jelly sample. On the far left is a bar showing the log-fold change in expression between "on" sites and "off" sites. Notable in this heatmap is the near-complete absence of a section of the metatranscriptome from all samples in this spring; this would correspond to genes only transcribed in the stable springs. Also notable is the highly expressed section at the top of the heatmap. The consistency of gene expression throughout the geysing cycle is further demonstrated in the figure of question 8.

Summary

These findings provide insights into the responses of *Synechococcus* and the broader microbial community to the unique conditions of geysing springs, highlighting the need for further research to elucidate the mechanisms by which these organisms withstand the challenges posed by unstable environmental conditions. Further research will be needed to clarify how *Synechococcus* and the rest of the community withstand the conditions of geysing springs. Post-translational modifications are a viable option for the community. Future research should involve more detailed studies of the environmental conditions in geysing springs, as well as the physiological and genetic adaptations of the microbial community and *Synechococcus*. Experimental studies could also be conducted to test microbial responses to simulated geysing cycles in the lab.