Diel fluctuations in nitrogen fixation in microbial mats from Great Sippewissette Marsh

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Microbial Diversity 2012

Abstract:
Microbial mats are highly structured and organized communities that employ a wide diversity of different metabolic strategies. Cyanobacteria, have been reported to be the organisms responsible for nitrogen fixation in these microbial mat associations. In previous studies of microbial mats, it has been consistently observed that the peak of nitrogen fixation occurs early in the morning, when oxygen levels are still low. For this study nitrogen fixation was studied over diel cycles in microbial mats from Great Sippewissett Marsh. The trend that was observed for microbial mats from Great Sippewissett differed from what has been previously reported. The microbial mats studied for the purpose of this experiment had peak of nitrogen fixation (110nmol ethylene cm^{-2}) occurring in the middle of the day at 13:00, when photon irradiance was at its daily maximum. The difference in the nitrogen fixation trend can be attributed to members of the Purple Sulfur bacteria group, which are located below the top Cyanobacterial layer of the mat. Transcripts of *nifH* showed the closest sequence similarity to *Halorhodospira* and *Allochromatiu*. These organisms were found to be the most dominant active members in the microbial community at times when nitrogen was being fixed. This study aims to link nitrogen fixation rate over the course of a diel cycle to gene transcript number and phylogeny for ammonium uptake using the *ntcA* gene, and nitrogen fixation using the *nifH* gene.

Introduction:
Microbial mats are present in a wide array of environments from thermal hot springs, salt marshes to polar deserts. These communities are highly structured and employ of a wide diversity metabolic strategies. A common feature of microbial mats is to have areas of high oxygen concentration, which is a result of photosynthetic activity. The photosynthetic function of mats is dominated by Cyanobacterial communities, which generate high oxygen concentrations during the day [4]. In addition, Cyanobacteria have been reported to be the organisms responsible for nitrogen fixation in these microbial mat associations [2]. It has been shown by researchers that gene expression for *nifH* (encoding the nitrogenase reductase subunit) is up regulated prior to nitrogen fixation. Then at twilight there is a burst of nitrogen fixation and transcript levels decrease as nitrogen fixation is occurring [5]. *NifH* has been used as a marker gene and researchers have been able to characterize nitrogen fixing organisms. The phylogeny of *nifH* is in general agreement with the small-subunit (SSU) rRNA gene-based phylogeny and large databases have been compiled for identification purposes [6]. Nitrogen fixation has been well studied in microbial mats compared to nitrogen uptake in these closely

associated communities. Nitrogen nutrition in cyanobacteria is regulated by *ntcA*, which is a transcriptional activator that is activated in response to low ammonium levels. This nitrogen stress response that is controlled by *ntcA* is present in all know species of Cyanobacteria. Since ammonium is the preferred and energetically most favorable source of nitrogen when it is not present cyanobacterial cells induce pathways that allow for growth on other inorganic forms of nitrogen [3]. Like with *nifH*, *ntcA* has been found to be in agreement with the small-subunit (SSU) rRNA gene-based phylogeny. This study aims to link nitrogen fixation rate over the course of a diel cycle to gene transcript number and phylogeny for ammonium uptake using the *ntcA* gene, and nitrogen fixation using the *nifH* gene.

Material and Methods:

Sample collection and experimental design:

A mat sample was collected from Great Sippewissett Marsh, which was roughly 43x43cm. The mat section was transported from Great Sippewissett to Loeb "outdoor laboratory". Samples were collected every two hours for RNA (flash frozen in liquid nitrogen), ion chromatography, temperature, oxygen profiles, photosynthetically active radiation measurements (PAR) and nitrogen fixation. Nitrogen fixation was measured as related to nitrogenase activity and ethylene production using acetylene reduction. Samples collected for nitrogenase activity were prepared in triplicate and were all 0.5cm in depth and 1.2cm in diameter. To each cored section of mat, 2ml of Great Sippewissett Marsh water was added. A dark control was prepared in parallel to the other samples. The dark control was necessary to observe the effect of photon irradiance on nitrogen fixation. Vials were incubated at *in-situ* conditions for 30 minutes prior to the addition of 10% of acetylene gas. Time point samples were then incubated for two hours, prior to sampling produced ethylene.

Acetylene Reduction Assay:

The production ethylene gas was measured in triplicate using a shimadzu FID-GC over a 30 hour time course experiment. Acetylene gas was formed by adding 0.5g of calcium carbide to an evacuated 130ml vial. Post vial evacuation 1ml of N_2 sparged milli-Q water was added and acetylene was immediately formed. 180 ml of acetylene was produced by the following the above experimental procedure. To determine the most appropriate concentration of acetylene to add to environmental microbial mat samples and experiment was conducted prior to the time course experiment where additions ranged from 1-10% of added acetylene. Based on the results it was concluded that 10% of headspace volume of acetylene was the optimal concentration to add to each extracted section of mat. The production of ethylene was then converted to a rate of nitrogenase activity/cm^{-2}.

Oxygen Profiles:

Oxygen profiles were generated using the UNISENSE O_2 microelectrode. The probe was calibrated to match *in situ* temperature conditions. Profiles started at the mat surface and were taken over a 2mm depth profile every two hours in 50μm increments.

DNA extraction:
DNA was extracted from Great Sippewissett mat samples using the MoBio Power Biofilm DNA Extraction Kit according to manufactures instructions. All DNA was quantified prior to bulk DNA PCR amplifications.

454 Barcoded Analysis:
Tag Pyrosequencing was performed on a representative microbial mat core from Great Sippewissett Marsh. Genes for the SSU rRNA were amplified using barcoded primers that contained Roche 454 Ti adaptor sequences as well as individual barcode sequences on each forward primer. The primer used specifically targets the 515F (5'-cgtatcgcctccctgcgccatcagxxxxxxxxgagtgycagmgccgcgtaa-3') and 907R (5'-ctatgcgccttgccagcccgctcagggccgycattcmtttragtt-3')region of the *E. Coli* 16s gene. Phusion HF polymerase (2X master mix) was used to amplify the 16s gene necessary for 16s amplification. PCR reactions were run on a MJ Thermocycler for Analysis of 454 data was preformed using Qiime. Quality control of sequences was implemented using the Qiime Workflow: Sequences that have a minimum length of 300 bp. Reference sequences were selected and aligned using the UCLUST algorithm via Qiime interface.

NtcA PCR, cloning, and sequencing:
DNA was extracted using MoBio Power Biofilm DNA Extraction Kit according to manufacturers protocols. *NtcA* was amplified from a microbial mat from Great Sippewissette Marsh using general cyanobacterial primers 1F and 4R (1F 5'-ATH TTY TTY CCN GGN GAY CCN GC-3' and 4R 5'-AT NGC YTC NGC DAT NGC YTG RT-3') which amplifies fragments that are 449 in length [3]. PCR reactions were run in 25µl with a final master mix concentration of 1x using Promega Master Mix. The reaction had 1 mmol L-1 each primer, and 0.2-10 ng genomic DNA. PCR reactions were run on a MJ Thermocycler for 40 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 52.5°C, and elongation for 1.5- min at 72°C following an initial 4-min denaturation step at 95°C. Optimal PCR amplification conditions were determined after running a temperature gradient to optimize samples for the most stringent annealing temperature at which amplification could be produced. Amplified fragments were gel purified and cloned into electrocompetent cells using Topo TA kit cloning procedures with a 3 hour ligation. DNA that was extracted from microbial mat samples and amplified was inserted into clone plasmids. Plasmids were sequenced using Sanger dideoxynucleotide chain termination method. Sequences were aligned using MOTHUR according to the reference *ntcA* generated database (from Dr. Anton Post's lab) prior to entering sequences into the ARB *ntcA* database. Sequences were then added to a maximum likelihood tree of *ntcA* genes from all know completely annotated Cyanbacterial genomes using ARB.

nifH RT- PCR, PCR, cloning, and sequencing:
DNA was extracted using MoBio Power Biofilm DNA Extraction Kit according to manufacturers protocols. Total RNA was extracted from flash frozen mat samples using MoBio Power Biofilm RNA Extraction Kit according to manufacturers

protocols. Total extracted RNA was resuspended in 25µl of H2O. Extracted RNA was run under nested PCR conditions with DNA primers to identify potential sources of DNA contamination post RNAse treatment of the total RNA extraction product. RNA was converted to cDNA using the Invitrogen cDNA kit. Which consisted of a 25 minute incubation at room temperature followed by one hour at 72°C and 10 minutes at 85°C. The converted cDNA was then used to amplify the *nifH* gene using two degenerate oligonucleotide PCR primers which are known to amplify a 460 bp segment of the *nifH* gene. This targeted gene was amplified using the nifH1 (5'-TGY GAY CCN AAR GCN GA-3') and nifH2 (5'-GCC ATC ATY TCN CC). Prior to the nifH1 and nifH2 amplification a less specific amplification was preformed using an additional set f degenerate primers consisting of nifH4 (5'-TTY TAY GGN AAR GGN GG-3') and nifH3 (3'-ATR TTR TTN GCN GCR TA-5') which were used for nested PCR based on conserved sequences outside of nifH1 nifH2 primer set [6]. After reverse transcription of extracted RNA, 2µl of the cDNA was added to a Promega PCR master mix concentration with a final concentration of 1x. The initial step of the PCR consisted of a denaturation step at 95°C for 1 min followed by annealing at 55°C 1 min, and extension at 72°C 1 min this PCR reaction was then followed by the second step of the nested which contained 1µl of amplified product. It is important to note that negative controls confirmed that the PCR results were from RNA and not from contaminating DNA. After the second round of PCR amplification, the amplified fragments were gel purified and cloned into electrocompetent cells using Topo TA kit cloning procedures with a 3 hour ligation step. DNA and cDNA that was extracted from microbial mat samples that were inserted into clone plasmids where then sequenced using the Sanger dideoxynucleotide chain termination method. Retrieved sequences were then aligned to a reference *nifH* database from [7]. Using MOTHUR. Sequences were then added to a maximum likelihood tree of *nifH* genes using ARB.

Incubations at 850nm:
Samples of mat were prepared in triplicate and were all 0.5cm in depth and 1.2cm in diameter. Cores were added to 10ml serum vials at which time 2ml of Great Sippewissett Marsh water was added and vials were crimped. All vials were placed into a 30°C incubator and only exposed to light at 850nm. After the initial 30 minute incubation acetylene was added to the headspace of each vial at a final concentration of 10% of the total headspace. GC measurements were taken after two, six and twelve hours of incubation. In total four different treatments were tested, a normal treatment, a dark control where mat received the same treatment as samples incubated at 850nm but where incubated in the dark and not exposed to any light source. A treatment was prepared where the green layer was removed from the cored section of mat and the fourth treatment was the addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to a final concentration was $2x10^{-5}$M.

Results and Discussion:
I. Community composition:
Based on 454 Pyrosequencing results the mat is dominated by Cyanobacteria at the phylum taxonomic level (Figure 2.). When the Cyanobacteria phylum is divided based on genus level sequence identification all sequences come back with the

highest similarity to filamentous *Cyanobacteria* that have the potential to fix nitrogen. For this section of mat there were no retrieved sequences that had the greatest similarity to heterocystousCyanobacteria. This suggests that the Cyanobacteria in this particular mat that are responsible for nitrogen fixation have to wait to be in an oxygen limited environment for fixation to occur. Additionally, the second greatest portion of the mat community is composed of Proteobacteria. Out of the Proteobacteria group 47% of the sequences have the greatest similiarty to Gammaproteobacteria which contains many sequences that are closely related to Purple Sulfur bacteria.

II. Diel paterns of acetylene reduction:
The results presented here differ from those that have been previously reported [1,2,5]. The highest rates of acetylene reduction were observed in the middle of the day; corresponding with the highest irradiance measurement as well as with the greatest oxygen concentrations (Figure 4.). Nitrogen fixation was not observed over time in the dark control and ethylene values remained consistently low. This trend was reproduced twice with fresh mat samples on different days. For both experiments similar nitrogen fixation trends were observed, confirming the reproducibility of this trend. The mat does follow expected oxygen profile trends where the mat is strongly anoxic at night and as the morning hours are approached and photosynthesis activity ramps up oxygen begins to accumulate and the draw of oxygen from upper water is lessened (Figure 3.)Since nitrogen fixation is greatest at the middle of the day when photosynthetic rates are also presumably the highest it is hypothesized that filamentous Cyanobacteria present in the green layer of the mat are not responsible for the observed nitrogen fixation. This hypothesis is based on the knowledge that typically oxygen concentrations are greatest at this time of the day and since filamentous Cyanobacteria to not have the ability to form anoxic regions it is unlikely that this group of organisms is responsible for the observed nitrogen fixation.

III. nifH functional gene analysis:
The nested PCR of total extracted RNA showed that extracted RNA was contamination free and contained no residual DNA (Figure 5.). RNA transcripts were amplified for two out of the three time points that were processed. Amplification of transcripts was not possible for the 7:00 am time point, which shows initial support for the hypothesis that potentially there was not the need for gene expression of *nifH* at this time in the day. The results of the phylogenetic analysis of *nifH* genes from DNA clone libraries and RNA transcript clone libraries are summarized in a neighbor joining tree (Figure6.). Branching patterns of the tree indicate that there are two distinct clades. The clade on the right of the tree is dominated by RNA *nifH* transcripts, which are identified by the green leaf color as opposed to DNA *nifH* sequences that have blue leaves. The phylogenetic identify of the RNA *nifH* transcripts are most closely related to *Halorhodospira* and *Allochromatium*, which are both identified as purple sulfur bacteria. In contrast the left side of the tree is more heavily dominated by DNA *nifH* sequences that have the greatest sequence similarity to *Nostoc, Methanothermobacter,* and *Methylobacter*. Having a dominance

of transcripts that are mostly closely related to Purple Sulfur bacteria is supporting evidence for the trend of nitrogenase activity observed in the acetylene reduction assay. Potentially for this system the Cyanobacteria are not the community members responsible for the diel trend of nitrogen fixation that is observed in Great Sippewissett microbial mats. Rather the presence of *nifH* gene transcripts most closely related to Purple Sulfur bacteria suggests that this group of organisms may be responsible for the bulk of nitrogen fixation in this environment.

IV. Incubation of Great Sippewissett mat at 850nm:
Mat sections that were incubated for 2, 6 or 12 hours showed similar trends in nitrogen fixation (Figure 7.). Nitrogenase activity was consistently the lowest under dark conditions. For sections that were treated with DCMU nitrogenase activity was slightly greater than that of sections were the Cyanobacterial green layer was removed prior to incubation. The most notable result for this experiment is the nitrogenase activity level associated with mat sections that were not altered. For every sampling time point nitrogen fixation was the greatest for this treatment. Though the trends are not as apparent with the other treatments it can be concluded from these treatments that for both the DCMU and green layer removal nitrogenase activity was greater than the dark samples. The fact that there was measureable nitrogenase activity under these incubation conditions is an additional line of evidence that the Purple Sulfur bacteria are responsible for the bulk of nitrogen fixation and not Cyanobacteria which do not have the ability to use light at 580nm.

V. NtcA Functional gene analysis:
NtcA sequences from clone libraries were found to have similarity to known *ntcA* sequences from sequenced Cyanobacterial genomes. The results of the phylogenetic analysis of *ntcA* genes from DNA clone libraries are summarized in a neighbor joining tree (Figure 8.). The *ntcA* sequences from the Great Sippewissett microbial mat community group together in three distinct clades. One group of sequences clusters with Microcoleus chthonoplastes (PCC 7420). The other two distinct groups of *ntcA* sequences do not show sequence similarity to any of the sequences in the *ntcA* ARB database. The fact that there are no know sequences that show high percent similarity to Great Sippewissett sequences is not that surprising because *ntcA* has solely been studied in marine systems. Finding new *ntcA* sequences will allow us to progress with the overall goals of this project. With these sequences we will be able to design more specific primers for future amplification work.

Conclusions and Future Work:
It can be concluded from this mini project that there are reproducible diel trends of nitrogen fixation in Great Sippewissett microbial mats. In past studies it has been consistently observed for microbial mats that the peak in nitrogen fixation occurs early in the morning when oxygen levels are still low. The trend that was observed in microbial mats from Great Sippewissett had the peak of nitrogen fixation (110nmol ethylene cm^{-2}) occurring in the middle of the day at 13:00 when irradiance was the greatest. The difference in the nitrogen fixation trend can be

attributed to members of the Purple Sulfur bacteria. More specifically based off *nifH* transcript sequence similarity *Halorhodospira* and *Allochromatium* were found to be active members in the microbial community at times when nitrogen fixation was active. To more definitively link nitrogen fixation to specific community members methods such as stable isotope probing should be implemented. Additionally, to better addresses the diel trend of gene expression of *nifH* and *ntcA* more specific primers should be developed based off from the clone library sequences generated in this project.

Acknowledgements:
I would like to thank everyone who made this amazing opportunity possible. Dan and Steve thank you the organization of this course and for all of your valuable advice along the way. Thank you to the TAs for all of their constant advice and support into the wee hours of the morning. I would also like to acknowledge all of my fellowclass mates for a wonderful experience. Dr. Anton Post was most helpful in project desgin as well as prviding valuable information about *ntcA* sequences as well as primers and the ARB database for *ntcA*. I would like to thank Dr. John Waterbury for the Lyngbya isolte from Great Sippewissett Marsh that was used as a control in this experiment for the experiments measuring nitrogen fixation while exposed to 850nm. The NSF and NASA provided my funding for the duration of this course.

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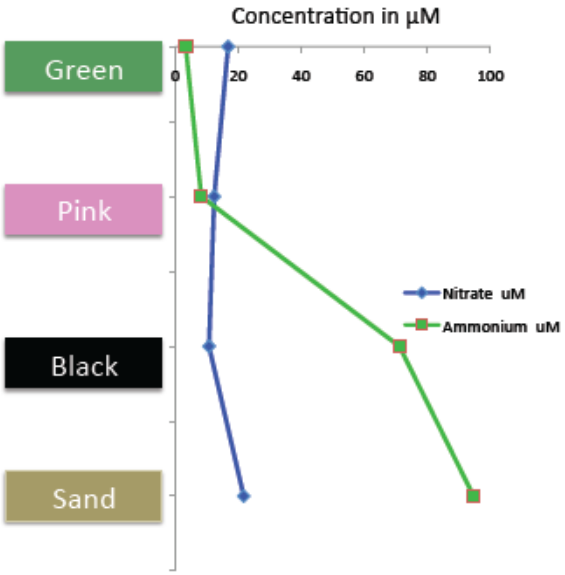
7. Zehr, J. P., B. D. Jenkins, et al. 2003. Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Envionmental Microbiology. 5(7): 539-554.



Adapted from Zwiebel

Figure 1. Nitrate and ammonium concentrations (µM) for different layers of Great Sippewissett microbial mat community. Ammonium concentrations increase starting in the black layer which indicates the location for denitrification. Nitrate concentrations remain similar throughout the depth profile of the mat.

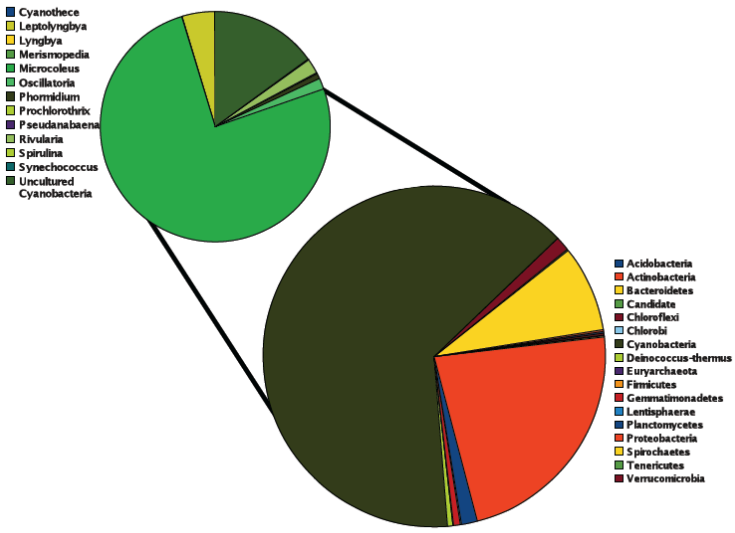


Figure 2. Phylogenetic relationship of 454 Pyrosequencing sequences to most similar reference sequences at the Phylum level (Pie chart on the right). The second pie chart is the Cyanobacterial Phylum sequences described at the genus level. Sequences were analyzed using the Qiime Workflow.

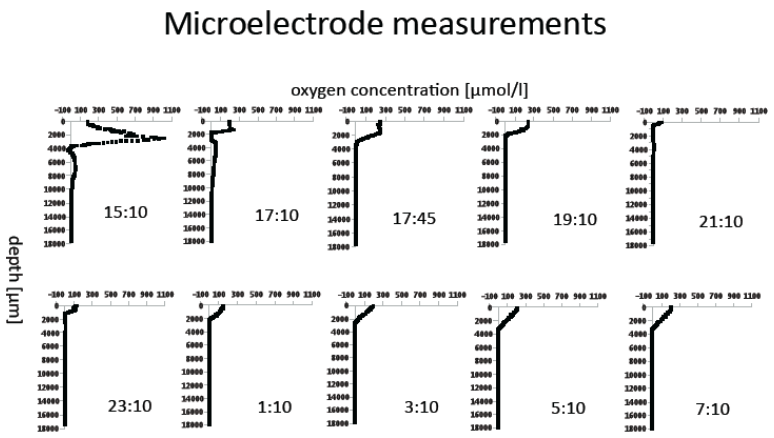


Figure 3. Diel Oxygen depth profiles in Great Sippewissett microbial mat community measured using a UNISENSE oxygen microelectrode. Measurements were taken in 50µm depth increments.

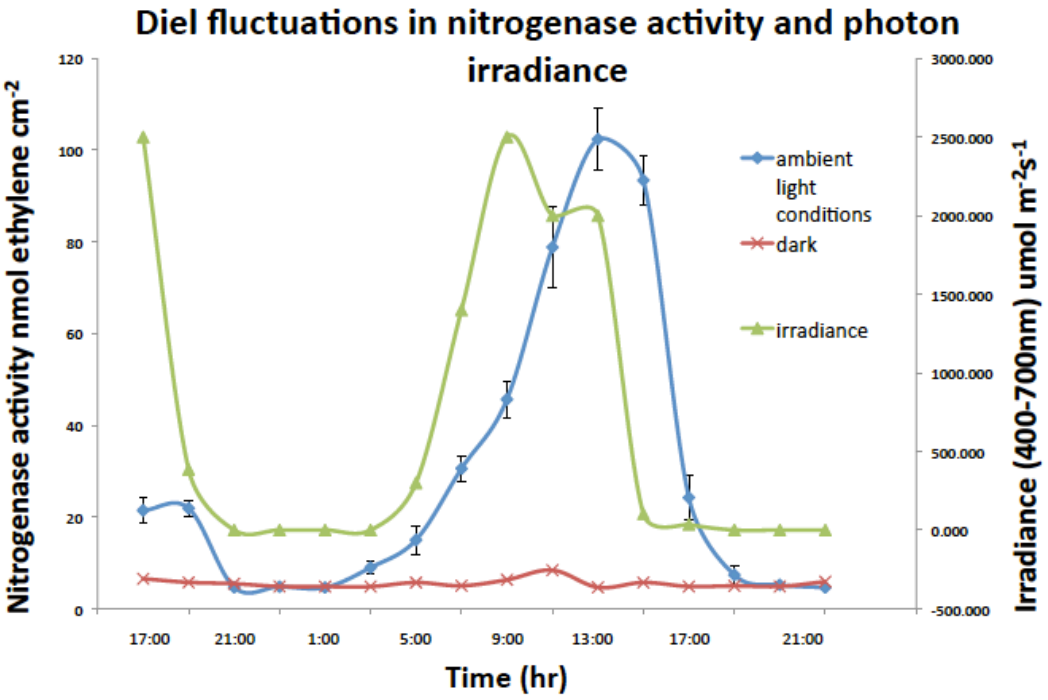


Figure 4. Diel patterns of acetylene reduction in Great Sippewissett microbial mat community. *In situ* nitrogenase activity and photon irradiance measured on July 11, 2012.

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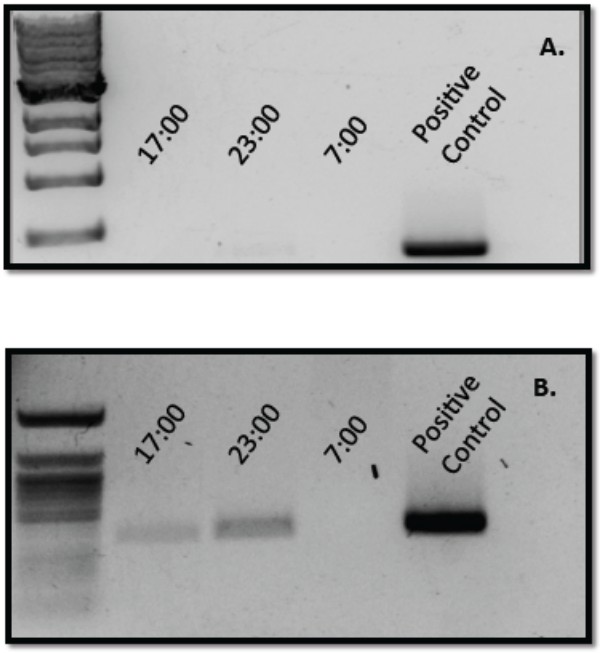

Figure 5. A.) Direct nested PCR of RNA samples extracted from Great Sippewissett mat at 17:00, 23:00 and 7:00. Extracted RNA that was used for RT-PCR was tested for DNA contamination. Positive control is DNA extracted from Great Sippewissett Microbial Mats . There is no band present for any of the time points indicating that extracted RNA is DNA contamination free. B.) PCR of cDNA from Great Sippewissett Microbial Mat samples from different times of the day. Amplification present for times points 17:00 and 23:00, there was no visible amplification for the 7:00 time point.

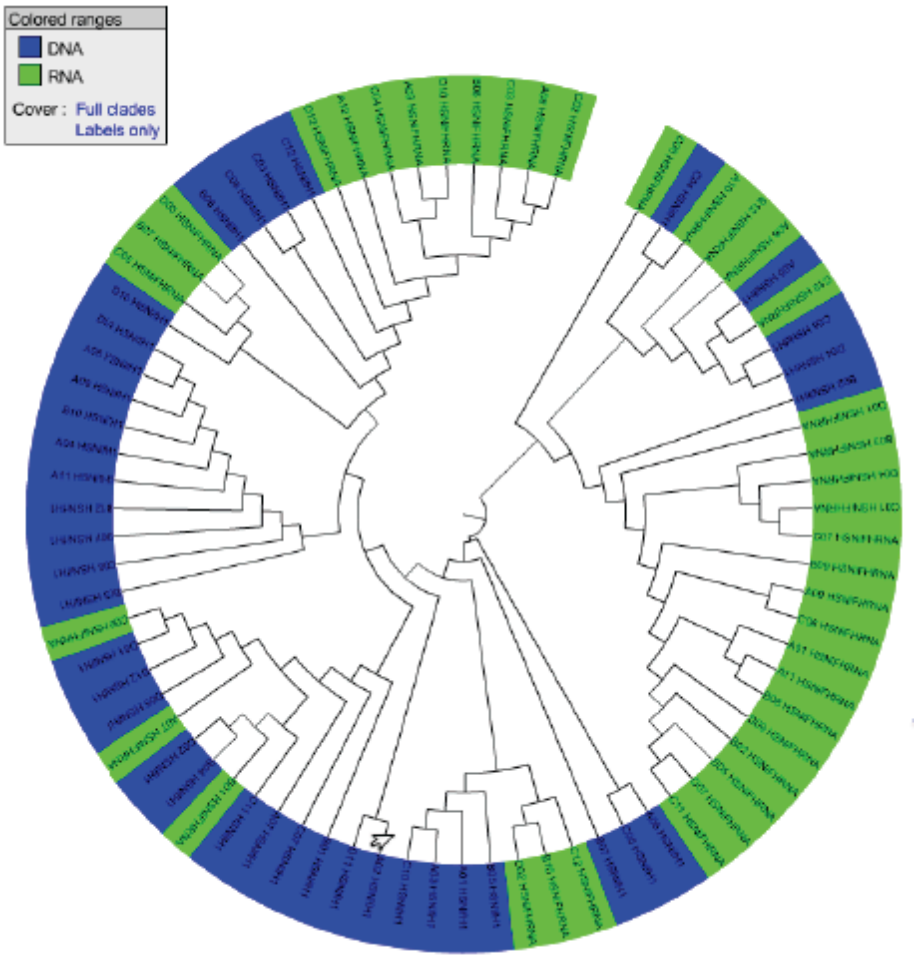

Figure 6. Phylogenetic relationship of *nifH* DNA (blue) and *nifH* RNA (green) sequences to each other. An external alignment was made using MOTHUR to align sequences to the Zher *nifH* database.

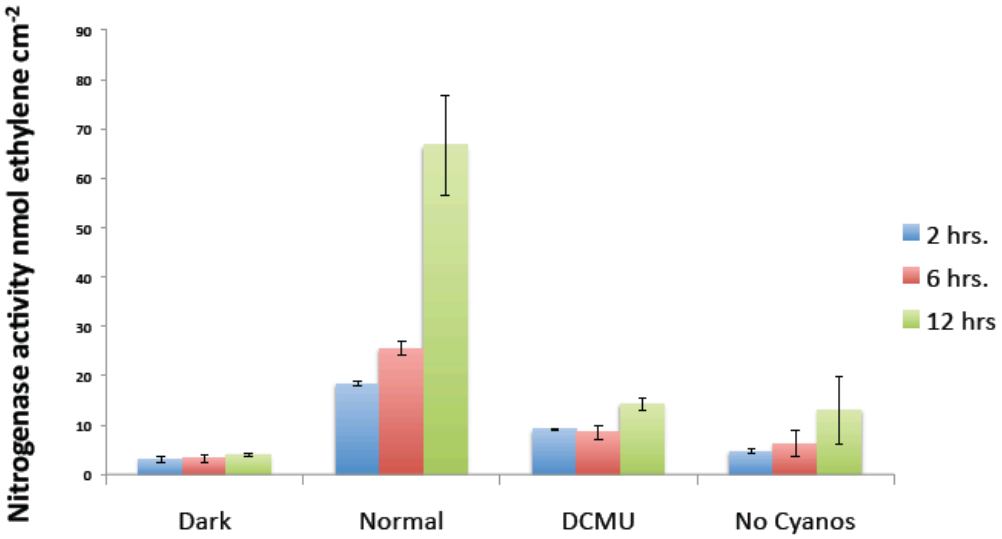

Figure 7. Patterns of acetylene reduction in Great Sippewissett microbial mat community when incubated in the dark, with DCMU, with the Cyanobacterial green layer of the mat removed, and without manipulation. All samples were incubated with a 850nm light source at 30°C for 2,6 or 12 hours prior to measuring acetylene reduction.

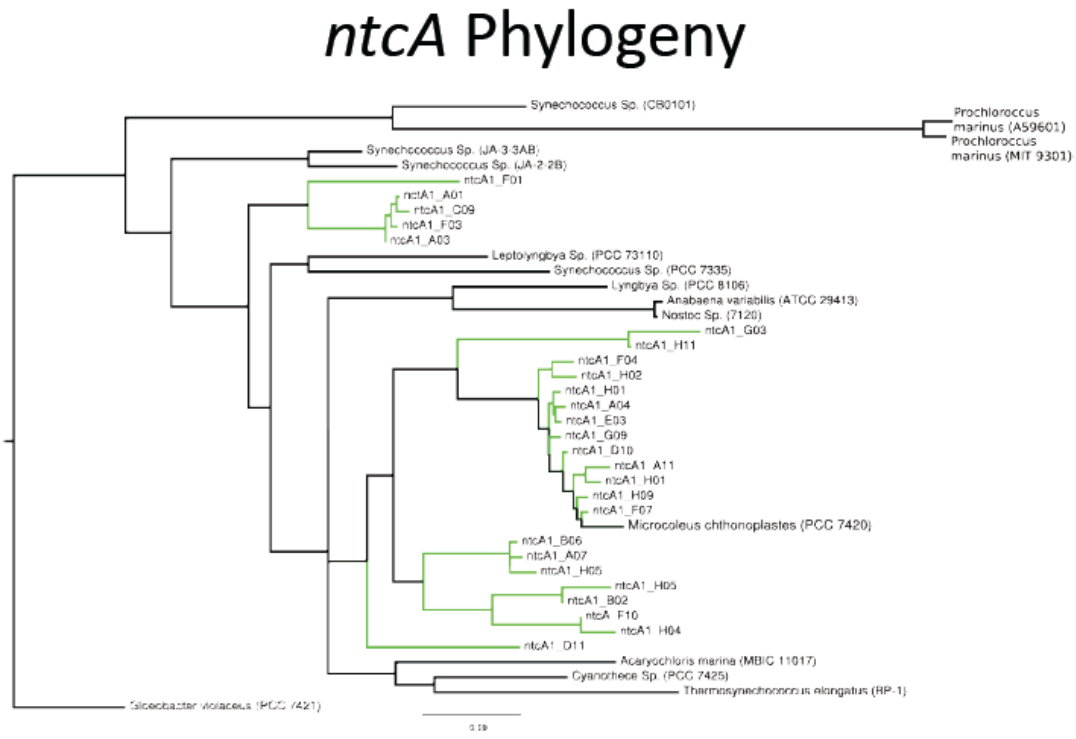

Figure 8. Phylogenetic relationship of *ntcA* isolate and closely related reference sequences from the Post lab *ntcA* ARB database. Minimum evolution phylogenies were constructed in ARB with Jukes–Cantor distance model. Accession numbers for reference sequences are listed in the figure (e.g., Microcoleus chthonoplastes (PCC 7420)).

Appendix:
454 Thermocycler program conditions:
Stage 1:
1x
98 C 60 sec

Stage 2:
10x
98 C 5 s
68 C 10 s
72 C 7 s

Stage 3:
12x
98 C 5 s
58 C 10 s
72 C 7 s

Stage 4:
72 C
21

then hold at 4 C

Heidi's sequence information:
454 Bracodes
HeidiOld: Extracted DNA from Great Sippewissett Marsh 4 July 2012. Same DNA that was used for nifH and ntcA DNA libraries
HeidiFront: DNA extracted from the front of a clover leaf using a sterile swab and lysis buffer from the power biofilm DNA extraction kit
HeidiBack: DNA extracted from the back of a clover leaf using a sterile swab and lysis buffer from the power biofilm DNA extraction kit

Clone Libraries:
1.HSNiFHRNA: Amplification of the nifH gene using nifH 3-4 and nifH1-2 primer sets. cDNA from the 3:00 time point of the diel nitrogen fixation experiment
2.nifH1 :DNA from mat DNA (the same that was used for HeidiOld pyrosequencing sample)
3.ntca1 : ntcA clones from from mat DNA (the same that was used for HeidiOld pyrosequencing sample) amplified using universal cyanobacterial primers for ntcA 1F and 4R
4. ntcA1: 2nd round of ntcA clones from from mat DNA (the same that was used for HeidiOld pyrosequencing sample) amplified using universal cyanobacterial primers for ntcA 1F and 4R