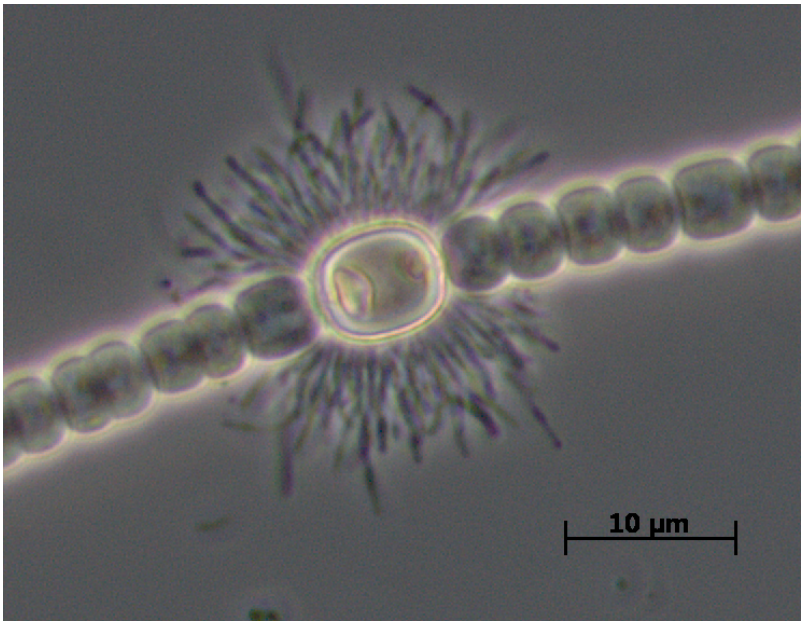

Substrate Exchange within the Anabaena-Epibiont Association Does H_2 Drive Symbiosis?

Laura Meredith
Program in Oceans, Atmospheres and Climate
Massachusetts Institute of Technology

MBL Microbial Diversity Course 2010
July 29th

Abstract

The cyanobacterium *Anabaena* differentiates its photosynthetic, vegetative cells into heterocystic, nitrogen-fixing cells protect its oxygen-sensitive nitrogenase from photosynthetic oxygen evolution. Epibiotic bacteria are found exclusively on the heterocysts of specific strains of *Anabaena*, and it has been postulated that they receive fixed nitrogen and/or carbon in exchange for respiring remaining O_2 around the heterocyst and aid their nitrogen-fixation. Nitrogen-fixation is accompanied by a necessary 1:1 ratio of H_2 production per molecule of N_2 fixed. The fate of the H_2 produced by *Anabaena*'s heterocysts is unknown and in this mini project the hypothesis that the epibionts utilize the H_2 to supplement their autotrohpic or heterotrophic growth is tested. This hypothesis could explain stable isotopic observations and would be a mutualistic exchange for both species. A series of substrate affinity tests and enzyme assays were performed and indicate that the epibionts may not be able to utilize hydrogen. A redox dye was employed to highlight the areas of respiration between the association. Varying the heterocyst nitrogenase metal co-factor (Mo, V, Fe) was found to vary the vegetative cell to heterocyst ratio in *Anabaena* and affects both the nitrogenase activity of the Co-Culture and putatively the preference for epibiont attachment. Good coverage of the epibiont genome was provided by 454 pyrosequencing and preliminary assessment indicates the species may not contain a hydrogenase, but may contain a nitrogenase and enzymes for metabolizing a number of proposed substrates that may be shared between *Anabaena* and epibiont. This work does not support the hypothesis that epibionts utilize the H_2 from nitrogen fixation within the heterocyst, but it lays the foundation with methods and with extensive sequence data to further explore the substrates that are exchanged and the motivator of this symbiosis.

Contents

1 Introduction

1.1 Motivation

Anabaena sp. are filamentous cyanobacteria that are able to accomplish both oxygen-evolving photosynthesis and oxygen-sensitive nitrogen fixation by separating the two processes in space, instead of in time (as do some other cyanobacteria). Vegetative cells photosynthetically fix carbon, a portion of which they provide to heterocysts to subsidize nitrogen fixation. In exchange, heterocysts provide vegetative cells with biologically available nitrogen. In aquatic ecosystems, the heterocysts of *Anabaena sp. SSM-00* are surrounded by epibiotic bacteria, which appear to be specific to that strain only [14]. Phylogenetically, the epibiont is an *Alphaproteobacterium* in the order *Rhizobiales*, some of which are known to be plant symbionts. Epibiotic bacteria have been implicated in siphoning off both carbon and nitrogen from *Anabaena* via their attachment to the heterocyst [12]. The association has been implied as mutualistic because the epibiotic associates might decrease the levels of O_2 around the oxygen-sensitive nitrogenases of the heterocyst by respiration of undefined compounds. Figure 1 depicts the association of the vegetative and heterocystic cells of *Anabaena*

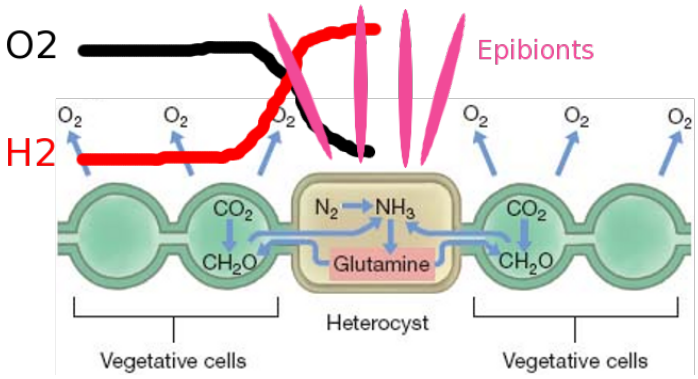

Figure 1: Schematic of *Anabaena* vegetative cells and heterocysts surrounded by epibiotic bacteria (pink). Expected O_2 (black) and H_2 (red) gradients near cells. Adapted from [7].

with the associated epibionts and the expected oxygen gradient.

Nitrogen fixation requires a stochiometric 1:1 loss of H_2 for every molecule of N_2 that is fixed. Cyanobacteria have been intensely studied to exploit this phenomenon for biological energy production [5]. One key issue is how to minimize the amount of H_2 lost to their own internal uptake hydrogenases [15], [8]. H_2 production by cyanobacteria has been estimated to constitute roughly 4% of the total annual emissions to the atmosphere [11], and measuring local concentrations of H_2 in ocean waters has been proposed as a method for quantification of the nitrogen fixation rate [9].

We know that heterocysts produce hydrogen during nitrogen fixation, but we don't know what the is fate of that hydrogen. The following three hypotheses are proposed as possible explanations:

- **heterocysts contain hydrogenases that intercept H_2 and recycle the energy internally before it can escape,**
- **epibionts consume the produced hydrogen, either for energy generation or for carbon fixation, or**
- **another group of microbes may attempt to utilize the escaping H_2, else it is lost to the atmosphere.**

An *Anabaena* and *Rhizobiales* epibiont culture (the Co-Culture) was incubated with ^{13}C bicarbonate and ^{15}N molecular nitrogen; the results showed that associated epibiont bacteria (but not unassociated epibiotic cells) were enriched strongly in the former and more weakly in the latter [2]. This suggests that the epibionts may consume both C and N from the *Anabaena* heterocysts; however, it does not exclude the possibility that epibiotic *Rhizobiales* might instead utilize the H_2 either to fix CO_2 or to boost growth on complex carbon substrates. It does not appear that anyone has tested the ability of the epibiont to grow on H_2/CO_2 alone or whether H_2 enhances its growth on other substrates. Therefore, I propose to test the second hypothesis in my mini project: **Epibiotic bacteria consume the H_2 that is produced by *Anabaena* during nitrogen fixation to drive energy generation or for carbon fixation.**

1.2 Outline

Epibiotic hydrogen-utilization will be tested by conducting a series of substrate affinity incubations. To date, pure cultures of the epibiont have only been grown in undefined, complex media. Hydrogen utilization might vary with the availability of other substrates in their environment. We wish to mimic the authentic association of *Anabaena* and epibiont in salty and oligotrophic environments, and therefore strive to reduce the complexity of the media used, while still sustaining growth, to determine whether the epibiont utilizes hydrogen in nature. Table 1 describes the combinations of substrates that will be tested, which can be loosely grouped as addressing the following scenarios:

1. does the epibiont use H_2 as an energy boost while metabolizing complex and undefined substrates,
2. does the epibiont utilize H_2 as an energy boost while metabolizing defined and expected metabolites supplied by *Anabaena*, and/or
3. does the epibiont utilize the energy from oxidizing H_2 to grow autotrophically on CO_2 supplied from the environment?

Epibionts will be grown on complex media and on saltwater media both with and without H_2 to address Scenario 1 and 3. To address Scenario 2, epibionts will be grown on a selection of possible defined and expected metabolites that are described here. Vegetative cells drive the production of **sucrose** via photosynthesis to deliver to heterocysts; if some sucrose escapes the heterocysts, it could be a carbon source to the epibiotic cells. Heterocysts deliver fixed nitrogen to the vegetative cells in the form of **glutamine** and **arginine**, which might be a carbon and/or nitrogen source to the epibionts. *Anabaena* has been observed to produce **glycolic acid** (or glycolate), but is also described as a experimental artifact [4]. If produced and excreted preferentially by heterocysts, it could be a carbon source for epibiotic bacteria. The outer membrane of the heterocysts of *Anabaena* include **glycolipids** that are described in detail by [1] and complex **polysaccharides**; however, such detailed substrates were not available so **severed heterocysts** will be used instead. It is likely that the epibionts can grow on a **sugar mix** of glucose, glycerol and succinate, so these compounds will be used as defined carbon sources as well. Because we don't know the nitrogen needs of the epibiotic bacterium, nitrogen in both fixed and molecular form were tested in selected cases. Nitrogenase and hydrogenase assays will be used to determine their activity in the cultures under different treatments and a redox dye will be employed to search for hot-spots of coupled respiration between the association.

Nitrogenase enzymes exist with Mo, V and Fe as metal co-factors [17]. The energy requirement and H_2 yield of these nitrogenases increases in the following order: Mo, V, Fe. Cultures were grown in media with different trace metal limitations to see if the identity of the nitrogenase impacts *Anabaena* or the Co-Culture.

The same cultures of *Anabaena* and epibiont have previously been characterized by their 16S ribosomal DNA [14]; however, this will be repeated to verify the identity of the cultures used. Whole DNA of the epibiont will be run on a 454 Life Sciences pyrosequencing plate in efforts to generate additional hypotheses regarding the driver of the *Anabaena*-epibiont association.

2 Materials and Methods

2.1 Cultures and Media

Cultures: The following three pure cultures were used in this investigation: 1) *Anabaena* sp. SSM-0 (DQ364237) and 2) *Anabaena* and epibiont as a gift from John Waterbury[1], and 3) the isolated epibiont, *Rhizobium* sp. WH2K (DQ364238), as a gift from Bradley Stevenson[2], which were all isolated in 2000 from School Street Marsh in Woods Hole. The reader is referred to [14] for phylogenetic trees of the original

[1]Woods Hole Oceanographic Institution
[2]Microbial Diversity Class of 2010; now at University of Oklahoma

isolates.

Media: Cultures of *Anabaena* alone and *Anabaena* with the epibiont (the Co-Culture) were grown in a version of Saltwater Oligatrophic (SO) medium (either SOX or $\frac{1}{2}$SO) as described in Appendix A. Trace metals molybdenum (Mo) and vanadium (V) were omitted from a preparation of AN medium (described in [16]), which is completely defined and lacks the natural seawater SO contains, that was used for nitrogenase metal co-factor experiments, while the normal amount of iron (Fe) was added. To make up the V and Mo media, a supplemental solution of each metal was prepared and added to the SO base by filter sterilization to 100 nM final concentrations. The epibiont alone was grown on either a Peptone-Yeast extract (PY) or Marine Agar (MA) medium as described in Appendix A.

2.2 Substrate Affinity Tests

Epibiont substrate affinity tests were performed by incubating at $30°C$ in the shaker to aerate the aerophiles in balch tubes crimped closed. Either SOX or SW Base were used as indicated in Table 1. The $\frac{1}{4}$xPY medium was made by adding peptone and yeast extract to either SOX or SW media at $\frac{1}{4}$ their full concentrations in 1xPY. A Marine Purity (MP) media has also been used to cultivate the epibionts, but was not used in this work. Fixed nitrogen (NH_3) was added in the form of $NaNO_3$ and NH_4Cl to final concentrations of 2.0 mM and 200 μM, respectively. In addition to CO_2 and complex carbon, the following other substrates were tested at 20 mM concentrations: sucrose, glycolic acid, a sugar mix[3], glutamine, and arginine. Epibionts were grown on heterocysts severed from the vegetative chain to represent epibiont metabolism of heterocyst glycolipids and polysaccharides. Heterocysts were obtained following the enrichment protocol found in Appendix B of [13], 2000 from 4 mL of the pure *Anabaena* culture. To the appropriate cultures, pure H_2 was added to approximately 25% the headspace volume[4]; N_2 was added with the same additional volume to ensure that oxygen levels were equal in the other cultures. Tubes were innoculated by adding 100μl of the epibiont culture to 5 mL of medium.

***Anabaena* and *Anabaena*/Epibiont substrate affinity tests** were performed by incubating 120 mL serum vials containing SOX medium as described in Table 2. In addition, 44 mg of sodium bicarbonate was added per liter of medium to mimic the 2.2 mM concentrations found in pure seawater so that *Anabaena* would be less likely to run out of CO_2 over the course of the closed-system experiment. Fixed nitrogen (NH_3) was added in the form of $NaNO_3$ and NH_4Cl to final concentrations of 2.0 mM and 200 μM, respectively. To the appropriate cultures, pure H_2 was added to approximately 25% the headspace; N_2 was added with the same additional volume to ensure that oxygen levels were equal in the other cultures. Serum vials were innoculated by adding about 1 mL of the epibiont culture to 50 mL of medium.

Changes in gas composition (*e.g.*, H_2, O_2, CO_2) of the headspace were monitored using a Shimadzu Gas Chromatograph (GC-2014) fitted with a Thermal Conductivity Detector (TCD). Growth of the epibiont cultures was monitored by measuring the Optical Density (OD) at 625 nm using a Bausch and Lomb spectrophotometer.

2.3 Enzyme Assays

Nitrogenase assays were performed by injecting acetylene into the headspace to a final 5% concentration and monitoring the production of ethylene in the headspace with the GC fitted with a Flame Ionization Detector (FID). At least four time points were recorded over which nitrogenase activity is directly proportional to the linear production of ethylene.

Hydrogenase assays were performed on 5 mL cultures in balch tubes by adding Benzyl Viologen (BV) to a final concentration of 1 mM, replacing aerobic headspace with N_2, and then adding to the headspace to

[3]The sugar mix was comprised of of glycerol, glucose, and sodium succinate - each at 6.5 mM concentrations.
[4]The 80:20 H_2:CO_2 gas was not used to avoid lowering medium pH.

scenario	purpose	C-source	N-source	H_2	media type
		1st Set			
1	H_2 + heterotrophy	complex C	complex N	-	SOX
1	H_2 + heterotrophy	complex C	complex N	+	SOX
1	H_2 + heterotrophy	complex C	complex N	-	SOX
1	H_2 + heterotrophy	complex C	complex N	+	SOX
3	N-fix + H_2 + autotrophy	CO_2	N_2 gas	-	SOX
3	N-fix + H_2 + autotrophy	CO_2	N_2 gas	+	SOX
3	H_2 + autotrophy	CO_2	NH_4^+	-	SOX
3	H_2 + autotrophy	CO_2	NH_4^+	+	SOX
		2nd Set			
2	H_2 + heterotrophy	sucrose	N_2 gas	-	SOX
2	H_2 + heterotrophy	sucrose	N_2 gas	+	SOX
2	H_2 + heterotrophy	glycolic acid	N_2 gas	-	SOX
2	H_2 + heterotrophy	glycolic acid	N_2 gas	+	SOX
2	H_2 + heterotrophy	sugar mix	N_2 gas	-	SOX
2	H_2 + heterotrophy	sugar mix	N_2 gas	+	SOX
2	H_2 + heterotrophy	glutamine	N_2 gas	-	SOX
2	H_2 + heterotrophy	glutamine	N_2 gas	+	SOX
2	H_2 + heterotrophy	arginine	N_2 gas	-	SOX
2	H_2 + heterotrophy	arginine	N_2 gas	+	SOX
2	H_2 + heterotrophy	severed heterocysts	N_2 gas	-	SOX
2	H_2 + heterotrophy	severed heterocysts	N_2 gas	+	SOX
		3rd Set			
1/2	H_2 + heterotrophy	sucrose + $\frac{1}{4}$complex C	N_2 gas	-	SW Base
1/2	H_2 + heterotrophy	sucrose + $\frac{1}{4}$complex C	N_2 gas	+	SW Base
1/2	H_2 + heterotrophy	glycolic acid + $\frac{1}{4}$complex C	N_2 gas	-	SW Base
1/2	H_2 + heterotrophy	glycolic acid + $\frac{1}{4}$complex C	N_2 gas	+	SW Base
1/2	H_2 + heterotrophy	sugar mix + $\frac{1}{4}$complex C	N_2 gas	-	SW Base
1/2	H_2 + heterotrophy	sugar mix + $\frac{1}{4}$complex C	N_2 gas	+	SW Base
1/2	H_2 + heterotrophy	glutamine + $\frac{1}{4}$complex C	N_2 gas	-	SW Base
1/2	H_2 + heterotrophy	glutamine + $\frac{1}{4}$complex C	N_2 gas	+	SW Base
1/2	H_2 + heterotrophy	arginine + $\frac{1}{4}$complex C	N_2 gas	-	SW Base
1/2	H_2 + heterotrophy	arginine + $\frac{1}{4}$complex C	N_2 gas	+	SW Base

Table 1: Description of Substrate Affinity Tests for Isolated Epibiont

innoculum	$+H_2$	$+NH_3$
Anabaena	-	-
Anabaena	+	-
Anabaena + epibiont	-	-
Anabaena + epibiont	+	-
Anabaena + epibiont	-	+
Anabaena + epibiont	+	+

Table 2: Description of Substrate Affinity Tests for *Anabaena* and *Anabaena* with

concentration of 25% following the procedure outlined by [10]. The extent of BV reduction was monitored by the change in absorption at 546 nm on the spectrophotometer. *Escheria coli*, known to have hydrogenases, was used as a positive control for the assay.

2.4 Imaging of Respiration Activity

Redox Sensor Green^{TM} reagent from Invitrogen's BacLight kit was used to visualize the sites of intense bacterial reductase activity within the *Anabena*-epibiont association to determine the basic redox locality between the two and to determine its variability to substrate changes. The reagent penetrates bacteria and will fluoresce green (490/520 nm) following its reduction within the cells. A second dye, propidium iodide, is included in the BacLight^{TM} kit as an indicator of membrane integrity; it stains the DNA of cells with compromised membranes and fluoresces red (490/635 nm). Zeiss filters FS38HE, FS43 and FS09 were used to excite BP 470/40, BP 545/25, and BP 450-490, respectively, for emission at BP 525/50 (green), BP 605/70 (red), and LP 515 (both green and red), respectively. Application of this method is described by [6].

2.5 Phylogenetic and Genomic Characterization

Verification of the strain identity of the received cultures of epibiont and *Anabaena* was done by pelleting 1 mL of cells by centrifugation, resuspending them in 1% Nonidet P40 (United States Biochemical Corp.), and boiling them for 5 minutes. Cell material was pelleted and only the supernatent was used as DNA template. PCR amplification of the 16S rDNA sequence was performed using standard techniques and gel electrophoresis was used to confirm successful amplification. After sequencing, the 16S stretch was compared with the published sequences for the epibiont (DQ364238) and *Anabaena* (DQ364237) strains using the ARB software package with SILVA 16S rDNA alignment and reference database.

Genomic sequencing by 454 Life Sciences technology was performed using template DNA from the original culture of epibiont from B. Stevenson. Six 1.5 ml samples of the culture were centrifuged for 10 minutes at 21,000 rpm; the pellets were resuspended in 1% Nonidet P40 and the MoBio Soil Extraction Kit was used to extract the DNA[5]. The concentration and quality of extracted DNA was verified using a NanoDrop. DNA was outsourced for 454 sequencing with coverage of $\frac{1}{5}$th of a plate. Sequence data was assembled using Newbler and preliminary investigations into the presence and/or absence of *Rhizobiales* genes of interest was performed using NCBI BLAST packages.

3 Results

3.1 Substrate Affinity Tests

Substrate affinity tests were conducted on the pure epibiont culture in three sets of experiments. Set 1 incubations addressed whether 1) hydrogen can be used to boost epibiont growth on complex media and whether 3) the epibiont can grow autotrophically on H_2 from *Anabaena* and CO_2 from the environment. Over the course of this seven day experiment, the epibiont only grew in full and dilute complex media, as shown by the 'PY' curves in Figure 2, which shows the growth curve of the epibiont as measured by increases in the optical density. The amount of growth in complex media is proportional to the concentration of complex substrates supplied. Solid lines denote cultures incubated without H_2, and it is clear from the dashed lines indicating cultures grown with H_2 that growth was not boosted by the presence of this high energy molecule. This strongly indicates that the answer to question one is 'no', hydrogen does not boost epibiont growth on complex media. From the same figure we find that the epibiont did not grow on Saltwater Oligotrophic (SO) media, even when supplemented with H_2 and/or fixed nitrogen. This indicates that the epibiont cannot support autotrophic growth when supplemented by H_2 - at least in these specific settings, which implies that the answer to question three is also 'no'.

[5]Kit protocol was followed, except a bead-beader was used for 1.5 minutes instead of the procedure listed in step 5.

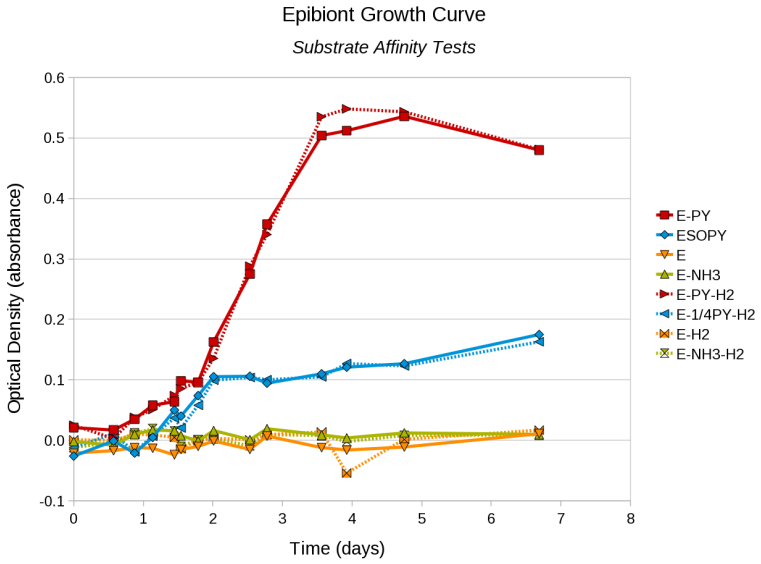

Figure 2: Growth curve of epibiont cultures under different substrate conditions

Growth information from OD measurements is corroborated by gaseous measurements of the culture headspace. Figure 3 tracks the changes in headspace H_2 and CO_2 of the epibiont cultures. Within our measurement limitations, there was no observable change in H_2 concentrations, meaning that the epibiont is not consuming H_2. On the other hand, the epibiont is clearly respiring the complex carbon substrates and driving up CO_2 concentrations, where CO_2 production is strongest for full PY, weaker for $\frac{1}{4}$PY and zero for SO cultures.

Cultures of *Anabaena* and of the Co-Culture were started in cultures with and without H_2 and/or fixed nitrogen as described in Table 2. The growth of *Anabaena* was generally low, and its filamentous nature did not lend itself to OD measures of growth rate. A fluorometer could have been used to measure growth, but in its absence we can use O_2 evolution as a proxy for growth as is shown for three of the six cultures in Figure 4-right, where the growth rates are remarkably similar in these three different cultures. Hydrogen concentrations in the headspace of the H_2-amended cultures are shown in Figure 4-left, where concentrations appear to increase for a few days before leveling off, although the significance of this increase is unknown. The Co-Culture grown with fixed nitrogen was observed after 1.5 weeks under the microscope and epibionts had not detached from the heterocysts that had not yet been discarded by *Anabaena*; this implies the epibionts may not be there for fixed nitrogen, or that they just couldn't physically detach.

Substrate affinity test Set 2 and 3 were conducted, as are described in Table 1. Unfortunately, no growth was observed in any tube of Set 2, which tested one or a few defined substrates in SO medium with and without hydrogen. This might not have been surprising because key nutrients could still be missing in such defined medium, except that Set 3 also had no growth, despite also including a $\frac{1}{4}$xPY culture that had grown in Set 1[6].

As a bit of an aside, it was observed repeatedly that detached heterocysts are found in the co-culture, usually completely surrounded by epibionts. As discussed later, the heterocysts can remain very reduced and the epibionts respiring strongly in that situation. To test whether the epibionts were only interested in the glycolipids and polysaccharides of the heterocyst, a pure *Anabaena* culture that was treated to remove vegetative cells and retain heterocysts was innocultated with epibionts. Even after heterocyst 'death' and starvation of their C-source by separation from the vegetative cells, the epibionts attached to free heterocysts.

[6]For the sake of simplicity Set 3 was prepared with the class Salt Water base instead of SOX medium so the $\frac{1}{4}$xPY was not an exact replicate in sets 1 and 3

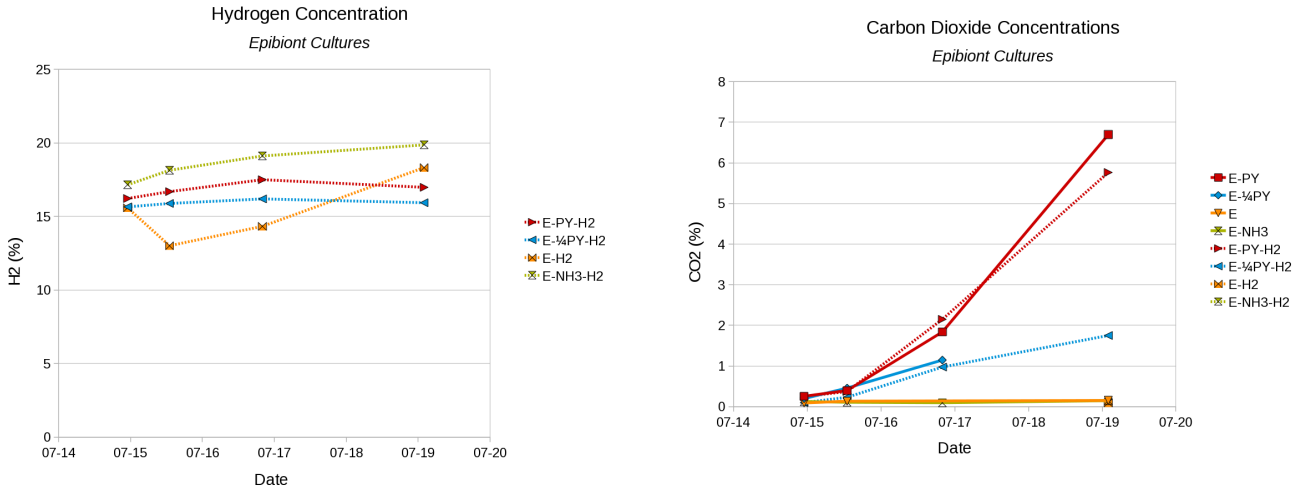

Figure 3: culture headspace gas concentrations: left) concentration of H_2 in headspace of epibiont cultures injected with 25% H_2 on day zero and right) concentration of CO_2 in headspace of epibiont cultures

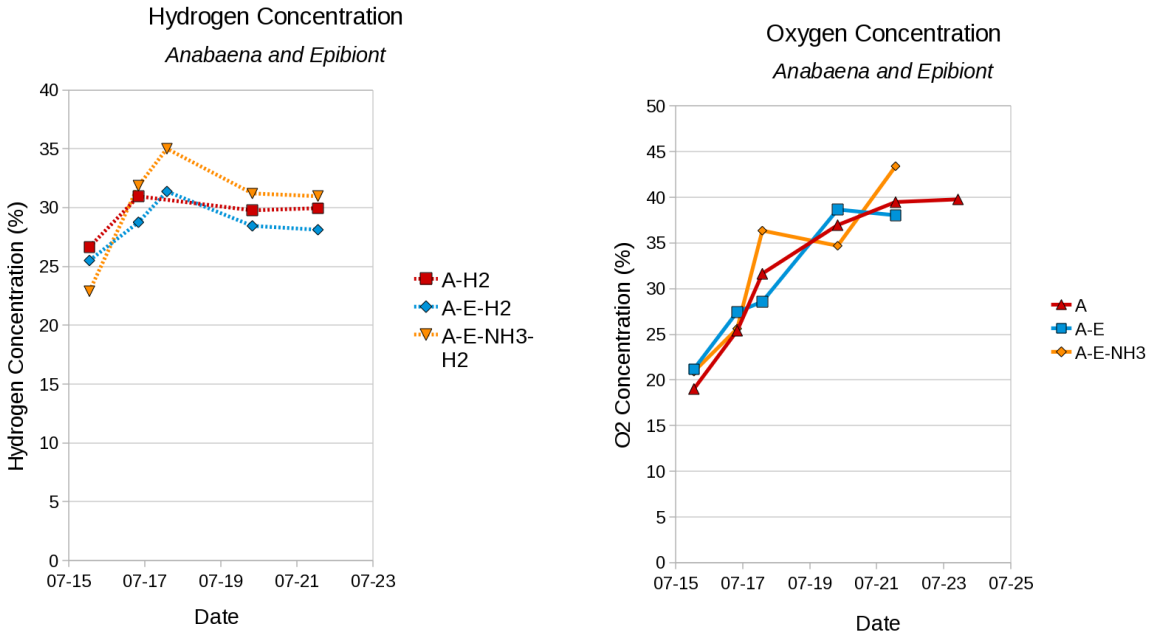

Figure 4: *Anabaena* and Co-Culture headspace gas concentrations: left) concentration of H_2 in headspace of *Anabaena* cultures injected with 25% H_2 on day zero and right) concentration of O_2 in headspace of select *Anabaena* and epibiont cultures.

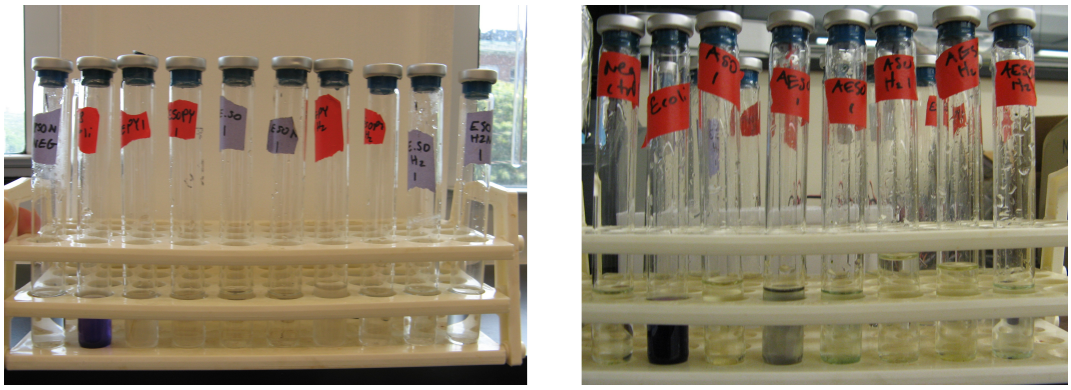

Figure 5: Hydrogenase assay results: left) epibiont hydrogenase assay result - only *E. coli* demonstrates hydrogenase activity and right) *Anabaena* and co-culture assay result - only *E. coli* and A-E demonstrates hydrogenase activity.

It would be interesting to test whether the epibionts are interested in metabolizing the outer contents of the heterocyst, or whether they are still able to function with the heterocyst in that state.

3.2 Enzyme Assays

Hydrogenase assays were performed on Set 1 of the epibiont cultures and on the *Anabaena* and co-cultures. For all assays the OD at 546 nm was recorded over time, but no appreciable changes were detected except for those also exhibiting color changes; therefore, only qualitative positive-negative results are reported.

Figure 5-left shows the end result of the hydrogenase assay on the epibiont cultures, where a strong purple color indicating hydrogenase activity was only observed in the positive control *E. coli* culture. That indication occurred within an hour, whereas only after a full day was a faint purple tint observed in the epibiont in full 1xPY medium, which does not indicate strong hydrogenase activity, but may be an interaction with other products in the complex media over time. Therefore, the hydrogenase assay was negative in all epibiont cultures.

The same test was applied to the *Anabaena* and *Anabaena* with epibiont cultures. Although we expect all *Anabaena* to have hydrogenases, especially within the nitrogen-fixing heterocysts, the positive result shown in Figure 5-right was only observed for the positive control and the *Anabaena* plus epibiont culture without added hydrogen or fixed nitrogen.

Nitrogenase assays were performed on the *Anabaena* and co-culture tubes. Over the course of six hours the relative strengths of nitrogenase activity were measured by observing the rate of acetylene reduction to ethylene. Figure 6 shows that, as expected, *Anabaena* cultures starved of nitrogen (A, A-E) exhibited nitrogenase activity, while those grown in nitrogen-rich medium do not (A-E-NH_3). Two other interesting trends are noted: 1) hydrogen is for the first time in the course of these experiments observed to affect cultures, here by increasing nitrogenase activity and 2) *Anabaena* with attached epibionts have higher nitrogenase activity than does *Anabaena* alone. For the sake of contrast, the acetylene reduction assay results for Set 1 of the epibiont cultures is shown in Figure 7. No nitrogenase activity is witnessed in the epibiont; however, the epibiont only had growth in complex media with fixed nitrogen. This makes the absence of acetylene reduction no less indicative of the capacity than did the same absence for the Co-Culture when grown with fixed nitrogen.

The *Anabaena* and Co-Culture were grown up under limiting trace-metal conditions to select for Fe, V and Mo nitrogenase co-factors as outlined in Table 3. The cultures were transferred three times over nine days to try to minimize residual metals in the final cultures. An acetylene reduction assay was performed on the cultures on day nine for about eight hours as shown in Figure 8. Unexpectedly, the Fe metal co-factor cultures outperformed the others in nitrogenase activity, especially markedly for the *Anabaena*-only culture,

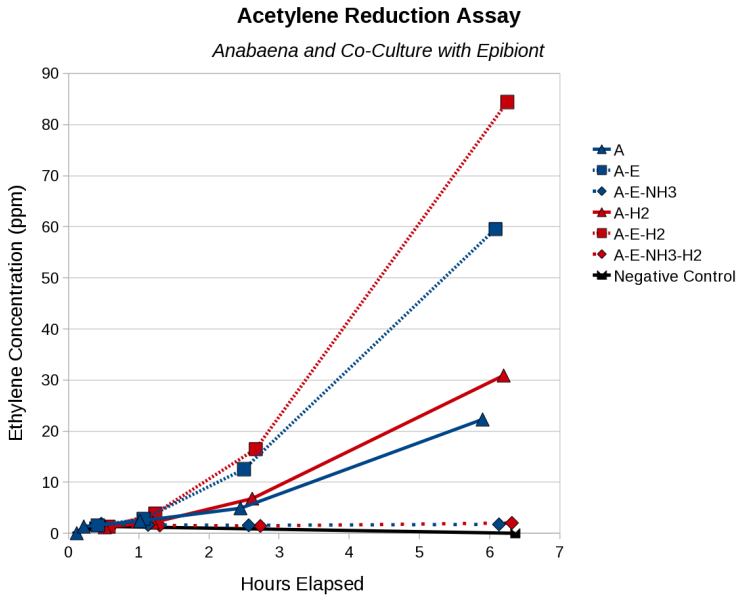

Figure 6: *Anabaena* and *Anabaena* with epibiont nitrogenase assay.

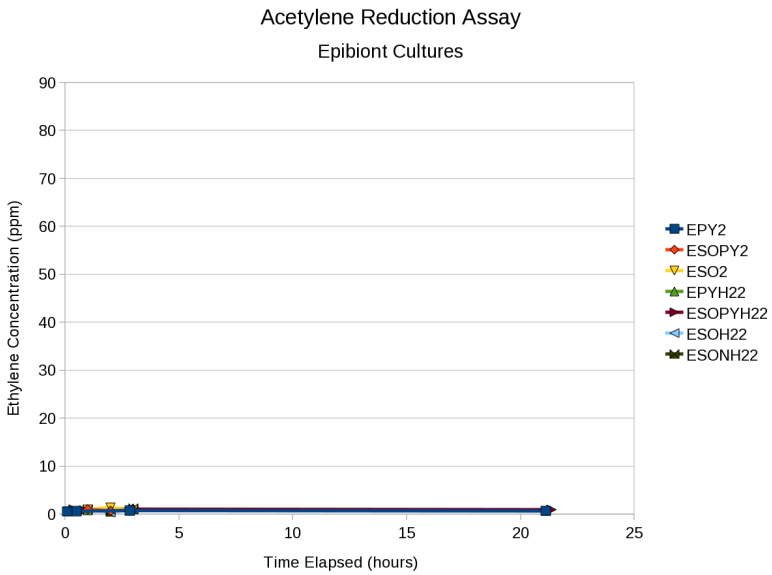

Figure 7: Epibiont Set 1 Cultures Nitrogenase Assay.

Innoculum	Metal Co-Factor
Anabaena	Fe
Anabaena + epibiont	Fe
Anabaena	V
Anabaena + epibiont	V
Anabaena	Mo
Anabaena + epibiont	Mo

Table 3: Description of cultures with limiting metal co-factors available for nitrogenase enzyme *Anabaena* and *Anabaena* with

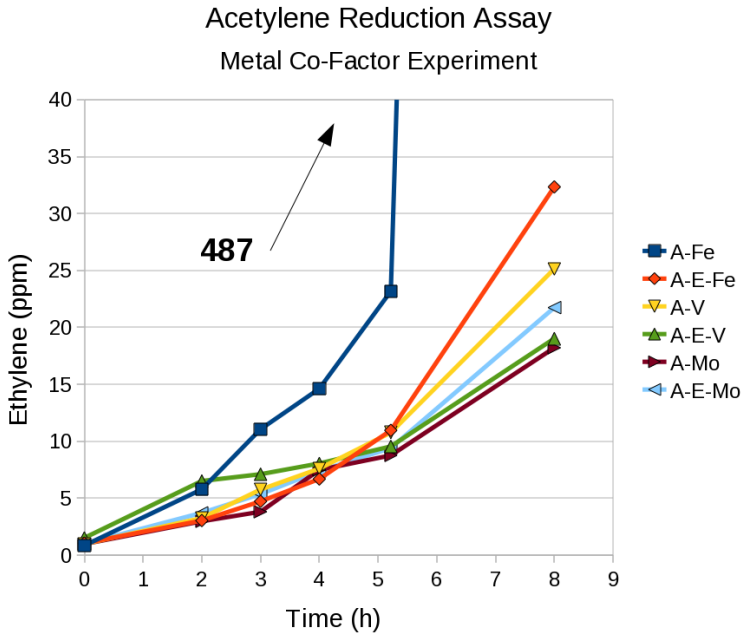

Figure 8: Nitrogenase assay on Anabaena and Co-Culture for cultures grown up with a limiting set of trace metals for the nitrogenase metal co-factor.

but also visibly for the Fe Co-Culture. All Vanadium and Molybdenum cultures of both *Anabaena* and the Co-Culture displayed similar nitrogenase activities.

Microscopy revealed interested trends in the morphology of Anabaena and attachment of epibionts under different metal co-factor treatments. The average number of vegetative cells per heterocyst was counted in the different cultures. The results indicated an increase in the number of vegetative cells per heterocysts from the least to most favorable metal co-factor (Fe, V, Mo) as illustrated in Figure 9 and listed in Table 4. In general, fewer vegetative cells are found per heterocyst for cultures with epibionts attached to the heterocyst versus pure Anabaena cultures. Additionally, it appeared that fewer epibionts were attached to young heterocysts (likely more subject to trace-metal restrictions) in the iron cultures, whereas the Vanadium and Molybdenum cultures had similar epibiont densities.

3.3 Imaging of Respiration Activity

Respiration activity of the Anabaena and epibiont Co-Culture was assessed using the Redox Sensor Green BacLight kit to visualize the locality of high respiration that could indicate the site of H_2 exchange. Propid-

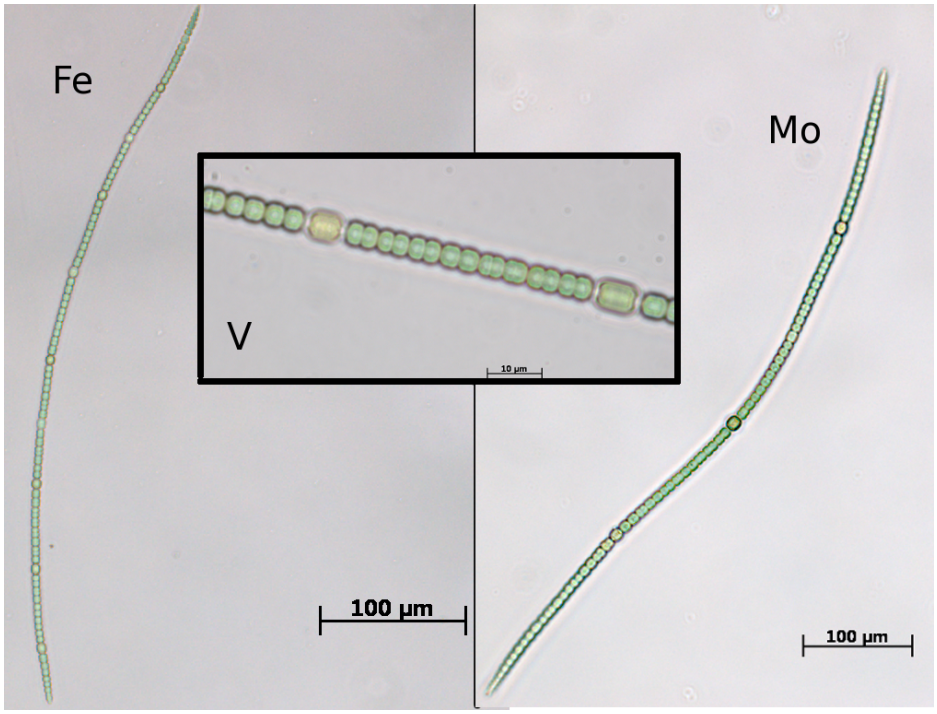

Figure 9: Microscopy illustrating trends of vegetative to heterocyst ratio between the various metal co-factor treatments to promote Fe-, Mo-, or V- nitrogenases.

Metal Co-Factor	Anabaena	Co-Culture	Epibionts Attached
Fe	16	13	fewer
V	21	15	same
Mo	22	19	same

Table 4: Average ratio of vegetative cells per heterocyst cell in cultures forced to use different metal co-factors. Qualitative observation of number of epibionts attached to young heterocysts for each co-factor.

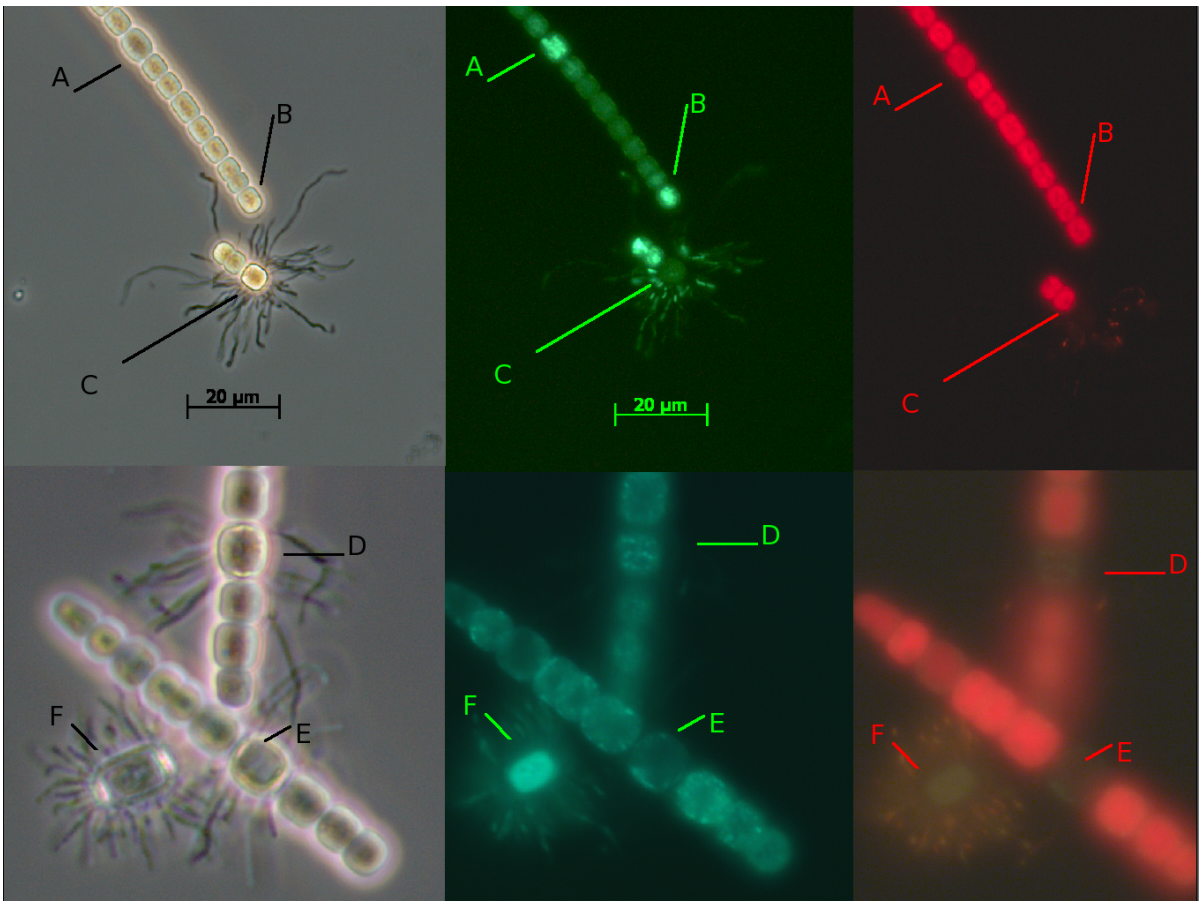

Figure 10: Visual results of Redox Sensor Green dye kit on Co-Culture; phase contrast in left plates, green redox dye fluorescence (490/520 nm) in center plates, and red membrane integrity dye fluorescence (490/625 nm) superimposed with cyanobacterial phycobillin autofluorescence (566/652 nm) in right plates. Heterocysts labeled A-F. Heterocysts C and F are partially and fully detached from the vegetative chain, respectively. Heterocysts A and B appear to be recently formed and do not have epibionts, whereas heterocysts D and E appear well-established and host many epibionts.

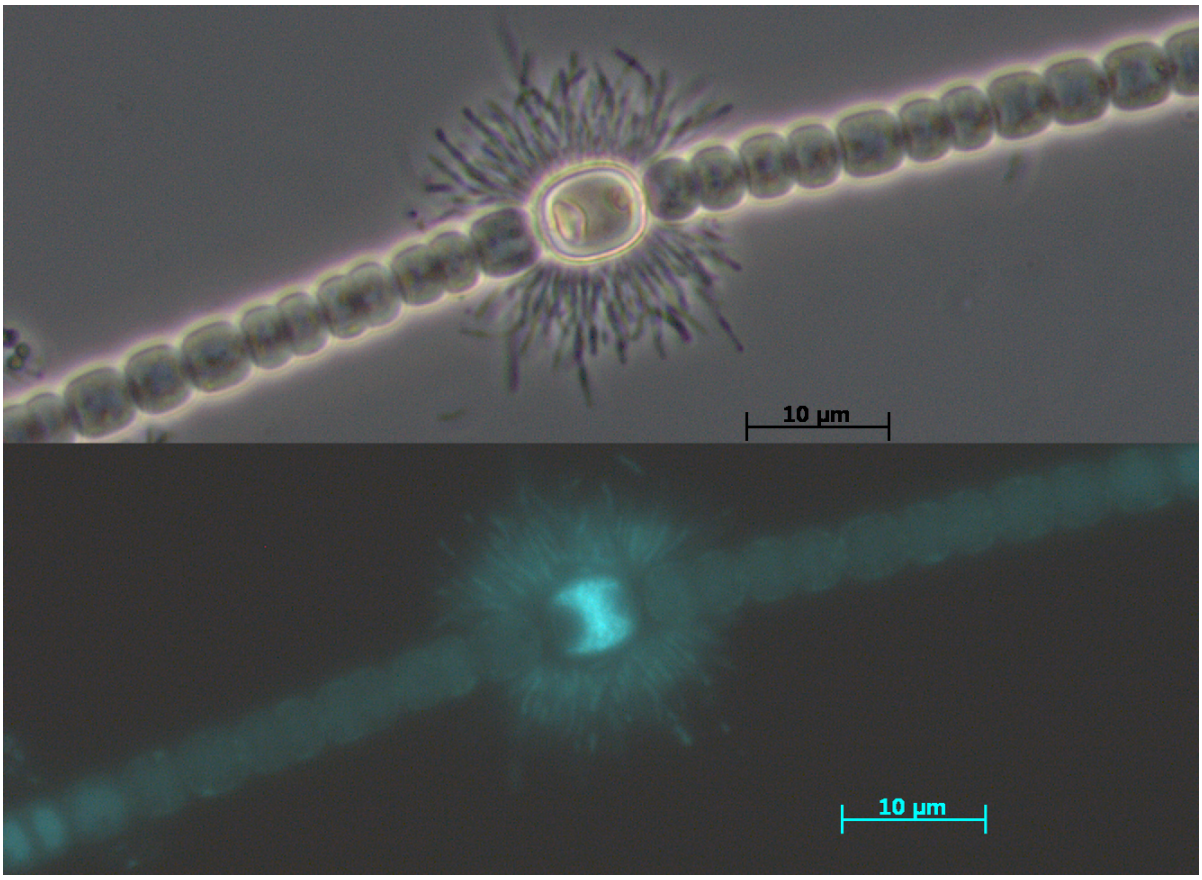

Figure 11: Visual results of Redox Sensor Green dye kit on Co-Culture; phase contrast in top plate and green redox dye fluorescence (490/520 nm) in bottom plate. Central heterocyst is highly reductive and attached epibionts exhibit high redox interaction with the dye.

ium idodide was used to indicate membrane integrity loss. Although highly reductive zones were apparent in heterocysts and their associated epibionts, opposing patterns were also found, and it was difficult to pinpoint clear trends.

Example image sets are shown in Figure 10, which shows a series of two microscope view fields under phase contrast (left), filters to pass green fluorescence (center), and filters to pass red fluorescence (right) as were described in the Methods section. Heterocysts in the figure are labeled as A-F. Heterocysts C and F are partially and fully detached from the vegetative chain, respectively. Heterocysts A and B appear to be recently formed and do not have epibionts, whereas heterocysts D and E appear well-established and host many epibionts. The epibionts surrounding heterocyst C show a clear trend of higher redox near the heterocyst and lower membrane integrity (cell death) farther from the heterocyst. Heterocysts C-F do not exhibit phycobillin autofluorescence, whereas A and B may have recently differentiated from vegetative cells and might retain more autofluorescence. Heterocyst F is completely dislodged from a filament, but still retains its strong reductive core and is completely surrounded by epibionts. This is not an isolated occurrence; many dislodged heterocysts were found completely surrounded by heterocysts. In contrast, heterocysts D and E are attached to the filament chain, but do not exhibit such strong reduction.

A different phase contrast and redox green set is shown in Figure 11 where the central heterocyst is highly reductive and its attached epibionts exhibit high redox interaction with the dye. The cyanophycin plugs, which are an amino acid polymer containing an aspartic acid backbone with arginine side groups, are

clearly visible at the poles of the heterocyst.

3.4 Phylogenetic and Genomic Characterization

The identity of the gifted strains of epibiont and *Anabaena* were verified by sequencing the PCR amplification product of their 16S rDNA sequence. The 16S stretches (over three dilutions), and the 16S obtained by the 454 run discussed below, were compared with the published sequences for the epibiont (DQ364238) and *Anabaena* (DQ364237) strains using the ARB software package with SILVA 16S rDNA alignment and reference database.

The $\frac{1}{5}$th of a 454 Life Sciences plate yielded high-quality sequence data on the epibiont that was assembled by Newbler with the metrics listed below:

- Total Reads = 181,090
- Total Bases = 70,021,298
- Peak Depth = 20
- Estimated Genome Size = 3.5 MB
- Aligned Reads = 99.5%
- Large Contigs = 245
- Average Contig Size = 13,340
- Largest Contig = 86,002

Lists of hydrogenase genes in the *Rhizobiales* species were searched using BLAST against the Newbler-assembled 454 sequence data on the epibiont; no significant matches were found with E values lower than 10^{-4}. Hydrogenase genes require a complex set of maturation, formation and expression proteins, such that one can look for these indicator genes in 454 data and search the neighborhood for additional hydrogenase units [3]. Though this avenue has not been pursued deeply yet, a preliminary search of *Rhizobiales* hydrogenase maturation proteins gave an E value of 10^{-5}, which may or may not indicate the presence of a functional hydrogase gene. Further investigations are needed.

Interestingly, the epibiont sequence data had gene fragments similar to *Rhizobiales* NifH nitrogenase genes, with two significant hits (E value of 10^{-40} and 10^{-5}). The following genes were found with H hits and E best E-value: beta-fructofuranosidase (sucrose metabolism) H=1, E=10^{-4}, glutaminase (glutamine metabolism) H=2, E=10^{-129}, arginase (arginine metabolism) H=8, E=10^{-8}, glocolate oxidase (glycolic acid metabolism) H=7, E=0, and dihydroxyacetone kinase H=10 and, E=10^{-16}. Details on these genes are given in Appendix B. This report will contain some discussion of the sequence data results, but due to time constraints and the sheer amount of data, additional research into these data will continue into the future.

4 Discussion

Does the epibiont love hydrogen the way I do? The data presented in this report indicate not. The substrate affinity tests only had growth in complex media and hydrogen did not affect their growth rate; therefore, the answer to our first substrate question, whether H_2 boosts their growth on complex media, is *no*.

The lack of growth also obscured the second and third set of tests, which aimed at testing different plausible substrates exchanged between epibiont and *Anabaena*; therefore, we don't know if hydrogen boosts the growth of the epibiont while they are metabolizing specific substrates from the heterocysts or not.

The third question was whether the epibiont could utilize H_2 from the heterocyst to fix CO_2 autotrophically, which could have been one possible alternative explanation for the nanoSIMS ^{13}C measurements by [2]. Though not dis-proven in this project, we saw no evidence that the epibiont could simply survive autotrophically in seawater with H_2 (with or without fixed nitrogen). Presumably, future attempts to pin down the substrate exchanged or sought out by the epibiont should be able to answer these questions conclusively, and the genomic information might help form hypothesis to test.

The curious increase in headspace hydrogen concentrations upon hydrogen addition shown in Figure 4 would be interesting to verify. We didn't expect H_2-amendments to increase H_2 concentrations in any of the hypothesis proposed thus far and don't exclude measurement error as the source of these results.

The hydrogenase assay was not very informative because 1) the epibiont only grew on complex media and may not express hydrogenase in those rich circumstances and 2) only one of six *Anabaena*-containing cultures gave a positive assay for hydrogenase, though we know they contain multiple forms, and therefore the trustworthiness of the assay as performed is questionable.

Nitrogenase assays were very interesting and might be a sensitive and informative way of probing the linkage between *Anabaena* and epibiont in the future. Only in the nitrogenase assay did we find that hydrogen boosted the nitrogenase activity of cultures of *Anabaena* and the Co-Culture. It is not clear whether this just enhanced the reducing atmosphere around the nitrogenase enzymes or whether the uptake hydrogenases in the heterocysts were empowered with additional fuel. It is also interesting that the presence of the epibionts clearly enhanced nitrogenase activity, implying that the association is mutualistic and that their function may indeed be to promote nitrogen fixation in exchange for some substrates from the heterocyst. The nitrogenase assay on the epibiont grown in complex media was relatively uninformative because we must grow it on N-free media to see expression of that gene. The sequence information indicates that the epibiont likely has nitrogenase genes and it would be interesting to test for nitrogen-fixation ability if they could be grown on N-free medium.

The metal co-factor tests were another interesting and informative way to explore the co-culture because varying the co-factor effectively varies the ATP requirement for N_2 fixation and the H_2 production during N_2 fixation in documented ways [17]. In this study we found that the iron nitrogenases had the highest nitrogenase activity, which was not expected. The heterocyst differentiation can be assessed visually and it was observed that more vegetative cells can grow per one heterocyst when the nitrogenase is the efficient Mo-type than can grow with the less efficient V-type or the least efficient Fe-type, which is rather intuitive. Additionally, it was observed that the presence of the epibiont makes the nitrogenase less efficient and thus fewer vegetative cells can get by per heterocyst. Also of note, it appeared that fewer epibionts were attaching to the heterocysts of the inefficient Fe-type; however, more time should be allotted to grow up these cultures in order to turn over lingering trace metals and more counts should be done to ensure these trends are reproducibly found.

Utilizing a redox dye to visualize the cellular areas and associations of maximum redox activity was very interesting. It has been observed for quite some time that the heterocysts are highly reducing and will precipitate tetrazolium dyes [12]. It appeared that the co-activity of the epibiont and heterocyst can be inferred by microscopy with these dyes; however, many exceptions to expected trends were observed and future use of this method should be used on simple, well-framed experiments. Optimally, if a certain substrate was known to stimulate growth, the dye could be used to test with and without addition of that substrate. Applying this method in more detail to free heterocysts with attached epibionts would be interesting; especially to epibionts attached to the heterocysts *post mortum*.

The sequence data for the epibiont is a unique opportunity to learn about this organism from the bottom up. Obviously, the data were not explored in much detail in this report, but work will continue after this

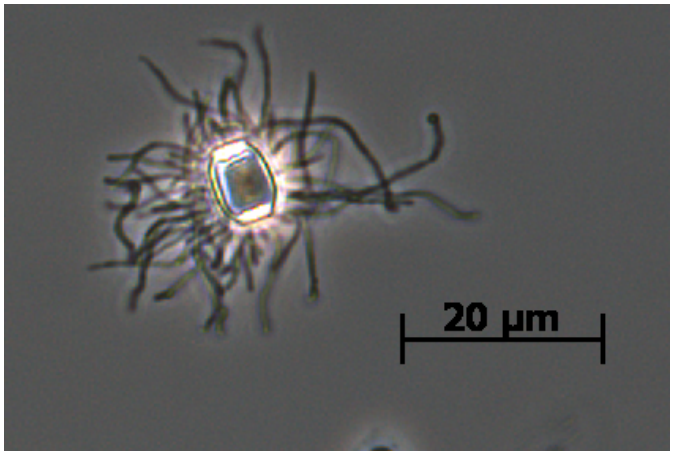

Figure 12: Free heterocyst of *Anabaena* with epibionts.

course to assess whether enough data is present to close the genome and if more is needed, how much? What can be said at this point is that the epibiont does not have a hydrogenase that has clearly popped out with preliminary hydrogenase searches, though a maturation protein might be there, which could suggest it is just cryptic. Interestingly, a nitrogenase is likely present, which might be expressed in nitrogen free media, which was not successfully tested in this work. Fairly good hits to genes for enzymes to metabolize a variety of potential substrates from the *Anabaena* heterocyst were found and more research into their viability could help form hypothesis to test in the future.

5 Concluding Remarks

In conclusion, the vast majority of the evidence presented in this paper indicate that hydrogen does not play a role in the association between the epibiont and *Anabaena*; instead, there is likely a transfer of specific substrates between the two. The epibiont exclusively attaches to this strain of *Anabaena* and it would be satisfying to determine the nature of their association and relationship. Methods described in this report, such as nitrogenase assays, variation of the metal co-factor and redox sensor green dye, should be of good use in this system in the future when hypothesis can be more directed by genomic evidence.

6 Acknowledgments

I want to sincerely thank our Course Directors Steve Zinder and Dan Buckley for coordinating this chaotic course into something short of a microbial masterpiece. Thanks for your ideas, conversations and for giving an atmospheric chemist a chance to explore her interests in a way not offered anywhere else. Immense thanks to Bekah for her unwavering kindness and support - you are the best. Thanks to our TAs Christina, Annie, Gargi, Heather and Chunk - without you, we are lost. Most of all, my gratitude to my wonderful classmates. You know who you are, and it's all of you. Thanks for patiently fielding my research questions (especially Yan and Kate) and to all of you for your company, friendship and humor. Thank you to Bradley Stevenson and John Waterbury for generously providing the cultures used in this study. Thank you to MBL; the Herbert W. Rand Fellowship and Scholarship Fund; Ron Prinn, MIT Center for Global Change Science; and the MIT Program in Oceans, Atmospheres and Climate, Houghton Fund.

Appendices

A Media

A.1 Saltwater Oligotrophic Media

A.1.1 1/2 Saltwater Oligotrophic (SO) Medium

1 Liter, autoclave seawater separately and add 1 ml vitamin mix to mqw mix after autoclaving (has been filter sterilized).

- 250 ml Double-distilled water
- 750 ml Filtered seawater
- 50 μM Na_2CO_3
- 0 $NaNO_3$
- 0 NH_4Cl
- 50 μM K_2HPO_4
- 0.5 ml Cyano Trace Metals
- 7.5 μM EDTA (disodium salt)

Cyano Trace Metals:

- 0.222 g/L $ZnSO_4$ x 7 H_2O
- 1.40 g/L $MnCl_2$ x 4 H_2O
- 0.025 g/L $Co(NO_3)_2$ x 6 H_2O
- 0.39 g/L $NaMoO_4$ x $2H_2O$
- 6.25 g/L Citric Acid Hydrate
- 6.0 g/L Ferric Ammonium Citrate (brown crystals)

A.1.2 Cyano SOX and SNAX Media

The 2xSOX (N-free) cyanobacteria medium used in the course contained:

- 250 ml Double-distilled water
- 750 ml Filtered seawater
- 20 μM Na_2CO_3
- 0 $NaNO_3$
- 0 NH_4Cl
- 20 μM K_2HPO_4
- 0.5 ml Cyano Trace Metals
- 0.5 μM EDTA (disodium salt)

- 1 ml 13-vitamin solution

Cyano Trace Metals:

- 0.222 g/L $ZnSO_4$
- 1.40 g/L $Mncl_2$ x 4 H_2O
- 0.025 g/L $Co(NO_3)_2$ x 6 H_2O
- 0.39 g/L $NaMoO_4$ x $2H_2O$
- 6.25 Citric Acid Hydrate
- 6.0 g/L Ferric Ammonium Citrate (brown crystals)

1000x 13-Vitamin Solution - See course lab manual, section 1.

Liquid media is prepped by adding ingredients to double-distilled water and autoclaved separately. The seawater is autoclaved in a glass container separate from the other ingredients. Following autoclaving the seawater and mineral ingredients are combined and dispensed into sterile culture vessels. Vitamins are added at this time.

A.2 Peptone-Yeast Media

A.2.1 1xPeptone Yeast (PY) Medium

Formula per 1 Liter H_2O

- 3.0 g Peptone
- 0.5 g Yeast Extract
- 15 g sea salts (sigma)

A.2.2 Marine Purity (MP) Medium

1 Liter

- 20 g NaCl
- 17 g AC broth (Difco)
- 8.0 g $MgSO_4\,x\,7H_2O$
- 0.6 g $CaCl_2\,x\,2H_2O$

Difco AC Broth - Formula per 1 Liter

- 20.0 g Proteose Peptone No. 3
- 3.0 g Beef Extract
- 3.0 g Yeast Extract
- 3.0 g Malt Extract
- 5.0 g Dextrose
- 0.2 g Ascorbic Acid

A.2.3 Marine Agar (MA) Medium

Difco Marine Agar 2216

- Approximate Formula* Per Liter
- Peptone ..... 5.0 g
- Yeast Extract ..... 1.0 g
- Ferric Citrate ..... 0.1 g
- Sodium Chloride ..... 19.45 g
- Magnesium Chloride ..... 8.8 g
- Sodium Sulfate ..... 3.24 g
- Calcium Chloride ..... 1.8 g
- Potassium Chloride ..... 0.55 g
- Sodium Bicarbonate ..... 0.16 g
- Potassium Bromide ..... 0.08 g
- Strontium Chloride ..... 34.0 mg
- Boric Acid ..... 22.0 mg
- Sodium Silicate ..... 4.0 mg
- Sodium Fluoride ..... 2.4 mg
- Ammonium Nitrate ..... 1.6 mg
- Disodium Phosphate ..... 8.0 mg
- Agar ..... 15.0 g

B Postive BLASTed Genes

Hydrogenase Large Subunit (hydrogen-oxidation) = zero

Hydrogenase Maturation Proteins (hydrogen-oxidation, 1 hit, E value 10^{-4})

1. NiFe hydrogenase maturation protein HypF -Rhizobium leguminosarum trifolii

Nitrogenase (nitrogen-fixation, 2 hits, E value of 10^{-40} and 10^{-5})

1. Mo-nitrogenase iron protein subunit NifH EC 1.18.6.1 IMGterm -Sinorhizobium meliloti
2. nitrogenase protein, NifH -Rhizobium etli
3. nitrogenase protein, NifH -Rhizobium etli
4. Mo-nitrogenase iron protein subunit NifH EC 1.18.6.1 IMGterm -Rhizobium etli

Glutaminase (glutamine metabolism) H=2, E=10^{-129}

1. L-glutaminase EC 3.5.1.2 IMGterm -Sinorhizobium meliloti

2. L-glutaminase EC 3.5.1.2 IMGterm -Agrobacterium tumefaciens str
3. L-glutaminase EC 3.5.1.2 IMGterm -Agrobacterium tumefaciens str
4. L-glutaminase EC 3.5.1.2 IMGterm -Rhizobium etli
5. L-glutaminase EC 3.5.1.2 IMGterm -Sinorhizobium medicae
6. L-glutaminase EC 3.5.1.2 IMGterm -Rhizobium etli

Glycerone Kinase (glycero-lipid metabolism)

1. homodimeric dihydroxyacetone kinase EC 2.7.1.29 IMGterm -Sinorhizobium meliloti
2. dihydroxyacetone kinase DhaK subunit EC 2.7.1.121 IMGterm -Sinorhizobium medicae
3. Glycerone kinase -Sinorhizobium medicae
4. homodimeric dihydroxyacetone kinase EC 2.7.1.29 IMGterm -Sinorhizobium medicae

Glycolate Oxidase (glycolic acid metabolism) H=7, E=0

1. PROBABLE GLYCOLATE OXIDASE SUBUNIT PROTEIN-Sinorhizobium meliloti
2. PROBABLE GLYCOLATE OXIDASE SUBUNIT PROTEIN-Sinorhizobium meliloti
3. glycolate oxidase subunit -Agrobacterium tumefaciensstr.

Dihydroxyacetone Kinase H=10 and, E=10^{-16}.

1. dihydroxyacetone kinase DhaL subunit EC 2.7.1.121 IMGterm -Sinorhizobium meliloti
2. dihydroxyacetone kinase DhaL subunit EC 2.7.1.121 IMGterm -Sinorhizobium meliloti
3. homodimeric dihydroxyacetone kinase EC 2.7.1.29 IMGterm -Sinorhizobium meliloti

Beta-Fructofuranosidase (sucrose metabolism) H=1, E=10^{-4}

1. beta-fructofuranosidase EC 3.2.1.26 IMGterm -Rhizobium etli
2. putative beta-fructofuranosidase protein -Rhizobium etli

Arginase (arginine metabolism) H=8, E=10^{-8}

1. probable ArgI2 arginase -Sinorhizobium meliloti
2. arginase EC 3.5.3.1 IMGterm -Sinorhizobiummeliloti
3. arginase EC 3.5.3.1 IMGterm -Agrobacterium tumefaciens str.

References

[1] Thorsten Bauersachs, Justine Compaor, Ellen C. Hopmans, Lucas J. Stal, Stefan Schouten, and Jaap S. Sinninghe Damst. Distribution of heterocyst glycolipids in cyanobacteria. *Phytochemistry*, 70(17-18):2034–2039, December 2009.

[2] Sebastian Behrens, Tina Losekann, Jennifer Pett-Ridge, Peter K. Weber, Wing-On Ng, Bradley S. Stevenson, Ian D. Hutcheon, David A. Relman, and Alfred M. Spormann. Linking microbial phylogeny to metabolic activity at the Single-Cell level by using enhanced element Labeling-Catalyzed reporter deposition fluorescence in situ hybridization (EL-FISH) and NanoSIMS. *Appl. Environ. Microbiol.*, 74(10):3143–3150, May 2008.

[3] August Bck, Paul W. King, Melanie Blokesch, Matthew C. Posewitz, and Robert K. Poole. Maturation of hydrogenases. volume Volume 51, pages 1–225. Academic Press, 2006.

[4] Brian Colman. Photosynthetic carbon assimilation and the suppression of photorespiration in the cyanobacteria. *Aquatic Botany*, 34(1-3):211–231, July 1989.

[5] Debabrata Das and T. Nejat Veziroglu. Hydrogen production by biological processes: a survey of literature. *International Journal of Hydrogen Energy*, 26(1):13 – 28, 2001.

[6] Marina G Kalyuzhnaya, Mary E Lidstrom, and Ludmila Chistoserdova. Real-time detection of actively metabolizing microbes by redox sensing as applied to methylotroph populations in lake washington. *ISME J*, 2(7):696–706, March 2008.

[7] Michael T. Madigan, John M. Martinko, Paul V. Dunlap, and David P. Clarck. *Brock Biology of Microorganisms*. Benjamin Cummings, 12 edition, 2008.

[8] M. Mochimaru H. Masukawa. Disruption of the uptake hydrogenase gene, but not of the bidirectional hydrogenase gene, leads to enhanced photobiological hydrogen production by the nitrogen-fixing cyanobacterium anabaena sp. PCC 7120. *Applied Microbiology and Biotechnology*, 58(5):618–624, April 2002.

[9] Robert M. Moore, Stephen Punshon, Claire Mahaffey, and David Karl. The relationship between dissolved hydrogen and nitrogen fixation in ocean waters. *Deep Sea Research Part I: Oceanographic Research Papers*, 56(9):1449–1458, September 2009.

[10] Ivonne Nijenhuis and Stephen H. Zinder. Characterization of hydrogenase and reductive dehalogenase activities of dehalococcoides ethenogenes strain 195. *Appl. Environ. Microbiol.*, 71(3):1664–1667, March 2005.

[11] Paul C. Novelli, Patricia M. Lang, Kenneth A. Masarie, Dale F. Hurst, Richard Myers, and James W. Elkins. Molecular hydrogen in the troposphere: Global distribution and budget. *Journal of Geophysical Research*, 104(D23):PP. 30,427–30,444.

[12] Hans W. Paerl and Patricia T. Bland. Localized tetrazolium reduction in relation to n2 fixation, CO2 fixation, and h2 uptake in aquatic filamentous cyanobacteria. *Appl. Environ. Microbiol.*, 43(1):218–226, January 1982.

[13] Bradley S. Stevenson. Enrichment and identification of a heterotrophic epibiont associated with an anabaena sp. isolated from school street marsh in woods hole, MA. Technical report, Microbial Diversity Summer Course, 2000.

[14] Bradley S. Stevenson and John B. Waterbury. Isolation and identification of an epibiotic bacterium associated with heterocystous anabaena cells. *Biol Bull*, 210(2):73–77, April 2006.

[15] Paula Tamagnini, Rikard Axelsson, Pia Lindberg, Fredrik Oxelfelt, Robbe Wunschiers, and Peter Lindblad. Hydrogenases and hydrogen metabolism of cyanobacteria. *Microbiol. Mol. Biol. Rev.*, 66(1):1–20, March 2002.

[16] John B. Waterbury, Joanne M. Willey, and Alexander N. Glazer Lester Packer. [6] isolation and growth of marine planktonic cyanobacteria. In *Cyanobacteria*, volume Volume 167, pages 100–105. Academic Press, 1988.

[17] Ying Zhao, Shao-Min Bian, Hui-Na Zhou, and Ju-Fu Huang. Diversity of nitrogenase systems in diazotrophs. *Journal of Integrative Plant Biology*, 48(7):745–755, 2006.