THERMO-ACIDOPHILIC ALGAE: pH AND METAL TOLERANCES

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THESIS ABSTRACT

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The class Cyanidiophyceae (the "cyanidia") includes three genera, the walled *Cyanidium* and *Galdieria* and the "naked" *Cyandioschyzon*. All of these algae are unicellular and asexual and live at high temperature and low pH. The cyanidia grow optimally at a pH of 2-3 but can tolerate a higher pH and lower their surrounding pH if it is above the optimal level. They can also tolerate high concentrations of potential toxins that are often found in their natural environments. This thesis shows that strains of cyanidia from Yellowstone National Park and other geographic locations have differing abilities to lower their surrounding pH and tolerate environmental toxins that are found in many environments in which they live. These unique characteristics of this class of algae allow them to be optimally adapted for life in extreme environments with few competitors.

This thesis includes unpublished co-authored material.

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CHAPTER I

INTRODUCTION

Cyanidiophyceae (the "cyanidia"), a class of unicellular, eukaryotic, asexual algae, are the only phototrophs that can live at both high temperature (35°-55°C) and low pH (0.5-4.0). Two of the three genera in this class, *Cyanidium* and *Galdieria*, have a cell wall, while the other, *Cyanidioschyzon*, is naked. In the two studies presented here, *Galdieria*-like walled cells and naked *Cyanidioschyzon* cells from Yellowstone National Park (YNP) and other geographic locations were tested for their ability to lower the pH of their surrounding medium and for tolerance to toxins found in their natural environments.

Cyanidia experience optimal growth at pH 2-3, but are able to maintain an internal pH of 6.6-7.0 (Beardall and Entwhistle 1984, Enami et al. 1986). The ability to maintain an internal pH near neutrality against an outside H^+ gradient is done by H^+ efflux, using PS I cyclic ATP generation (Kura-Hotta and Enami 1984, Enami and Kura-Hotta 1984). This unique characteristic of the cyanidia enables them to live in acid hot springs, soils, and vents in which the external pH can be as low as 0.0-4.0. The work described in Chapter II focuses on the ability of strains from YNP, Japan, New Zealand, and the Philippines to lower the pH of their medium from 6.0, 5.5, or 5.0 to or towards a more optimal pH of 2.5-3.0. My goal was to examine differences among strains in their ability to tolerate a high initial pH and to lower the pH to a more optimal level. I also examined the relationship between growth and lowering of pH over the duration of the experiments.

Chapter III focuses on tolerance to toxins found in thermo-acidic environments in YNP and other geographic locations. Cyanidia from YNP, Japan, New Zealand, Iceland, and the Philippines were tested for tolerance to arsenite, arsenate, aluminum, and mercury. The special focus of this study was comparing arsenite and arsenate tolerance of strains from YNP springs with high and low arsenic. One strain from a spring in YNP rich in arsenite has been shown to have the ability to oxidize the more toxic arsenite to the less toxic form, arsenate (Lehr et al. 2007b, Qin et al. 2009). Aluminum is also a common component of YNP environments, and was tested because Al ions are solubilized under acidic conditions and often inhibit growth of organisms (Nagasaka et al. 2002). Differences have been shown between strains of the same species with respect to tolerance to mercury, arsenic, and other compounds, presumably due to the conditions of the different environments from which the strains were isolated (Albertano and Pinto 1986). The experiments in Chapter III were designed to determine whether or not there were differences among strains in tolerances to toxins whose concentrations vary between habitats.

The following two studies, Chapters II and III contain unpublished co-authored material. The research, analysis, and writing are primarily my own work.

CHAPTER II

THE LOWERING OF pH IN CONFINED ENVIRONMENTS BY THERMO-ACIDOPHILIC ALGAE (CLASS: CYANIDIOPHYCEAE)

This chapter contains unpublished material that was co-authored with Richard W. Castenholz . The experiments, analysis, and writing is primarily my own work.

Introduction

In confined environments, such as microbial mats, endolithic niches, and batch cultures in flasks, most algae and cyanobacteria during photosynthesis and growth raise the pH of their medium by using free CO_2 for their autotrophic metabolism and growth (Fogg et al. 1973, Miller et al. 1988). Some of these phototrophs (especially cyanobacteria) also have the ability to take up HCO_3^- (bicarbonate ion) and convert it to CO_2 by intracellular carbonic anhydrase (Gross 2000, Gao and Zou 2001). If the initial pH of the medium is much lower than optimal (i.e., below ~ 7), growth may not occur in many of the cyanobacteria, or if metabolically competent at that pH, they may gradually raise the pH of their medium, after a lag, to a more optimal pH that allows maximal photosynthesis and growth (Giraldez et al. 1997). In a thermophilic cyanobacterium, *Synechococcus* sp. that was maintaining a visible population at pH 4.5 in a YNP hot spring (without other photosynthetic competitors), sustained growth did not occur in

culture except above pH 6.5, although transient growth (0-~10 h) occurred at pH 5 and 6, but cells failed to complete division and DNA synthesis (see Kallas and Castenholz 1982).

In contrast to other members of the Rhodophytan algae, some species in the class Cyanidiophyceae and order Cyanidiales are capable of growing with a low environmental pH, but maintain an internal pH of about 6.6-7.0 (Beardall and Entwhistle 1984, Enami et al. 1986). In their natural environment of acid hot springs or soils the external pH ranges from 0.0 to 4.0, and temperatures from 35-40 to 56°C, although cells may be recovered by culture enrichment from water sites up to a pH of ~6 even though no biofilm of cyanidia was visible (unpublished data). In the laboratory, Kura-Hotta and Enami (1981) showed that one Japanese strain of *Cyanidium* was able to lower the pH from 3.9 down to 3.0 in a few minutes at a low photon flux (~ 8 μmol photons m^{-2} s^{-1}) at 45°C in medium that was unbuffered, using PS I cyclic ATP generation. This also occurred in the dark, using respiratory ATP generation, but the process was slower (Kura-Hotta and Enami 1984). In all cases the H^+ efflux that was required to maintain an internal pH near neutrality against a strong outside H^+ gradient was dependent on ATP generation (Kura-Hotta and Enami 1984, Enami and Kura-Hotta 1984).

According to some authors the phylogeny of the Cyanidiales extends to the base of the Rhodophytan lineage at about 1.3-1.5 10^9 years (Yoon et al. 2002, 2004, 2010). The genetic characters of the cyanidia suggest enough separation from other Rhodophyta to elevate the group to the class Cyanidiophyceae or the subphylum Cyanidiophytina (Yoon et al. 2006). Saunders and Hommersand (2004) have proposed that the cyanidia be elevated to the phylum Cyanidiophyta separate from the phylum Rhodophyta.

Although several groups of algae and protists have a few species that inhabit extreme acidic waters (e.g., photosynthetic diatoms, euglenoids, green algae), the "cyanidia" are the sole photosynthetic microbial inhabitants of volcanic, acidic waters at temperatures above ~40-45°C.

In our study, we tested several strains of cyanidia obtained from YNP, Japanese, Philippine, and New Zealand hot springs to determine their ability to lower the pH of their medium to lower pH levels from 6.1, 5.5 and 5.0, values well above the normal pH in which cyanidia form visible populations in nature. If pH could be lowered, we asked to what lower level of pH? We were particularly interested in determining whether or not these strains varied in their ability to change the external pH of the medium in which they are growing..

Materials and methods

Cultures used

The clonal cultures used in the experiments and their sources and times of isolation are shown in Table 1.1. Clonal isolation was done by spreading dilute field-collected material (in liquid phase) on standard medium at pH 2.5, solidified by 8.0 gL^{-1} Sigma agargel™ (A3301), a mixture of phytagel and agar. This medium solidifies better at low pH than agar does alone. Plates were incubated at 40-43°C under about 30 μphotons m^{-2} s, with light provided by coolwhite fluorescent lamps. Single colonies were removed with a watchmaker's forceps, after about 7-14 days with a small piece of agargel on which they occurred (to avoid desiccation of the cells), and transferred to

loose-cap 15 ml capacity tubes with about 5 ml of liquid medium. The new liquid culture of suspended cells was then spread again on new plates and the procedure repeated. Axenicity was tested on solidified medium with 0.5 gL^{-1} yeast extract, and also by visual examination under 1000x oil immersion phase contrast. The culture designations in Table 1.1 are as follows: 1A (the most common isolate in YNP) is walled and *Galdieria*-like; 1B was less common, and was the naked *Cyanidioschyzon* type (both identical using 18S rDNA and *rbcL* sequences); IIIA and IIIB are from Japan and more closely related to *Galdieria maxima*, as is type V from New Zealand; type IV is from New Zealand and more closely related to *Galdieria sulphuraria* (Toplin et al. 2008).

Medium and maintenance

The standard culture medium and its preparation are described in Toplin et al. 2008. Except for the experimental levels, the standard external pH was 2.5. Experimental pH levels are shown in Table 1.2. The pH was measured and adjusted with an Accumet AB15 pH meter, with further checks by colorpHast 2.5-4.5 paper strips. Cultures were maintained in 50 ml or 125 ml cotton-plugged Erlenmeyer flasks with 30 ml or 75 ml medium at 30-50 μmol photons $m^{-2}s^{-1}$ in controlled temperature incubators at 40-43°C.

Experimental procedure

Six experiments that differed in starting pH were conducted using 50 ml cotton-plugged Erlenmeyer flasks with 30 ml medium. The external pH in control cultures was

2.5, and the initial pH in controls were 6.1, 5.5, or 5 at the beginning of different experiments. Triplicate flasks were used for each strain and condition. The chlorophyll *a* absorbance of the inoculum ranged between 0.04 and 0.39. Duplicate flasks were used for the control at pH 2.5, since this pH was quite stable. In all cases the temperature was 40-43°C, and the photon flux produced by coolwhite fluorescent lamps with continuous illumination at 80-85 μmol photons m^{-2} s^{-1}. The average duration of the experiments was 30 days, with a range of 27-35 days. Chlorophyll *a* was used a proxy for biomass of the cyanidia. The entire 30ml of culture was vacuum-filtered on a GF/F glass fiber filter, filter-washed with pH 7 medium to prevent pheophytin formation, and extracted with 5 or 10 ml of optical grade DMSO. After 24 h in darkness at 12°C, the clear extract was read at 664-665 nm (Chl *a* maximum) and 750 nm (to subtract from 665 nm maximum for possible turbidity). This measurement was recorded as yield, and was used to determine whether there were differences in growth rates among the treatments.

Statistical analysis

Analysis of variance (ANOVA) was used to determine whether or not there were significant effects of treatment and strain and their interaction. The interaction term is of particular importance because significance indicates that different strains performed differently in the different pH environments. That is, there was a genotype by treatment interaction (GXE) in the ability to alter pH. In the statistical models we treated both treatment and strains as fixed effects because we were specifically interested in these particular strains and environments. If there was a significant effect of either strain or environment, we then used Tukey's HSD to examine differences among them. To

determine whether there were differences between the initial pH and the final pH we performed matched-pairs t-tests. The statistical analyses were performed with JMP Pro 9.0.2 (SAS 2010).

Results

Experimental results

Experiment 1, with a beginning pH of 6.1, had the lowest yields during the 30± day experimental period and the strains were least able to lower their pH during the duration of the experiment (Table 1.2). There was a significant strain effect, treatment effect, and strain by treatment interaction (Fig. A.8). Strain 5506 had a significantly higher yield than 5578 and 5508 regardless of treatment, and yield in 5585 was intermediate and not significantly different from the strains with high or low yield (Fig. A.8). There was also a significant difference between treatments, showing that the cells in the control flasks (pH 2.5) had higher yields than those in the experimental flasks (pH 6.1) (Fig. A.8). There was also a significant strain by treatment interaction, indicating the strains had different yields under different treatments (pH levels). Strains 5506 (Geyser/2001) and 5585 (Nymph/2001) had similar high yields at pH 2.5, but 5585 had lower yield than 5506 at pH 6.1 (Fig. A.8). For the control group with starting pH of 2.5, there was a significant difference between the start and finish pH, with the mean difference being -0.17±0.02 pH units, t_{13}=-8.38, P<0.0001. There were also differences among strains in their ability to change the pH (F5.60, P=0.0197), with stains 5506 (Geyser/2001) and 5585 (Nymph/2001) making the biggest change and strain Norris

Dragon Spring the least. For the treatment group with starting pH of 5, there was a significant difference between the start and finish pH, with the mean difference being -1.44±0.11 pH units, t_{13}=-12.99, P<0.0001. There were also differences among strains in their ability to change the pH of the medium (11.81, P<0.0001), with stain 5506 (Geyser/2001) making the biggest change and strain 5578 (Lemonade/2001) the smallest.

Experiment 2 with a lower beginning pH of 5.5, had higher yields and lower final pH levels than in experiment 1 (Tables 1.2). There was a significant strain effect and treatment effect, but the strain by treatment interaction was not significant (Fig. A.9). Strain 5506 (Geyser/2001) was the strain with the highest yield, and was significantly different from 5508 (Dragon/2001) and 5578 (Lemonade/2001), but not 5585 (Nymph/2001), which had the second highest yield (Fig. A.9). Strains had significantly higher yields at pH 2.5 than pH 5.5. But there were not significant differences in the strain's reactions to the different pH levels (Fig. A.9). There was a significant difference between the pH at the beginning and the end of the experiment, with the mean difference being -0.73±0.16 pH units, t_{19}=-4.60, P=0.0002. However, there was not a significant difference among strains, F=2.65, P=0.08.

In experiments 3, 4, and 5 the strains were all started at pH 5.0 and were able to lower their final pH levels below pH 4 and many to the near optimal pH of ~2.5, with greater yields, than in the previous experiments (Table 1.2).

In experiment 3, strain, treatment, and the strain by treatment interaction were all significant (Fig. A.10). Strain 5506 (Geyser/2001) and 5585 (Dragon/2001) had significantly higher yields, and the yields from all the other strains were not significantly different from each other. In general, the strains had higher yields at pH 2.5, and lower at

pH 5 (Fig. A.10). There was a significant difference between the initial and final pH, with the mean difference being -0.97±0.17 pH units, t_{19}=-5.62, P<0.0001. However, there was not quite a significant difference among strains, F=2.79, P=0.07 in their ability to change the pH.

In experiment 4 there was only one treatment (pH 5), so only strain effects were measured (Fig. A.11). The strain effects were significant, with 5506 (Geyser/2001) again as the highest yielding strain. It was significantly better than 5578 (Lemonade/2001), Nymph Creek (2010), and 5508 (Dragon/2001) (Fig. A.11). There was a significant difference between the initial and final pH, with the mean difference being -1.98±0.09 pH units, t_{20}=-23.13, P<0.0001. There were also significant differences among strains, F=16.24, P<0.0001. Strains 5506 (Geyser/2001) and 5585 (Nymph/2001) changed the pH the most and strain 5508 (Lemonade/2001) changed the pH the least.

Strain effects were significant in experiment 5, but treatment and the strain by treatment interaction were not (Fig. A.12). Strain 5578 (Lemonade/2001) was the highest yielding strain, and was significantly higher than Norris Dragon Spring (2010), 5508 (Dragon/2001), Lemonade Creek (2010), and Nymph Creek (2010). Strains did not have significantly different yields at the two pH levels (Fig. A.12). For the control group with starting pH of 2.5, there was a significant difference between the initial and final pH, with the mean difference being -0.26±0.07 pH units, t_{13}=-3.67, P=0.0028. There were also differences among strains in their ability to affect pH (F125.47, P<0.0001). Strains 5506 (Geyser/2001) and 5578 (Lemonade/2001) decreased pH the most, and strain Nymph Creek (2010) the least. For the treatment group with starting pH of 5, there was a significant difference between the initial and final pH, with the mean difference being -

1.68±0.15 pH units, t_{20}=-11.15, P<0.0001. There were also differences among strains in thier ability to affect pH (F17.75, P<0.0001). Strains 5506 and 5578 made the biggest change, and strain Nymph Creek (2010) the least.

Experiment 6, which had an initial pH of 5.0, included strains from New Zealand, Japan, the Philippines, and one from YNP, showed much lower yields (Table 1.2). Only 3 of the 8 strains in this experiment had any yield at pH 5. And these also lowered their pH below 4 (Table 1.2, Fig. A.7). None of the three strains from Japanese springs showed any yield or ability to lower the pH of the medium during the experimental time, and only one of the three from New Zealand (5704) showed a yield that resulted in a lowering of pH (3.4). Only the strain from the Philippines showed substantial yield and a lowering of pH to 3.0. All strains of the six experiments maintained the pH of the controls at 2.5 or lower, in a few cases even to 1.9 (mean=2.32 for all six experiments with a standard error of ±0.028) (Table 1.2). Strain, treatment, and strain by treatment interaction effects were significant (Fig. A.13). 5704 from New Zealand was significantly higher yielding than any other strain. In general, the strains yielded better at pH 2.5 than pH 5, but 5704 from New Zealand yielded even more in pH 5 than it did in pH 2.5 (Table 1.2, Fig. A.13). For the control group with starting pH of 2.5, there was a significant difference between the initial and final pH, with the mean difference being -0.24±0.09 pH units, t_{18}=-2.64, P=0.0167. However, there were no differences among strains in their ability to afect pH (F2.19, P=0.1180). For the treatment group with starting pH of 5, there was a significant difference between the initial and final pH, with the mean difference being -0.66±0.16 pH units, t_{20}=-4.07, P=0.0006. There were also differences among strains in their ability to

change the pH (F244.68, P<0.0001). Strains 5704 (NZ) and 5774 (Philippines) made the biggest change, and strains 5678 (Japan) and 5706 (Japan) the least.

Relationship between strain, yield, and ability to lower pH

In an experiment with two strains in separate flasks, growth began only after about 300 h (~ 12 days) when pH had begun to decrease from 5.5 to 4.8 and 5.2 with a final arrival at pH 3.1 or 3.5 at about 600 h (25 days) (Fig. A.1). Yield was compared to final pH in Figure 1. There was a correlation (r= -0.85, P < 0.0001) between high yield and low final pH, with a higher yield corresponding to a lower pH. This was expected, because the lowering of pH represents a gradually increasing growth rate resulting in a higher yield (Fig. 1, Appendix A).

Lemonade Creek (LC), Nymph Creek (NC), and Norris Dragon Spring (NDS), all isolated in 2010 were compared to the corresponding strains 5578, 5585, and 5508, respectively, and isolated in 2001. The strains from 2010 often performed similarly in response to the corresponding strains from 2001. For example, the Lemonade Creek strain (LC) was not significantly different from its corresponding strain, 5578, in two of the three experiments when they were run together (Table 1.2, Appendix A). The Norris Dragon Spring strain from 2010 (NDS) was also not significantly different from its corresponding strain, 5508, in two of the three experiments (Table 1.2, Appendix A). The new Nymph Creek strain (NC), by contrast, was significantly different from 5585 (the older Nymph Creek strain) in all three experiments in which they were run together (Table 1.2, Appendix A). All the above were morphologically the same (*Galdieria*-like

with cell wall), except 5610 which was a morphologically a naked *Cyanidioschyzon* although both morphotypes have indistinguishable 18S-rDNA and rbcL sequences.

Table 1.1. Culture isolates used in the experiments, sources, temperatures, pH values, and dates of clonal isolation. Brief descriptions of the sources are in text. Types are described in Toplin et al. (2008) Locations of the sources and types of the strains are in Toplin et al. (2008). Strains are briefly decribed in Methods.

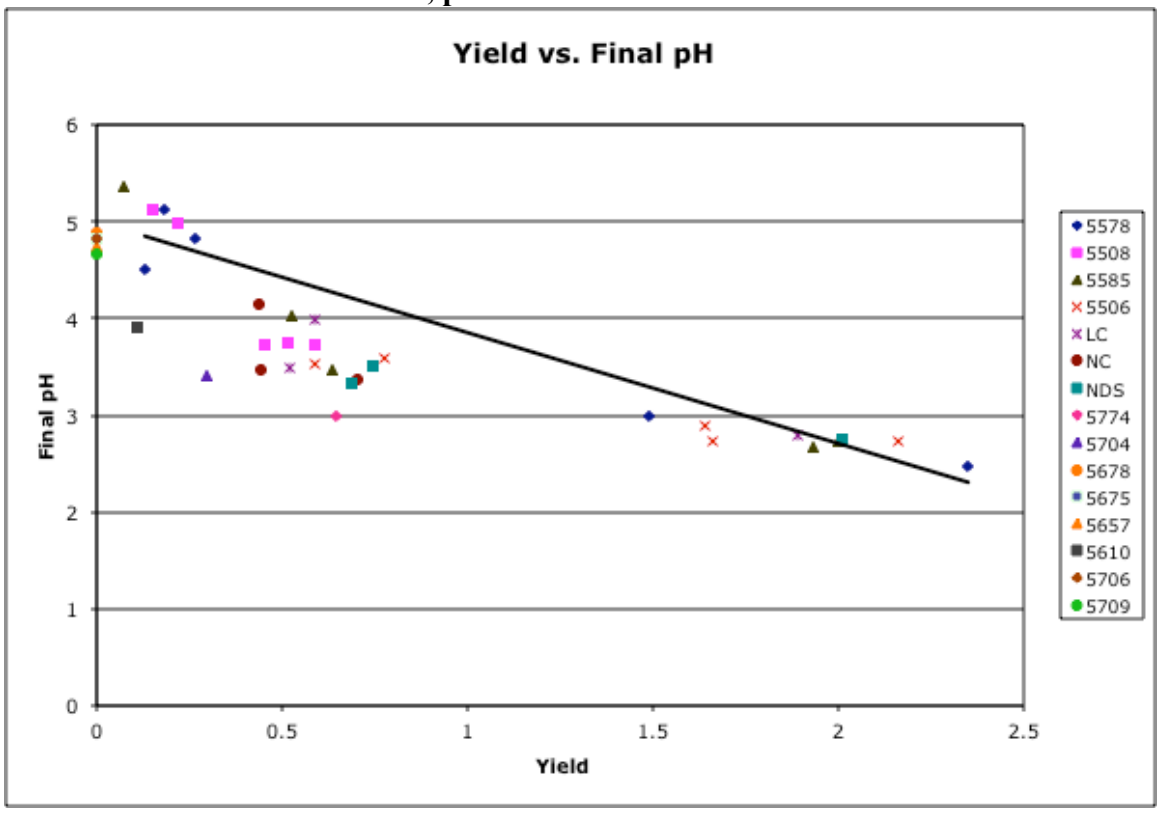
CCMEE No.	Type	Source	Collection Temp	Collection pH	Isolation yr
5578	1A	Lemonade Creek, YNP	40º	2.2	2001
LC	1A?	Lemonade Creek, YNP	48°	2.2	2010
5508	1A	Norris Dragon Spring, YNP	45º	1.9	2001
NDS	1A?	Norris Dragon Spring, YNP	40º	3.0	2010
5585	1A	Nymph Creek, YNP	40º	3	2001
NC	1A?	Nymph Creek, YNP	42°	2.8	2010
5506	1A	Norris Geyser Basin, YNP	40º	1.0	2001
5774	Gald.-like	Taal Volcano, Philippines	40º	2.9	2006
5704	V	Waimangu, NZ	Unknown	Unknown	2005
5678	IIIB	Kusatu, Japan	45º	1.9	2003
5675	IIIB	Nakabusa, Japan	Unknown	Unknown	2003
5657	IIIA	Owakudani, Japan	>45°	2.5	2003
5610	IB[1]	Sylvan Crust, YNP	40º	4	2001
5706	IV	Craters of the Moon, NZ	Unknown	Unknown	2005
5709	V	Whaka, NZ	30°	4	2005

[1]naked *C. merolae* morphotype

Table 1.2. Results from six experiments with beginning and final pH values, percentages of yield, and yield (expressed as corrected chlorophyll *a* absorbance of total biomass in 35 ml of culture). Standard error of the mean for each experiment is shown for final pH and yield.

Exp #	Strain	Start pH	Final pH	% Yield	Yield
1	5578	6.1	4.83 ± 0.06	12	0.27 ± 0.08
2	5578	5.5	5.12 ± 0.11	57	0.18 ± 0.03
3	5578	5.0	4.51 ± 0.10	13	0.13 ± 0.01
4	5578	5.0	3.00 ± 0.07	N/A	1.49 ± 0.11
5	5578	5.0	2.47 ± 0.03	153	2.35 ± 0.04
3	LC	5.0	3.48 ± 0.11	68	0.52 ± 0.10
4	LC	5.0	2.79 ± 0.08	N/A	1.89 ± 0.06
5	LC	5.0	3.98 ± 0.05	124	0.59 ± 0.04
1	5508	6.1	5.13 ± 0.07	7	0.15 ± 0.01
2	5508	5.5	4.99 ± 0.16	38	0.22 ± 0.02
3	5508	5.0	3.74 ± 0.29	133	0.51 ± 0.15
4	5508	5.0	3.72 ± 0.16	N/A	0.45 ± 0.07
5	5508	5.0	3.74 ± 0.04	90	0.59 ± 0.04
3	NDS	5.0	3.32 ± 0.05	237	0.69 ± 0.10
4	NDS	5.0	2.76 ± 0.04	N/A	2.01 ± 0.10
5	NDS	5.0	3.50 ± 0.08	99	0.75 ± 0.10
1	5585	6.1	5.37 ± 0.03	2	0.07 ± 0.01
2	5585	5.5	4.02 ± 0.02	55	0.53 ± 0.02
3	5585	5.0	3.47 ± 0.10	27	0.64 ± 0.12
4	5585	5.0	2.73 ± 0.07	N/A	2.00 ± 0.39
5	5585	5.0	2.67 ± 0.06	109	1.93 ± 0.15
3	NC	5.0	3.47 ± 0.05	142	0.44 ± 0.03
4	NC	5.0	3.37 ± 0.15	N/A	0.70 ± 0.23
5	NC	5.0	4.15 ± 0.38	109	0.43 ± 0.19
1	5506	6.1	3.52 ± 0.13	18	0.59 ± 0.10
2	5506	5.5	3.58 ± 0.37	18	0.78 ± 0.25
3	5506	5.0	2.90 ± 0.15	80	1.64 ± 0.38
4	5506	5.0	2.73 ± 0.04	N/A	2.16 ± 0.24
5	5506	5.0	2.74 ± 0.02	85	1.66 ± 0.53
6	5774	5.0	3.00 ± 0.01	137	0.64 ± 0.02
6	5704	5.0	3.40 ± 0.09	24	0.30 ± 0.09
6	5678	5.0	4.88 ± 0.04	0	0
6	5675	5.0	4.82 ± 0.06	0	0
6	5657	5.0	4.75 ± 0.05	0	0
6	5610	5.0	3.91 ± 0.07	35	0.11 ± 0.01
6	5706	5.0	4.83 ± 0.04	0	0
6	5709	5.0	4.66 ± 0.04	0	0

Figure 1. The relationship between the final pH and yield (as mean from 3 flasks) of all experiments; color and symbol for each strain. Standard error of the mean listed in Table 1.2. Correlation -0.85, p<0.0001*.



Discussion

How cyanidia tolerate high pH and lower the pH of their surroundings

In most of the six experiments, effects of strain, treatment, and strain by treatment interaction on yield were significant (Appendix A). Strain effects were significant in every experiment, showing that strains grow differently from each other regardless of treatment (Appendix A). Treatment effects were significant in every case except experiment 5, with strains growing significantly better at pH 2.5 than pH 5. This supports previous work that states the optimum pH for cyanidia is 2-3 (Brock 1978). The strain by treatment interaction was significant in all except two of the experiments: it was not significant in experiment 2 or 5 (Appendix A). It is notable that experiments 3 and 5 were run with the same strains and conditions, but in experiment 5 neither the treatment nor strain by treatment interaction were significant, but they were significant in experiment 3. These results are anomalous, and could possibly be due to differences in inoculum between the two experiments. Overall, it appears that there might be significant differences abilities to tolerate a high pH between strains in YNP, however, more work could be done to look into the differences in growth ability at high pH between strains from YNP.

When more recent isolates (2010) of strains from Norris Dragon Spring, Lemonade Creek, and Nymph Creek were compared to the older isolates (2001) 5508, 5578, and 5585, respectively, they did not always perform the same, with respect to yield at high pH. Older and newer isolates of Norris Dragon Spring and Lemonade Creek

strains each had significantly different yields in only one of three experiments, but Nymph Creek older and newer isolates were significantly different in every case. Any differences between older and newer isolates could be due to changing environmental conditions that might affect the ability of cyanidia to tolerate a high pH, or possibly, that the strains collected in 2010 were different from those collected in 2001.

There were significant differences in strain's ability to lower their pH levels in three of the five experiments that were run with YNP strains. When we looked at the beginning and final pH values for the six experiments, we found that in every case there was a significant difference in the pH values at the start and the end of the experiment. Therefore, in every experiment, the cyanidia were lowering the pH significantly. There were also significant differences among strains in this ability in four of the six experiments. Strain 5506 (Geyser/2001) lowered the pH the most in experiments 1, 4, and 5. This is notable because this culture was isolated from an extreme soil site, in which it might be able to lower the surrounding pH, due to the semi-enclosed environment.

The majority of our tests were on YNP strains, but in experiment 6, we used strains from hot springs from around the world. Where a strain was from mattered. Strains from New Zealand (5704) and the Philippines (5774) were most able to lower the pH, while strains from Japan (5678, 5706) were least able. It is also notable that the treatment effects and strain by treatment interaction were significant for experiment 6 with respect to yield, the only experiment using strains from locations other than YNP (Fig. A.13). Yield at pH 2.5 was much higher overall than at pH 5 in this experiment, and yield varied considerably by strain, with strains from Japan yielding less well. The

YNP strains, measured in experiments 1-5, were all able to lower the pH of the medium to a somewhat lower value and those starting at pH 5.0 had high enough yields to lower the pH, some to about 2.5-3.0 (Appendix A). The strain from the Taal Volcano lake in the Philippines (experiment 6) also showed a similar ability (Fig. A.13). The mechanism for the reduction in pH was presumably accomplished by the previously described mechanism of rapid ATP-dependent H^+ efflux (Enami and Kura-Hotta 1984, Kura-Hotta and Enami (1981, 1984), Enami et al. 2010).

Why cyanidia lower their surrounding pH

In nature and in culture, the optimum pH for growth of the cyanidia appears to be between 2 and 3 (Brock 1978). Therefore, attaining this range in the surrounding medium is ideal and gives the best opportunity for growth. For example, the ability to lower the pH could represent a survival strategy in some natural situations, such as pH 5-6 soil pockets near more acidic environments that have acquired cells of "cyanidia" through earlier high water, rain spatter, insect movement, or other vectors. It is possible that these habitats are more abundant in YNP than other locations such as Japan. We have shown that some cyanidia can lower their pH in a small, enclosed environment. Soil pockets at pH 5-6 in YNP might provide a similar habitat to our experimental conditions, in which the cyanidia can lower their surrounding pH to a more optimal level (e.g., Norris Geyser Basin "Extreme Site" from which strain 5506 was isolated). Cyanidia in Japan may not experience these same conditions or may not have had similar mutations, and therefore, have not adapted to tolerate and lower a pH of 5-6.

Given that there are no other phototrophic taxa in these extreme environments

with high temperature and low pH, it would seem that only the ancestors of the cyanidia were able to escape competition by other phototrophs in the thermal-acidic habitat. Few prokaryotic phototrophs inhabit acidic volcanic waters, thermal or non-thermal, although a few cyanobacteria are known from pH 4-4.5 in geothermal springs, but this range does not appear to be optimal for growth (Kallas and Castenholz 1982). A few cyanobacteria are also known from pH 4 in soils (Belnap 2001), and even at pH 2.9 in a single lake by a lignite mining area (Steinberg et al. 1998). Lowering their surrounding pH could be an important adaptation that allows cyanidia to live in and tolerate environments that are not optimal for growth.

Bridge

The previous chapter focused on the ability of cyanidia to lower the pH of their surrounding medium from pH 5-6 to a more optimal pH of 2-3. This tolerance and ability to alter the pH of their environment has adaptive value for cyanidia, allowing them to live in a variety of conditions. In the next chapter, cyanidia were tested for their tolerance to toxic compounds found in varying concentrations in their habitats in YNP and other locations. Chapter III shows that tolerance to arsenite, arsenate, aluminum, and mercury varies among strains of the same 18S and rbcL phylotype that live within different environments and geographic areas.

CHAPTER III

TOLERANCE TO ARSENIC, METALS AND OTHER ENVIRONMENTAL FACTORS BY CULTURE STRAINS OF THERMO-ACIDOPHILIC CYANIDIALES FROM YELLOWSTONE NATIONAL PARK

This chapter contains unpublished material that was co-authored with Tyler Roberts and Richard W. Castenholz. The experiments, analysis, and writing is primarily my own work.

Introduction

The order Cyanidiales (or Class Cyanidiophyceae) of the Rhodophyta (red algae) comprise the only group of phototrophs that occur in acidic thermal environments (upper limit, 56°C) and pH levels (0-4). Few acidophilic prokaryotic phototrophs occur at pH 4 or below in nature or in the lab, and none in these volcanic waters. The three morphologically described genera of the Cyanidiales are *Cyanidium, Galdieria,* and *Cyanidioschyzon*, all unicellular. The first two genera have a rigid wall and divide internally to form 4 or more daughter cells. *Cyanidioschyzon* lacks a wall and divides by "binary cytokinesis" (Merola et al. 1981). Previous studies have assessed the responses of various Cyanidiales to various metals and metaloids that occur naturally in acidic environments (Brock 1978, Albertano and Pinto 1986, Pinto et al. 2003, Nagasaka et al.

2002, 2004, Lehr et al. 2007a,b, Qin et al. 2009, Castenholz and McDermott 2010). In the earliest study listed (Brock 1978) it is uncertain whether the all the walled strains used or observed were true *Cyanidium* or *Galdieria*, since before 1981 these thermo-acidophiles were all regarded as *Cyanidium caldarium* (Merola et al. 1981).

We assessed the tolerance of a number of culture strains of the "cyanidia"(i.e. the Cyanidiales) isolated from a variety of acid environments in Yellowstone National Park (YNP) to arsenite (As III), arsenate (As V), aluminum, and mercury, all common components and features of many YNP acidic environments (see below). Since 18S-rDNA (nuclear) and rbcL (chloroplastic) sequences link all of the predominate *Galdieria* morphotypes from YNP as 99% to 100% similar to *Cyanidioschyzon merolae*, it is the main purpose of the present study to determine whether the culture isolates are different from each other using phenotypic (physiological) characters, which if consistent, are genetically based, since the experiments were "common garden" situations. The potential toxins tested in this study are common constituents of many of the YNP acidic habitats from which many cultures were isolated. The special focus is on different tolerances to arsenite (As III) by strains from high and low arsenite sites. Arsenite is known to be oxidized to the less toxic arsenate (As V) by at least one strain of the "cyanidial" culture collection (Lehr et al. 2007b, Qin et al. 2009).

Materials and methods

Cultures used

The clonal cultures used in the experiments and their sources and times of

isolation are shown in Table 2.1. Clonal isolation was done by spreading dilute field-collected material (in liquid phase) on standard medium at pH 2.5, solidified by 8.0 gL^{-1} Sigma agargel™ (A3301), a mixture of phytagel and agar. It solidifies better at low pH than agar alone. Plates were incubated at 40-43°C under about 30 μphotons m^{-2} s^{-1}, with light provided by coolwhite fluorescent lamps. Single colonies were removed with a watchmaker's forceps, after about 7-14 days with a small piece of agargel on which they occurred (in order to avoid desiccation of the cells), and transferred to loose-cap 15 ml capacity tubes with about 5 ml of liquid medium. The new liquid culture of suspended cells was then spread again on new plates and the procedure repeated. Axenicity was tested on solidified medium with 0.5 gL^{-1} yeast extract, and also by visual examination under 1000x oil immersion phase contrast. The cultures designations in Table 2.1 are as follows: 1A (the most common isolate in YNP) is walled and *Galdieria*-like; 1B was less common, and was the naked *Cyanidioschyzon* type (both identical using 18S rDNA and *rbcL* sequences).

Medium and maintenance

The standard culture medium and its preparation are described in Toplin et al. 2008. The standard external pH was 2.5. Cultures were maintained in 50 ml or 125 ml cotton-plugged Erlenmeyer flasks with 30 ml or 75 ml medium at 30-50 μmol photons $m^{-2}s^{-1}$ in controlled temperature incubators at 40-43°C.

Experimental procedure

The chlorophyll *a* absorbance of the inoculum ranged between 0.04 and 0.39. Duplicate flasks were used for the control at pH 2.5, since this pH was quite stable. In all cases the temperature was 40-43°C, and the photon flux produced by coolwhite

fluorescent lamps with continuous illumination at 80-85 μmol photons m^{-2} s^{-1}. The average duration of the experiments was 30 days, with a range of 27-35 days. Chlorophyll *a* was used a proxy for biomass of the cyanidia. The entire 30ml of culture was vacuum-filtered on a GF/F glass fiber filter, filter-washed with pH 7 medium to prevent pheophytin formation, and extracted with 5 or 10 ml of optical grade DMSO. After 24 h in darkness at 12°C, the clear extract was read at 664-665 nm (Chl *a* maximum) and 750 nm (to subtract from 665 nm maximum for possible turbidity). This measurement was recorded as yield, and was used to determine whether there were differences in growth rates among the treatments.

Arsenite (As III) tolerance

Four experiments were conducted using 50 ml cotton-plugged Erlenmeyer flasks with 30 ml medium. Controls used pH 2.5 Cyanidium medium. Experimental concentrations were 0.4, 0.6, and 1.0mM $NaAsO_2$. Triplicate flasks were used for each strain and condition. The temperature was 40-42ºC, and the photon flux produced by coolwhite fluorescent lamps with continuous illumination was ~80-85 μmol photons $m^{-2}s^{-1}$. The average duration of the experiments was 15 days, with a range of 13-20 days. The flasks were swirled once per day. No additional CO_2 was added.

Arsenate (As V) tolerance

Two experiments were conducted using the same conditions as the arsenite experiments. Experimental arsenate concentrations were 20, 30, and 40mM Na_3AsO_4. NaCl (40 mM) was used in the medium as a second control in one experiment to ascertain the possible effects of high sodium. The two experiments were run for 21 and 22 days.

Arsenic speciation

Water samples were taken from Norris Dragon Spring (from the source and three sites downstream) and from Lemonade Creek (two sites) and later tested for total arsenic arsenite, and arsenate. Arsenite was calculated as the difference between total arsenic and arsenate. The following procedure was used for this arsenic speciation procedure.

Preparation

-1.5 g of sodium borohydride and 0.05 g of sodium hydroxide were weighed into a 50 ml centrifuge tube. Three tubes were prepared so there would be extras in the field.

-Tris buffer was prepared with 2 M Tris, and set to pH 6.0. Three 50 ml centrifuge tubes were filled with the buffer.

-Other supplies included concentrated hydrochloric acid (three 1 ml tubes), approximately 20 50-ml centrifuge tubes (2 for each sample), a P-1000 "pipetman", a P-200 "pipetman", pipet tips, a squirt bottle, and d.H_2O (two, 500-ml bottles).

In the field

-Five ml of filtered water (0.22 μm filter with 10 ml syringe) were pipetted from each sample into a 50-mL centrifuge tube.

-One ml of Tris buffer was added and then shaken.

-One ml of the borohydride solution was added slowly. (If borohydride is added too quickly, the solution would bubble out of the tube).

The tops of the tubes were left open to vent off arsine for 1 minute, then closed and shaken vigorously. Again, the tubes were opened to vent for 1 minute. Shaking and venting was repeated for 10 minutes.

-Finally, 100 μl of conc. HCl was added to stop the reaction. Three samples were taken for each sample site for later speciation.

In the lab

-For measurements of total arsenic, a 1 mg/ml As in 2% NaOH atomic absorption standard solution (Acros Organics), and for measurements of As(III), a 0.5% (w/v) solution of sodium arsenite (Ricca Chemical) were diluted appropriately. All other materials were purchased from Fisher Scientific.

Sample preparation for total arsenic measurements

-A reducing solution was prepared by mixing 10 g of KI, 5 g of ascorbic acid and 100 ml of 18MΩ H_2O. Samples and standards were pre-reduced by adding 2.00 ml of the reducing solution, 5.00 ml of HCl (trace metal grade), a suitable volume of sample or standard and 18MΩ H_2O to a final volume of 10.00 ml. The reduction was allowed to proceed at room temperature for a minimum of 1 hour prior to analysis.

Sample preparation for As(V) measurements

-A suitable volume of sample or standard and 5.00 ml of 1.0M Tris-HCl (adjusted to pH 6.4) were mixed and then diluted with 18MΩ H_2O to a final volume of 10.00 ml.

Instrument parameters

-The concentration of total arsenic and As(V) were determined by using hydride generation-atomic absorption spectroscopy. The absorbance was measured at a wavelength of 193.7 nm and a mixture of 0.6% $NaBH_4$ and 0.5% NaOH was used for reduction to arsine.

Aluminum tolerance

Two experiments were conducted. Controls were Cyanidium medium with 300mM NaCl to allow for the possible effects of maximum chloride in the experiments

with highest Al concentration. Experimental aluminum concentrations were 100mM, 200mM, and 300mM ($AlCl_3$). The two experiments were run for 17 days each.

Mercury tolerance

Two experiments were conducted. Controls were in Cyanidium medium. Experimental mercury concentrations in the first experiment were 2μM, 3.5μM, and 5μM ($HgCl_2$). Concentrations for the second experiment were 1μM, 2μM, and 3μM. The first experiment ran for 14 days and the second for 16 days.

Statistical analysis

Analysis of variance (ANOVA) was used to determine whether or not there were significant effects of treatment and strain and their interaction. The interaction term is of particular importance because significance indicates that different strains performed differently in the different environments. That is, there was a genotype by treatment interaction (GXE). In the statistical models we treated both treatment and strains as fixed effects because we were specifically interested in these particular strains and environments. If there was a significant effect of either strain or environment, we then used Tukey's HSD to examine differences among them. The statistical analyses were performed with JMP Pro 9.0.2 (SAS 2010).

Results

Arsenite (AsIII) tolerance

The first experiment showed some yield for all strains at all concentrations of arsenite. Strain, treatment, and the strain by treatment interaction were all significant. YNP Norris Dragon Spring strain 5508 was the highest yielding at all concentrations of arsenite (Table 2.2, Fig. B.10). YNP Sylvan Crust strain 5610 (naked Cyanidioschyzon) was the least tolerant of arsenite, but was not significantly different from strain 5578 from Lemonade Creek or strain 5584 from Nymph Creek (Table 2.2, Fig. B.10). The strains had the highest yields in the control medium, followed by 0.4mM arsenite, and worst in 0.6mM and 1.0mM arsenite (Fig. B.10).

In the second experiment, which included a subset of the strains in experiment one plus an additional treatment, strain, treatment, and the strain by treatment interaction effects were all significant. Lemonade Creek strain 5578 was the highest yielding strain had higher yields for controls and in the presence of arsenite, except for the highest arsenite concentration (1.0 mM) (Table 2.2, Fig. B.11). Norris Dragon Spring (5508) and Sylvan Crust strain 5610 (naked) were less tolerant of arsenite, and not significantly different from each other (Fig. B.11). Strains had the highest yields in both regular medium and with 1mM NaCl, showing that the sodium in sodium arsenite was not an inhibitory factor (Fig. B.11).

The thrid experiment included 5610 as the others had, but also two recently (2010) collected strains, again, all three effects tested were significant. Strain 5610 (Sylvan/2001) was least tolerant of arsenite, with the newer two strains (LC site 1 and NDS site 1) showing higher yields, with NDS site 1 as the highest (Table 2.2, Fig. B.12). Strains had the highest yields in regular medium, and then progressively lower yields with increasing arsenite concentrations (Fig. B.12).

Arsenate (AsV) tolerance

In the first arsenate experiment, treatment effects and the strain by treatment interaction were significant, and strain effects were very nearly significant (P=0.056). 5602 (Rabbit Creek, YNP) was the highest yielding strain and the only strain that had a significant yield with an arsenate concentration of 40mM (Table 2.2, Fig. B.13). However, it was only significantly different from the two lowest yielding strains, 5584 (Nymph/2001) and 5610 (Sylvan/2001). Strain 5508 (Dragon/2001) had the highest yield with an arsenate concentration of 20mM (Fig. B.13). Strains had the highest yields in regular medium, and progressively lower yields with higher arsenate concentrations (Fig. B.13).

In the second experiment, all three effects tested were significant. Norris Dragon Spring (NDS) was the most tolerant to arsenate and had significantly higher yields than any other strain (Fig. B.14). Most other strains did not show any yield in the presence of 30mM and 40mM arsenate (Table 2.2, Fig. B.14). It should be pointed out that arsenate concentrations were many times those of arsenite because the lesser toxicity of arsenate.

Strains had equally high yields in regular medium and the NaCl control, showing that the sodium in sodium arsenate was not inhibitory (Fig. B.14).

Aluminum tolerance

The first experiment showed significant effects of strain, treatment, and the strain by treatment interaction. Norris Dragon Spring (2010) was significantly higher yielding than any other strain, and had extremely high yields in the control medium (Fig. B.15). Strains had similar high yields for all concentrations of aluminum (100, 200, 300mM) (Table 2.2, Fig. B.15). Strains had highest yields in regular medium, followed progressively by NaCl, 100, 200, and 300mM aluminum (yields at all treatments was significantly different) (Fig. B.15).

In the second experiment, the effects of strain, treatment, and the strain by treatment interaction were also significant. Strain 5585 (Nymph/2001) was the highest yielding strain and grew significantly better than the other strains in the control medium (Table 2.2, Fig. B.16). Strains showed generally similar tolerances to aluminum, and showed progressively more inhibition with higher concentrations of aluminum (Fig. B.16).

Mercury tolerance

In the first mercury experiment, the effects of strain, treatment, and strain by treatment interaction were significant. Strains 5602 (Rabbit/2001) and 5506 (Geyser/2001) were the highest yielding strains because they had very high yields in the

regular medium, and only 5614 (Sour/2001) had any yield at a mercury concentration above 2μM (Table 2.2, Fig. B.17). Strain 5610 (Sylvan/2001) was the only strain that did not show any yield at any mercury concentration. Strains had significantly higher yields in regular medium, followed by 2μM mercury, and worst in both 3.5 and 5μM mercury (Fig. B.17).

Strain, treatment and their interaction were again significant in the second experiment. Since the second experiment was conducted with lower mercury concentrations, most of the strains showed yields at many concentrations (Table 2.2, Fig. B.18). Strains 5506 (Geyser/2001) and 5585 (Nymph/2001) were both significantly higher yielding than the other strains (Table 2.2, Fig. B.18). Strain 5585 (Nymph/2001) had the highest yield at a mercury concentration of 3μM, and again 5610 (Sylvan/2001) was one of the lower performing strains that showed low yields at all mercury concentrations (Fig. B.18). Strains had highest yields in regular medium, followed by 1μM mercury, and worst at both 2 and 3μM mercury (Fig. B.18).

Arsenic speciation

Norris Dragon Spring- all sites showed much more arsenite than arsenate, with arsenate levels slowly increasing away from the source (0.12μM at source to 1.05μM at Site 4) (Table 2.3).

Lemonade Creek- arsenite levels were slightly higher than arsenate at both sites, but total arsenic was very low (1.27μM and 1.28μM at Sites 1 and 2, respectively) compared to Norris Dragon Spring (24.45μM or 0.0245 mM at source)(Table 2.4).

Location and properties of Dragon Spring and the Lemonade Creek site

Dragon Spring is at 44°43'54'' N and 110°42'39'' W. The pH was stable at about 3.0 to 3.1 (Skorupa 2012). The salinity, using conductance as a proxy, was about 2.08 Ms, which would correspond to about 2 g L^{-1} (Boyd 2007), very similar to the salinity of the Cyanidium medium. Jackson et al. (2001) have shown that arsenite is similarly high as in our results (Table 2.3). However, further downstream than we have sampled, their arsenite values decreased with greater distance and arsenate increased. Hg values in the water were 0.5-1.0 μM (Boyd 2007) lower, but similar to the concentrations used in the experiments. Al concentrations in this spring were measured at about 0.120 mM. Much higher concentrations were used in the experiments, since it was known that at least some "cyanidia" were known to have very high tolerances to aluminum (Nagasaka et al. 2002).

The site used for sampling on Lemonade Creek is 44°48'04'' N. and 110°43'44'' W. Skorupa (2012) measured values of pH 2 to 3. The conductance was about 2.5 Ms giving a probable salinity of somewhat over 2 Gl^{-1} (Ball et al. 2006). Total arsenic was about 3% of that found in Dragon Spring (Table 2.3 and 2.4), and in the study made by Skorupa (2012) arsenic and P were undetectable. Hg was about 2.5 μM in Lemonade Creek (Ball et al. 2006) and Al was about 0.4 mM (Skorupa 2012).

In the Norris Geyser Basin there is an acid soil site in which the salinity of the interstitial water was ~ 11 Ms and contained ~ 16 μM Hg and ~ 8 mM Al (Soil Analytical Lab., Plant and Soil Sci. Dept., Montana State U.). Strain 5506 and several other "cyanidia" were isolated from this "Extreme Site" (Toplin et al. 2008).

Table 2.1. Culture isolates used in the experiments, sources, temperatures, pH values, and dates of clonal isolation. Brief descriptions of the sources are in text. Types are described in Toplin et al. (2008).

CCMEE No.	Type	Source	Collection Temp	Collection pH	Isolation yr	Experiments
5506	IA	Norris Basin, YNP	40º	1.0	2001	As(III), Hg
5508	IA	Norris Dragon Spr, YNP	45º	1.9	2001	As(III), As(V), Al, Hg
5578	IA	Lemonade Crk, YNP	40º	2.2	2001	As(III), As(V), Al, Hg
5584	IA	Nymph Crk, YNP	42°	3	2001	As(III), Hg
5585	IA	Nymph Crk, YNP	42°	3	2001	As(III), Hg
5602	IA	Rabbit Crk Source, YNP	52°	<4	2001	Hg
5610	IB	Sylvan Crust, YNP	40º	4	2001	As(III), As(V), Al, Hg
5614	IA	Sour Crk, YNP	41°	1.9	2001	Hg
LC	IA	Lemonade Crk, YNP	48°	2.2	2010	As(III), As(V), Al
NC	IA	Nymph Crk, YNP	42°	2.8	2010	As(III), As(V), Al
NDS	IA	Norris Dragon Spr, YNP	40º	3.0	2010	As(III), As(V), Al
LC site 1	IA	Lemonade Crk, YNP	46°	2	2011	As(III)
NDS site 1	IA	Norris Dragon Spr, YNP	45°	3	2011	As(III)

Table 2.2. Results from all experiments with toxin concentration, percentages of yield, yield (expressed as corrected chlorophyll *a* absorbance of total biomass in 35 ml of culture). Standard error of the mean for each experiment is shown for yield.

Strain	Toxin	Concentration	Exp #	% Yield	Yield
5506	As (III)	0	1	100	0.97 ± 0.07
		0.4mM		30	0.29 ± 0.01
		0.6mM		17	0.16 ± 0.01
		1.0mM		18	0.18 ± 0.03
5508	As (III)	0	1	100	0.86 ± 0.03
		0.4mM		89	0.76 ± 0.10
		0.6mM		74	0.63 ± 0.01
		1.0mM		57	0.49 ± 0.09
5578	As (III)	0	1	100	0.84 ± 0.14
		0.4mM		22	0.19 ± 0.04
		0.6mM		18	0.15 ± 0.01
		1.0mM		14	0.02 ± 0.01
5584	As (III)	0	1	100	0.66 ± 0.02
		0.4mM		61	0.39 ± 0.02
		0.6mM		21	0.14 ± 0.03
		1.0mM		14	0.09 ± 0.00
5610	As (III)	0	1	100	0.66 ± 0.02
		0.4mM		29	0.19 ± 0.01
		0.6mM		10	0.07 ± 0.01
		1.0mM		2	0.01 ± 0.01
5508	As (III)	0	2	100	0.11 ± 0.00
		1.0mM NaCl		135	0.15 ± 0.03
		0.4mM		97	0.10 ± 0.01
		0.6mM		91	0.10 ± 0.00
		1.0mM		61	0.07 ± 0.00
5578	As (III)	0	2	100	0.29 ± 0.10
		1.0mM NaCl		164	0.47 ± 0.12
		0.4mM		57	0.17 ± 0.06
		0.6mM		65	0.19 ± 0.06
		1.0mM		3	0.01 ± 0.00
5610	As (III)	0	2	100	0.09 ± 0.00
		1.0mM NaCl		97	0.09 ± 0.00
		0.4mM		32	0.03 ± 0.02
		0.6mM		14	0.01 ± 0.01
		1.0mM		0	0
NDS site 1	As (III)	0	3	100	0.59 ± 0.06
		0.4mM		23	0.14 ± 0.03
		0.6mM		15	0.09 ± 0.00
		1.0mM		9	0.05 ± 0.01
LC site 1	As (III)	0	3	100	0.23 ± 0.03
		0.4mM		40	0.09 ± 0.00
		0.6mM		33	0.08 ± 0.00
		1.0mM		3	0.01 ± 0.00

Table 2.2 (continued)

5610	As (III)	0	3	100	0.08 ± 0.03
		0.4mM		7	0.01 ± 0.00
		0.6mM		0	0
		1.0mM		0	0
5506	As (V)	0	1	100	0.83 ± 0.38
		20mM		53	0.44 ± 0.02
		30mM		17	0.14 ± 0.00
		40mM		0	0
5508	As (V)	0	1	100	0.25 ± 0.02
		20mM		347	0.86 ± 0.06
		30mM		88	0.22 ± 0.02
		40mM		0	0
5578	As (V)	0	1	100	1.00 ± 0.69
		20mM		14	0.14 ± 0.01
		30mM		7	0.07 ± 0.03
		40mM		1	0.01 ± 0.01
5584	As (V)	0	1	100	0.55 ± 0.01
		20mM		30	0.16 ± 0.00
		30mM		17	0.10 ± 0.00
		40mM		0	0
5602	As (V)	0	1	100	1.80 ± 0.30
		20mM		13	0.23 ± 0.01
		30mM		9	0.16 ± 0.04
		40mM		4	0.07 ± 0.03
5610	As (V)	0	1	100	0.49 ± 0.02
		20mM		57	0.28 ± 0.01
		30mM		0	0
		40mM		0	0
5614	As (V)	0	1	100	0.98 ± 0.08
		20mM		30	0.29 ± 0.01
		30mM		12	0.11 ± 0.06
		40mM		0	0
5508	As (V)	0	2	100	0.42 ± 0.12
		40mM NaCl		86	0.36 ± 0.02
		20mM		3	0.01 ± 0.00
		30mM		0	0
		40mM		0	0
NDS	As (V)	0	2	100	1.02 ± 0.16
		40mM NaCl		65	0.66 ± 0.17
		20mM		29	0.30 ± 0.04
		30mM		4	0.04 ± 0.02
		40mM		1	0.01 ± 0.00
5578	As (V)	0	2	100	0.55 ± 0.02
		40mM NaCl		81	0.44 ± 0.04
		20mM		0	0
		30mM		0	0
		40mM		0	0

Table 2.2 (continued)

LC	As (V)	0	2	100	0.32 ± 0.06
		40mM NaCl		177	0.57 ± 0.03
		20mM		31	0.10 ± 0.03
		30mM		5	0.01 ± 0.01
		40mM		0	0
NC	As (V)	0	2	100	0.16 ± 0.01
		40mM NaCl		120	0.20 ± 0.00
		20mM		18	0.03 ± 0.01
		30mM		0	0
		40mM		0	0
5610	As (V)	0	2	100	0.25 ± 0.01
		40mM NaCl		98	0.24 ± 0.00
		20mM		0	0
		30mM		0	0
		40mM		0	0
5508	Al	0	1	100	0.17 ± 0.01
		300mM NaCl		47	0.08 ± 0.00
		100mM		49	0.08 ± 0.00
		200mM		23	0.04 ± 0.01
		300mM		11	0.02 ± 0.01
NDS	Al	0	1	100	0.73 ± 0.02
		300mM NaCl		50	0.36 ± 0.05
		100mM		34	0.25 ± 0.06
		200mM		11	0.08 ± 0.01
		300mM		4	0.03 ± 0.01
5578	Al	0	1	100	0.20 ± 0.04
		300mM NaCl		121	0.24 ± 0.03
		100mM		98	0.20 ± 0.01
		200mM		56	0.11 ± 0.01
		300mM		0	0
LC	Al	0	1	100	0.15 ± 0.01
		300mM NaCl		122	0.19 ± 0.01
		100mM		91	0.14 ± 0.01
		200mM		64	0.10 ± 0.01
		300mM		17	0.03 ± 0.01
NC	Al	0	1	100	0.13 ± 0.00
		300mM NaCl		82	0.11 ± 0.01
		100mM		55	0.07 ± 0.01
		200mM		37	0.05 ± 0.00
		300mM		0	0
5610	Al	0	1	100	0.16 ± 0.02
		300mM NaCl		46	0.07 ± 0.01
		100mM		68	0.11 ± 0.00
		200mM		35	0.05 ± 0.00
		300mM		0	0

Table 2.2 (continued)

5508	Al	0	2	100	0.50 ± 0.06
		300mM NaCl		39	0.20 ± 0.01
		100mM		28	0.14 ± 0.00
		200mM		20	0.10 ± 0.00
		300mM		4	0.02 ± 0.00
NDS	Al	0	2	100	0.25 ± 0.05
		300mM NaCl		60	0.15 ± 0.03
		100mM		33	0.08 ± 0.01
		200mM		21	0.05 ± 0.00
		300mM		3	0.01 ± 0.00
5578	Al	0	2	100	0.24 ± 0.02
		300mM NaCl		63	0.16 ± 0.02
		100mM		58	0.15 ± 0.01
		200mM		27	0.07 ± 0.00
		300mM		3	0.01 ± 0.00
5585	Al	0	2	100	1.36 ± 0.20
		300mM NaCl		12	0.17 ± 0.04
		100mM		6	0.09 ± 0.02
		200mM		4	0.06 ± 0.02
		300mM		1	0.02 ± 0.01
NC	Al	0	2	100	0.20 ± 0.01
		300mM NaCl		89	0.17 ± 0.01
		100mM		55	0.11 ± 0.01
		200mM		32	0.06 ± 0.00
		300mM		11	0.02 ± 0.02
5610	Al	0	2	100	0.35 ± 0.06
		300mM NaCl		53	0.18 ± 0.02
		100mM		47	0.16 ± 0.00
		200mM		26	0.09 ± 0.00
		300mM		17	0.06 ± 0.05
5506	Hg	0	1	100	1.94 ± 0.19
		2μM		29	0.57 ± 0.20
		3.5μM		0	0
		5μM		0	0
5508	Hg	0	1	100	1.10 ± 0.38
		2μM		73	0.81 ± 0.11
		3.5μM		0	0
		5μM		0	0
5578	Hg	0	1	100	1.03 ± 0.24
		2μM		25	0.26 ± 0.00
		3.5μM		0	0
		5μM		0	0
5584	Hg	0	1	100	0.50 ± 0.01
		2μM		37	0.18 ± 0.02
		3.5μM		0	0
		5μM		0	0

Table 2.2 (continued)

5602	Hg	0	1	100	2.15 ± 0.22
		2μM		61	1.31 ± 0.05
		3.5μM		0	0
		5μM		0	0
5610	Hg	0	1	100	0.22 ± 0.04
		2μM		0	0
		3.5μM		0	0
		5μM		0	0
5614	Hg	0	1	100	0.84 ± 0.02
		2μM		69	0.58 ± 0.12
		3.5μM		29	0.24 ± 0.01
		5μM		0	0
5506	Hg	0	2	100	1.25 ± 0.22
		1μM		87	1.08 ± 0.11
		2μM		29	0.37 ± 0.13
		3μM		0	0
5508	Hg	0	2	100	0.29 ± 0.03
		1μM		137	0.40 ± 0.05
		2μM		62	0.18 ± 0.06
		3μM		44	0.13 ± 0.08
NDS	Hg	0	2	100	0.18 ± 0.02
		1μM		98	0.17 ± 0.01
		2μM		49	0.09 ± 0.00
		3μM		7	0.01 ± 0.01
5578	Hg	0	2	100	0.21 ± 0.02
		1μM		18	0.04 ± 0.01
		2μM		12	0.02 ± 0.00
		3μM		8	0.02 ± 0.00
5585	Hg	0	2	100	0.94 ± 0.05
		1μM		77	0.73 ± 0.27
		2μM		55	0.52 ± 0.11
		3μM		36	0.34 ± 0.19
5610	Hg	0	2	100	0.28 ± 0.02
		1μM		9	0.02 ± 0.01
		2μM		4	0.01 ± 0.00
		3μM		0	0
5614	Hg	0	2	100	0.71 ± 0.17
		1μM		39	0.28 ± 0.07
		2μM		10	0.07 ± 0.01
		3μM		0	0

Table 2.3. Results of arsenic speciation for Norris Dragon Spring. Site 1 is 0.2m from the source at 45°C; Site 2 is 0.8m at 37°C; Site 4 is 2.5m at 42°C; all sites at pH ~3.0.

	Total As (μM)	Arsenate (μM)	Arsenite (μM)
Source	24.45 ± 1.00	0.12 ± 0.05	24.33
Site 1	30.32 ± 0.48	0.74 ± 0.22	29.58
Site 2	28.38 ± 0.65	1.19 ± 0.03	27.19
Site 4	30.86 ± 0.45	1.05 ± 0.09	29.81

Table 2.4. Results of arsenic speciation for Lemonade Creek. The 2 sites are >20 and 22m from the main source (pH 2.0), 46° and 45°C, respectively.

Source	Total As (μM)	Arsenate (μM)	Arsenite (μM)
Site 1	1.27 ± 0.03	0.50 ± 0.02	0.77
Site 2	1.28 ± 0.05	0.48 ± 0.08	0.80

Discussion

Tolerance to arsenic

The strains from Norris Dragon Spring were generally more tolerant to arsenite than strains from Lemonade Creek. In the first experiment, 5508 (Dragon/2001) had a significantly higher yield and tolerated arsenite better than 5578 (Lemonade/2001) (Fig. B.10). In the second experiment, 5578 (Lemonade/2001) was higher yielding than 5508 (Dragon/2001), but did not grow as well as 5508 in the presense of the highest concentration of arsenite (1.0mM) (Fig. B.11). Experiment 3 showed that Norris Dragon Spring (2010) had a much higher yield than Lemonade Creek (2010) in the control medium, and a similar yield in the presence of arsenite (Fig. B.12). The two strains from Lemonade Creek were severely inhibited by 1.0mM arsenite, whereas the two strains from Norris Dragon Spring always showed some yield at this concentration (Appendix B). The higher tolerance shown by strains from Norris Dragon Spring is adaptive because arsenite concentrations are more than 30 times higher in Norris Dragon Spring than Lemonade Creek, as shown in the arsenic speciation experiment (Table 2.3). Similar arsenite concentrations and pH levels were found at various distances from the source of Dragon Spring with arsenate increasing greatly after about 4-5 meters, indicating probable biological oxidation of the arsenite (Jackson et al. 2001).

Arsenate tolerance was much higher than arsenite tolerance in all strains tested, as we used much higher concentrations in the two arsenate experiments. In the first experiment, strain 5602 (Rabbit/2001) was the only strain with a significant yield at the highest concentration of arsenate (40mM), and 5508 (Dragon/2001) had the highest yield at 20mM arsenate (Fig. B.13). In the second experiment, Dragon/2010 had the highest

yield and had the highest tolerance to arsenate (Fig. B.14). It had a considerably higher yield than any other strain in 20mM arsenate. It is notable that in both experiments, a strain from Norris Dragon Spring (2001 and 2010) had a significantly higher yield than other strains in the presence of 20mM arsenate, as this environment has high arsenic levels and this strain may be able to transform arsenite to arsenate as a detoxification strategy.

Norris Dragon Spring strain 5508 has been shown to have the ability to oxidize the highly toxic arsenite to less toxic arsenate (Lehr et al. 2007b, Qin et al. 2009). The detoxification strategy employed by this alga may use two different methods that are coupled: arsenite oxidation to the less toxic arsenate, which, however, would be taken up by the phosphate permeases (since phosphate is low in the milieu of Dragon Spring, and subsequently when the oxidized arsenate is taken up and re-reduced to arsenite and methylated in the cytosol (Qin et al. 2009)). The final product would presumably be TMA(III) (trimethyl arsine) a volatile gas that would leave the cell passively.
It has also been shown that the oxidation of As(III) to As(V) in Dragon Spring is due to biotic rather than abiotic processes (Langner et al. 2001). Thus, to be able to live in this spring, it is very important that strains tolerate high levels of arsenite, and also perhaps that they have the ability to oxidize arsenite to the less toxic form. The "cyanidia" appear to contribute significantly to arsenic cycling in this environment.

Tolerance to aluminum and mercury

"Cyanidia" have extremely high tolerances to aluminum, as shown in the two experiments in which aluminum concentrations were as high as 300Mm, which was also

shown by Nagasaka et al. (2004). In experiment 1, Dragon/2010 was the highest yielding strain, but only had significantly higher yields than the other strains in the control medium and 300mM NaCl (Fig. B.15). Strains all had similar tolerances to the aluminum concentrations, and had progressively lower yields with increasing concentrations. It is also noteworthy that 300 mM NaCl was inhibitory, so some of the inhibition at various aluminum chloride concentrations could have been due to inihibition caused by large chloride concentrations. Experiment 2 showed similar results to experiment 1. Strain 5585 (Nymph/2001) was the highest yielding strain, but only because it had an extrememly high yield in the control medium (Fig. B.16). All strains showed similar tolerances to aluminum, and again 300mM NaCl was inhibitory but the yield in this medium was not significantly different from the yield in 100mM aluminum chloride (Fig. B.16).

The high tolerance to aluminum may be due to an energy-dependent Al-efflux mechanism (Yoshimura et al. 2000) and an Al-sequestering mechanism mediated by iron-storage particles (Nagasaka et al. 2002). *Cyanidium caldarium* cells contain electron-dense bodies, which have high levels of Fe and P, and might be used in the detoxification of Al. Al has a high affinity for phosphate ions, which could facilitate its deposition and sequestration in the electron-dense bodies (Nagasaka et al. 2002).

Mercury tolerance was very low in most strains, but mercury was not completely inhibitory at concentrations $\geq 2\mu$M. In the first experiment, both 5602 (Rabbit/2001) and 5506 (Geyser/2001) were high yielding because they had high yields in the control medium (Fig. B.17). Strain 5614 (Sour/2001) was the most tolerant to mercury, and was the only strain that had any yield at a mercury concentration higher than 2 μM.

Tolerances were low across all strains to these higher concentrations and, notably, the naked 5610 (Sylvan/2001) did not show any yield at any mercury concentration (Fig. B.17). The second experiment used lower concentrations and the strains showed much higher yields in the presence of mercury (Fig. B.9, Fig. B.18). Both 5506 (Geyser/2001) and 5585 (Geyser/2001) had high yields in the control medium and at the three mercury concentrations (Fig. B.18). Strain 5585 (Geyser/2001) was the most tolerant of the highest mercury concentration (3 μM). Again, 5610 (Sylvan/2001) was one of the lower yielding strains and had very low tolerance to mercury (Fig. B.18). Differences in toxicity limits to mercury and other metals were shown to exist among different strains of the same species, probably due to the concentration of toxins in the environments from which the various strains were isolated (Albertano and Pinto, 1986). These differences between strains presumably reflect conditions of each strain's native environment.

Overall, tolerances to all compounds tested varied widely across the strains tested. Some of these differences appeared to be consistent when strains were run in multiple experiments with the same toxin. For example, Norris Dragon Spring strains generally showed higher tolerance to arsenite and arsenate than other strains, and the naked *Cyanidioschyzon* strain 5610 (Sylvan/2001) had low tolerance to every potential toxin that we tested. Significant strain effects also showed that strains have inherently different growth rates, regardless of what treatment is used. Thus, there seem to be significant genetic differences in tolerances even among strains from YNP that are shown to be 99% to 100% similar using 18S-rDNA and rbcL sequences.

CHAPTER IV

CONCLUSIONS

The two studies presented here show that cyanidia have a unique set of adaptations that allow them to thrive in thermo-acidic environments. They are able to tolerate pH levels of 5-6 and lower this pH in their surrounding medium to 2-3, a level that is optimal for growth. These algae are also able to tolerate many toxins that are found at high concentrations in their natural environments. We found significant differences between strains both within YNP and between other geographic locations in ability to lower their pH and tolerance to various compounds.

Chapter II showed that strains from YNP were better able to tolerate high pH and lower their surrounding pH than strains from the other geographic locations. Growth and lowering of the pH were correlated, showing that strains that were best at lowering the pH also had the highest yield. Therefore, tolerance to high pH and ability to lower the surrounding pH could be an important adaptation that allows cyanidia to live in harsh environments with few competitors. Chapter III also showed consistent variations between strains in their tolerance to the compounds tested. Strains from locations high and low in arsenite had tolerances that reflected their environmental conditions. Tolerances to other compounds differed between strains, with the naked *Cyanidioschyzon* showing much lower tolerance to the toxins compared to walled strains. The two studies showed that there are significant differences in ability to tolerate and lower a high pH, and tolerances to various environmental toxins vary by geographic location, even among

strains that have been characterized as 99%-100% similar by 18S-DNA and rbcL sequencing.

APPENDIX A

CHAPTER II SUPPLEMENTARY INFORMATION

Figure A.1. Sample growth experiment for two strains (CCMEE 5506 and 5508) with start at pH 5.5 in a 75 ml culture. Yield is chlorophyll *a* absorbance of 5 ml of uniformly mixed culture at each time point.

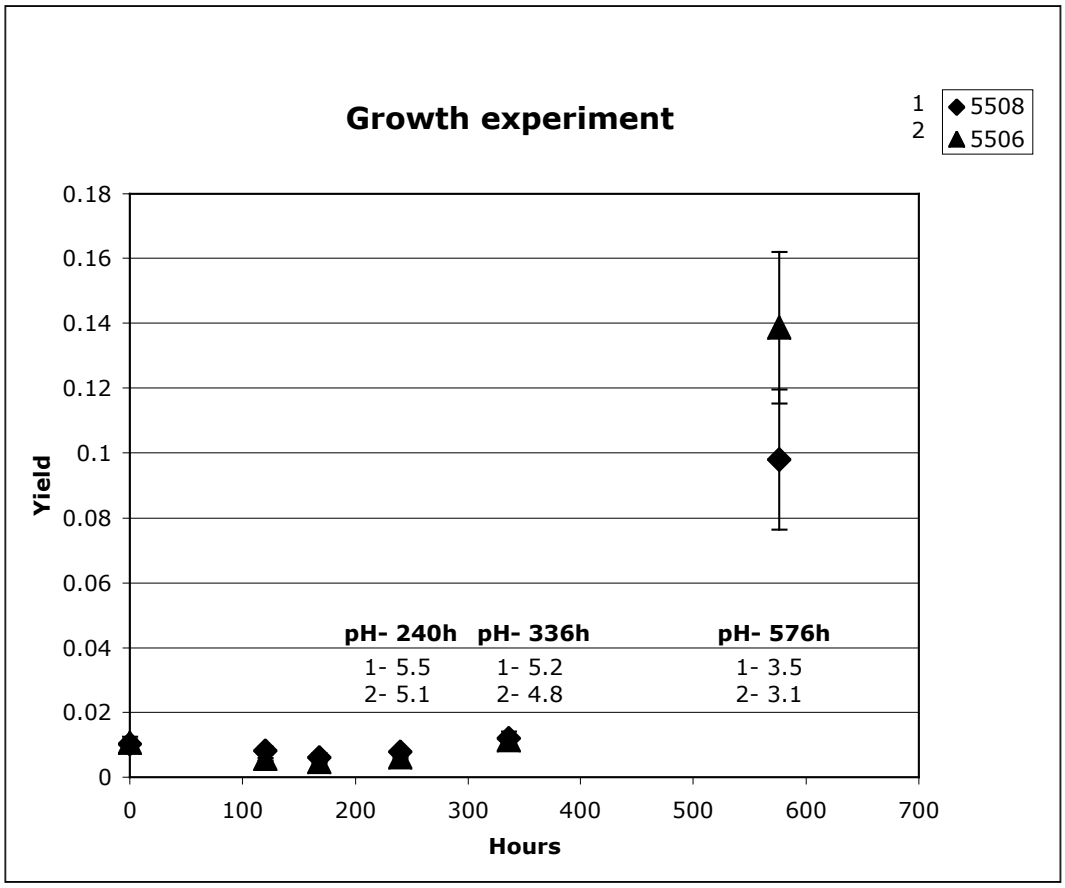


Figure A.2. pH experiment 1 indicating start and mean of final pH. Yield as chlorophyll *a* absorbance of entire 35 ml culture with S.E. for triple flasks.

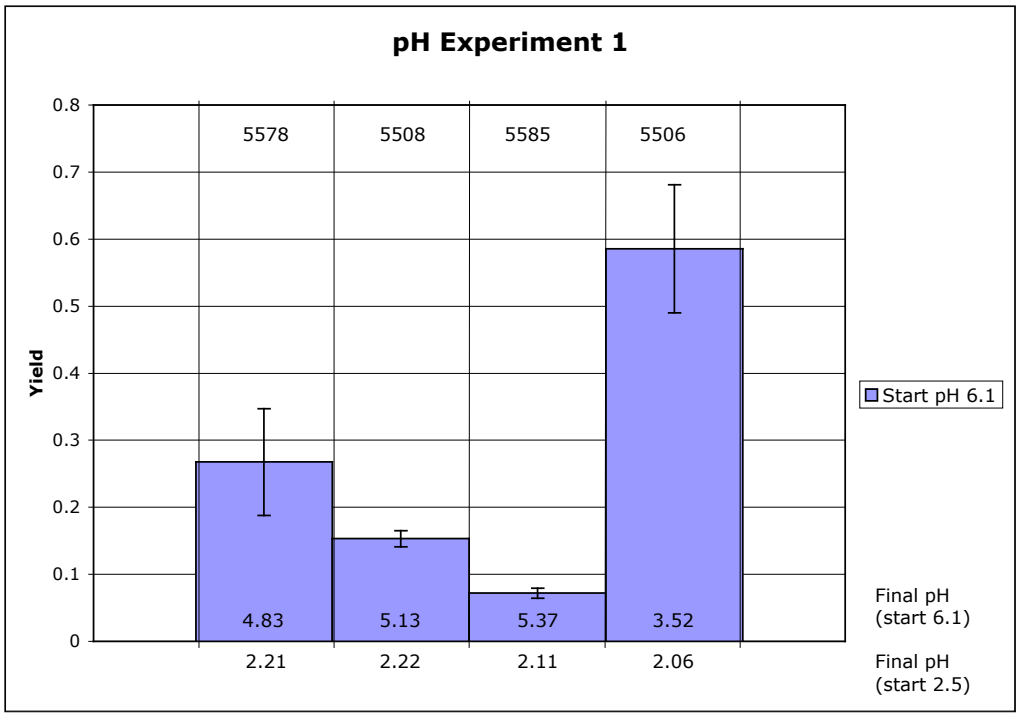


Figure A.3. pH experiment 2 indicating start and mean of final pH. Yield as chlorophyll *a* absorbance of entire 35 ml culture with S.E. for triple flasks.

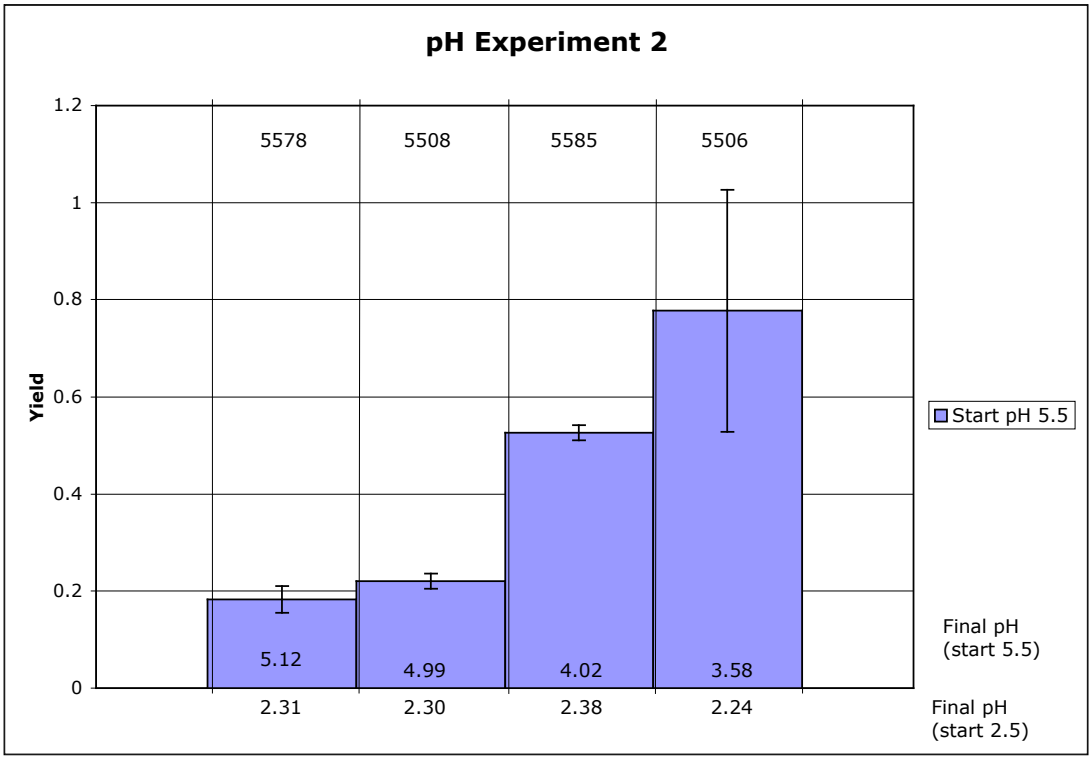


Figure A.4. pH experiment 3 indicating start and mean of final pH. Yield as chlorophyll *a* absorbance of entire 35 ml culture with S.E. for triple flasks.

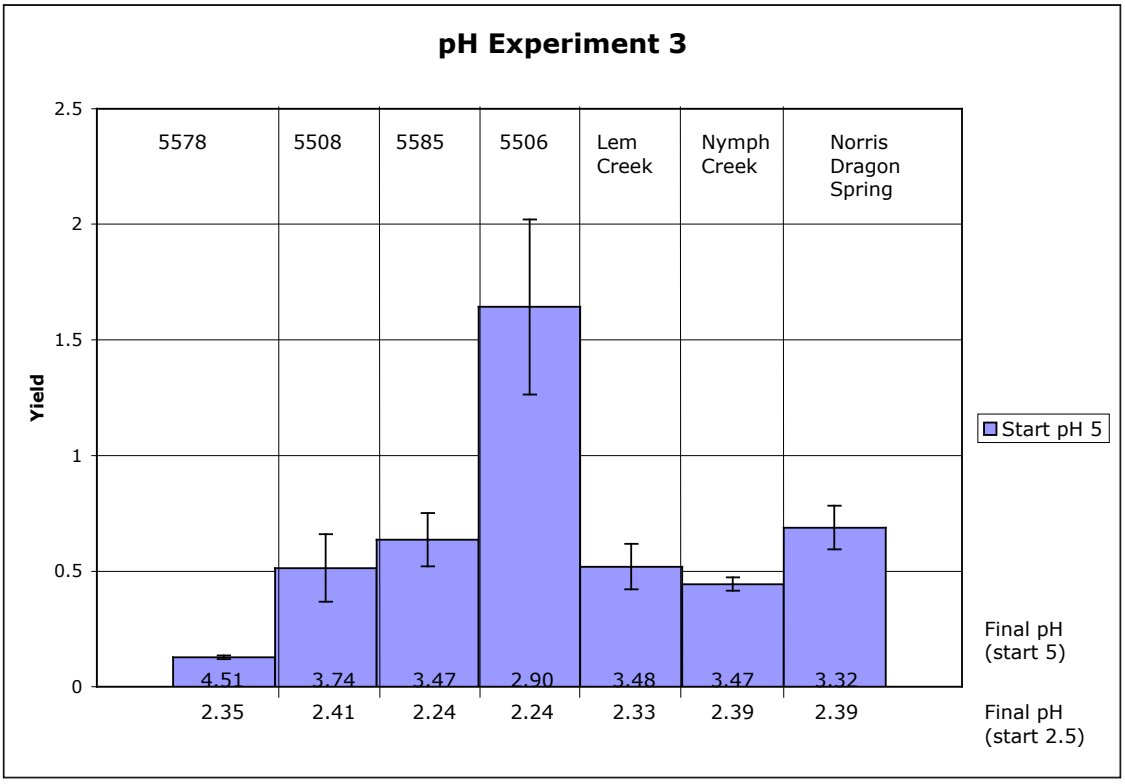


Figure A.5. pH experiment 4 indicating start and mean of final pH. Yield as chlorophyll *a* absorbance of entire 35 ml culture with S.E. for triple flasks.

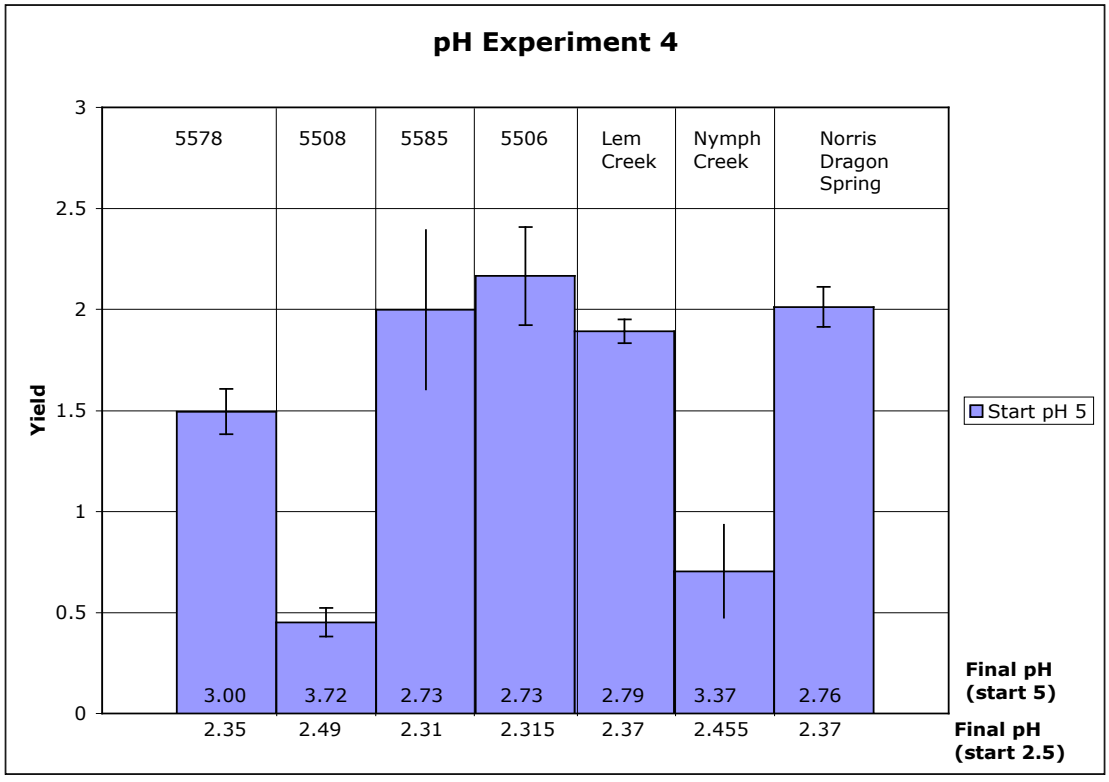


Figure A.6. pH experiment 5 indicating start and mean of final pH. Yield as chlorophyll *a* absorbance of entire 35 ml culture with S.E. for triple flasks.

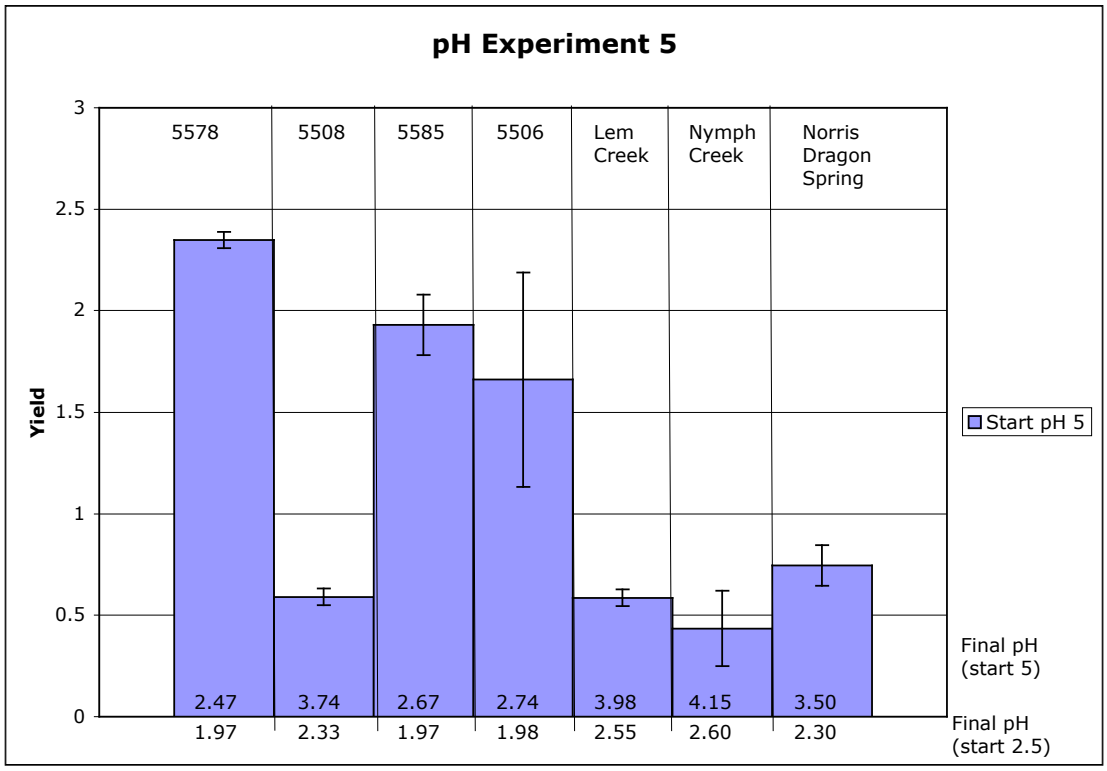


Figure A.7. pH experiment 6 indicating start and mean of final pH. Yield as chlorophyll *a* absorbance of entire 35 ml culture with S.E. for triple flasks.

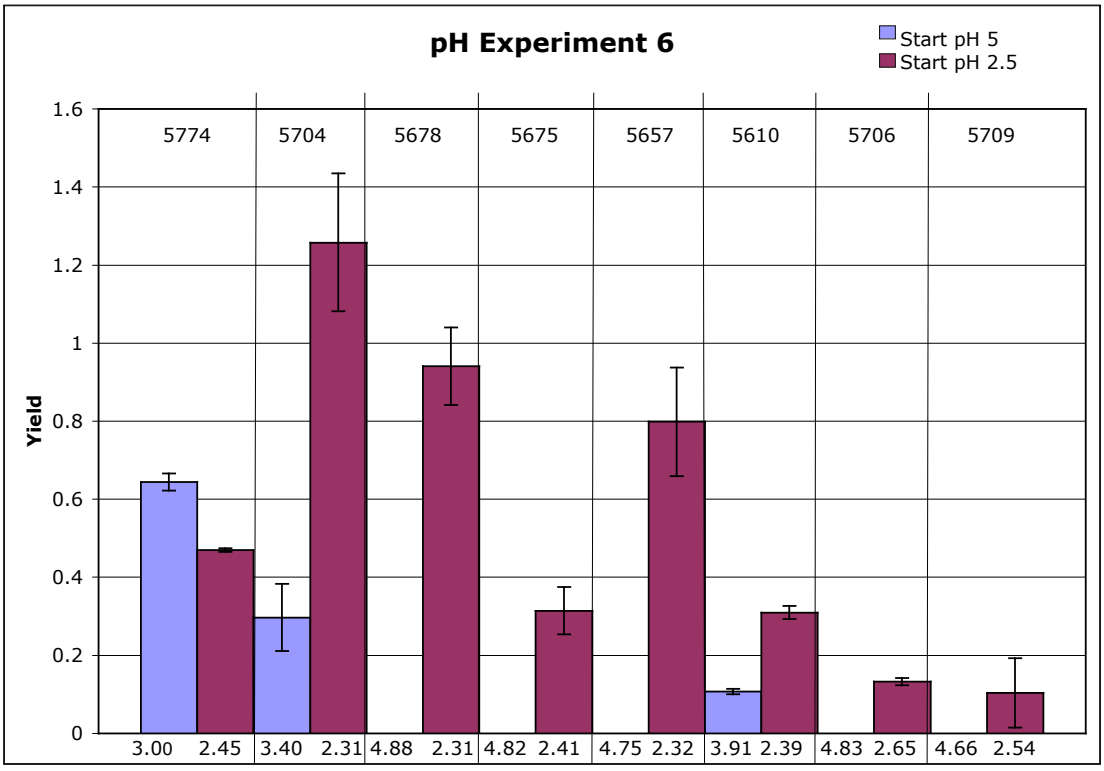
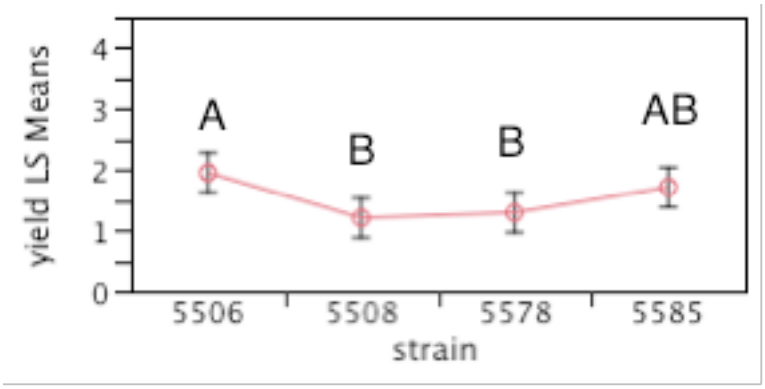


Figure A.8. pH experiment 1. A. Variation among strains in yield ($F_{3,7}$= 5.48, P = 0.013). B. Differences between pH treatments in yield ($F_{1,7}$ = 283.71, P < 0.0001). C. The treatment by strain interaction ($F_{3,7}$= 3.75, P = 0.041).



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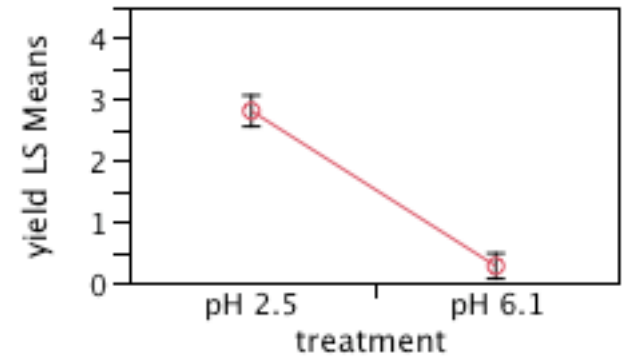
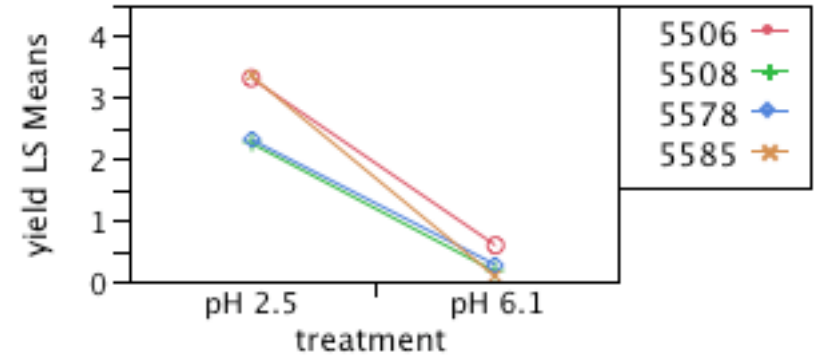
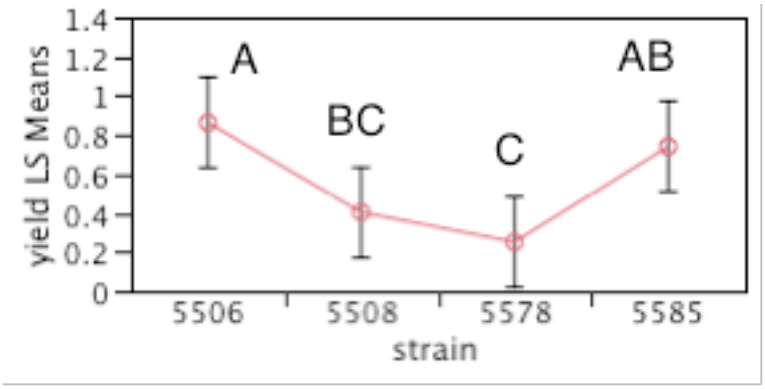


B.



C.

Figure A.9. pH experiment 2. A. Variation among strains in yield ($F_{3,7}$ = 7.21, P = 0.0050). B. Differences between pH treatments in yield ($F_{1,7}$= 6.66, P = 0.024). C. The treatment by strain interaction ($F_{3,7}$ = 0.45, P = 0.72).



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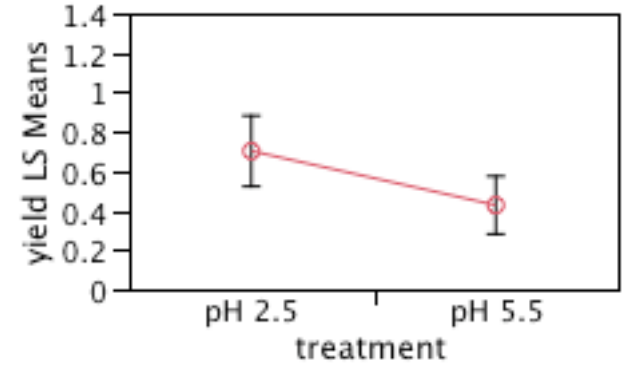

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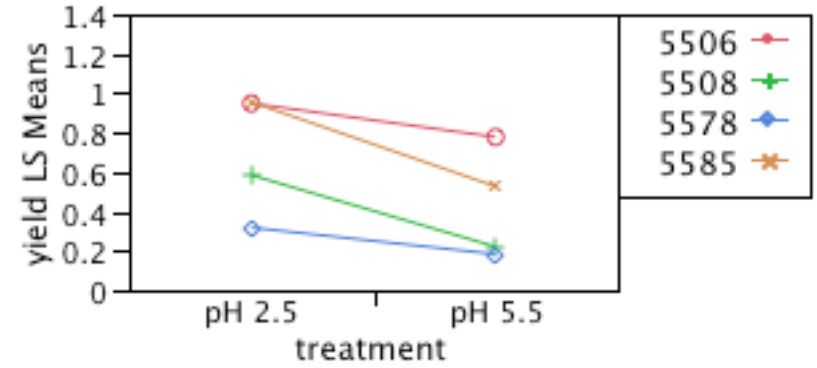
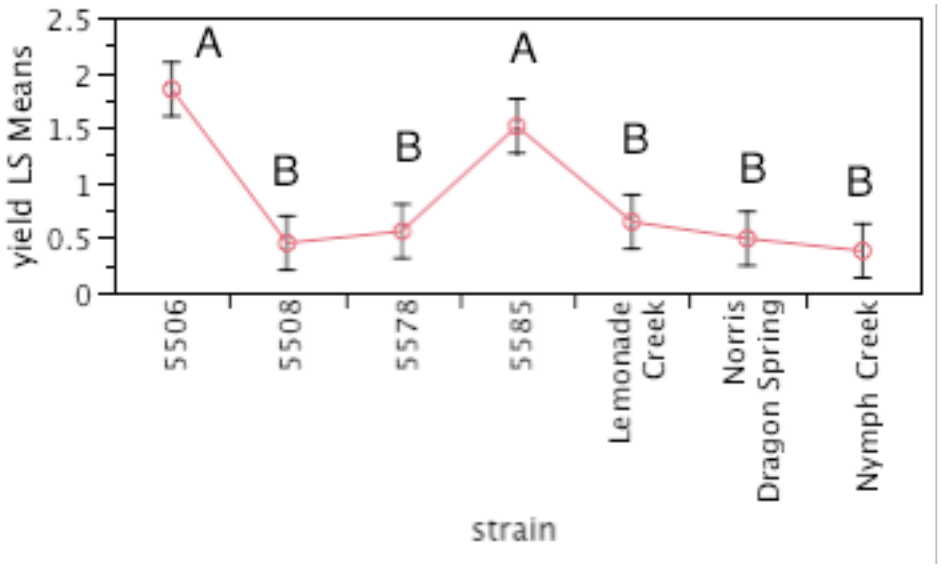


C.

Figure A.10. pH experiment 3. A. Variation among strains in yield ($F_{6,7}$= 24.83, P < 0.0001). B. Differences between pH treatments in yield ($F_{1,7}$ = 17.43, P = 0.0004). C. The treatment by strain interaction ($F_{6,7}$ = 9.75, P < 0.0001).



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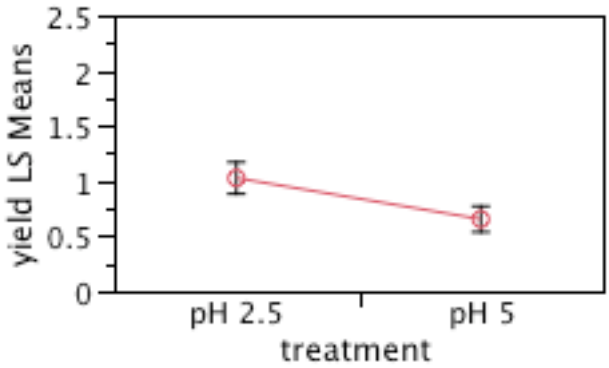

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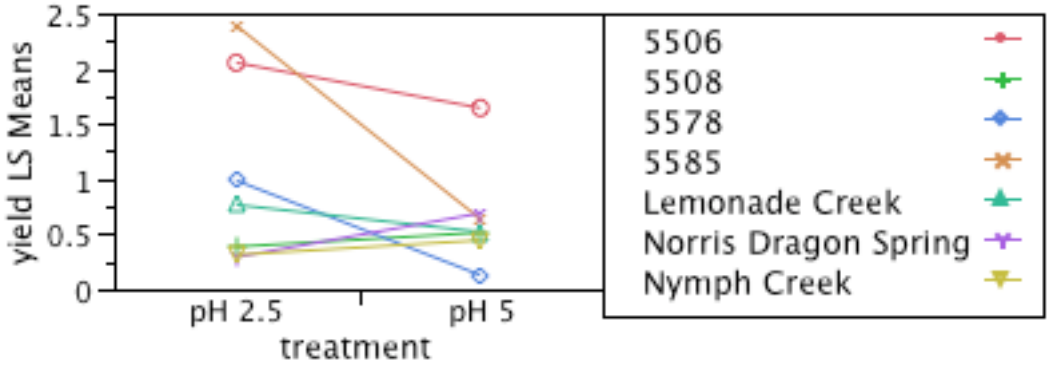


C.

Figure A.11. pH experiment 4. A. Variation among strains in yield ($F_{6,6}$ = 11.08, P = 0.0001).

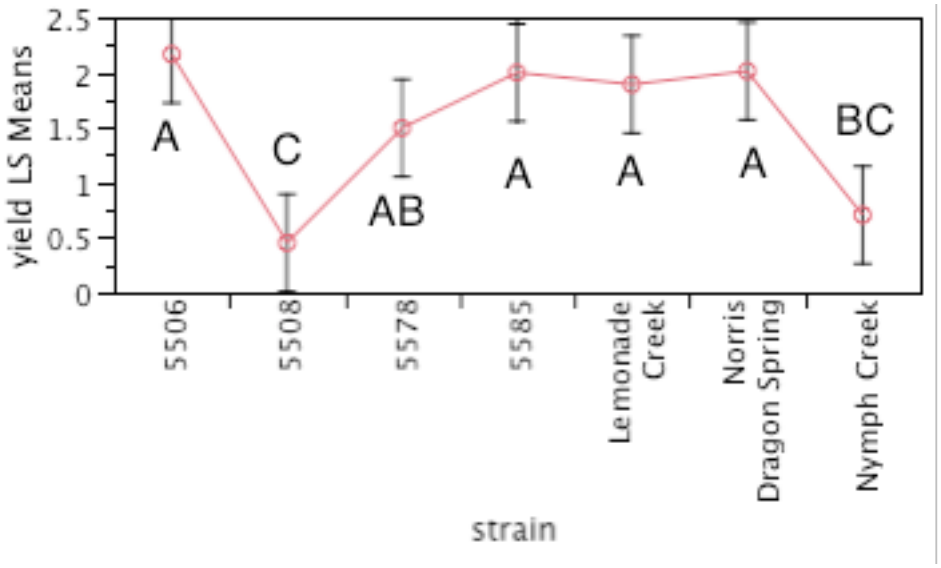
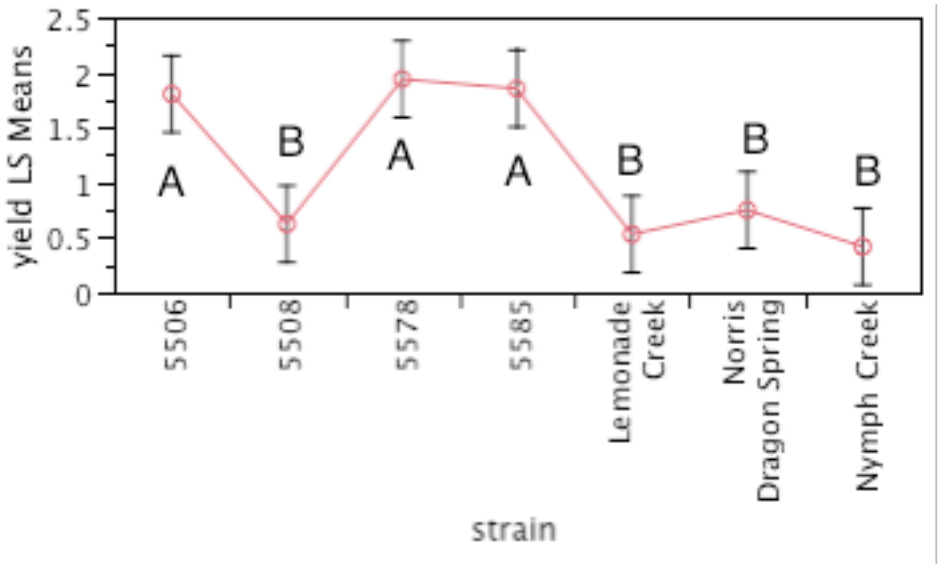


Figure A.12. pH experiment 5. A. Variation among strains in yield ($F_{6,13} = 17.22$, $P < 0.0001$). B. Differences between pH treatments in yield ($F_{1,7} = 0.73$, $P = 0.40$). C. The treatment by strain interaction ($F_{6,7} = 1.05$, $P = 0.42$).



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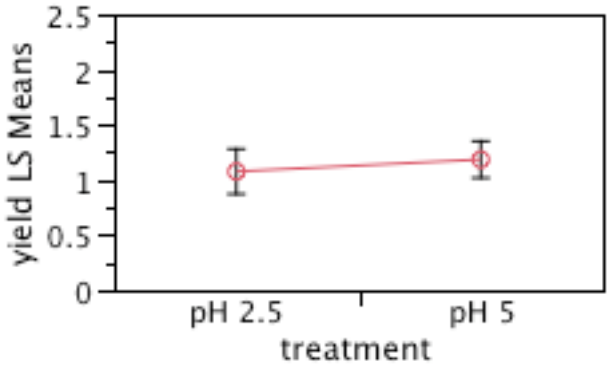

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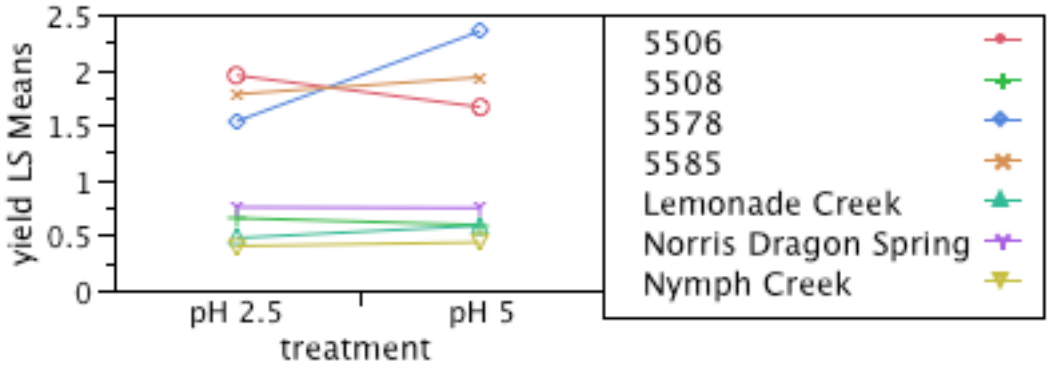
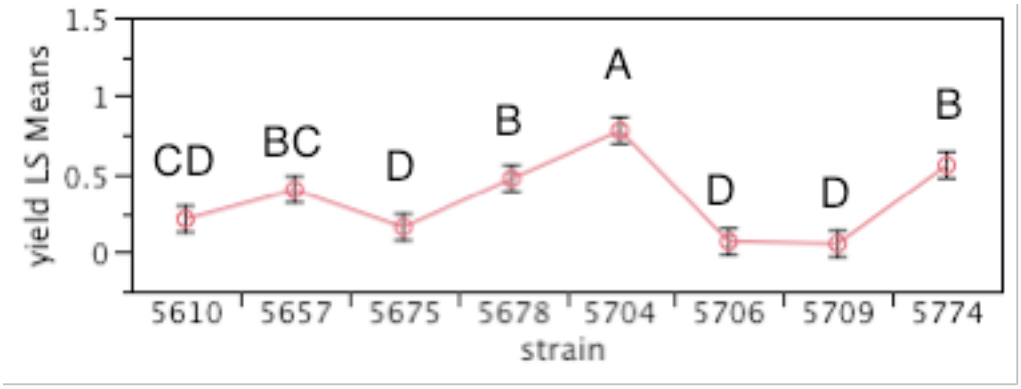


C.

Figure A.13. pH experiment 6. A. Variation among strains in yield ($F_{7,15} = 40.21$, $P < 0.0001$). B. Differences between pH treatments in yield ($F_{1,15} = 202.45$, $P < 0.0001$). C. The treatment by strain interaction ($F_{7,15} = 27.97$, $P < 0.0001$).



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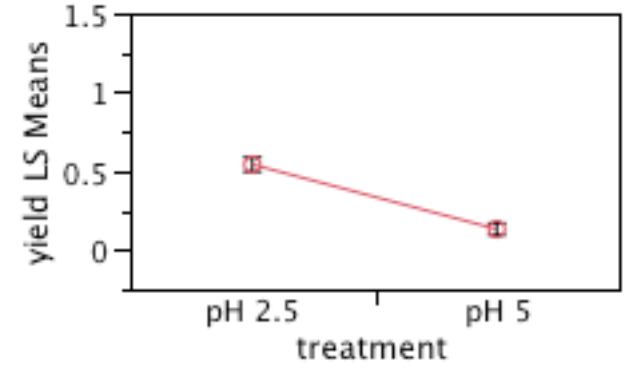

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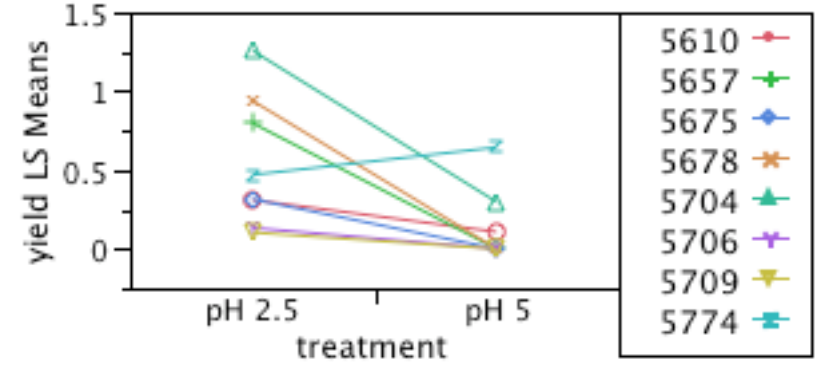


C.

APPENDIX B

CHAPTER III SUPPLEMENTARY INFORMATION

Figure B.1. Arsenite tolerance experiment 1. Experimental concentrations of 0.4, 0.6, and 1.0mM $NaAsO_2$. Yield as chlorophyll *a* absorbance of entire 35 ml culture with S.E. for triple flasks.

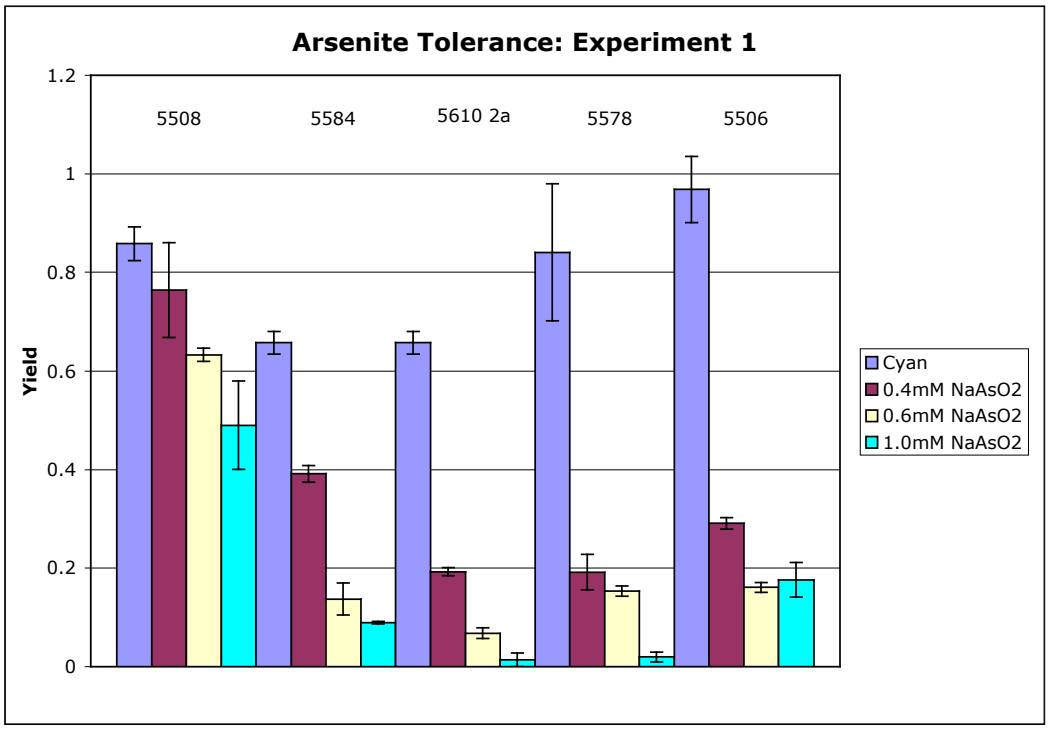


Figure B.2. Arsenite tolerance experiment 2. Experimental concentrations of 0.4, 0.6, and 1.0mM $NaAsO_2$ and 1.0mM NaCl. Yield as chlorophyll *a* absorbance of entire 35 ml culture with S.E. for triple flasks.

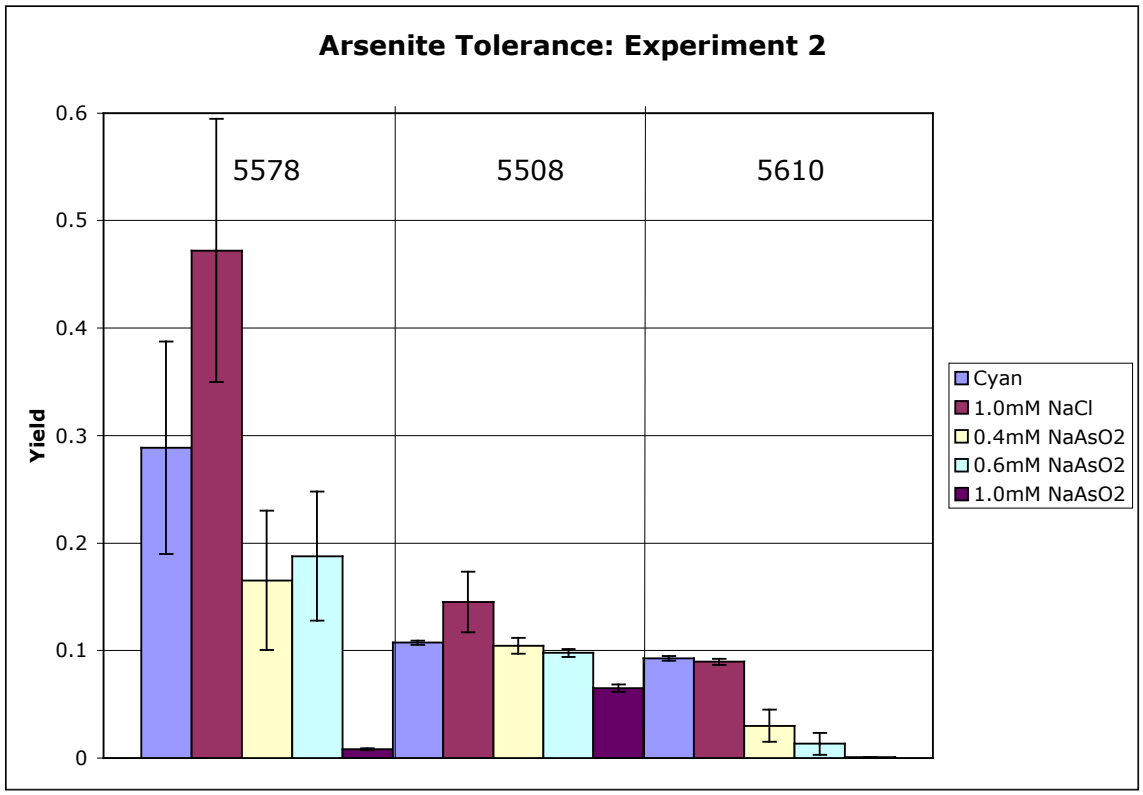


Figure B.3. Arsenite tolerance experiment 3. Experimental concentrations of 0.4, 0.6, and 1.0mM $NaAsO_2$. Yield as chlorophyll *a* absorbance of entire 35 ml culture with S.E. for triple flasks.

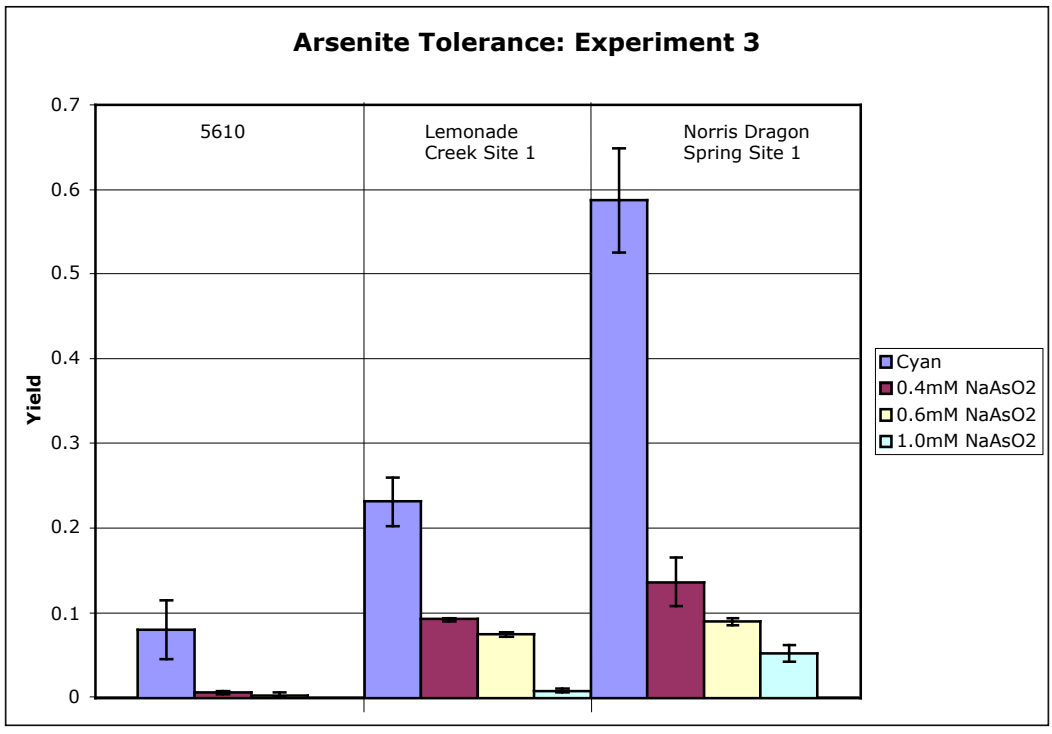


Figure B.4. Arsenate tolerance experiment 1. Experimental concentrations of 20, 30, and 40mM Na_3AsO_4. Yield as chlorophyll *a* absorbance of entire 35 ml culture with S.E. for triple flasks.

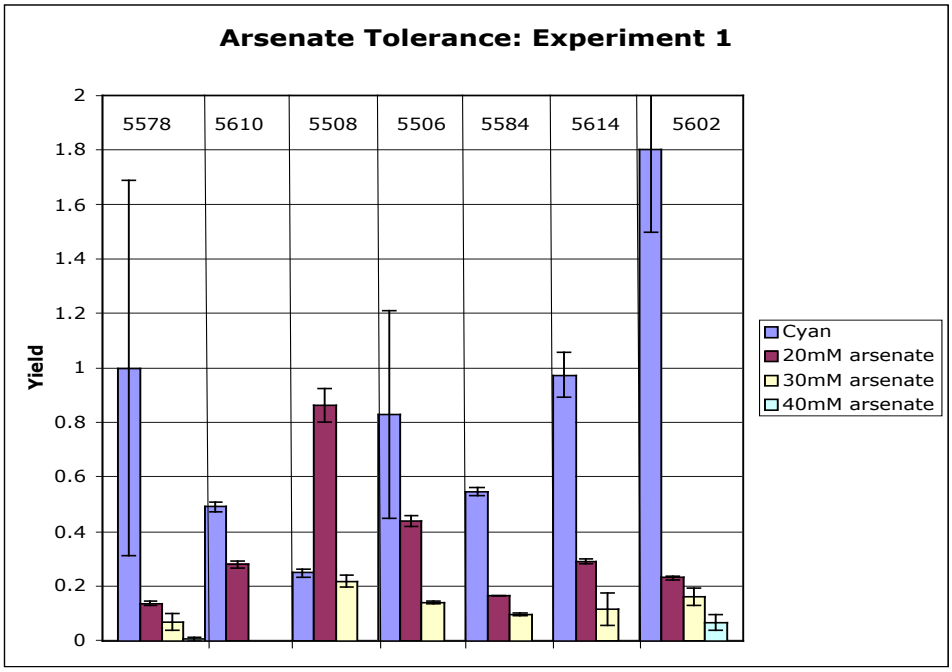


Figure B.5. Arsenate tolerance experiment 2. Experimental concentrations of 20, 30, and 40mM Na_3AsO_4 and 40mM NaCl. Yield as chlorophyll *a* absorbance of entire 35 ml culture with S.E. for triple flasks.

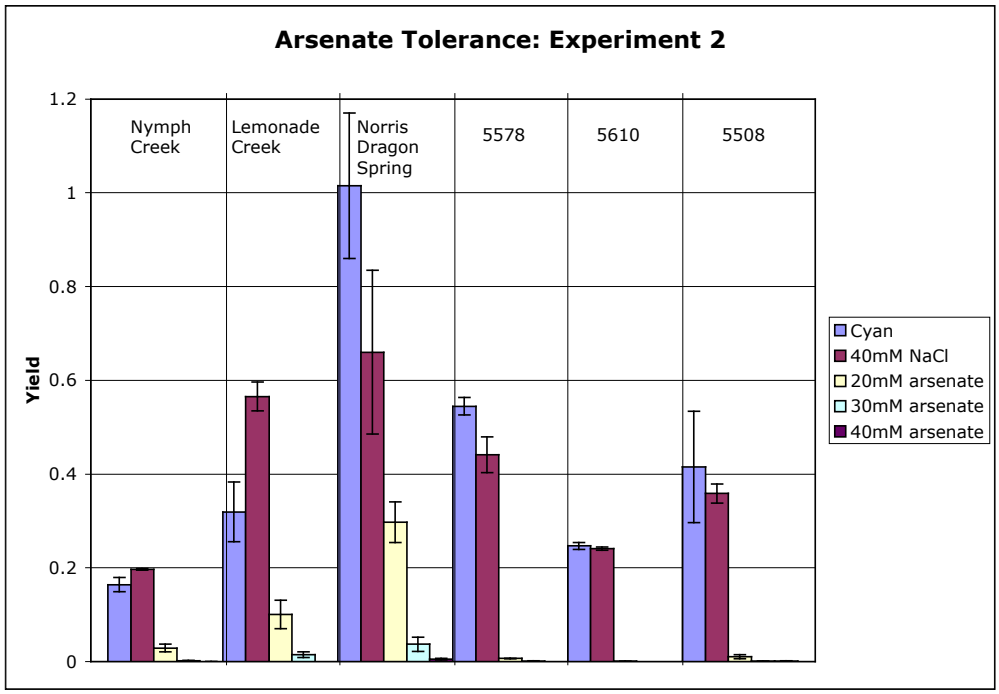


Figure B.6. Aluminum tolerance experiment 1. Experimental concentrations of 100, 200, and 300mM $AlCl_3$ and 300mM NaCl. Yield as chlorophyll *a* absorbance of entire 35 ml culture with S.E. for triple flasks.

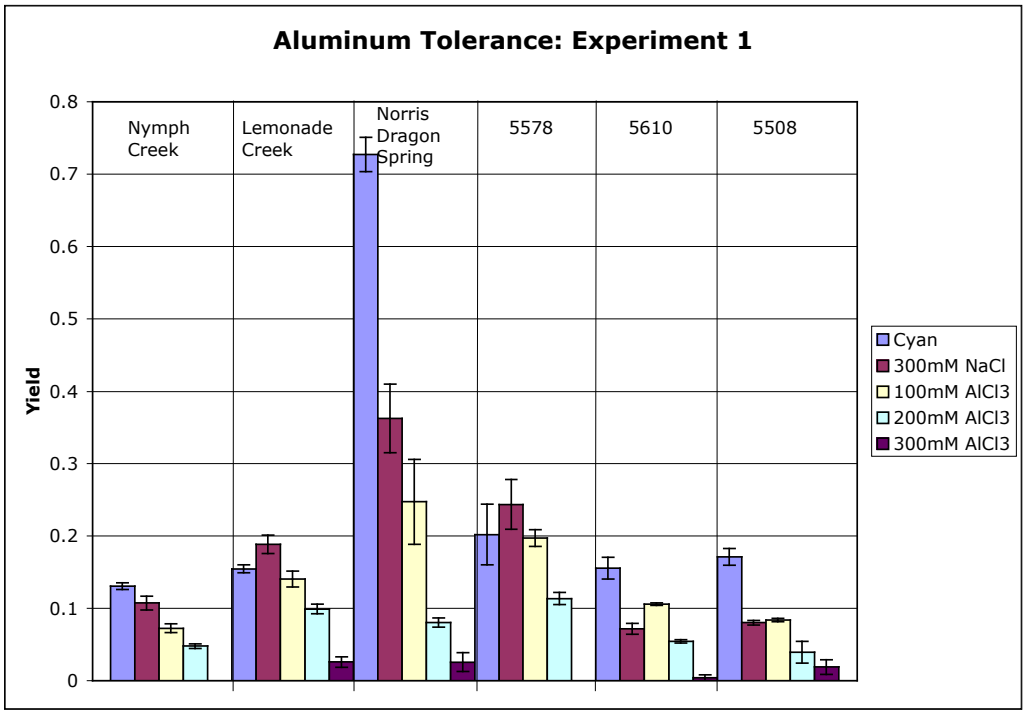


Figure B.7. Aluminum tolerance experiment 2. Experimental concentrations of 100, 200, and 300mM $AlCl_3$ and 300mM NaCl. Yield as chlorophyll *a* absorbance of entire 35 ml culture with S.E. for triple flasks.

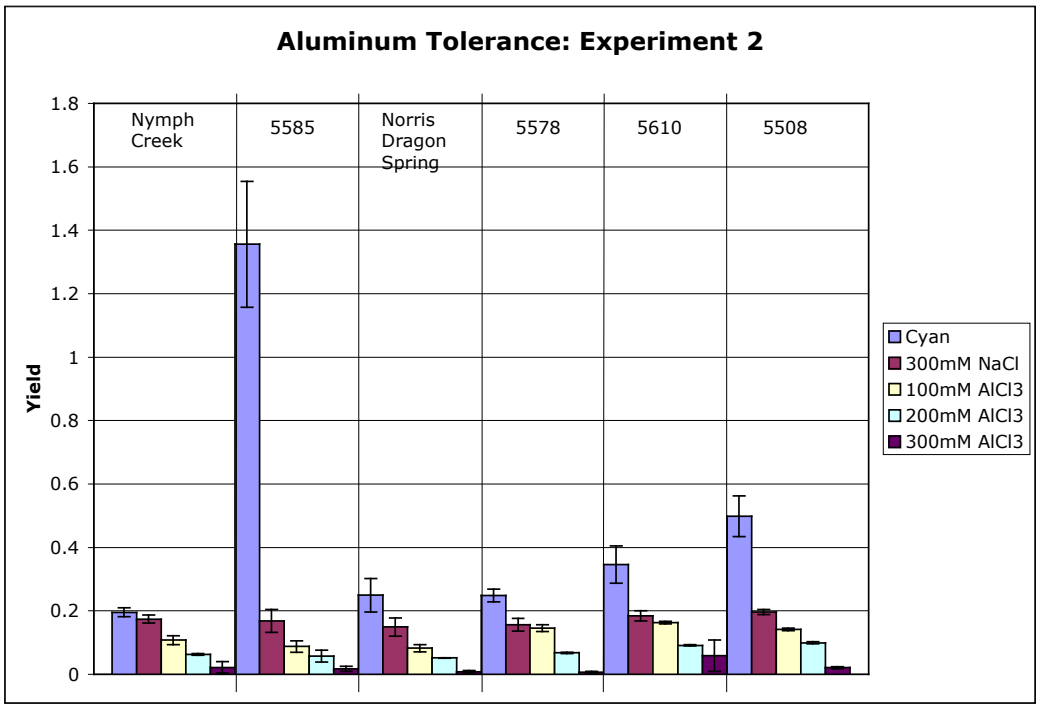


Figure B.8. Mercury tolerance experiment 1. Experimental concentrations of 2, 3.5, and 5μM $HgCl_2$. Yield as chlorophyll *a* absorbance of entire 35 ml culture with S.E. for triple flasks.

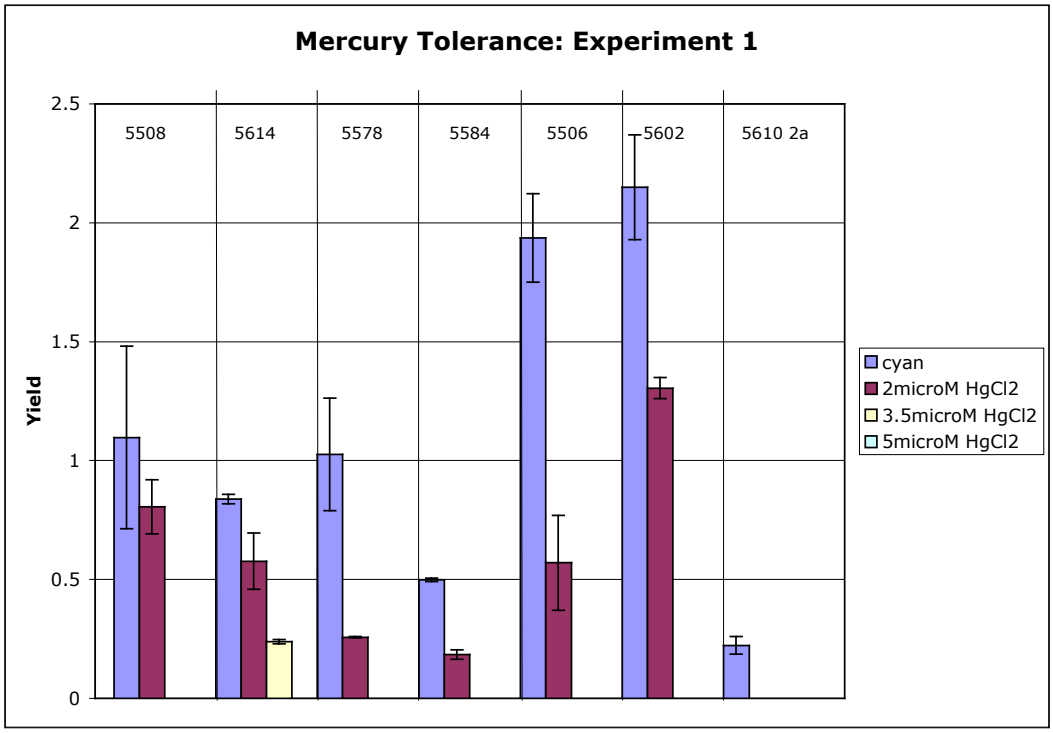


Figure B.9. Mercury tolerance experiment 2. Experimental concentrations of 1, 2, and 3μM $HgCl_2$. Yield as chlorophyll *a* absorbance of entire 35 ml culture with S.E. for triple flasks.

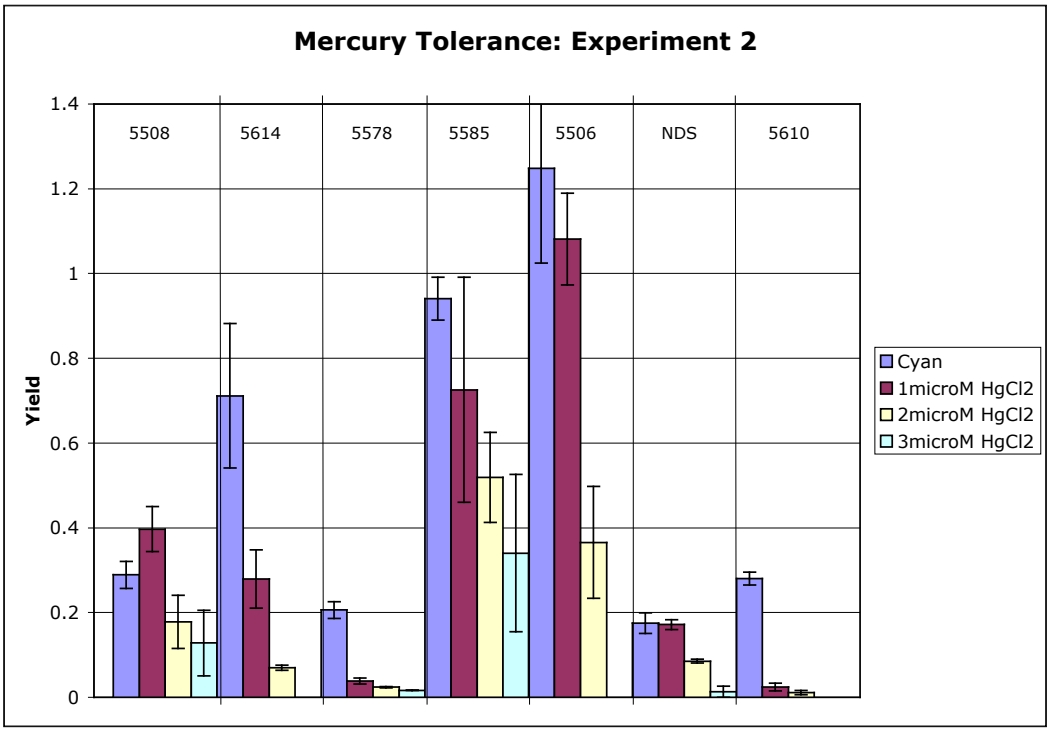
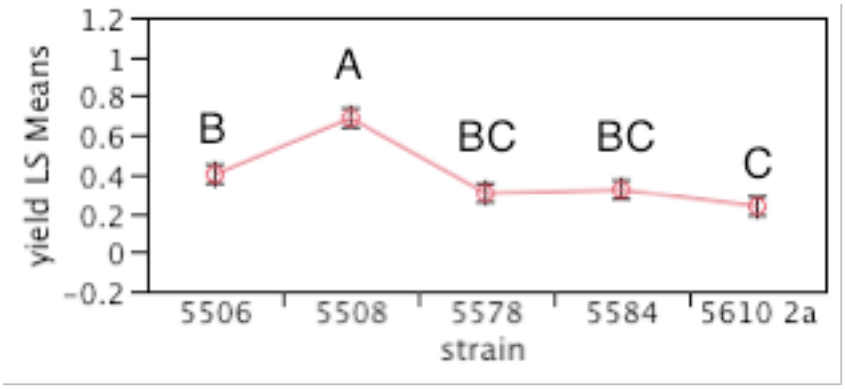


Figure B.10. Arsenite experiment 1. A. Variation among strains in yield ($F_{4,19}$ = 51.91, P < 0.0001). B. Differences between treatments in yield ($F_{3,19}$= 168.98, P < 0.0001). C. The treatment by strain interaction ($F_{12,19}$ = 5.53, P < 0.0001).



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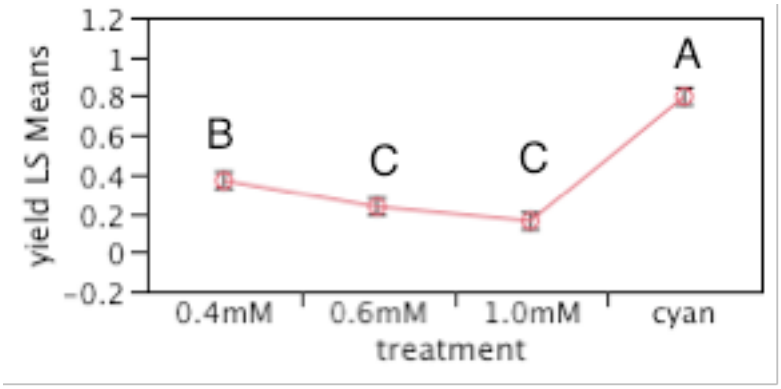

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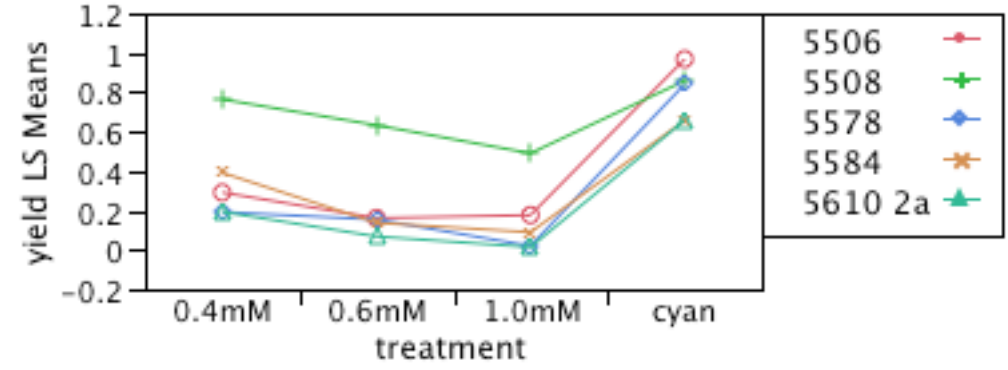
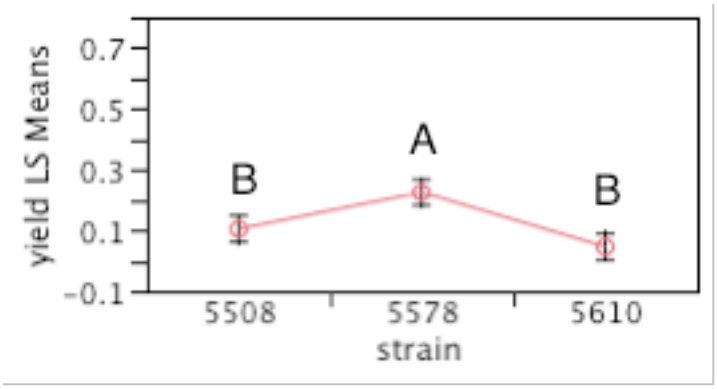


C.

Figure B.11. Arsenite experiment 2. A. Variation among strains in yield ($F_{2,14}$ = 18.59, P < 0.0001). B. Differences between treatments in yield ($F_{4,14}$ = 8.35, P = 0.0001). C. The treatment by strain interaction ($F_{8,14}$ = 2.94, P = 0.015).



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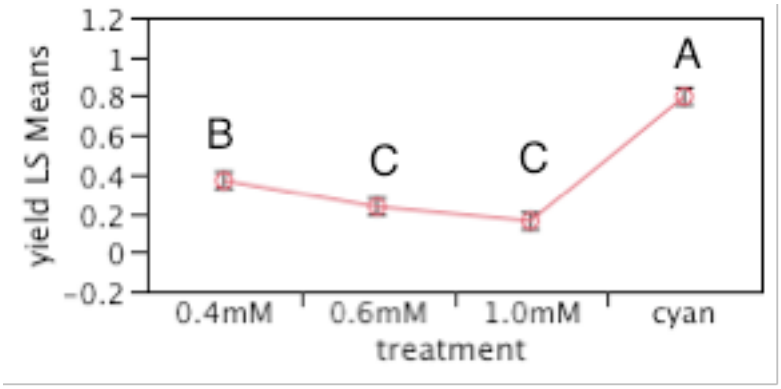

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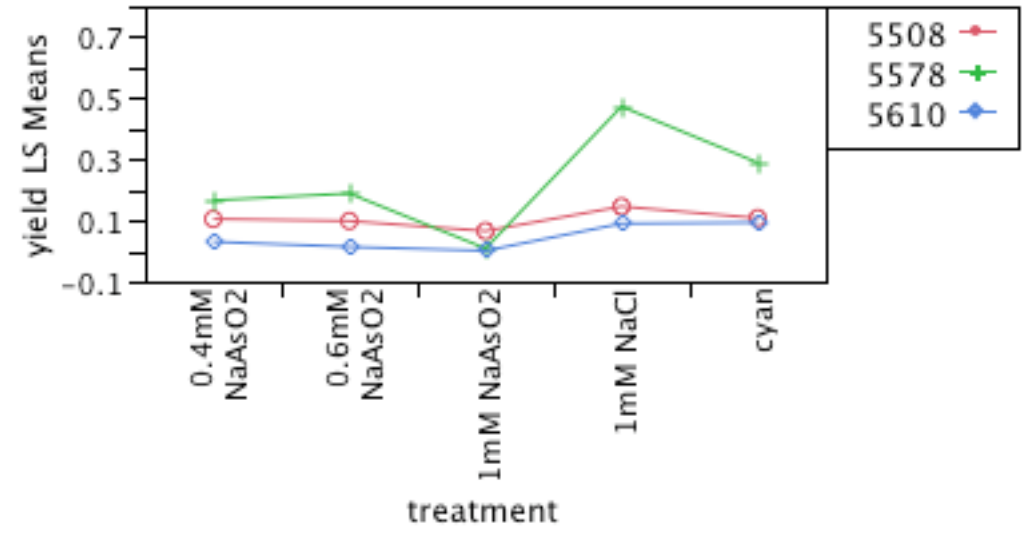
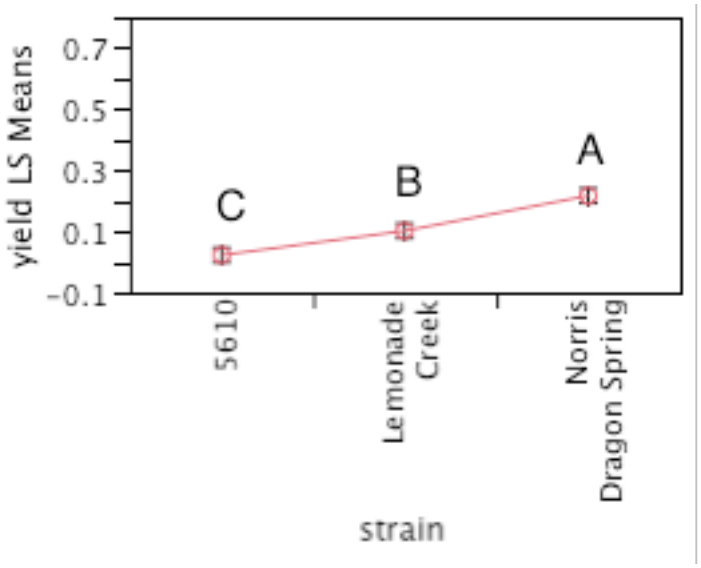


C.

Figure B.12. Arsenite experiment 3. A. Variation among strains in yield ($F_{2,11}$ = 67.63, P < 0.0001). B. Differences between treatments in yield ($F_{3,11}$ = 85.14, P < 0.0001). C. The treatment by strain interaction ($F_{6,11}$ = 21.95, P < 0.0001).



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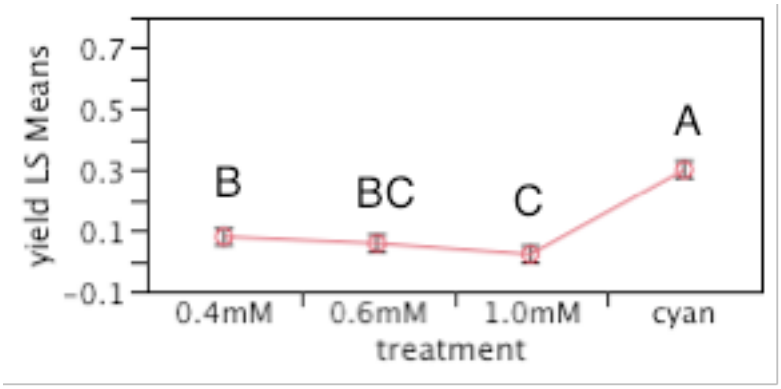

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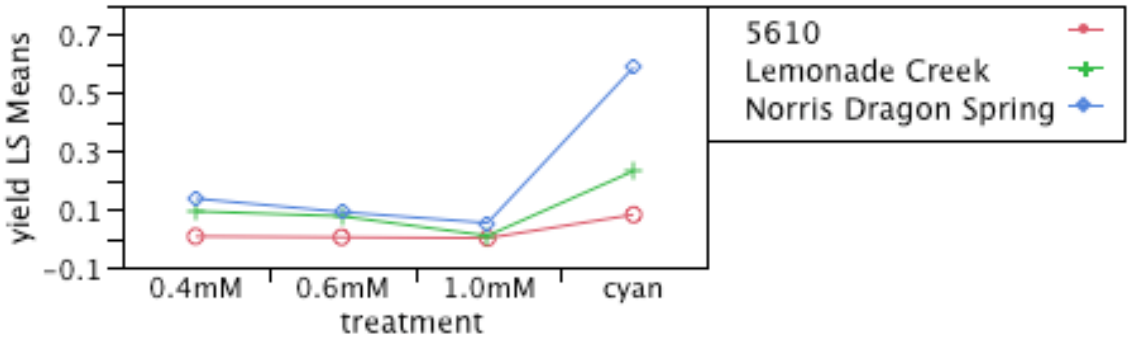
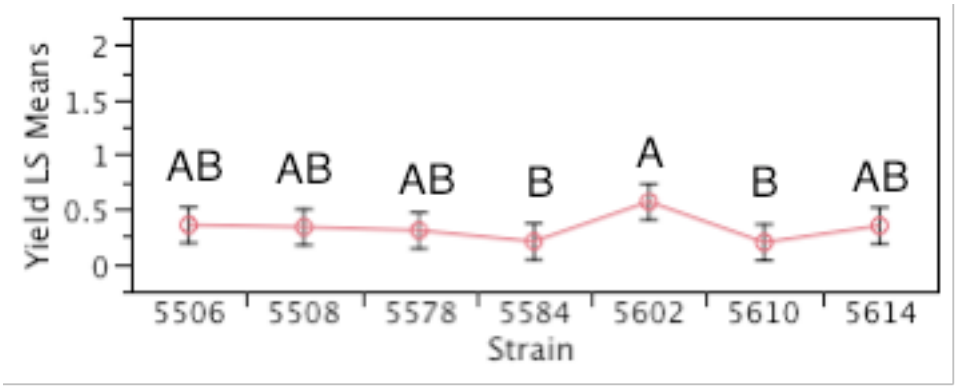


C.

Figure B.13. Arsenate experiment 1. A. Variation among strains in yield ($F_{6,27}$ = 2.37, P = 0.056). B. Differences between treatments in yield ($F_{3,27}$ = 37.07, P < 0.0001). C. The treatment by strain interaction ($F_{18,27}$ = 3.37, P = 0.0020).



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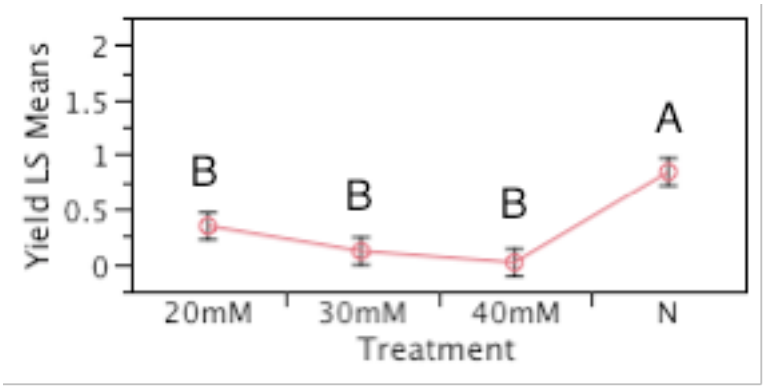

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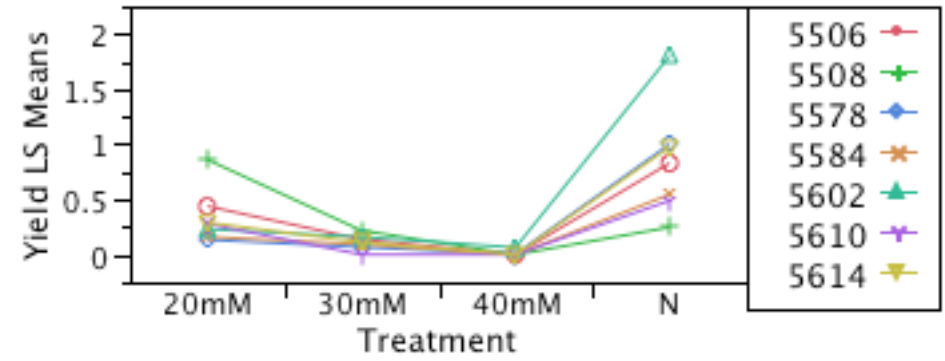
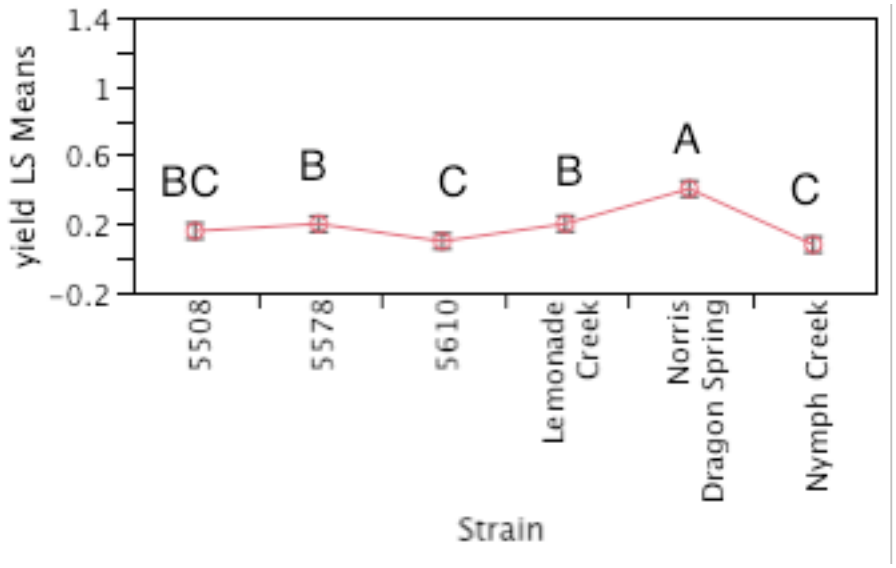


C.

Figure B.14. Arsenate experiment 2. A. Variation among strains in yield ($F_{5,29}$ = 25.56, P < 0.0001). B. Differences between treatments in yield ($F_{4,29}$ = 112.79, P < 0.0001). C. The treatment by strain interaction ($F_{20,29}$ = 6.88, P < 0.0001).



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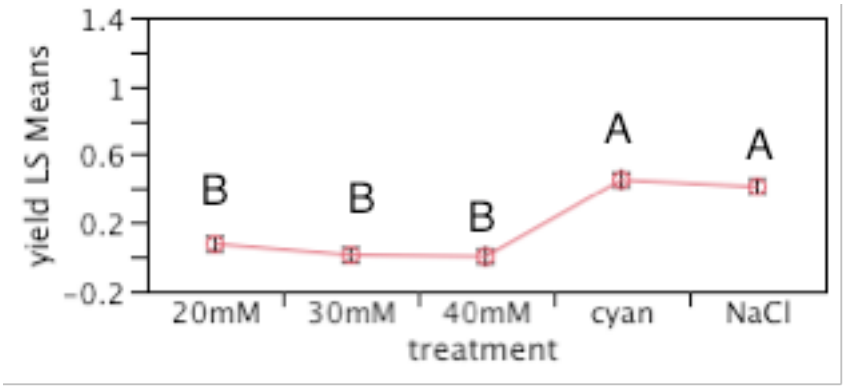

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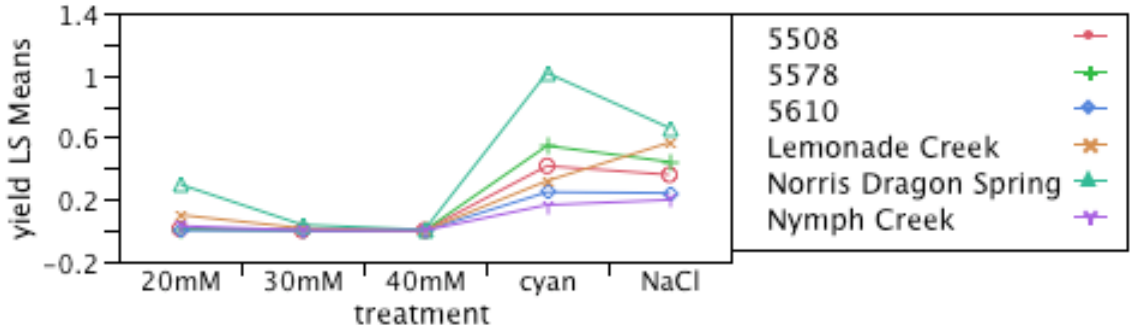
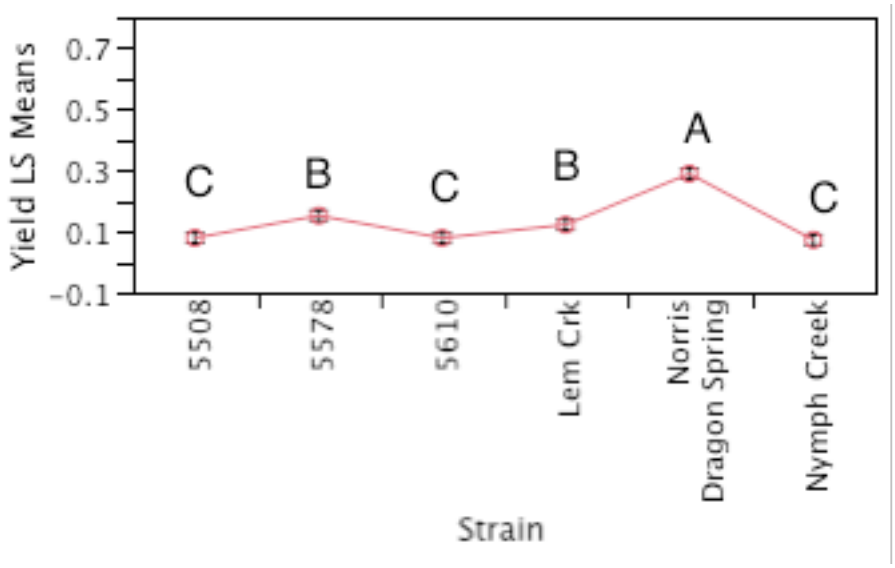


C.

Figure B.15. Aluminum experiment 1. A. Variation among strains in yield ($F_{5,29}$ = 94.34, P < 0.0001). B. Differences between treatments in yield ($F_{4,29}$ = 146.01, P < 0.0001). C. The treatment by strain interaction ($F_{20,29}$ = 26.01, P < 0.0001).



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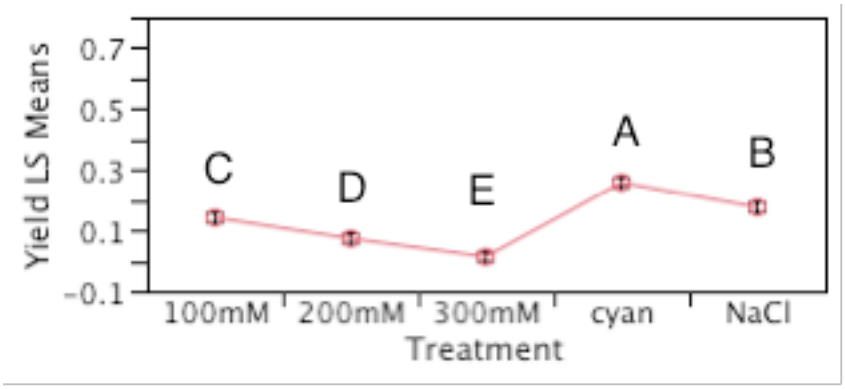

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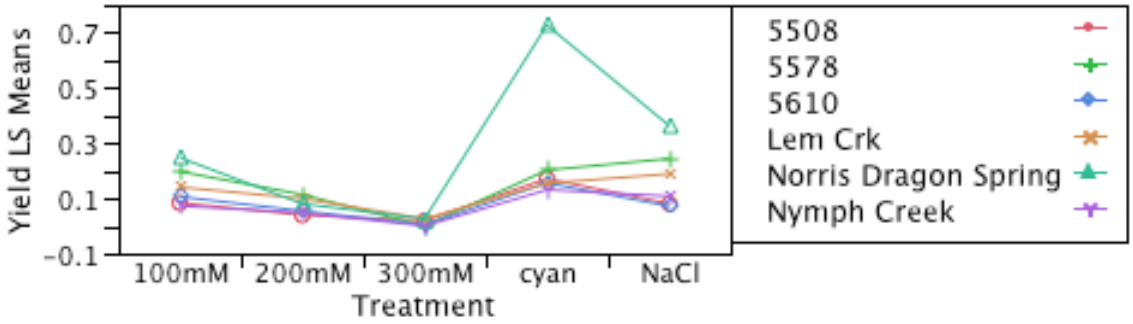
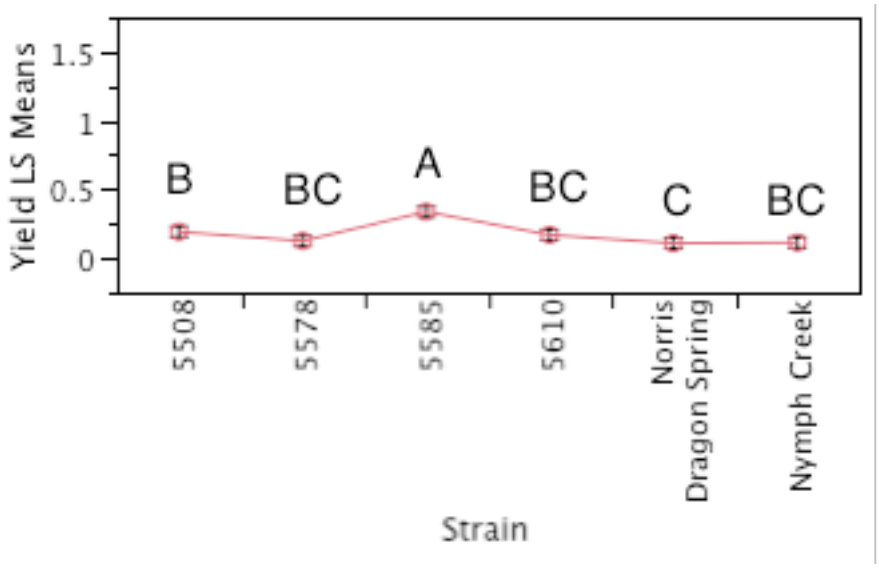


C.

Figure B.16. Aluminum experiment 2. A. Variation among strains in yield ($F_{5,29}$ = 19.69, P < 0.0001). B. Differences between treatments in yield ($F_{4,29}$ = 103.42, P < 0.0001). C. The treatment by strain interaction ($F_{20,29}$ = 20.87, P < 0.0001).



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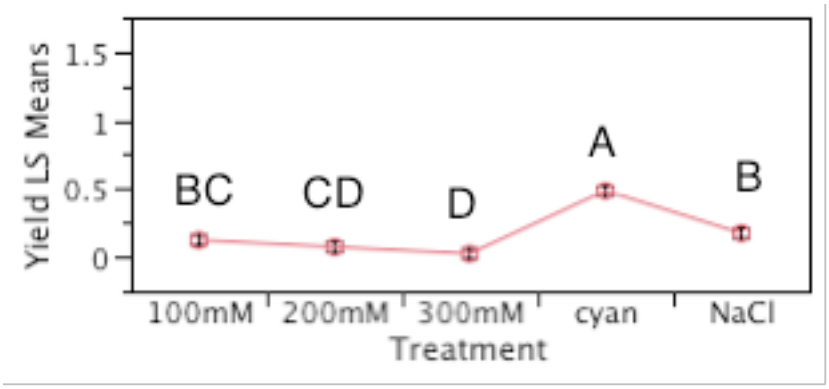

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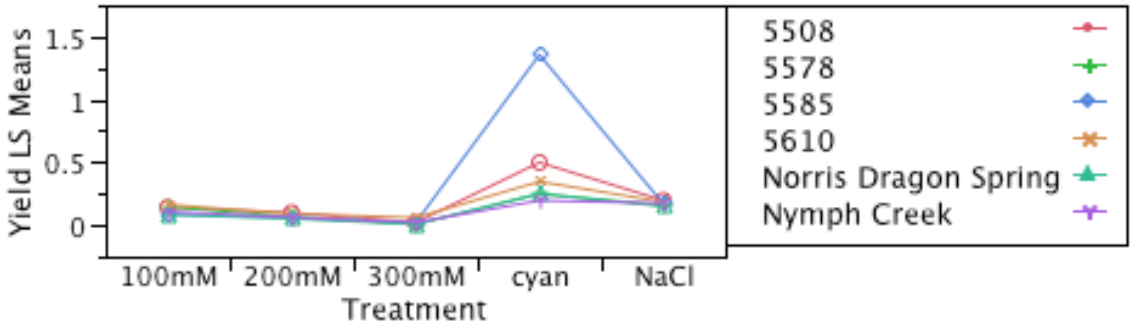
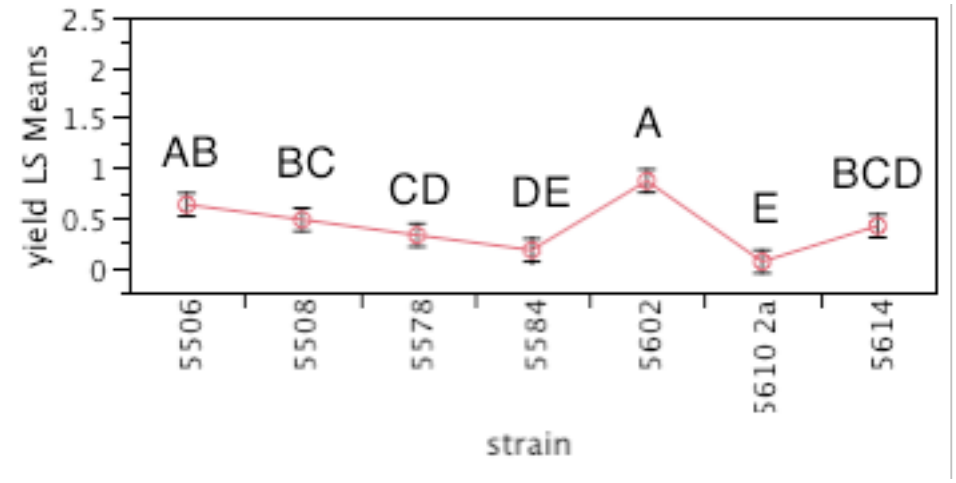


C.

Figure B.17. Mercury experiment 1. A. Variation among strains in yield ($F_{6,27}$ = 23.35, P < 0.0001). B. Differences between treatments in yield ($F_{3,27}$ = 148.48, P < 0.0001). C. The treatment by strain interaction ($F_{18,27}$ = 10.53, P < 0.0001).



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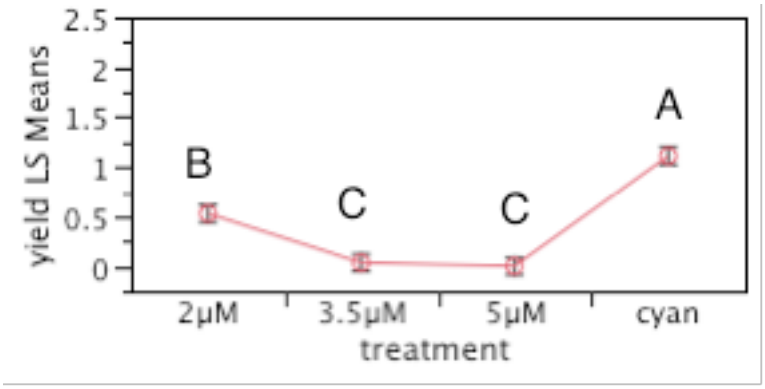

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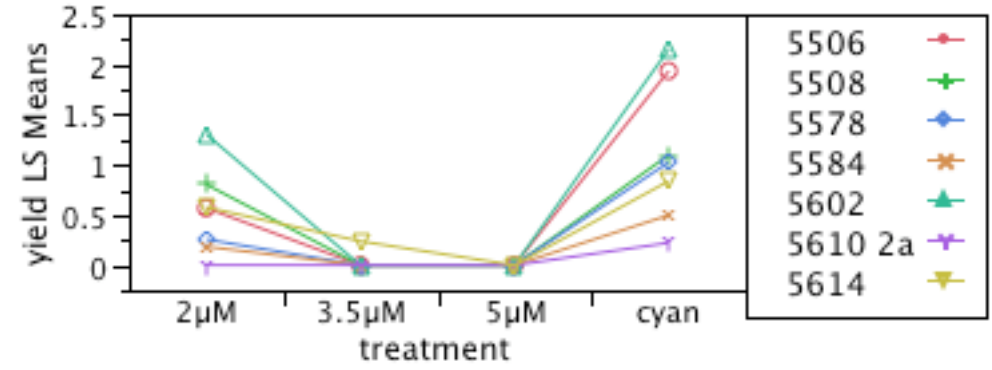
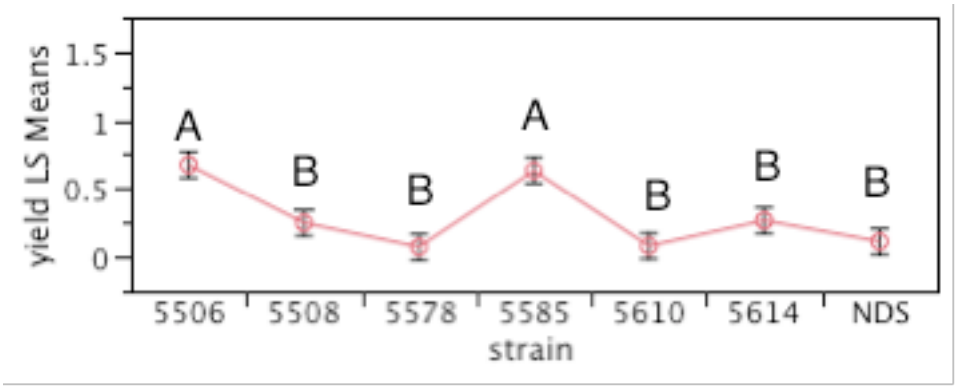


C.

Figure B.18. Mercury experiment 2. A. Variation among strains in yield ($F_{6,27}$ = 29.54, P < 0.0001). B. Differences between treatments in yield ($F_{3,27}$ = 36.39, P < 0.0001). C. The treatment by strain interaction ($F_{18,27}$ = 4.62, P < 0.0001).



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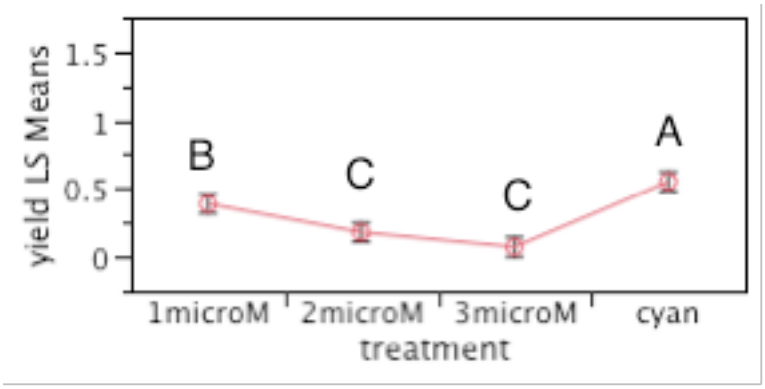

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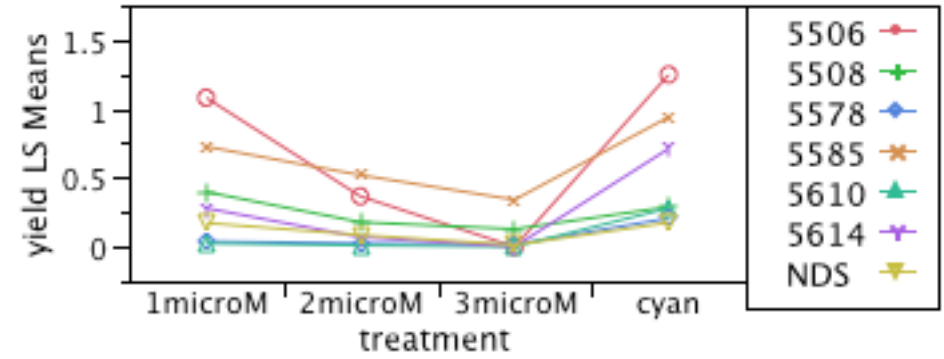

C.

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