AGRICULTURAL RESEARCH FOUNDATION
FINAL REPORT
FUNDING CYCLE 2020 – 2022

TITLE: Marvelous Microalgae: The Role of Photosynthetic Microorganisms in the Biology of Nursery Retention Pond Water

RESEARCH LEADER: Luisa Santamaria
COOPERATORS: Maria Marlin - Faculty Research Assistant
Martha Sleeper -Everde Growers, Forest Grove, OR
Chris Ames - Kraemer's Nursery, Mt. Angel, OR

ABSTRACT:

Cyanobacteria are bacteria that are capable of photosynthesizing, just like plants—and that's unusual in the world of microorganisms. In addition, some cyanobacteria can even fix nitrogen. Nurseries can be a unique setting, often with areas that provide optimal growing conditions (moisture, nutrients, light) for these microorganisms. In this project, we developed a robust protocol for isolating cyanobacteria from nursery recycling ponds. In addition, we were successful in culturing these photosynthetic microorganisms in both solid and liquid media. We conducted studies to determine if our isolates could reduce the concentrations of phosphate and nitrate in solutions. In addition, we wanted to visualize the growth curve of our isolates when introduced to excess phosphate or toxic glyphosate. During these experiments, we also monitored the effect that the microalgae had on the pH of the solution. Cyanobacterial growth was positively correlated to a rise in pH, occasionally resulting in a pH increase of almost 3 values on the scale! We further demonstrated that our cyanobacterial isolates could reduce nitrate and phosphate ions from an aqueous solution; sometimes by over 90%! Finally, two of our three isolates were able to grow when introduced to toxic glyphosate solutions. This study merely scratches the surface of the potential we believe these microorganisms possess to clean water and contribute to the overall nursery recycling pond ecosystem.

OBJECTIVES:

Objective 1: Our first objective is to isolate and culture photosynthetic microorganisms that survive in the contaminated water of nursery retention ponds. We will select three isolates and study their morphology and growth characteristics.

Objective 2: Our second objective is to delve deeper into the ability of these microorganisms to reduce ammonia (nitrogen) and phosphates (phosphorus) in an aqueous solution. We will also investigate the effect microalgae have on water quality as well as study their ability to survive in glyphosate, a common herbicide found in contaminated water.

PROCEDURES:

Objective 1:

The COVID-19 pandemic arrived just as we had secured our funds and scheduled our nursery trips to collect water samples. Travel and laboratory access suddenly became heavily restricted. However, during 2020 and early 2021, we managed to complete our nursery visits for water collection. Depending on the size of the nursery's recycling pond, we collected between 5-10 samples, varying the depth of collection. Water samples were collected in sterile containers; water temperature and pH were also recorded. One nursery was troubled with cyanobacteria growing in a production area experiencing overwatering. We collected water from this area as well.

Isolation process and culture media: We first wanted to visualize the diversity of microalgae growth, both bacterial and eukaryotic, from the water samples. To accomplish this, we pipetted a small amount (between 1-2 mL) of each water sample onto solid BG-11 media (basic algal culture media). We used a sterile bacterial spreader to ensure the samples were distributed evenly around their respective plates. Three replicate plates per sample were conducted. These plates were sealed and placed under LED light (between 2800-3500 lux) to allow for microalgae to grow. Samples were incubated at room temperature (23℃) for 2 weeks, at which time ample green colonies were observed on the plates.

Cleaning cultures on selective media: However, we weren't certain if the green colonies were eukaryotic microalgae or cyanobacteria. We wanted to select only cyanobacteria and continue the purification process with these isolates. To accomplish this, we transferred colonies and grew them on a selective media. This selective media was made by adding 100 mg/L of cycloheximide to our basic Z8 and BG-11 media. Cycloheximide inhibits protein synthesis in eukaryotic cells (e.g. algae) but allows for the growth of prokaryotic organisms.

The purification of these colonies took several months; cyanobacteria can sometimes occur with fungal or bacterial symbionts. Purification involved multiple, sequential transfers of a very small amount of cell growth to fresh media. Once pure cyanobacteria cultures were growing readily on solid media, we transferred them to liquid media (again, BG-11). Finally, we selected the three isolates to study in our research project. We characterized these isolates based on their morphology. We then purchased three UTEX Photobioreactors with the corresponding UTEX RGB-LED Lighting Platform (Culture Collection of Algae at the University of Texas, Austin). Throughout the remainder of our project, we maintained our cultures in these units (Photo 1). Photobioreactors were filled with a 1:1 mixture of sterile deionized water and BG-11 liquid media. Then, 350 mL of fresh culture was added to its respective bioreactor, and bioreactors were placed on the benchtop.

Objective 2:

Protocol for Measuring Phosphate and Ammonia Uptake by Cyanobacteria Isolates

Cultures were grown in phosphate-free liquid media for 1 week, to deplete any remaining phosphate stores. After 1 week, 10 mL of culture were transferred to individual flasks that had been dry-autoclaved. Standard solutions were mixed using Hach® Phosphate Standard Solution (PO_4); we

prepared 0.25 mg/L, 0.5 mg/L, and 1 mg/L phosphate solutions. Sterile deionized water was used for control. 50 mL of respective phosphate standard solutions were then added to the flasks. Then, the pH was taken and recorded for 'Week 0'. Individual samples were then filtered using a vacuum pump. To measure and track free phosphate in the solution over time, reagents from Hach® Phosphate Test Kit (Model PO-23) were used for phosphate extraction. Then, the phosphate concentration of each solution was measured against a known standard at 880 nm using a Thermo Scientific™ Orion™ AquaMate 8000 UV-Vis Spectrophotometer. Data collection continued on a weekly basis for 2-4 weeks, depending on how long the culture survived. The same process with the same concentrations of nitrate standards was used to measure nitrogen uptake by cyanobacteria. We used reagents from Hach® Nitrogen Ammonia Test Kit (Model NI-SA) to extract nitrogen ammonia (NH3—N). After extraction, the nitrate-ammonia concentration of each solution was measured at 655 nm, per test kit instructions. The percent change in the concentration of phosphate/nitrate was calculated from the initial values and the final values. The following equation was used:

$$\frac{Final\ concentration - Initial\ concentration}{Initial\ concentration} \text{ x } 100$$

Protocol for Measuring Growth in Glyphosate Solutions and Phosphate Solutions

Glyphosate is an herbicide of 'moderate concern' according to the Oregon Water Quality Pesticide Management Team, and nurseries are no exception to industries that could potentially contribute to water contamination. We wanted to investigate the tolerance of our cyanobacteria isolations to the chemical. However, since we did not have an economic or efficient way to extract glyphosate at the small concentrations we were testing, we designed the experiment differently to simulate real-world growing conditions in nursery recycling ponds. 50 mL of actively growing cultures from the bioreactors were added to individual flasks. 50 mL of regular Z8 media was then added to each flask, and cultures were allowed to acclimate to their environment for 24 hours (slow shaking, 120 rpm). We prepared standard solutions of glyphosate by diluting Gly Star® Plus (365 grams glyphosate/L) in sterile deionized water. The same concentrations noted above for phosphate and ammonia were also used. After 24 hours, 50 mL of respective glyphosate standard solutions were added to respective flasks (to represent runoff from herbicide application). Again, sterile deionized water was used for the control. The pH of each solution as well as the optical density at 750 nm was measured for 'Day 0'. These measurements were repeated 3 times a week for 2 weeks and then plotted vs. time.

We repeated this process using phosphate standard solutions as well, except we expanded data collection to 3 weeks. Experimental set-up is represented in Photo 2. We knew from our previous experiment that cyanobacteria can grow and reduce the levels of phosphate in solution, but we wanted to take it a step further and visualize their growth curve. We again simulated a real-world environment that would be high already in nutrients before receiving additional phosphorus from runoff. The procedure in the previous paragraph was utilized, except phosphate standards were used instead of glyphosate standards.

Growth Rate Calculations

Growth rate (μ) was calculated for cyanobacteria growth in glyphosate & phosphate experiments based on the growth curve that was generated. The equation derived in Widdel (2007) was used; namely:

$$\mu = \frac{2.303\ (log\ OD2 - log\ OD1)}{(t2 - t1)}$$

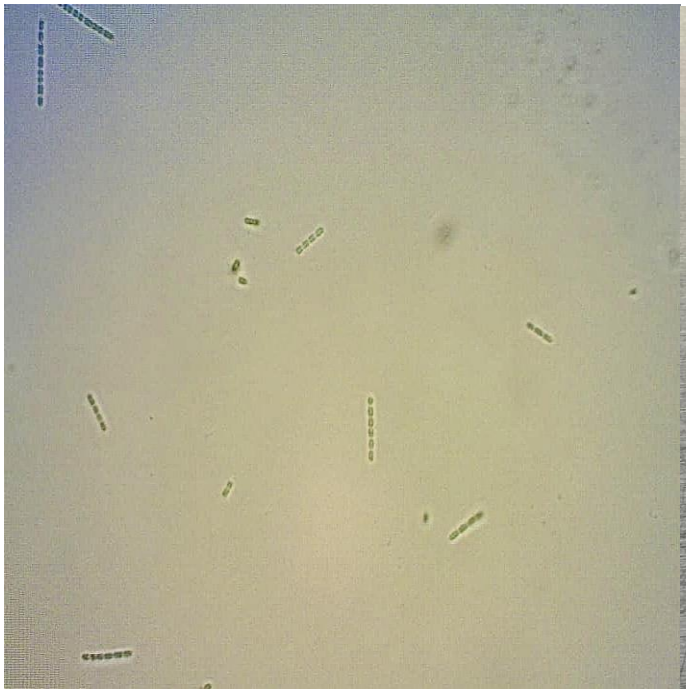
Optical density (OD) was measured at 750 nm, and we calculated growth rate during the best approximation of the exponential phase, based on the curve. Since we did not measure mass or cell density, the growth rate is a measure of turbidity over time. Turbidity is correlated to cell density; so, while not an exact measure of cell growth, it is accepted and commonly used.

SIGNIFICANT ACCOMPLISHMENTS:

We discovered vast diversity of cyanobacteria in different nursery retention ponds throughout the Willamette Valley. What is more, cyanobacteria were present in the water despite the season! (One of our isolates is from water collected in the middle of January.)

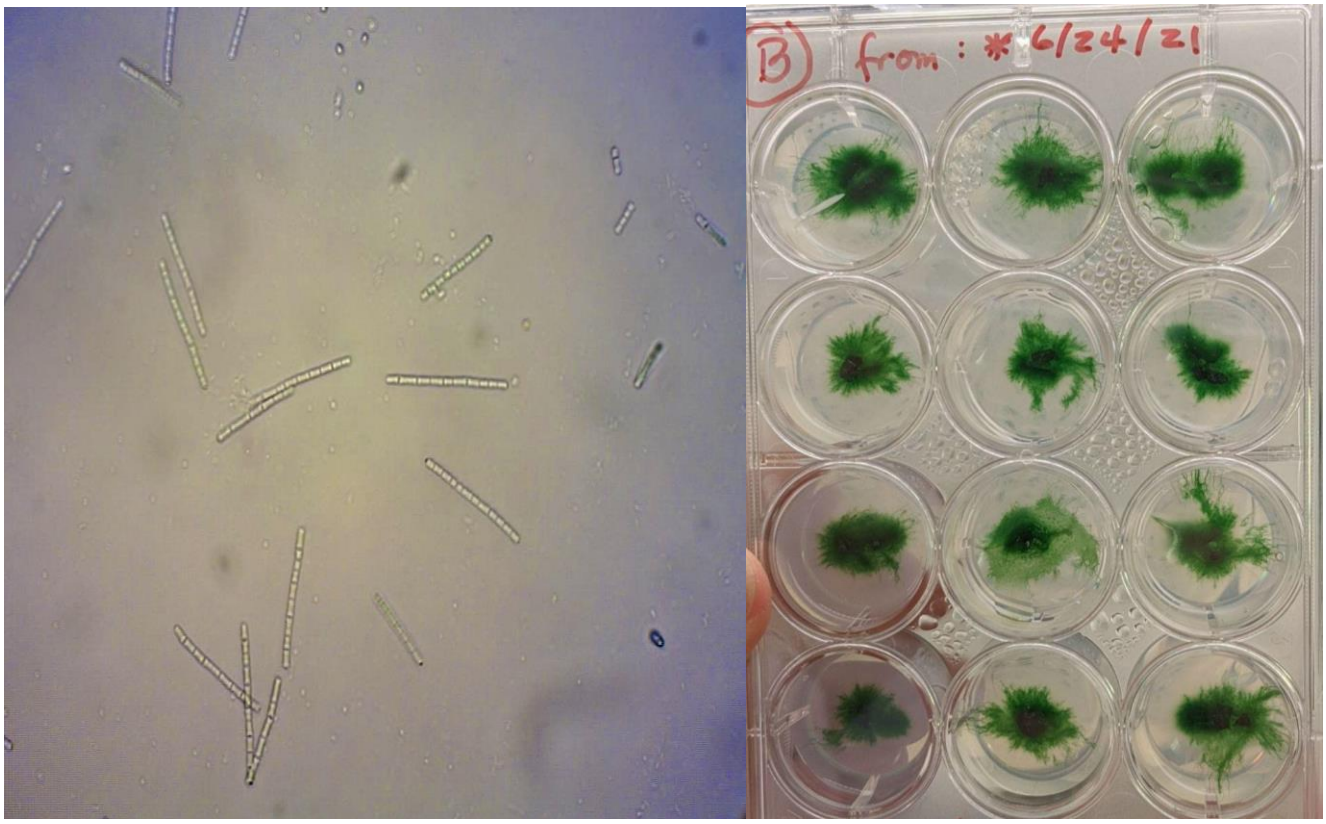
Isolate A Background

Isolate A was isolated from a sample collected at a nursery recycling pond in Mt. Angel, Oregon. The sampling took place on July 21st, 2020. At the time of sampling, the water was 74.8°F, with a pH of 7.60.
Based on morphology, we have identified this isolate as a member of the Nostocaceae family. A picture of Isolate A under the microscope at 40x (left) and growing on solid media (right) is included below.



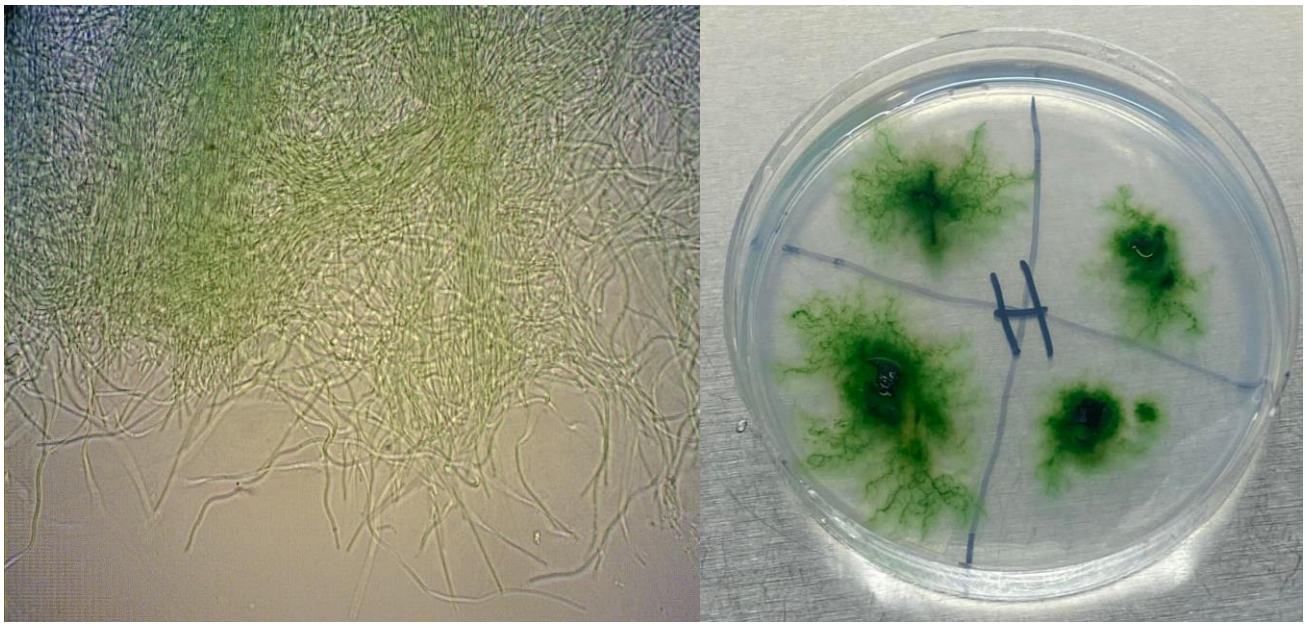
Isolate B Background

Isolate B was isolated from a sample collected at a nursery in Aurora, Oregon on March 3rd, 2020. This specific nursery did not have a recycling pond per se, but they had a lot of areas of standing water in their production areas. These were like small recycling ponds, as runoff accumulated in these areas. We took samples of the cyanobacteria growing prolifically in these puddles. The temperature that day was 51°F with 85% humidity. Based on morphology, we have identified this isolate as another member of the Nostocaceae family, potentially in the genus *Anabaena* family. A picture of Isolate B under the microscope at 40x (left) and pure culture growing on solid media (right) is included below.



Isolate H Background

Isolate H was isolated from a nursery recycling pond in Cornelius, Oregon. The sampling took place on January 28th, 2021. At the time of sampling, the water was 43.7 °F, with a pH of 8.42. Based on morphology, we have identified this isolate as a *Planktothrix* species. A picture of Isolate H under the microscope at 40x (left) and growing on solid media (right) is included below.



Phosphate Uptake

Phosphate-starved cells were transferred to flasks containing phosphate standard solutions of varying concentrations (0.25 mg/L, 0.5 mg/L, and 1 mg/L). Concentration of phosphate was monitored weekly via chemical digestion and spectrophotometer analysis of filtrate against a known standard.
The filtrate from all three isolate control treatments demonstrated a significantly higher phosphate concentration after 4 weeks (Table 1). This is impressive, signaling that even after a week of growing without phosphate, some stores remained. In addition, it is likely that the cells eventually died and released phosphate into the solution.

Isolate A saw percent increases instead of decreases in phosphate in all treatments, however, there were fluctuations over the four weeks. The fluctuations contained sharp increases (peaks) and then decreases. We hypothesize that the peaks in phosphate concentration represent death of cells. However, some remain alive and continue to utilize the phosphate present in the water. This is represented by the decline in phosphate concentration. However, soon very little is left, and the cells begin to perish again.

Isolate B and H removed 88% and 98% of the phosphate, respectively, from the 1 mg/L phosphate standard solution (Table 1)! There was a modest decrease in phosphate concentration in the 0.5 mg/L standard solutions as well for both isolates. Isolate H also contributed to a 56% decrease in phosphate in the 0.25 mg/L standard solution.

Table 1. Percent (%) change in phosphate concentration of solutions after 4 weeks. Positive values represent a percent increase, while negative values represent a percent decrease.

Isolate + Treatment	Isolate A	Isolate B	Isolate H
Control (sterile water)	255%	1,450%	1,100%
0.25 mg/L phosphate standard solution	1,528%	11%	-56%
0.5 mg/L phosphate standard solution	226%	-59%	-58%
1 mg/L phosphate standard solution	174%	-88%	-98%

Nitrate Uptake

Isolate A began the nitrate uptake experiment with elevated amounts of nitrates already in solution. Although heterocysts were not readily observed under the microscope, this isolate potentially could have been fixing nitrogen while growing in the nitrogen-starved media for the week prior to the start of the experiment. Regardless, it easily reduced nitrate when re-introduced to this molecule; after two weeks the concentration of ammonia-nitrate was almost negligible in all solutions. Isolates B and H performed similarly in ammonia solutions as they did in phosphate solutions; they did not begin with elevated nitrate concentrations. Both isolates could remove and utilize ammonia-nitrate across all concentrations (Table 2).

Table 2. Percent (%) change in ammonia-nitrate concentration of solutions after 3 weeks.
Positive values represent a percent increase, while negative values represent a percent decrease.

Isolate + Treatment	Isolate A	Isolate B	Isolate H
Control (sterile water)	-1,122%	1,300%	319%
0.25 mg/L ammonia-nitrate standard solution	-1,203%	-93%	7%
0.5 mg/L ammonia-nitrate standard solution	-560%	-98%	-29%
1 mg/L ammonia-nitrate standard solution	-2,696%	-79%	-95%

Growth Curves and Growth Rates in Excess Phosphate

Cyanobacteria were grown in normal Z8 media, which contains phosphate as a nutrient source. After 24 hours, they were then introduced to additional phosphate solutions. Isolate A demonstrated consistent growth, despite the addition of phosphate. Figure 1 shows a similar growth curve for all concentrations of phosphate standard as well as the control. The growth rate during the exponential phase of growth was highest in the solution containing 1 mg/L of phosphate (Figure 4a). The lowest growth rate was found in the control treatment, indicating that the cyanobacteria grew better when introduced to excess phosphate.

Isolate B also shows a tolerance for phosphate. Interestingly, the control treatment and the 1 mg/L treatment peaked earlier than the 0.25 mg/L and 0.5 mg/L treatments (Figure 2). The growth rate during the exponential phase of Isolate B was similar, despite the differing concentrations of phosphate in the solution (Figure 4b).

Finally, Isolate H experienced steady growth in the presence of excess phosphate (Figure 3). The cyanobacteria growing in 1 mg/L phosphate experienced a slight stationary phase, but quickly recovered. In fact, the cyanobacteria in 1 mg/l phosphate displayed the highest growth rate during the exponential phase (Figure 4c).

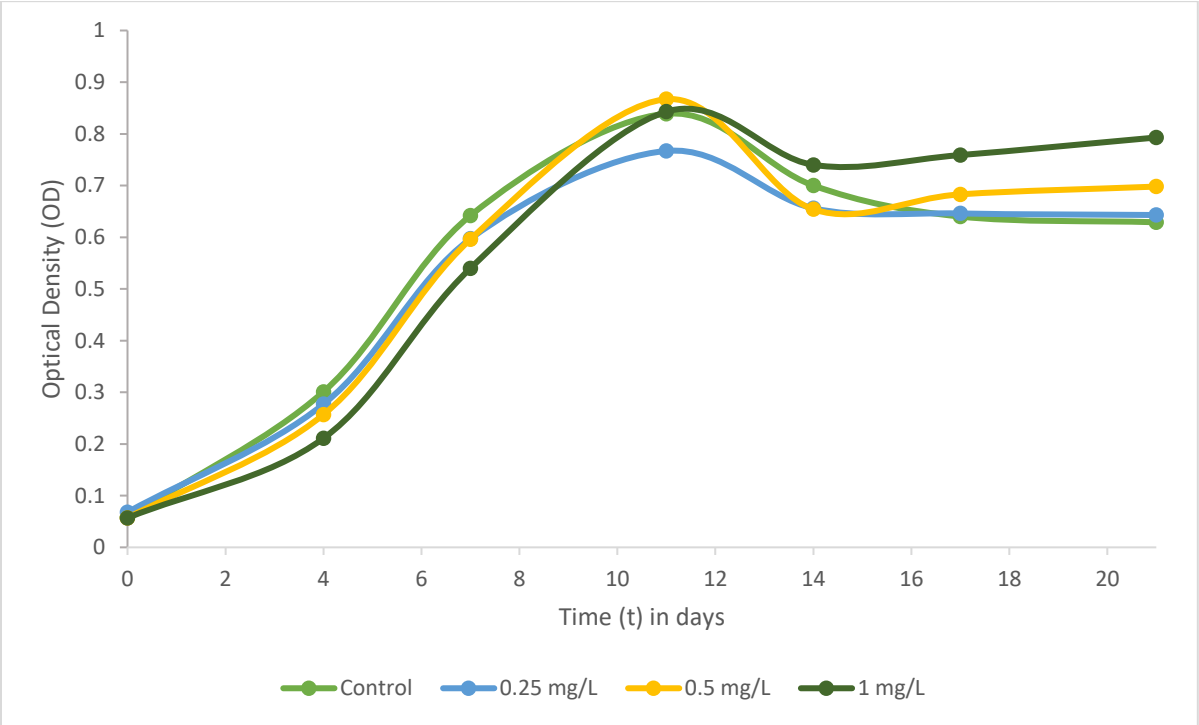


Figure 1. Growth curve for Isolate A in differing phosphate concentrations. Cyanobacteria cultures were grown in BG-11 media and then supplemented with three different concentrations of phosphate standard solutions. Sterile deionized water served as a culture. OD of the liquid cell culture was measured 3 times a week at 750 nm and plotted over time.

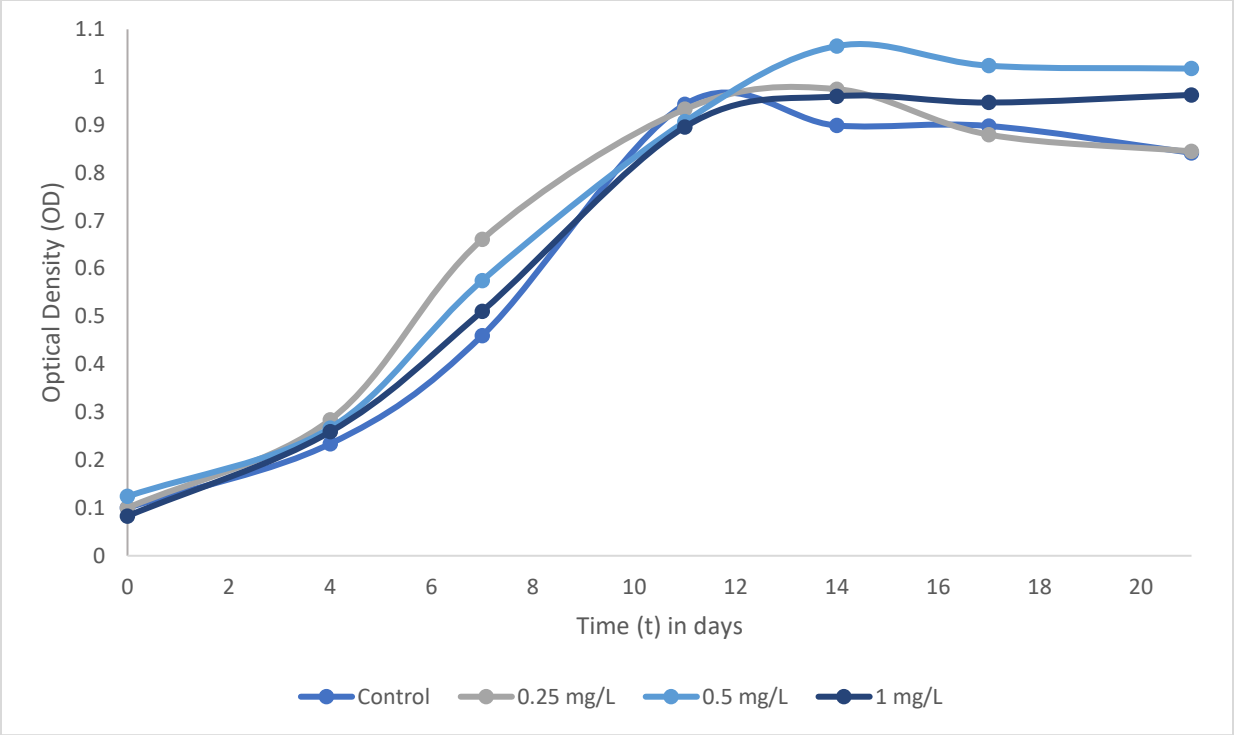


Figure 2. Growth curve for Isolate B in differing phosphate concentrations. Cyanobacteria cultures were grown in BG-11 media and then supplemented with three different concentrations of phosphate standard solutions. Sterile deionized water served as a culture. OD of the liquid cell culture was measured 3 times a week at 750 nm and plotted over time.

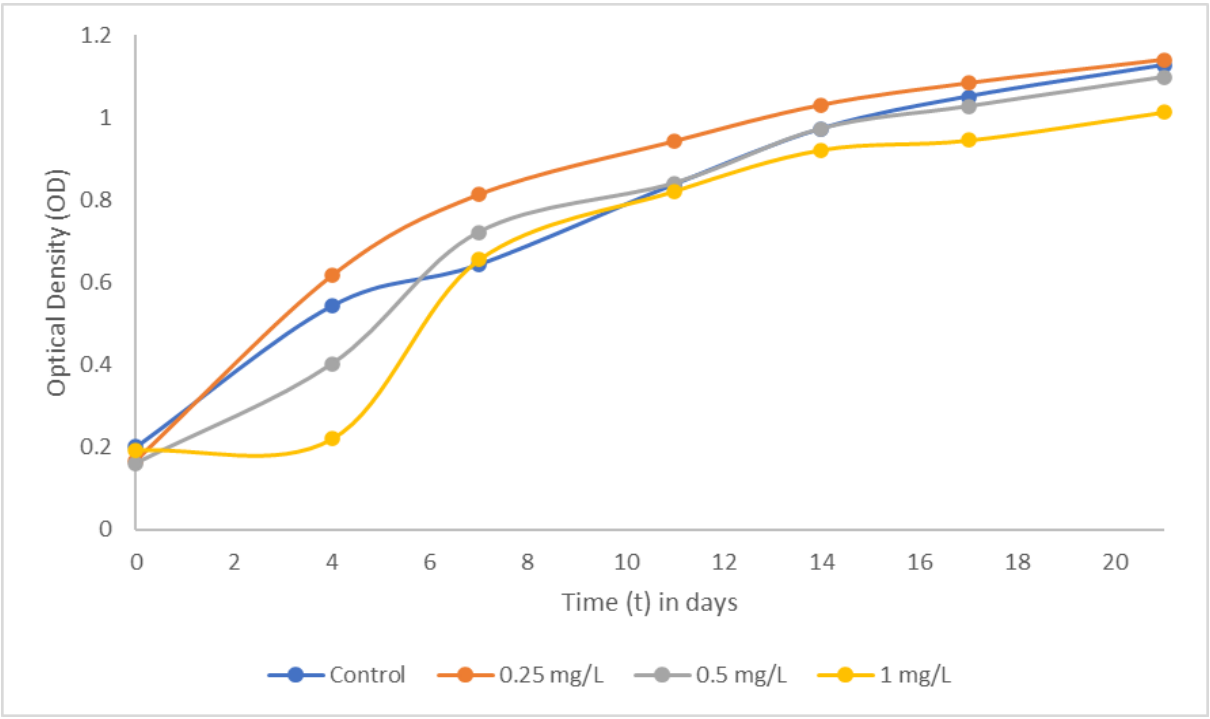


Figure 3. Growth curve for Isolate H in differing phosphate concentrations. Cyanobacteria cultures were grown in BG-11 media and then supplemented with three different concentrations of phosphate standard solutions. Sterile deionized water served as a culture. OD of the liquid cell culture was measured 3 times a week at 750 nm and plotted over time.

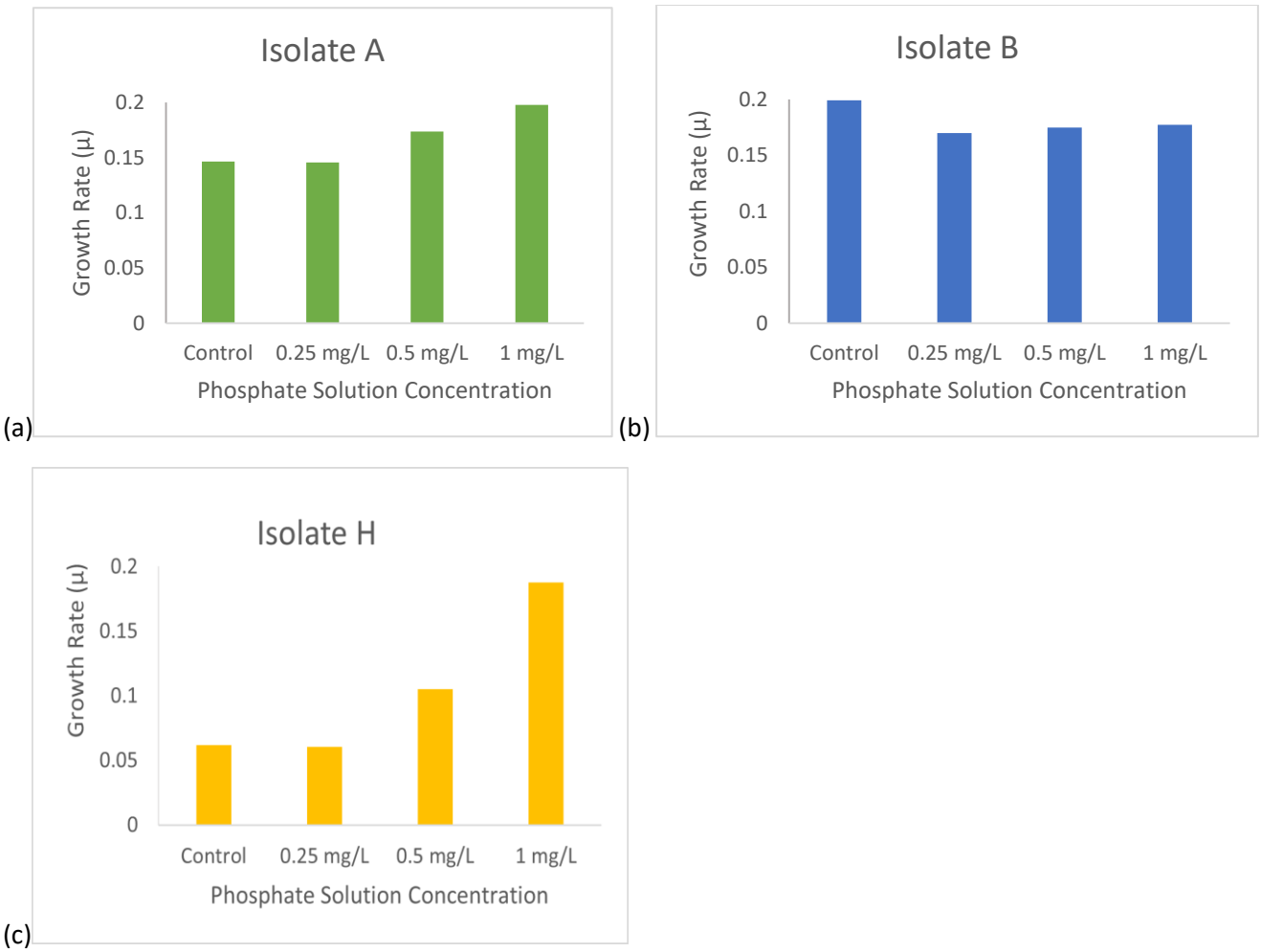


Figure 4. Growth rate during exponential phase of cyanobacteria isolates when grown in excess phosphate solutions. Exponential phase (7 days, from Day 4- Day 11) was estimated by the growth curve in the preceding figures. Growth rate is measured in terms of optical density (turbidity) over time.

Growth Curves and Growth Rates in Glyphosate Solutions

Our three cyanobacteria isolates tolerated the presence of glyphosate in solution. While the growth curves were flatter, signaling lower growth, there was still positive growth and not instant death. During the first week, Isolate A demonstrated an increase in growth in all solutions except for the 0.25 mg/L glyphosate solution (Figure 5). The control treatment had the highest growth rate, and the growth rate in 0.25 mg/L of glyphosate was the lowest for this isolate (Figure 8a).

While there was an initial decrease, Isolate B experienced positive growth in all concentrations of glyphosate (Figure 6)! As expected, the growth rate decreased as the concentration of glyphosate increased (Figure 8b). Perhaps most remarkably, the growth rate of the control was the lowest, suggesting that the presence of glyphosate simulated growth.

Isolate H peaked quickly in both the control and glyphosate treatments (Figure 7). It then experienced a steady decline in growth, including in the control. It is possible that the culture as a whole was not in optimal health. The growth rate for 3 of the 4 solutions were negative (Figure 8c); the 1 mg/L had a very small positive growth rate over the 6 days.

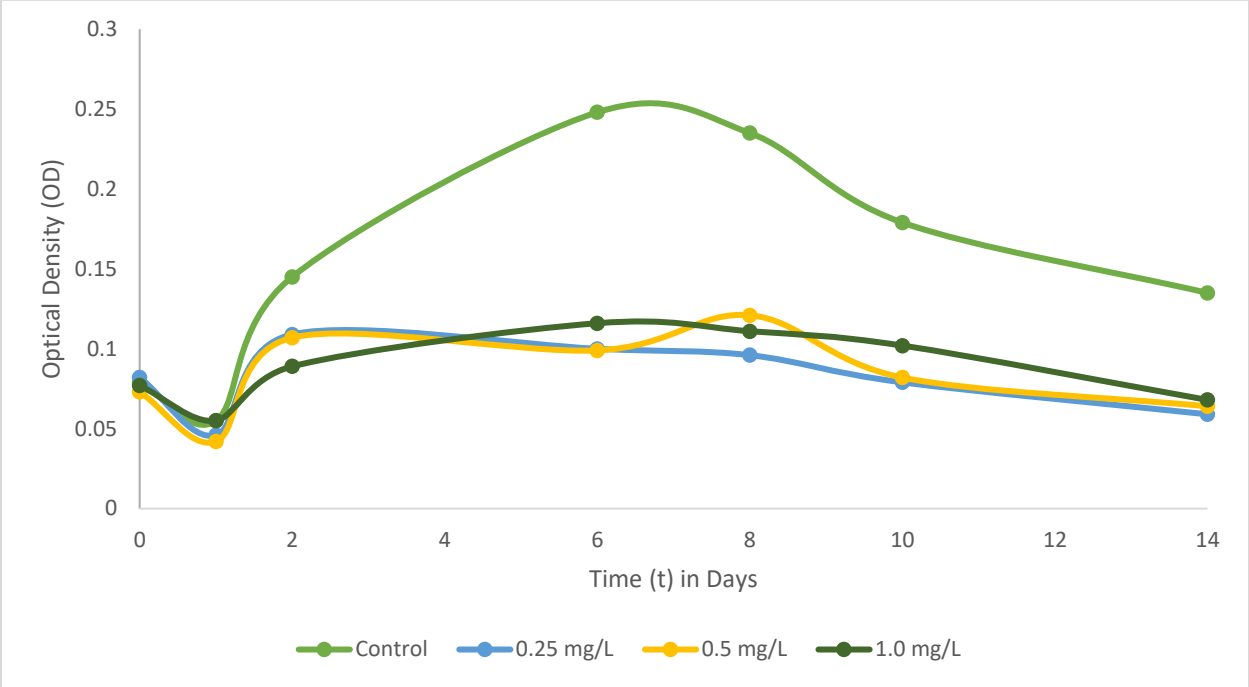


Figure 5. Growth curve for Isolate A in differing glyphosate concentrations. Cyanobacteria cultures were grown in three different concentrations of glyphosate, with sterile deionized water serving as a culture. OD of the liquid cell culture was measured 3 times a week at 750 nm and plotted over time.

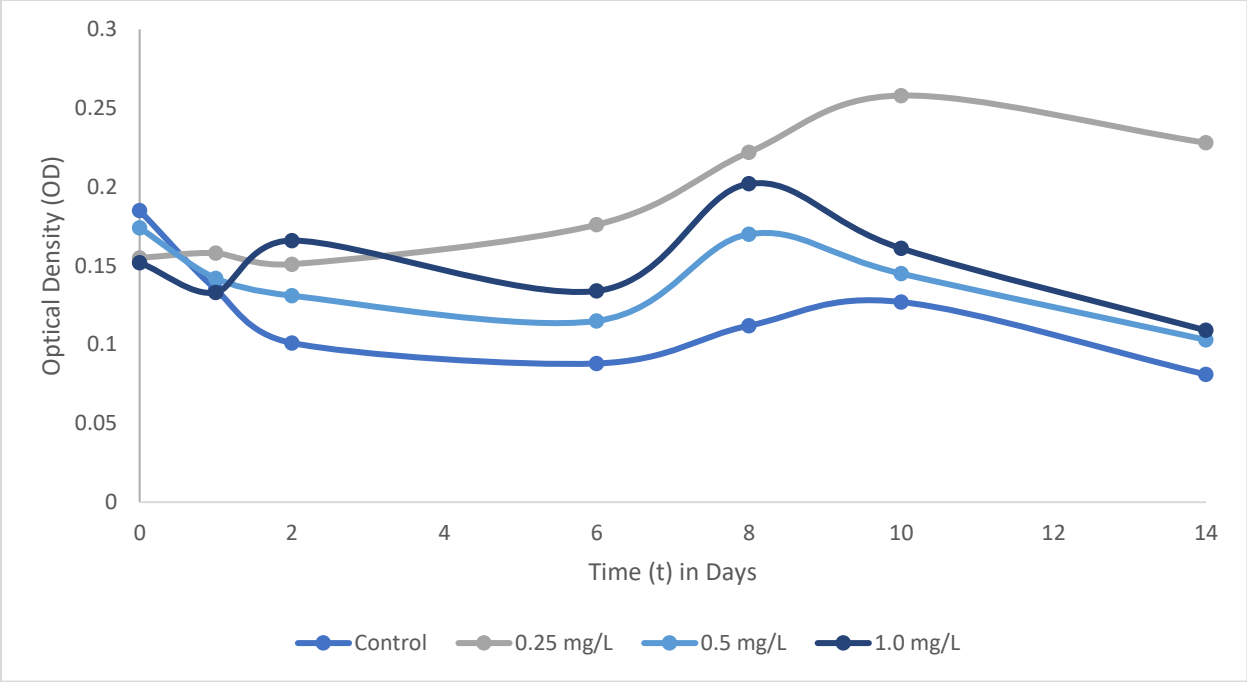


Figure 6. Growth curve for Isolate B in differing glyphosate concentrations. Cyanobacteria cultures were grown in three different concentrations of glyphosate, with sterile deionized water serving as a culture. OD of the liquid cell culture was measured 3 times a week at 750 nm and plotted over time.

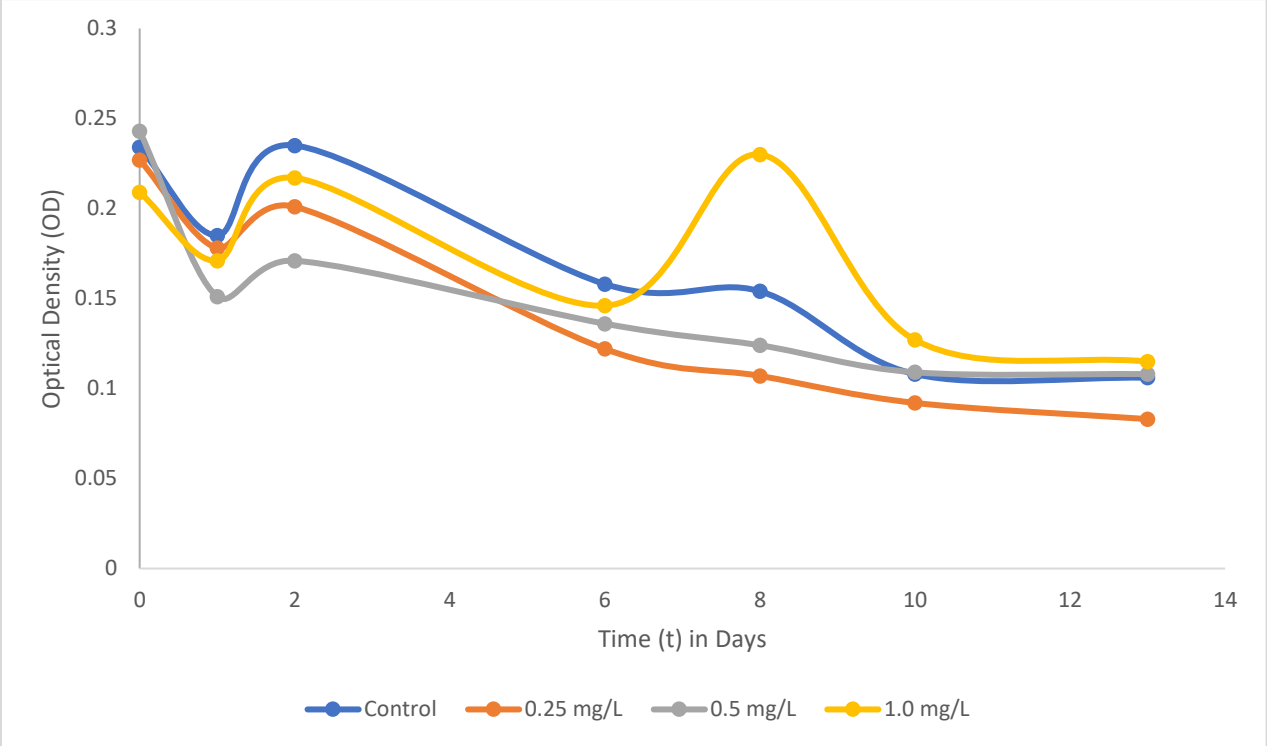
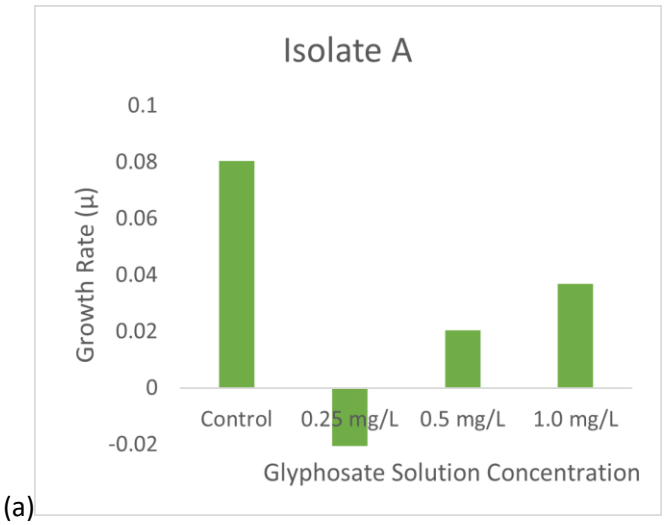
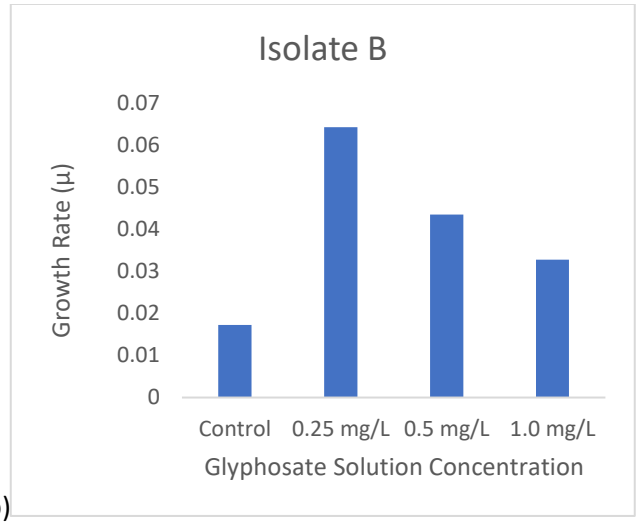


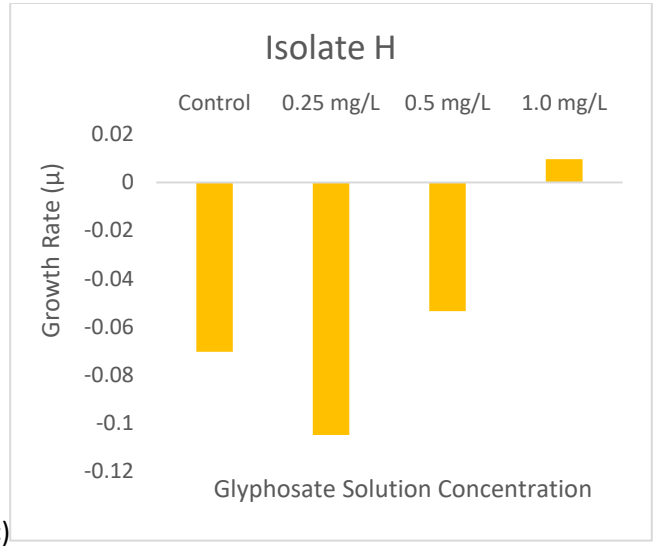
Figure 7. Growth curve for Isolate H in differing glyphosate concentrations. Cyanobacteria cultures were grown in three different concentrations of glyphosate, with sterile deionized water serving as a culture. OD of the liquid cell culture was measured 3 times a week at 750 nm and plotted over time.



(a)



(b)



(c)

Figure 8. Growth rate during exponential phase of cyanobacteria isolates when grown in glyphosate solutions. Exponential phase (6 days, from Day 2- Day 8) was estimated by the growth curve in the preceding figures. Growth rate is measured in terms of optical density (turbidity) over time.

Overall Effect of Cyanobacteria on pH:

In the phosphate and nitrate uptake experiments, in general, pH correlated with the concentration of phosphate/nitrate in the solution. In other words, as the concentration of phosphate/nitrate decreased, so did the pH. In phosphorus and glyphosate growth curve experiments, we saw dramatic changes in the pH of solutions for isolates A and B (Tables 3 and 4). Isolate H resulted in only small increases in pH. In addition, the pH followed the direction of cyanobacterial growth in these two

growth curve experiments; an increase in growth (turbidity) corresponded to an increase in pH. Interestingly, for all three isolates, pH did not differ between the various concentrations and the control. This indicates that cyanobacteria influence the pH of the water, regardless of extra nutrients (phosphorus) or toxic chemicals (glyphosate).

Table 3. Fluctuations in pH of solutions when cyanobacteria were grown in excess phosphate. pH was taken at Day 0 and is indicated below as 'Starting pH'. This value is contrasted with the highest pH recorded over the course of the entire experiment to demonstrate the effect of cyanobacterial growth on this parameter, despite the addition of phosphate.

	Isolate A		Isolate B		Isolate H	
	Starting pH	**Highest pH recorded**	**Starting pH**	**Highest pH recorded**	**Starting pH**	**Highest pH recorded**
Control (sterile water)	7.88	10.35	8.65	9.94	10.28	10.39
0.25 mg/L phosphate	7.88	10.38	8.58	10.35	10.32	10.42
0.5 mg/L phosphate	7.83	10.43	8.58	10.36	10.28	10.53
1 mg/L phosphate	7.84	10.45	8.54	10.44	10.34	10.5

Table 4. Fluctuations in pH of solutions when cyanobacteria were grown in glyphosate. pH was taken at Day 0 and is indicated below as 'Starting pH'. This value is contrasted with the highest pH recorded over the course of the entire experiment to demonstrate the effect of cyanobacterial growth on this parameter, despite the presence of glyphosate.

	Isolate A		Isolate B		Isolate H	
	Starting pH	**Highest pH recorded**	**Starting pH**	**Highest pH recorded**	**Starting pH**	**Highest pH recorded**
Control (sterile water)	9.94	11.38	10.94	10.98	10.88	11.53
0.25 mg/L glyphosate	10.24	11.22	10.88	10.94	11.1	11.50
0.5 mg/L glyphosate	10.37	11.15	10.9	11.20	11.07	11.47
1 mg/L glyphosate	10.31	11.31	10.79	11.15	10.44	11.48

BENEFITS & IMPACT:

Cyanobacteria are ubiquitous all around us. Nurseries are an especially attractive environment for these marvelous microalgae, as nutrients and water are plentiful both on the ground and in the recycling pond. This study has provided insight into the ecology of cyanobacteria in nursery recycling ponds and the potential they have to remove phosphate and nitrate from the water.

The growth rate in phosphate experiment is especially relevant for the Willamette Valley. One of the managers of a nursery we visited explained to us that phosphate is very high in the soil already. Add some runoff to that mix, and the concentration goes up even more! Cyanobacteria can grow readily in phosphate, so managing phosphate concentrations should be a critical control point for all nurseries. But on the flip side, our isolates had the ability to utilize that phosphate and decrease the phosphate concentration in the water. This can have bioremediation implications for the future, and more research is warranted. Isolate B in particular shows potential, as it was able to grow in the presence of glyphosate!

Growth of cyanobacteria was positively correlated with an increase in pH. This can have far-reaching consequences on the pond ecosystem, as plankton and other larger organisms could be sensitive to pH. However, it's important to balance this with the potential of cyanobacteria to clean excess phosphate and nitrate from the water. The next step will be to investigate how to harness these microbe's water-cleaning abilities without the resulting bloom of growth.

An additional impact from this project presents itself in the form of successful student research and project design. Funds from this project were used to support Katie Gregor, a student attending Oregon State University. She initiated a project to grow cyanobacteria in large quantities by designing her own bioreactor (Photo 3)! She will co-author an article in the June 2022 Sustainability issue of *The Digger* Magazine.

ADDITIONAL FUNDING RECEIVED DURING PROJECT TERM:

There has been no additional funding received during the project term.

FUTURE FUNDING POSSIBILITIES:

We will continue to advocate for cyanobacteria and their role in nursery recycling ponds. In addition to the results discussed above, we found several exciting aspects of our cyanobacteria. Isolate A produced a vibrant blue pigment (Photo 4), and we look forward to exploring possibilities for pigment extraction and use. We are actively searching for potential collaborators who work in this field and who will be willing to aid us in continuing this avenue of research. In addition, we have at least ten isolates in pure culture, all from nursery recycling ponds. During our microscopic characterization of them, we identified some unique structures on some of these species. Among these structures are heterocysts, which are present in species that fix nitrogen, as well akinetes, structures that act as energy reserves in some cyanobacteria species. We have received a grant from Oregon Department of Agriculture and Oregon Association of Nurseries to hire a summer intern in 2022. We are planning on engaging this

intern with our project, by using the cultures isolated during this study as a source of potential biofertilizer or some bioproducts (pigments or secondary metabolites). In the future, we will use the data generated to apply for future grants at the local, state, or even national level.

As identified in the original project proposal, potential investors in our research include freshwater aquaculture, pharmaceutical, and agrochemical business. In addition to serving as a potential biofertilizer, our native cyanobacteria species might be found to produce anti-pathogenic compounds or other metabolites that could have a relevant application in agriculture. Such examples include chemicals that can degrade herbicides or trap heavy metals. We hope that companies who work closely with nurseries will be interested in future collaboration.

REFERENCES:

Oregon Department of Agriculture Water Quality Pesticide Management Team: https://www.oregon.gov/oda/programs/Pesticides/Water/Pages/AboutWaterPesticides.aspx

Widdel, F. (2007). Theory and measurement of bacterial growth. *Di dalam Grundpraktikum Mikrobiologie*, *4*(11), 1-11.

PHOTOS:

Photo 1. Representative example of our bioreactor set-up. Bioreactors were filled with a 1:1 ratio of growth media and sterile deionized water. Liquid cyanobacteria culture was then added, and bubbling took place with aquarium bubblers. Bioreactors were placed on the bench top and kept at room temperature.

Photo 2. Example setup for determining growth rate in excess phosphate. Isolates were grown for 24 hours in normal media. Then, 5- mL of differing concentrations of excess phosphate were added to the flasks. From left to right: control treatment (sterile water), 0.25 mg/L phosphate solution, 0.5 mg/L phosphate solution, and 1 mg/L phosphate solution. This picture was taken on Day 21; all cultures are still alive and green!

Photo 3. Katie, our student intern in part supported by this grant, poses with her bioreactor that she built from scratch!

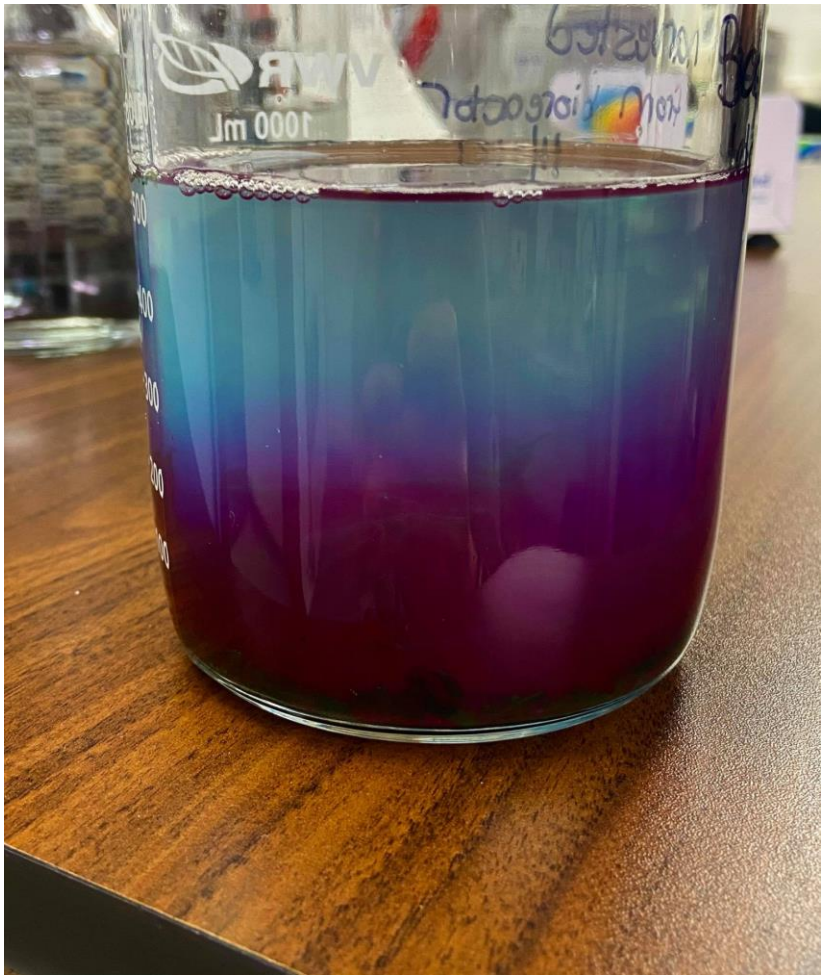


Photo 4: Isolate A produces a vibrant pigment that can be extracted for further investigation.