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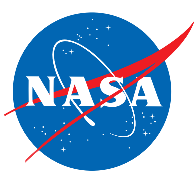

MICROALGAE

PROTOCOLS

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Author

Dr. Daniel Kainer, *Director, Lone Star College Biotechnology Institute*

Editors

Karen Whitfield, *NASA Education Coordinator, Oklahoma State University*

Stephanie Burks, *Biology Instructor, Hinds Community College*

Alicia Baturoni Cortez, *Co-Investigator, NASA Office of STEM Engagement*

Christine Kainer, *Genetics Laboratory Manager, Mercury Laboratory Services*

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Dr. Julie Gonzales, *Biotechnology Program Chair,* and the Biotechnology and Cellular and Molecular Biology *students of Des Moines Area Community College*

Lyndsay Baker, *MicroAlgae Principal Investigator*

Ashley Shuey, Jerica Bidic Bohnstengel and Devin Ledesma, *students, Des Moines Area Commuity College*

Emmanuel Patelidas, *Student, University of Illinois at Chicago*

Hope Calogero, *Student, Fond du Lac Tribal & Community College*

Hernando Hernandez, *Student, University of Texas at Tyler*

Joshua Livermore, *Kemin Industries*

Matthew Huber, *Algae Research Supply Company*

Stephen Wedgwood

Michelle Gray, *Boeing*

Matthew Hoffman, *Boeing*

Dr. David Nobles, Jr., *Curator, UTEX Culture Collection of Algae*

MICROALGAE INTRODUCTION

Introduction

When thinking about the exciting scientific initiatives at NASA, algae don't typically come to mind. However, insight into nature can come from unexpected places. Algae, which most people view as a nuisance that often shows up in swimming pools and bird baths, is actually a group consisting of many thousands of species that exhibit an incredibly diverse array of interesting properties. Many people don't realize that a variety of products—from thickeners in ice cream and fertilizers that help crops grow, to nutritional supplements and ingredients in cosmetics—all come from one or more of these amazing algal species! NASA scientists realized a long time ago that the long history of algae-based discoveries on earth could be continued on space-based missions.

The word "algae" refers to an incredibly extensive and diverse group of aquatic organisms. There are two types: macroalgae and microalgae. The large multicellular macroalgae are found in freshwater environments such as ponds and streams, as well as in marine environments. These organisms are easily visible to the naked eye and tend to be measurable in inches, although giant kelp in the ocean can grow to well over a hundred feet in length. In contrast, microalgae are tiny single-celled organisms that grow in aqueous (*i.e.*, watery) environments and are measurable in micrometers (a micrometer is one one-millionth of a meter). Microalgae are commonly found in bogs, marshes, and swamps, although species may exist in a variety of habitats.

In 2018, NASA astronauts aboard the International Space Station (ISS) performed experiments[1] on *Chlamydomonas reinhardtii* (aka "Chlamy"), an algae species that has provided scientists with insights into the secrets of life for many decades. This particular mission involved experiments that attempted to identify the genetic attributes necessary for optimizing Chlamy's growth in a microgravity environment. In addition, a number of experiments were designed to determine whether stresses linked to the microgravity environment onboard the ISS would trigger Chlamy to produce valuable compounds that could be utilized either by astronauts in space or by people on Earth.

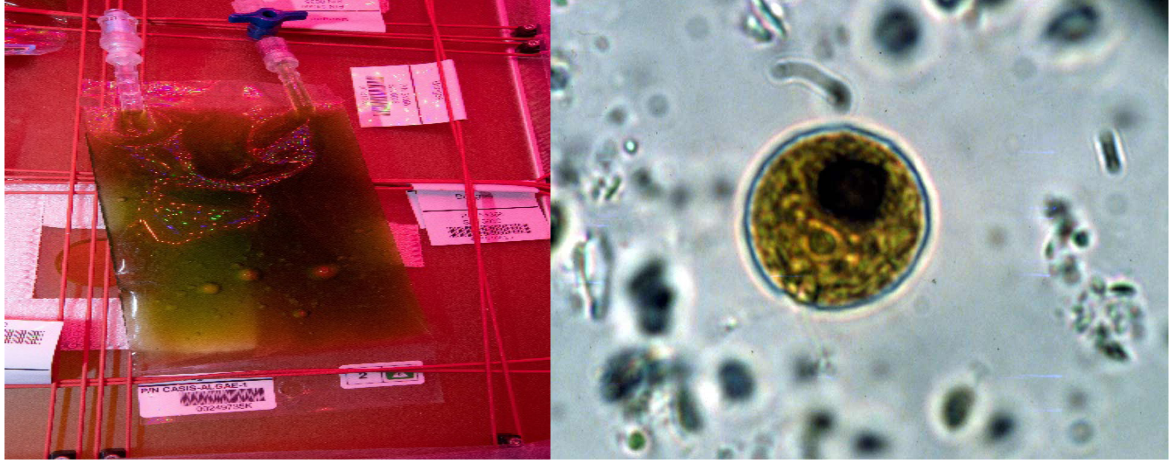


Figure 1. (left) Bag containing *Chlamydomonas reinhardtii* aboard the International Space Station[2]; (right) Micrograph of *Chlamydomonas reinhardtii*[3]

While NASA continues its important work with Chlamy, we now find ourselves at the beginning of a new algae project – a project that *you* can be a part of! The new alga (no, that is not a typo – did you know that *alga* is the singular form of the word *algae?)* at the center of this new mission is called *Haematococcus pluvialis*. Those algae species sure can be tongue twisters, can't they? Why don't we borrow a page from the Chlamy scientists and call *Haematococcus pluvialis* "Pluvi?" Whew…that is easier for *all* of us!

You may be asking the question, “Of all of the algal strains that NASA could have chosen to study aboard the ISS, why did they choose Pluvi?” In order to answer that question, let’s start with some seemingly unrelated questions:

1) Have you ever wondered why salmon, shrimp and flamingos are pink?

2) Have you ever thought about the types of things that NASA does to help keep its ISS astronauts healthy despite them living in the hazardous environment of space?

Let’s address these questions one at a time:

1) Salmon, shrimp and flamingos are pink because they eat Pluvi and other strains of algae that make a compound known as **astaxanthin**. Astaxanthin is one of the most potent antioxidants known to science[4], and has a deep red color. Organisms that consume large quantities of it tend to be either red or pink in color.

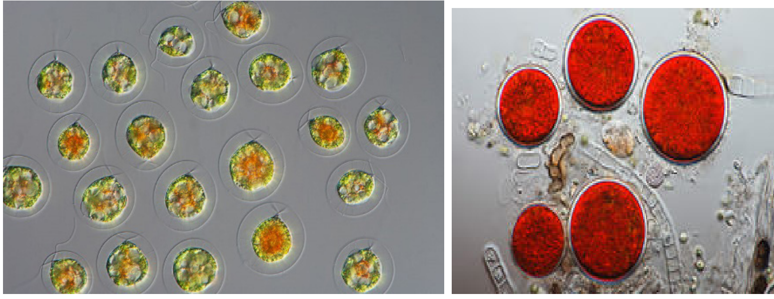


Figure 2. (left) *Haematococcus pluvialis* in early-stage astaxanthin production[5]; (right) *Haematococcus pluvialis* in late-stage astaxanthin production[6]

2) Astronauts aboard the International Space Station spend many consecutive months in space, and they face challenges that those of us on earth don’t have to face. For example, the microgravity environment that they live in places stress on their muscles and bones because they don’t have to work as hard as they do on earth where they constantly experience a gravitational field. As a result, these tissues often atrophy[7] unless the astronauts pay close attention to their nutrition and exercise regimens. In addition, they are constantly exposed to much higher levels of radiation than most of us because they don’t have the protection of Earth’s atmosphere while they are in space. Astaxanthin, a very potent antioxidant, can promote astronaut health aboard the ISS and generate tremendous long-term benefits for them.

Figure 3. Astronaut Steve Lindsey exercises while aboard the International Space Station[8]

The next question you may be asking is, “Why does Pluvi make astaxanthin in the first place?” It turns out that a number of species naturally contain astaxanthin. When conditions are favorable, Pluvi is a green alga and when they are stressful, the rapid increase in astaxanthin production turns the algae bright red. For Pluvi, astaxanthin production increases significantly when the microorganism is under stress. There are many types of stressors that can trigger the shift from green to red, but in most cases intense light is one of the stressors (typically in combination with a second stressor like nutrient deprivation or high salt concentration). During its “stressed out” phase, Pluvi changes from either a **non-motile** (*i.e.*, not capable of movement) cell or a **motile biflagellated** (*i.e.*, capable of movement because of the presence of two swimming appendages called **flagella**) cell to a **cyst** (*i.e.*, a tough, protective capsule capable of surviving harsh environments) filled with astaxanthin and therefore red in color. While, as you can imagine, the biological details of Pluvi’s life cycle are quite complex[11], astaxanthin essentially serves as a potent “sunscreen” for Pluvi that protects it from intense light and other stressors.

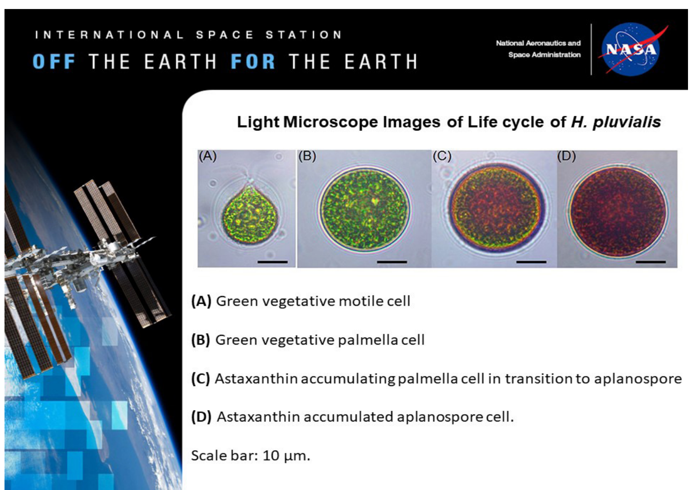


Figure 4. Light Microscope Images of *Haematococcus pluvialis* life cycle morphological stages[12]

So, you may be wondering why we care so much about astaxanthin, from a human perspective. While the jury is still out on the specific health benefits as an antioxidant, evidence suggests that it can serve as an enormous boost for many aspects of health. The primary benefit of any antioxidant is to neutralize **free radicals** (highly reactive molecules with unpaired electrons that are generated via normal biological processes). Free radicals cause *oxidative* damage to tissues and DNA and anti*oxidants* help prevent this damage. Astaxanthin is a potent antioxidant, being 6,000 times more effective than vitamin C, 550 times more effective than vitamin E and 40 times more effective than beta-carotene[13]. A number of studies indicate that this potent antioxidant activity can result in a number of health benefits including:

1) Brain health by preserving cognitive function[14].
2) Male fertility by helping improve sperm quality[15].
3) Reduction in eye fatigue[16].
4) Reduction in artherosclerosis[17].
5) Enhanced memory[18].
6) Demonstrated multiple beneficial effects when it comes to upping exercise performance and preventing injury[19].
7) Enhanced immune response[20].

8) Inhibition of proliferation (rapid increase) and spread of cancer cells[21].

9) Improved skin appearance and health[22].

10) Decreased chronic inflammation[23].

11) Improved joint health and reduced pain associated with rheumatoid arthritis[24]

12) Exhibited a number of anti-aging effects[25].

Figure 5. (left) Nutraceuticals are food supplements that enhance human health and well-being[9]; (right) Astaxanthin is a popular nutraceutical because of its powerful antioxidant properties[10].

Studies exemplified by the list above suggested that astaxanthin may hold a number of benefits for human health. For that reason, NASA has decided to investigate the potential that astaxanthin may hold for astronaut health on long duration space exploration.

MICROALGAE PROJECT ONE

Project I:

Establish Cultivation Conditions by Building a Classroom "VEGGIE" Unit

Since 2013, the ISS has employed a specialized growth chamber known as "VEGGIE" in order to carry out experiments on growing fresh produce, algae, and other organisms that require precise growth conditions[26]. The range of applications has been quite diverse, including the production of fresh lettuce and other greens[27] for direct astronaut consumption to an extended study of the alga "Chlamy" described earlier. The objective of this project is to build a cost-effective replica in your classroom where you can conduct your very own Pluvi experiments along with the ISS astronauts. Any data that you generate during these experiments under the influence of Earth's gravity (unless your classroom just happens to have been built in low earth orbit) can then be compared with their data generated under similar conditions in a microgravity environment!

If you don't have an opportunity to work on these experiments while the actual mission is going on (or if you simply would like to continue experimenting with algae), you can do any of these experiments at any time, including with the "space algae" that NASA returns to earth from the Space Station!

Because you will be conducting experiments that mirror the Pluvi experiments on the International Space Station which occur in the VEGGIE growth chamber, you will need to build our own low-cost VEGGIE unit for the classroom! The design below is developed to keep costs at a minimum. Feel free, however, to make changes, improvements, etc. as you see fit!

Material List

Classroom VEGGIE Frame	**Quantity**
10-foot ¾" PVC pipes	2
¾" PVC side outlet elbows	8
¾" PVC tees	2
PVC cutter	1
Classroom VEGGIE Walls	**Quantity**
Clear heavy- or medium-duty 70x70 in. shower curtain	1
White duct tape **OR**	1 roll
Muslin clips	12
Classroom VEGGIE Door	**Quantity**
Clear 25x25 in. vinyl carpet runner	2 linear feet
Industrial strength Velcro	2 patches
Tools/Misc.	**Quantity**
Scissors	1 pair
Yard/meter stick	1
Clamp lamp	1

Figure 6. Materials needed for construction of a classroom VEGGIE unit

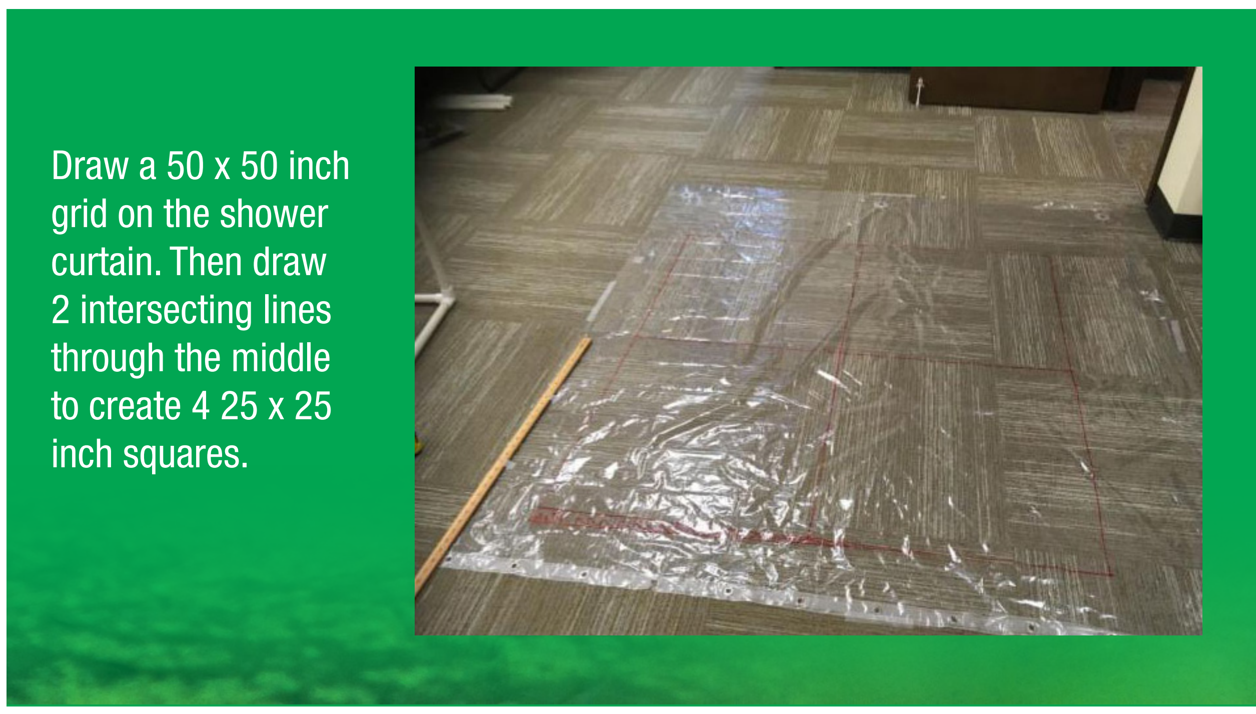


Figure 7. Step 1 of Classroom VEGGIE assembly: Create 4 25” X 25” squares by drawing 2 intersecting lines in the middle of the curtain.

Figure 8. Step 2 of Classroom VEGGIE assembly: Cut away extra shower curtain material.

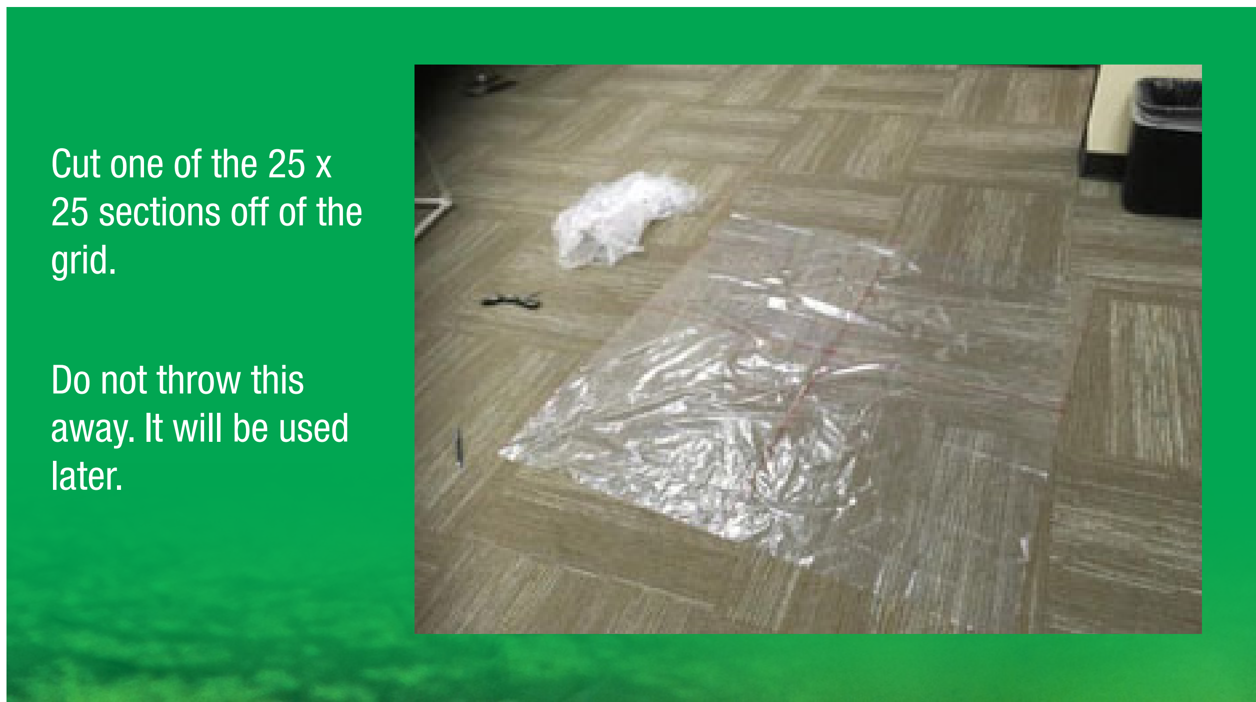


Figure 9. Step 3 of Classroom VEGGIE assembly: Cut away a 25” section of the shower curtain and put it away for future use.

Figure 10. Step 4 of Classroom VEGGIE assembly: Attach the previously-discarded cutaway to the edge of the remaining shower curtain as seen in the image above.

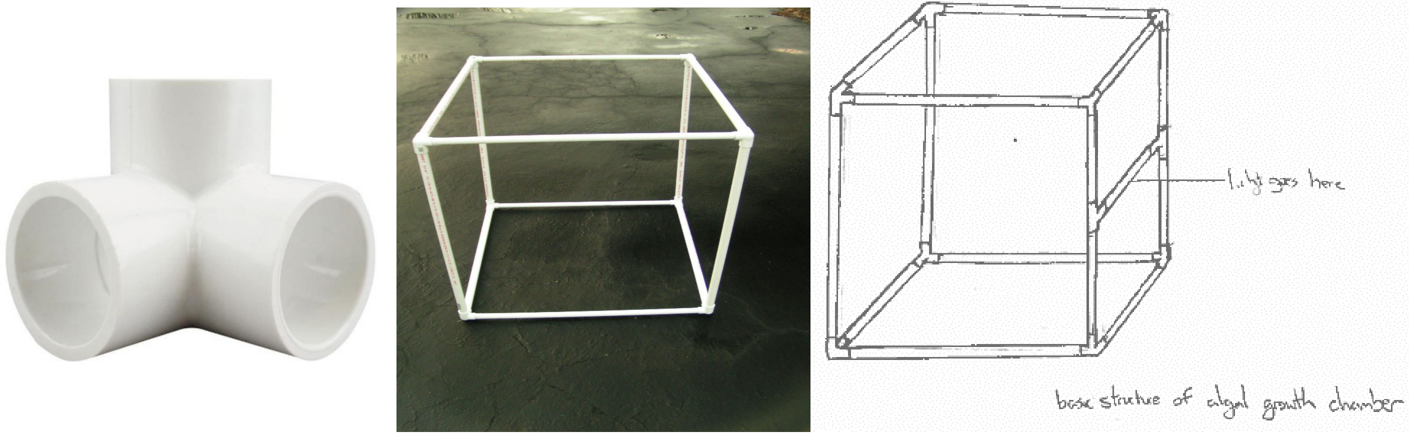


Figure 11. Step 5 of Classroom VEGGIE assembly: Assemble VEGGIE frame by inserting 13 ¾” PVC pipes (18” long) into 8 ¾” side outlet elbows[28] (left) to create a cube. Finished frame is shown in the middle image**.**

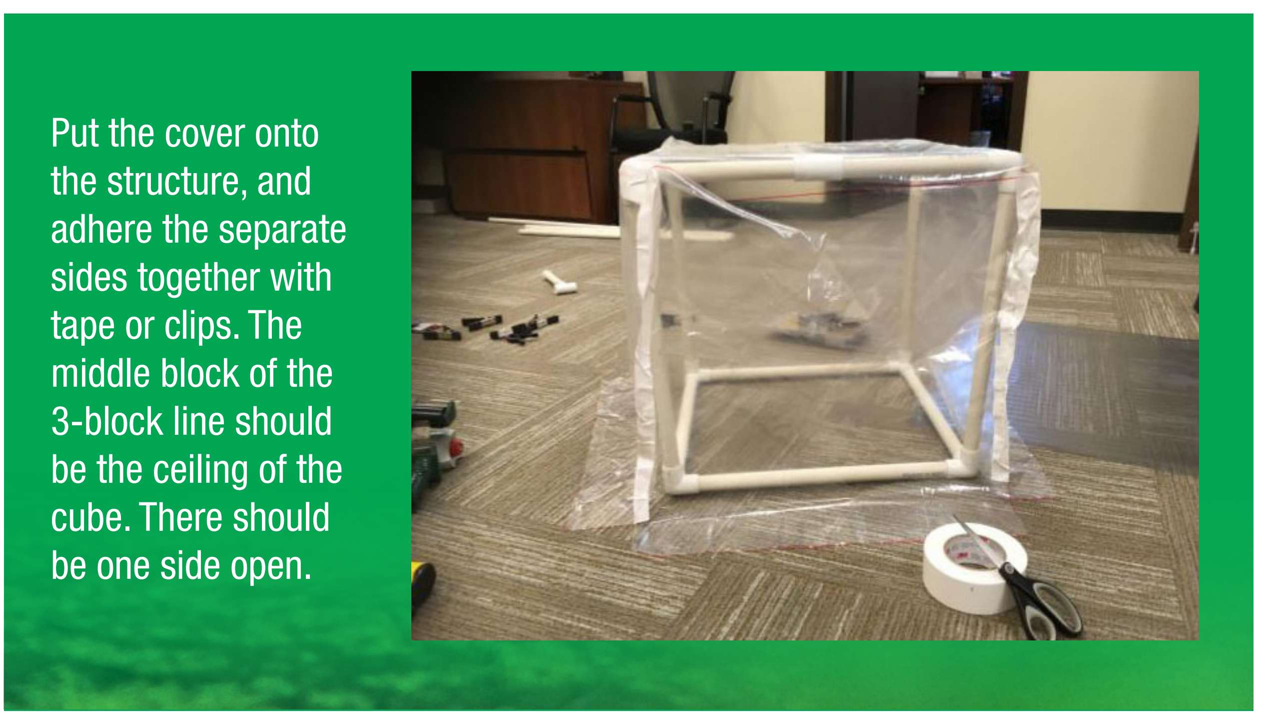


Figure 12. Step 6 of Classroom VEGGIE assembly:
Attach modified shower curtain to PVC frame (as described above).

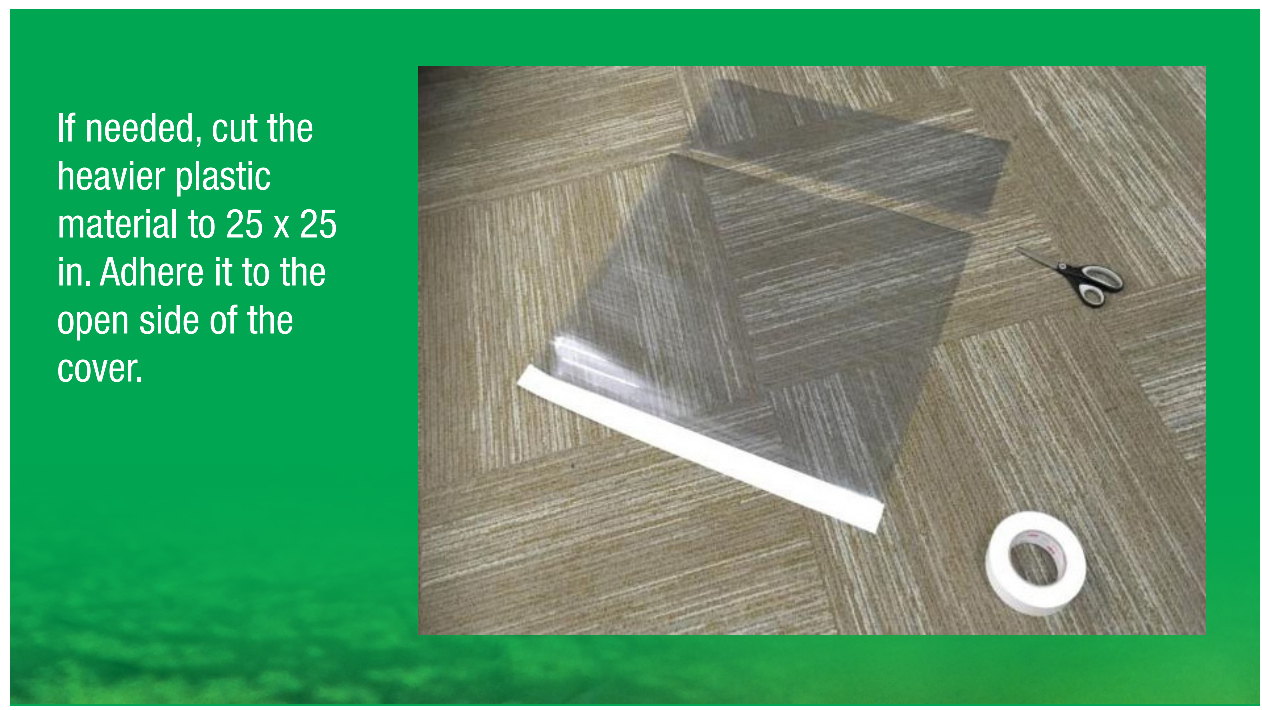


Figure 13. Step 7 of Classroom VEGGIE assembly: Assemble and install Classroom VEGGIE door;
Slit door flap for easy open and close

Figure 14. Step 8 of Classroom VEGGIE assembly: Attach velcro to Classroom VEGGIE door.

Figure 15. Assembled Classroom VEGGIE unit.

The experiments you will be performing are based on the experiments carried out by ISS crew and will vary depending upon the hypothesis that you, your classmates and/or your teacher would like to test. Examples of such experiments will be discussed later. For now, take a look at Figure 16 and begin to think about possibilities. What type of light will you use to stress the algae? How far will you position that light from the algae samples? How many algae cultures will you grow in your VEGGIE unit and what will the differences be between them so that you can answer your question of interest?

The goal here is not to provide you with a "step-by-step" set of experiments to perform. Instead, the goal is for you to be a scientist, design and perform one or more quality experiments, and add to the insights gained by the ISS algae mission being carried out in 2019. Following the conclusion of the ISS experimental mission, the algae payload will be returned to Earth in cold stowage, after which a fraction of it will by **cryopreserved** (*i.e.*, frozen in liquid nitrogen to save it for future applications). The remaining "space algae" will be made available to educational institutions (middle schools, high schools, colleges and universities) so that teachers and professors around the United States can make it available for their students to conduct further research.

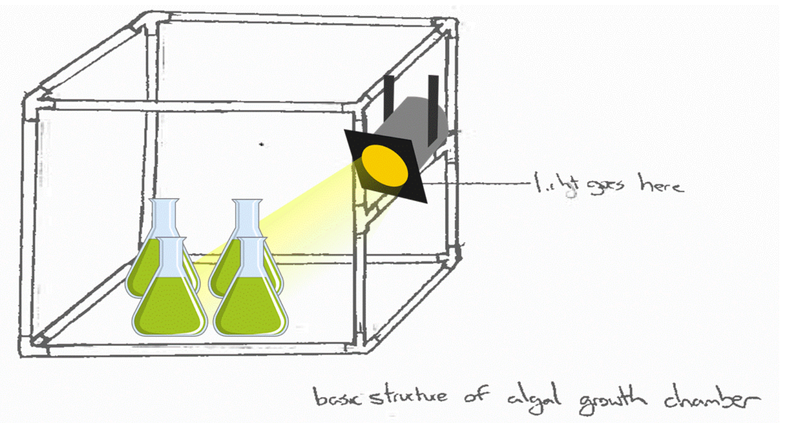


Figure 16. Addition of light and (optional) support bar to Classroom VEGGIE unit. To add the light bar, cut the rear two vertical PVC pipes with a PVC cutter and connect them with two ¾" tees, as shown in the picture. The light can then be clipped to this rear bar and the algae flasks/bags can be positioned accordingly. A small clamp lamp[29] (such as the one shown on Figure 16's right), can be obtained at your local hardware or department store and works well with this setup.

MICROALGAE PROJECT TWO

Project II

Establishing Optimal Lighting Conditions

One thing that all algae species have in common is that they are **autotrophs**, which is a fancy scientific word that means “self-feeder.” What this means is autotrophs are organisms that make their own food. The vast majority of known autotrophs, including all algae species, do so via a process known as **photosynthesis** (which literally means “making with light”). All photosynthetic organisms (including algae) require light, water, trace nutrients and carbon dioxide to power the processes of life. Through photosynthesis, algae convert CO_2 into glucose and other sugars. These sugars are then broken down into lipids, which serve as source material to produce membranes for new algal cells. If the algae are starved of nutrients, the fatty acids are converted into lipids. Astaxanthin is derived from lipids and Pluvi generates astaxanthin when stressed by conditions such as nutrient deprivation.

In order to understand the basics of photosynthesis, we have to understand the properties of light itself and how these properties are exploited by algae to make food.

The **electromagnetic spectrum** (Figure 17) describes and categorizes the range of wavelengths over which electromagnetic radiation extends. Interestingly, when most people think about light, they think about the visible light that our eyes are capable of detecting. A quick glance at the electromagnetic spectrum diagram indicates that this portion of the spectrum is a very small fraction of the whole. Similarly, the fraction of the spectrum that is useful to photosynthetic organisms such as algae is comparably small. Biologists refer to the fraction of the electromagnetic spectrum that powers photosynthesis as **photosynthetically active radiation (PAR)**.

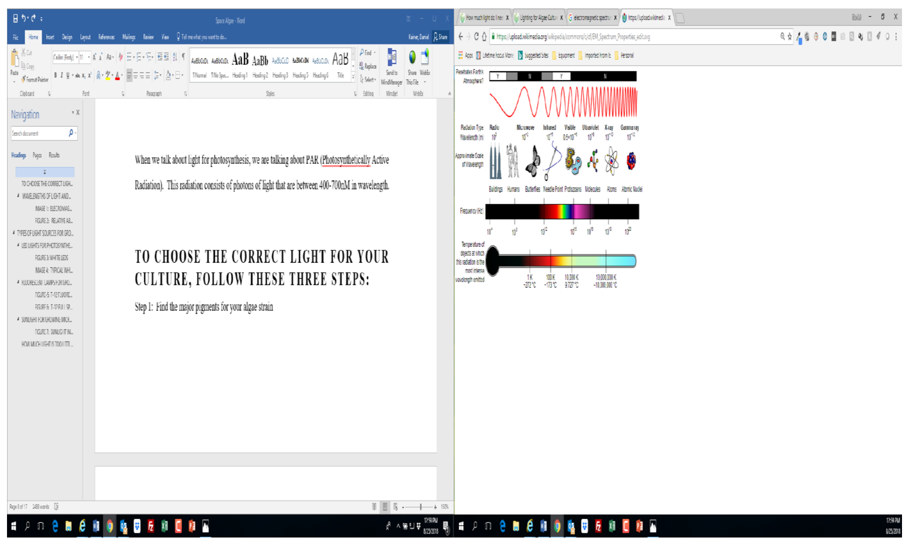


Figure 17. The Electromagnetic Spectrum.[30] Additional information and activities related to determining appropriate wavelengths of light can be found in Appendix A. Determining appropriate light intensity, resources and activities are available in Appendix B.

Determining appropriate light intensity

1) To get started, you'll need to determine, through researching the scientific literature, the optimal light intensity at which the strain of interest thrives. According to UTEX.org (a very comprehensive algae resource hosted by the University of Texas), Pluvi should be exposed to a maximum of 3200 **lux** with a 12 hours light/12 hours dark cycle. The easiest way to ensure that your algae are exposed to a cycle of appropriate duration would be to add a simple timer capable of controlling how long the light is on and how long the light is off. A simple timer capable of doing exactly this can be obtained from your local hardware store for $7-$10. Understanding the various ways to measure light is challenging, but let's give it a try. If this turns out to be too challenging, no worries – a light meter (available for as little as $20) can give you a direct measurement of light and you can adjust accordingly!

   a. A lux is a measurement of light intensity that corresponds to one **lumen** per square meter.

   b. A lumen (lm) is a measure of the total quantity of *visible* light (~400-800 nm) emitted by a light source.

      i. A lumen is the measurement of **luminous flux**. Flux is the amount "stuff" that passes perpendicularly through a surface in a given time. Luminous flux refers to the amount of visible light passing through a surface and can be thought of as the rate at which energy from visible light travels through a surface. The more energy from visible light that passes through the surface, the greater the luminous flux. The more direct that light passes through the surface (optimal angle is perpendicular to the surface) the greater the luminous flux.

      ii. **Radiant flux** is the flux for all electromagnetic radiation (light). Luminous flux is different from radiant flux because luminous flux only includes the spectrum of the electromagnetic radiation that the human eye detects (visible light).

      iii. Finally, the relationship between the intensity of the light projected onto a surface is inversely proportional to the square of the distance from the light source, a relationship known as the **inverse square law** (illustrated in Figure 18). Simply put, this law describes the phenomenon observed when, for example, doubling the distance between a light source and a target, one reduces the light intensity by 4 (since $2^2=4$)

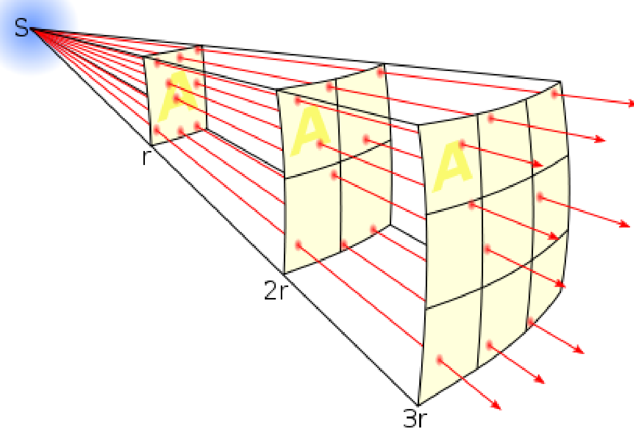


Figure 18. The inverse square law[32]: light intensity reduces inversely by the square of the distance from the light source.

iv. Let's sum up what we need to know for our Pluvi (remember Pluvi?) experiment, with respect to light intensity:

   a. When we double the distance between our light source and our Pluvi target, we reduce the light intensity (and therefore the number of lumens per unit area).

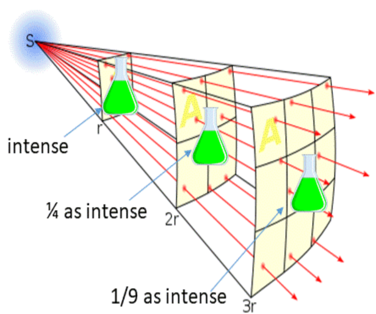


Figure 19. The light intensity received by Pluvi will dramatically decrease as its container is moved away from a light source because of the inverse square law

   b. You can typically find the number of lumens generated by the bulb that you choose to use as your light source for the experiment. Try to use these concepts to find the optimal intensity for your Pluvi. Your class can even do an experiment testing the inverse square law and recording its effects on Pluvi growth rates!

   c. When it comes to light sources your main choices are shown in Figure 20.

Sunlight	Incandescent	Fluorescent	LED

Figure 20. Types of light sources that are suitable for Pluvi experiments[34,35,36,37]

Because typical examples of each of these sources are full spectrum, they should each work for growing all the types of algae, including Pluvi, as long as the intensity isn't too high and/or they don't produce mainly green light. You may want to base your decision on what is already available or most easily obtainable.

Light-Based Algae Experiments

In the following open-ended protocols students will conduct controlled experiments to formulate and test individual hypotheses about light requirements affecting the growth of Pluvi.

Here are some sample hypotheses:

- Does the type of light source influence Pluvi's growth characteristics?
- What light intensity level provides optimal Pluvi growth?
- Are there different light requirements for supporting maximal growth versus astaxanthin production in Pluvi?

Review the light requirements for Pluvi growth and design an experiment and hypothesis to predict the growth characteristics of Pluvi under different types of lighting environments.

Materials (per experiment). Multiply the amount shown in the following table by the number of experimental set ups desired to determine the total amount of each item required.

Item	**Quantity** *(see note above)*
Haematococcus pluvialis culture	1 (variable size; determine based on goals)
Quart-sized zip seal bags	1 per culture
500 mL bottle of MES-Volvox (or BG-11) medium	1
Sterile tubes for initiating culture	3-5
Optional	
Aquarium air pump or air stone	1

1. Obtain a sample of *Haematococcus pluvialis* from UTEX: (https://utex.org/products/mes-volvox-medium) or a biological/scientific supplier. An internet search will provide additional options.
2. Grow culture in MES-Volvox medium in indirect sunlight or gentle lighting until the culture takes on a noticeable green hue. To ensure culture viability, start your first culture in only a few milliliters of medium. Use a "stair step approach" where each time you expand the culture, you inoculate in a volume no more than ten-fold the volume of the inoculation volume:
    a. Example: Inoculate 1 mL of dark green algae culture into 9 ml of medium (total of 10 mL).
    b. Once this culture becomes dark green, inoculate 5 mL of this culture into 45 mL of medium (50 mL total). Repeat this process until you have enough algae to initiate your experiment.
    c. Optimally, the experiment should occur in the classroom VEGGIE unit described in Project I. If not, identify a relatively undisturbed area in the classroom where the culture will experience similar conditions over the course of the experiment.
    d. To best simulate the VEGGIE environment, you can grow the algae in a large plastic bag.

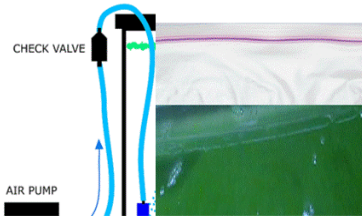


Figure 21. Schematic of Culture Bag/Air Pump Setup for Classroom VEGGIE unit (image created by the author specifically for this publication)

e. The closest simulation would be to leave it open slightly so a tube from an aquarium pump can be inserted and continuously bubble the culture (as shown in Figure 21). However, this creates a risk of spillage, so only choose this option if you think you can avoid a spill.

f. If you are worried about a spill, leave the bag completely sealed (make sure there is an air gap of at least half of the total volume in the bag). Once or twice a day, open the bag and let some new air in. Afterwards, seal the bag and gently rock the culture back and forth. This will both agitate and aerate the culture, thereby helping to ensure the health of the culture.

g. Take a picture of the culture every 1-2 days making note of the time. When comparing different cultures under different light conditions, compare the intensity of the green culture. Remember, darker green means more algae growth than a lighter green culture!

h. Monitor the growth of the algae for 25 days. If necessary, add a small amount of MES-Volvox medium to replenish nutrients.

Before moving on, a brief discussion of modeling the culture conditions aboard the ISS is in order. The main thing to keep in mind is that when studying something as complex as a living organism, there are many variables that can impact how it behaves in an experiment. As a result, the more differences there are between the setup in the “Classroom VEGGIE” and the ISS VEGGIE, the less confidence you will have when you try to compare your experimental results with those of the crew. While the gravitational differences between your classroom and the Space Station are pretty much a given, there are many variables that you *can* control, including:

- Keeping your culture on a 12 hours light/12 hours dark cycle
- Agitating your culture occasionally:
  - » If possible, aerate your culture with a small aquarium pump and air stone
  - » If you decide to keep your bags sealed to avoid spills, you should “poke” the bag occasionally, by opening it slightly and gently agitating the liquid inside by rocking it back and forth (without spilling it of course!)

MICROALGAE
PROJECT THREE

Project III

Determining Approximate Pluvi Culture Concentrations

So far, the density of the Pluvi cultures has simply been estimated by evaluating the “greenness” of the culture and evaluating whether or not the intensity of the green color is increasing over time. Because Pluvi is a green alga, a culture that demonstrates an increasing color intensity is a sign that it is growing and healthy. A culture that maintains a constant green intensity is not growing, and a culture that exhibits a decreasing green intensity is probably dying, because dead algal cells break down the chlorophyll pigments that give cells their green color in the first place. While this approach is a good approximation to culture behavior, it is a completely qualitative approach, which means that it can only provide relative data in terms of such things as one culture being more dense than another, or a particular culture changing its density relative to past time points. If this approach is taken, take photographs of the culture(s) over time and display those photographs side by side with the time points clearly indicated.

One simple method to change cell density measurements from qualitative to semi-quantitative (i.e., measurements that provide an approximation of the actual measurement of culture density, albeit at a relatively low level of precision) involves the use of a Secchi disk (Figure 22). Secchi disks are opaque disks attached to a ruler or tape measure used to estimate the transparency of water by measuring the depth at which the disk disappears from view from the surface. These disks were created in 1865 by Angelo Secchi[38] and are typically attached to a long rope for measuring the clear depth of bodies of water. They can be modified for use in small algae cultures by attaching a small version of this disk to a plastic ruler (Figure 22).

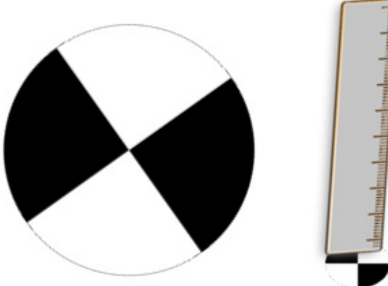


Figure 22. Secchi Disk. An internet search will provide a printable Secchi disk or you can make your own. Attach the disk, at a right angle, to a small plastic ruler, as shown on the image (modified from[39]) on the right.

In order to develop a proper semi-quantitative assay using a Secchi disk, you will need to have enough culture to perform dilutions and measure each diluted sample with the Secchi disk. This process will be much easier with the following materials:

1. 50 mL centrifuge tube[40] and rack[41]. They are inexpensive and available online.

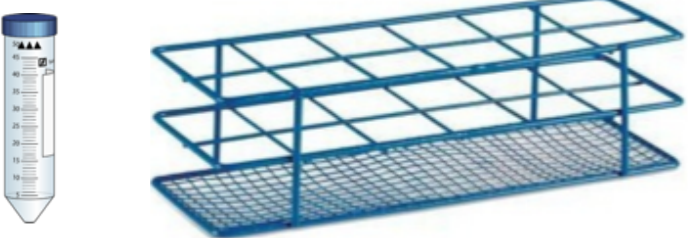


Figure 23. Centrifuge tube and rack.

2. Secchi disk attached to ruler (Figure 22)
3. Distilled water (available from your local grocery store)

Materials

Item	Quantity
50 mL centrifuge tubes	5 per culture to be measured
Test tube rack	1
Plastic ruler	1
Secchi disk (printed)	1
Distilled water	1 large bottle

Follow these steps:

1. Label 5 tubes: 1X, 0.8X, 0.6X, 0.4X & 0.2X
2. Add the following amounts of distilled water to the tubes:

Tube	mL Distilled Wa-ter	mL Pluvi	Depth of Secchi Disk Disappearance
1X	0	50	
0.8X	10	40	
0.6X	20	30	
0.4X	30	20	
0.2X	40	10	

3. Lower the Secchi disk into each tube until you are no longer able to see the white and black surface of the disk. Record the depth at which it disappears in each tube.
4. Plot a graph and demonstrate the relationship between the relative concentration of algae in each tube and the depth at which the Secchi disk disappeared. Your graph should look something like this:

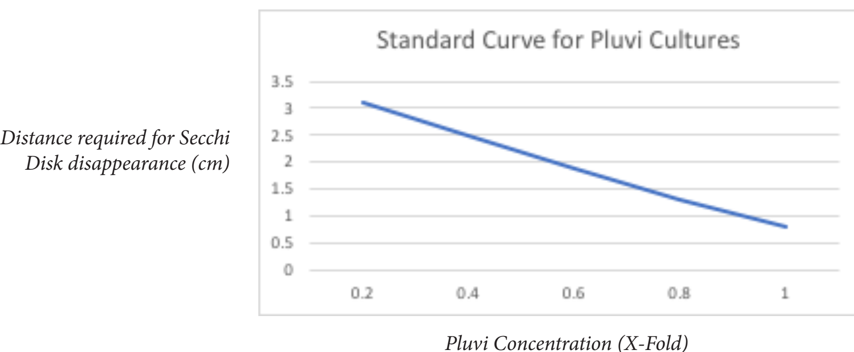


Figure 24. Standard curve graph.

5. The graph is known as a standard curve, which is a graph that plots the relationship between (in this case) relative Pluvi concentrations and the depth at which the Secchi disk disappears.

6. This curve will allow you to estimate the concentration (relative to the reference 1X culture) for additional cultures going forward. You can estimate via the process of interpolation, or estimating data points that fall within a range that has already been graphed:

   a. You have a culture of unknown concentration and test it with the Secchi disk.

   b. The Secchi disk disappears when you submerge it 2 cm below the surface.

   c. You then estimate the concentration of this tube via interpolation:

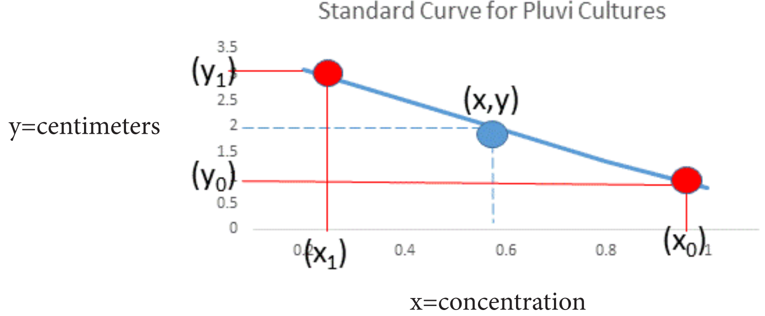


Figure 25. Standard curve for Pluvi cultures.
Curve used to estimate the relative Pluvi concentrations.

   d. As shown in the above figure, you can simply estimate the concentration by approximating where the dotted line hits the x axis

   e. The concentration of Pluvi in the previously unknown sample is approximately 0.58X, based on a visual inspection of where the interpolation line (blue dotted line) crosses the x axis

   f. Conversely, if you want a little bit more precision, you can determine the value mathematically:

   i. Choose two points towards either end of the standard curve (red dots)

      ii. These points have (x,y) values and the slope of the line can be determined

      iii. $\frac{x-x_0}{y-y_0} = \frac{x_1-x_0}{y_1-y_0}$, $x = x_0 + (y-y_0)\frac{x_1-x_0}{y_1-y_0} = \frac{x_0(y_1-y)+x_1(y-y_0)}{y_1-y_0}$

MICROALGAE
PROJECT FOUR

Project IV

Determining Conditions for Astaxanthin Production in Pluvi

Astaxanthin belongs to a larger class of chemical compounds known as terpenes[42], which are essential precursors to what are known as **essential oils** (*e.g.*, ginger, lavender, mint, etc.). Essential oils tend to have an aroma that people associate with the plant from which they are derived. Next time you get a chance, go smell a mint or lavender plant. Do you like what you smell? You are smelling the unique combination of terpenes that the plant synthesizes. Terpenes are synthesized in many different types of organisms, but plants and algae exhibit a much greater diversity of terpene production than any other class of organisms.

Although there are many thousands of different terpene molecules in nature, they all derive from a precursor molecule: **isopentenyl diphosphate (IPP)**. All carotenoids, the subclass of terpenes that astaxanthin belongs to, derive from IPP and its isomer, dimethylallyl diphosphate (aka dimethylallyl pyrophosphate)[43].

CH_3 H_2C O O P O P O O O^- O^- O^- ↔ CH_3 H_2C O O P O P O O O^- O^- O^-

Figure 26. (left) Isopentenyl diphosphate[44] (sometimes referred to as isopentenyl pyrophosphate) structure. Also referred to as IPP, (right) dimethylallyl diphosphate[45] (sometimes referred to as dimethylallyl pyrophosphate or DMAPP), which is a natural isomer of IPP.

Although the specific details of terpene synthesis are complex, essentially what happens is that these molecules assemble, one five-carbon unit at a time, in a "head to head" fashion. Interestingly, an important rule of chemistry that helps scientists make sense of how this huge and diverse class of molecules can be assembled is known as the **isoprene rule**[46], which simply states that the five carbon compounds described here (known, conveniently, as **isoprenes**) always attach "head to tail" and never "head to head" or "tail to tail." This basic principle is shown below in Figure 27. You may notice that this "isoprene rule" is violated in the molecule (and important micronutrient) known as beta carotene because there is a "tail to tail" attachment where the C4 carbon of one isoprene bonds with the C4 carbon of the adjacent isoprene. This "violation" of the isoprene rule causes beta carotene to be classified as an **irregular terpene**. We point this out because beta carotene is a precursor for astaxanthin.

Figure 27[46] (top left) An isoprene showing its head (carbon #1) and tail (carbon #4). The isoprene rule states that only "head to head" assembly of terpenes occur, such as in (top middle) myrcene or in (top right) the ringed terpene limonene.

Head Tail C_1 C_2 C_3 C_4 C_5

C1 C4 CH_2 H_3C CH_3 Myrcene

CH_3 C1 C4 C4 C3 H_3C CH_2 Limonene

β-Carotene

The last bit of biochemistry (yep, did you realize that you were learning some pretty challenging biochemistry while learning about Pluvi?) that we need to discuss is the steps involved in the conversion of beta carotene into astaxanthin. Relax, there are only 4 changes required. Can you spot them in Figure 28? The two double bonded oxygen atoms are what are known as **ketones** and enzymes known as **ketolases** catalyze their addition. The two –OH groups are known as **hydroxyl** groups and are added thanks to the action of enzymes known as **hydroxylases**. The specific enzymes that carry out these modifications differ between organisms that make astaxanthin. In Pluvi's case, it appears that the first two modifications involve ketolases in the chloroplast, while the second two steps involve hydroxylases in the cytoplasm. This appears to be the reason why the entire cell becomes red – it fills up with newly-synthesized astaxanthin!

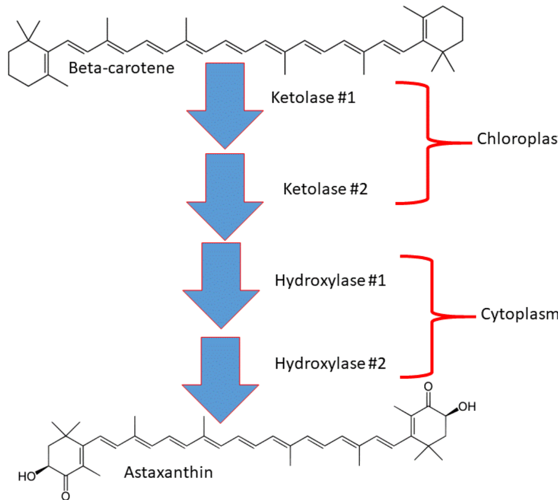


Figure 28. Pathway for astaxanthin production from beta-carotene in Pluvi[47].

ISS Experiment Ground Study: Triggering and Detecting Astaxanthin Production in Pluvi

The crew will be following this plan for 25 days during their mission. Plan accordingly if you want to do your experiments in parallel.

<u>Events</u>

1. Algae unloaded and placed into NODE 1 each morning
2. Algae is stored in a cargo transfer bag at the end of each day to mimic a night schedule
3. While on orbit, photographs will be sent every fourth day
4. Color change expected by 20th day on orbit
5. On Day 25, algae is to be packed up and placed into cold stowage waiting to be sent back to ground

The ISS astronauts will be testing their hypothesis using a single variable: the microgravity environment. Exposing Pluvi to that environment will serve as a stressor that will induce astaxanthin synthesis. If you want to mimic the ISS experiment here on the ground, you'll do things the same as the crew, with the difference being 1G versus MicroG. We recommend that you set up a corresponding sample with no other stressors than exposure to gravity to compare to the astronauts' sample. Then create additional samples to expose your Pluvi to different stresses. You also have the freedom to test multiple variables on a single sample. Examples of stressors which people have been able to use to trigger astaxanthin production in Pluvi include:

1. High light intensity
   a. Look at changing the distance between the light source and the algae and/or the type of lighting used between your growing culture and your stressed culture.
2. High salt (However, not so much that it kills your algae!)
   a. You can try a "dose-response" curve for salt, which will require separate small cultures, each with a different amount of salt added to the MES-Volvox medium.
3. Nitrogen (or other nutrient) deprivation
   a. You could grow your Pluvi in BG-11 medium instead of MES-Volvox. Go to https://utex.org/products/bg-11-medium for either ordering information or recipes to make it yourself.
   b. You could then stress your Pluvi in BG-11 (-N) medium. Go to https://utex.org/products/bg-11-n-medium for either ordering information or recipes to make it yourself.
4. Elevated temperature (Again, not so high that your poor Pluvi dies!)
   a. You will have to get creative for this approach. If your school has an old incubator, you could culture your control Pluvi in your VEGGIE chamber.
   b. Your stressed algae could then grow in your incubator (making sure to get as many non-temperature conditions as close to those in the VEGGIE unit as possible).

In this section you will decide on the stresses to which you'll expose your Pluvi. These experiments are open-ended, so you will be directly involved in the experimental design. As such, this materials list is designed to simply be a guide. You will need to determine the amounts and types of materials that fit the experiment that *you* design.

Materials

Item	Quantity
Light source (narrow wavelength or higher intensity bulb, depending on variable being tested)	1 per culture
Salt – for stress tests to try to induce astaxanthin production (NaCl or KCl). You should do a "dose response" so you will need to prepare a few batches of media, each with a different salt concentration	1 bottle NaCl and/or KCl

Nitrogen deprivation – the easiest approach for this would be to purchase 1 bottle of BG-11 medium and 1 bottle of BG-11 (-N) medium from UTEX.	1 bottle BG-11 & 1 bottle BG-11(-N)
Temperature – small incubator, small refrigerator, classroom space for room temperature incubation	To be determined based on particular experimental requirements

To do a proper experiment, you will likely need to have multiple cultures/bags of Pluvi placed under different specific conditions. One thing that you might not have taken into consideration is that different algae-containing bags tend to look the same as each other and must therefore be properly labeled. It is equally important to keep documentation during your experimentation that includes the date, procedures, and any notes, photos, or observations you choose to include.

MicroAlgae Culture Bags

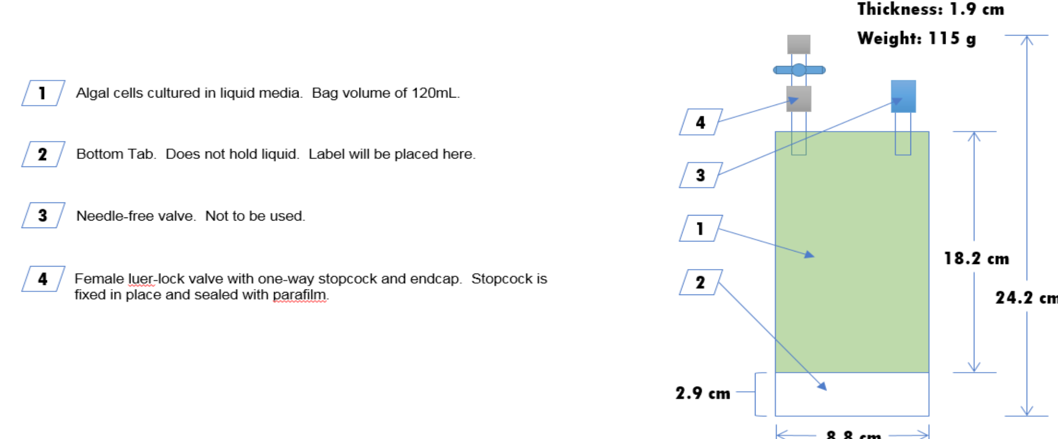


Figure 29. Schematic of bags to be used on ISS mission to contain Pluvi (provided by NASA's Microalgae mission planning team members)

Experiment Timeline

Day	Procedure	Data to Collect (photos, video, measurements, etc.
1		

Figure 30. Sample research log for documenting your experiments.

MICROALGAE PROJECT FIVE

Project V

Advanced Algae Quantitation Experiments

In this section we will discuss some simplified versions of methods that professional algae scientists (also known as **phycologists**) engage in every day. As you know, scientists spend much of their time taking measurements of the objects of their study. Such measurements, however, can be challenging when working with an organism like Pluvi, which is only approximately 5 microns (10^{-6} m) in diameter. In this section, we will discuss three methods for estimating algae growth in a culture: direct cell counting, spectrophotometry and dry weight measurements. The equipment and materials listed may not be common to most high school or community colleges. You may want to investigate whether your local colleges/universities or industries have a loan program.

Materials

Direct cell counts	Quantity
Compound light microscope	1
Hemocytometer	1
Spectrophotometry	**Quantity**
Visible spectrophotometer	1
Cuvette	6-12
Dry Weight	**Quantity**
Drying oven	1
Single pan balance	1
Physiological saline (0.9% saline solution)	500 mL
Photosynthetic Pigment Quantitation	**Quantity**
Filter paper	3-5
95% ethanol or 90% acetone	1 L
Chromatography tank	1
TLC plates (silica gel)	1-5
#2 pencil	1
Capillary tubes or Pasteur pipettes	1 box

A **hemocytometer** (Figure 31) is a thick glass microscope slide with a gridded depression that helps facilitate the accurate counting of cells. This device, as its name implies, was originally designed for blood cell counting, but its applications have expanded to most types of animal cell culture, as well as algae cultivation.

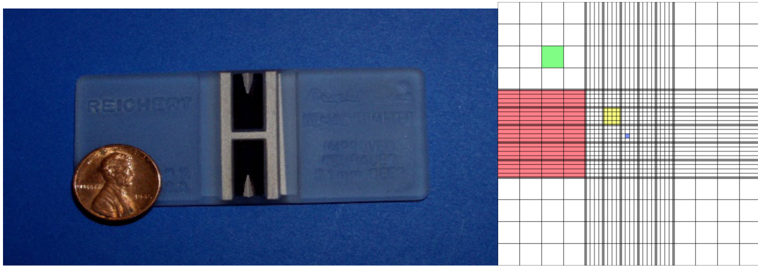


Figure 31. Hemocytometer used for algal cell counts. (Left) Hemocytometer slide with penny as a size reference. (Right) Schematic showing embedded grid on hemocytometer slide that helps with accurate counting.

Professional grade hemocytometers can be rather expensive, but lower quality models that are perfectly well-suited for the projects discussed here are available for less than $20 on online. Proper use is outlined below:

1) Clean the hemocytometer slide with ethanol solution. **Important**: *Don't* wipe the slide with abrasive cloths, even paper towels! Instead, gently *dab* the slide with lens paper (that doesn't scratch) until the ethanol is removed from the slide. Be careful with this step – hemocytometers are easily scratched, especially on the grid region shown in Figure 31.

2) Place a glass cover slip directly over the grid region of the slide.

3) Resuspend your algae culture by gently pipetting up and down several times.

4) Take a small amount of homogeneous culture in a pipette. Place the tip of the pipette over the indentation and gently add suspended culture until the grid region is filled under the cover slip.

5) Rotate the hemocytometer and repeat steps 3 and 4 on the other side of the grid. This will allow you to take two measurements and either gain confidence about the accuracy of your count or tip you off into starting over if your counts are wildly different.

6) Place the hemocytometer on the stage of a microscope and find the highest magnification that you can see both the grid and the algal cells clearly in.

7) Only count the cells in each set of sixteen corner squares.

8) Average the corner cell counts.

9) Multiply the average count by 10,000 (10^4).

10) Repeat steps 7-9 for the second grid. If the two results are similar, take the average of the two results. If they are very different, you most likely made a mistake with re-suspending your culture, adding the sample to the slide or something else. In this case you should repeat the entire process so you can be confident in your counts.

Cell counts, as described above, is a highly visual method for estimating the state of your culture. If you carry out this procedure every day or two, you will be able to track the growth of your culture. Like any other microorganism, algae will **plateau**, or cease to increase in number any further due to consumption of nutrients, accumulation of waste products, etc.

If cultures remain in a plateau phase for too long, they will likely deteriorate or even crash. Therefore, monitoring of cultures in this way can help ensure that cultures are tended to on an appropriate schedule. When this happens, cultures should be harvested, fed and/or split, depending on the goals of the project.

A **spectrophotometer** is an instrument that measures the intensity of light of a specific wavelength that strikes a detector after it passes through a sample. Many organic molecules, including many that are important in biology, absorb specific wavelengths of visible or ultraviolet light very efficiently. This happens because wavelengths of light are associated with amounts of energy and, because of the specific fashion by which particular molecules' electrons are arranged into orbitals. Certain amounts of energy will excite electrons from an orbital of lower energy to an orbital of higher energy. Since all matter contains atoms and all atoms contain electrons and all electrons are in orbitals of energy levels, there will be some specific wavelengths of light that contain just the right amount of energy needed to excite one or more electrons in a particular molecule.

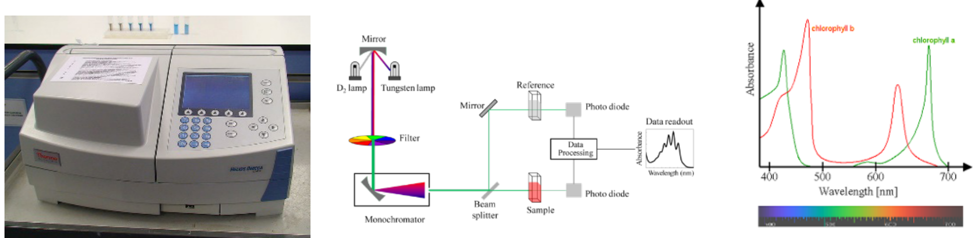


Figure 32. Spectrophotometry. (Left) Photograph of a typical, benchtop, laboratory-based spectrophotometer. (Middle) Schematic of spectrophotometer functionality, from light generation to wavelength selection to light detection and conversion of raw data into an analyzable signal. (Right) Chlorophyll a and b absorption spectrum.

The specific wavelength that molecules absorb efficiently can be determined by generating an **absorption** spectrum (Figure 32, right). This spectrum is generated by focusing a range of wavelengths through a sample containing the molecule to be measured and recording the absorbance of light at each wavelength. Simple spectrophotometers require this process to be done manually, but more sophisticated models can carry out a **wavescan,** whereby the wavelengths are generated and the absorbance levels recorded automatically.

As shown in Figure 32, chlorophyll a and chlorophyll b have slightly different absorption spectra. The peaks that are generated by this process demonstrate the wavelengths that correspond to the **absorption maxima**, or wavelengths that correspond to maximum light absorption, of these molecules. The wavelength that corresponds to the absorption maximum for a molecule is typically the wavelength that is used to measure the concentration of a molecule in a spectrophotometric assay. The amount of light that is absorbed by the sample at this absorption maximal wavelength is proportional to the concentration of the substance being analyzed. A simplified procedure is outlined below:

1) Pipette enough of the algae sample from the same source that you used for the hemocytometer analysis to fill approximately 80% of a clean cuvette that is compatible with your spectrophotometer.

2) When you are trying to determine the concentration of algae using a spectrophotometer, you are actually measuring the absorbance of chlorophyll in the living cells. Interestingly, chlorophyll exhibits different absorbance properties depending upon its specific environment. Setting your spectrophotometer to read at 670 nm should give you a reasonably good measurement of your algae culture.

3) Use 1 ml of the culture medium as blank. Consult your spectrophotometer instructions on how to do this with the particular model you are using. This will ensure that if the medium itself has any substance(s) that also absorb at 670 nm, they won't contribute to the overall absorbance and therefore won't give you an inaccurate measurement of your algae.

4) Record the absorbance reading as "Day 0" in a lab notebook. If you see that your spectrophotometer is reading 1.8 or higher, your sample is too concentrated and needs to be diluted. You can dilute your sample with distilled water and repeat the process until you have a steady reading that is well under 1.8.

5) Repeat this process each day and monitor the growth of your sample. If you couple the spectrophotometer data with hemocytometer data, you will eventually be able to correlate spectrophotometric absorbance with cell counts.

The final method to quantitate your algae culture is to measure its **dry weight**. This measurement could be particularly relevant for a species like Pluvi because the total amount of dry weight could correspond to the amount of astaxanthin being produced. The process is as follows:

1) Weigh an empty centrifuge tube (with lid) and record the value.

2) Take a sample of algae and place it in the tube and secure the screw top lid.

3) Balance the centrifuge by placing a tube with a similar amount of water in a tube directly across the rotor from your sample. If you don't do this, the centrifuge will be unbalanced and that can be dangerous – for either the centrifuge or even the people in the room, depending upon the speed that the centrifuge is spinning!

4) Following centrifugation, decant the supernatant, rinse the remaining pellet with 0.9% saline solution (*i.e.*, physiological saline).

5) Resuspend the algae pellet in the saline solution by gently pipetting it up and down and repeat steps 1 and 2.

6) After this washing step, decant the supernatant and place the sample in a heated incubator (adjusted to 42° C). Dry the algae pellet for 48-72 hours.

7) Weigh this tube with your dried algae sample (with screw cap on) on a single pan balance and record the weight.

8) Subtract the weight of the empty tube obtained in step one from the total weight of the tube plus algae obtained in step 7. This value corresponds to the weight of the dried algae biomass.

Record the values of your hemocytometer, spectrophotometric and/or dried biomass measurements together in a lab notebook. If you continue to do this over time, you will not only observe how your culture behaves via these three separate, but related, parameters, but you will see how the different measurements correlate with each other.

Analysis of Algal Pigments with Thin-Layer Chromatography (TLC)

Chromatography is a collection of techniques designed to separate molecules in a complex mixture based on the physical and chemical properties associated with those molecules. There are numerous types of chromatographic systems. These range from low-cost simple paper chromatography methods to sophisticated (and expensive!) high performance liquid chromatography-mass spectrometry systems that cost hundreds of thousands of dollars. What all of these systems have in common is that they exploit the differential interaction of molecules with the components of a **stationary phase** (*i.e.*, a component of a system that does not move) with a **mobile phase** (*i.e.*, a component of a system that does move).

One of the more convenient and low-cost types of chromatography that illustrates this mobile phase/stationary phase interaction quite well is called **thin-layer chromatography** (TLC). TLC consists of a solid plate coated in some type of thin coating, which serves as the stationary phase and an organic solvent that moves along the plate and serves as a mobile phase. The plate is placed upright in a chamber and a small amount of organic solvent is added to the bottom of the tank (Figure 33). Capillary action drives the movement of the organic solvent up the plate.

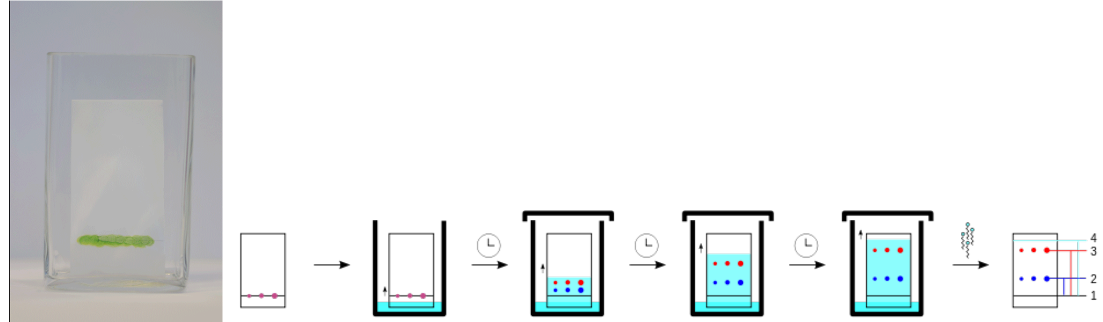
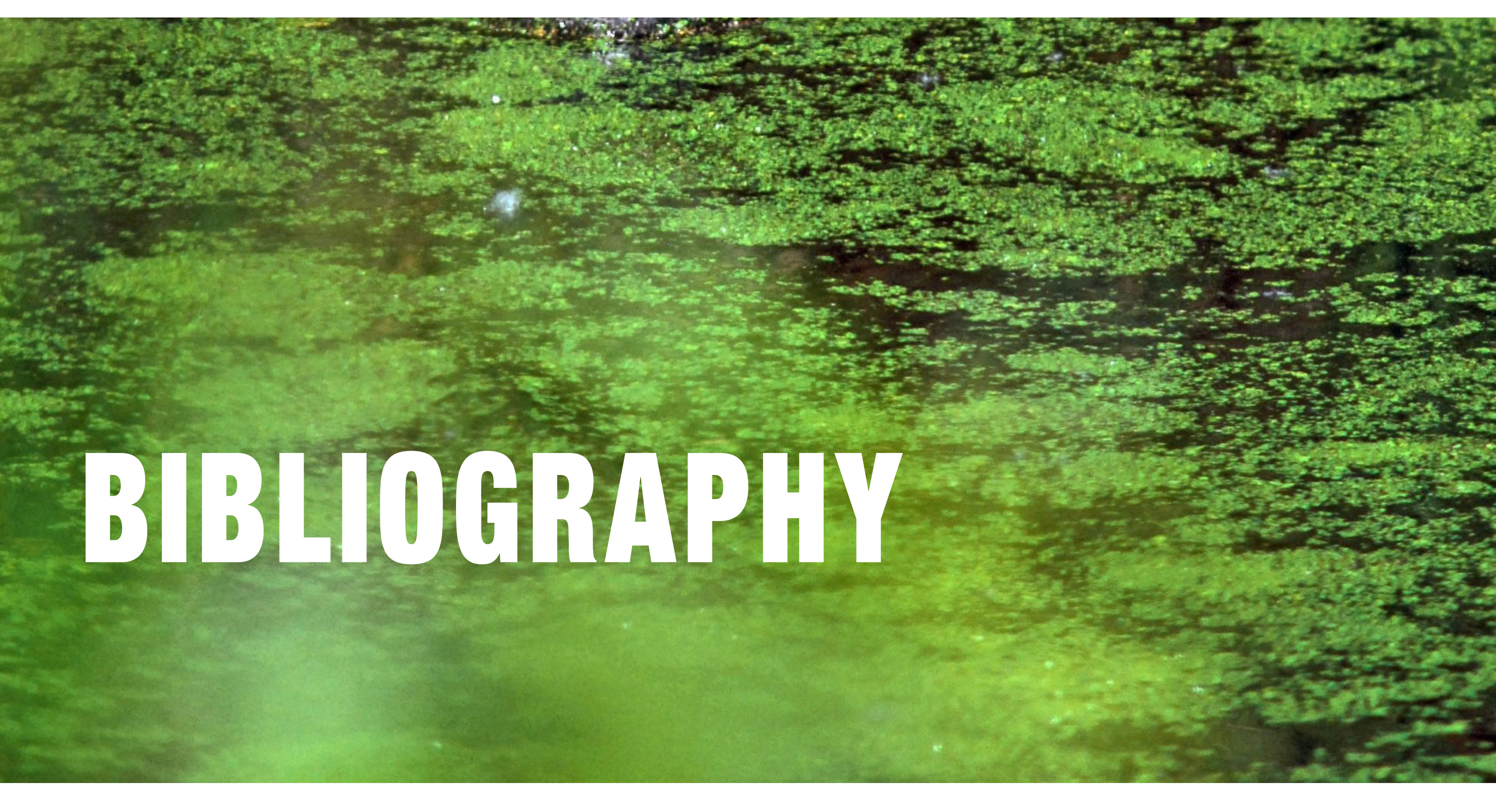


Figure 33. Thin-Layer Chromatography setup. (Left) TLC tank and plate.
(Right) Schematic of TLC separation principles.

Molecules that interact more strongly with the stationary thin layer will move slowly, while molecules that interact more strongly with the mobile organic solvent will move more quickly. The overall effect, then, is a separation of the individual components of a complex mixture. We will carry out an experiment in which **lysed** (*i.e.*, broken open) algal cells will be subjected to TLC (thin-layer chromatography, not tender loving care – breaking open cells is required for thin-layer chromatography but wouldn't be considered very loving for these poor algae cells!). Because algae are full of photosynthetic pigments like chlorophylls and xanthophylls, which are visible to the naked eye, and because TLC will separate these pigments from each other quite readily, this experiment will result in a highly visible outcome that should be relatively easy for the student to interpret the results.

1) ***IMPORTANT NOTE*** Breaking open algal cells can be challenging because of their strong cell walls. You can extract algal pigments without breaking open the cells; however, if you choose to do this, you will likely only be able to extract 20-25% of the pigments. Without specialized equipment like a sonicator, the best way to lyse the cells is to boil the sample in the organic solvent that will be used for TLC. **This can result in safety concerns, so only do this when wearing proper personal protective equipment such as a lab coat and proper eye protection!**
2) The following extraction and chromatography steps should be carried out in a fume hood. If this isn't possible, carry the procedures out in a well-ventilated area and minimize direct exposure to the solvent as much as possible.
3) The most common solvents to use in TLC experiments for algae are 90% acetone, methanol and 95% ethanol. Acetone is less effective than the other two and methanol is poisonous, so we suggest using 95% ethanol.
4) If you choose to boil the sample in the solvent, boil the sample in 95% ethanol for five minutes.
5) Allow the algae sample to incubate in the solvent (in the dark and at room temperature), if possible for as long as 24 hours.
6) While the algal pigments are extracting, set up the TLC system:
    a. Only handle TLC silica gel plates with gloved hands and forceps. These plates are easily scratched, so handle with care!
    b. Measure 1.5 cm from the bottom of the plate and on both sides. Gently, without scratching the plate, use a pencil to draw a straight line from 1.5 cm from the left of the plate to 1.5 cm from the right of the plate, with the pencil mark being 1.5 cm above the bottom of the plate.
    c. Draw evenly-spaced dots along the length of the pencil line, one dot for each sample that you want to analyze on the plate. For smaller TLC plates, you should draw no more than 5-6 dots and run no more than 5-6 samples. Larger plates can accommodate more samples. The goal is to not have samples so close together on the plate that they run together during the separation process.
7) Gently mix the algal **lysate** (*i.e.*, solution of broken-open algae cells)
8) Use a capillary tube or Pasteur pipette to remove a small amount of lysate from your sample tube and spot it directly on one of the dots drawn on the bottom of your TLC plate. Repeat this step for each sample you want to run. You can spot samples from different lysed cultures and/or you can run different amounts of sample on each dot. If needed, you can spot a small amount on sample of a dot, allow it to dry for a minute or two, and then spot additional sample on the same spot.
9) Add enough solvent to the bottom of the TLC chamber to cover the bottom of the chamber. Don't add much more than the minimum amount to get full coverage of the bottom.
10) Using forceps or a gloved hand, pick up the TLC plate from the top and gently place it into the tank such that the solvent at the bottom of the tank wets the bottom of the plate.
11) Place the chamber lid on the top of the chamber to minimize solvent evaporation.
12) Observe the upward migration of the separating solvents as the thin-layer chromatography process progresses.
13) Use forceps or a gloved hand to remove the TLC plate from the chamber once the leading edge of the solvent reaches 1 cm below the top of the plate.
14) Allow plate to dry for 5 minutes.
15) Take a picture of the plate – samples will degrade over time and will eventually no longer be visible on the plates themselves.



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APPENDIX A

Appendix A

I. *Determining appropriate wavelengths of light.*

The specific wavelengths of light that drive photosynthesis in an organism depends on which photosynthetic pigments are present in that organism, as each pigment absorbs a very narrow range of specific wavelengths. It is crucial that any light source used for an algal strain contains wavelengths corresponding to the PAR for that species. Figure 34 (below) shows a few examples of how various photosynthetic pigments absorb very different wavelength combinations.

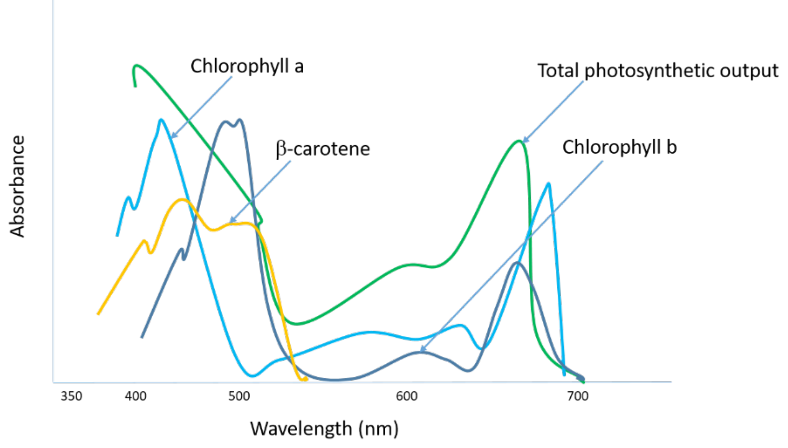

Figure 34. Light Absorption with Various Photosynthetic Pigments (drawn by the author specifically for this publication)

Using light sources containing wavelengths beyond the specific PAR rarely negatively impact the culture. Therefore, using full spectrum lights, such as grow lights, will typically suffice. When making a decision regarding what type of light to choose for your culture, you must take the following into consideration:

1) Do some research regarding the major photosynthetic pigments contained within your algal strain.

2) Identify the peak wavelengths absorbed by those pigments. As a thought experiment, answer the following question: How can you tell that most algae and all plants do *not* utilize wavelengths associated with the color green (~520-560 nm)?

   With this “puzzle” in mind, look up the photosynthetic pigment phycoerythin and answer 2 questions:

   a. What type of algae species is this pigment found in?

   b. What is different about these species compared to most algae that you have probably heard about before?

3) Choose a light source that emits these wavelengths. Most full-spectrum lights will meet this need.

APPENDIX B

Appendix B

i. A **luminosity function** (Figure 35) describes the relative sensitivity of the human eye to specific wavelengths of visible light.

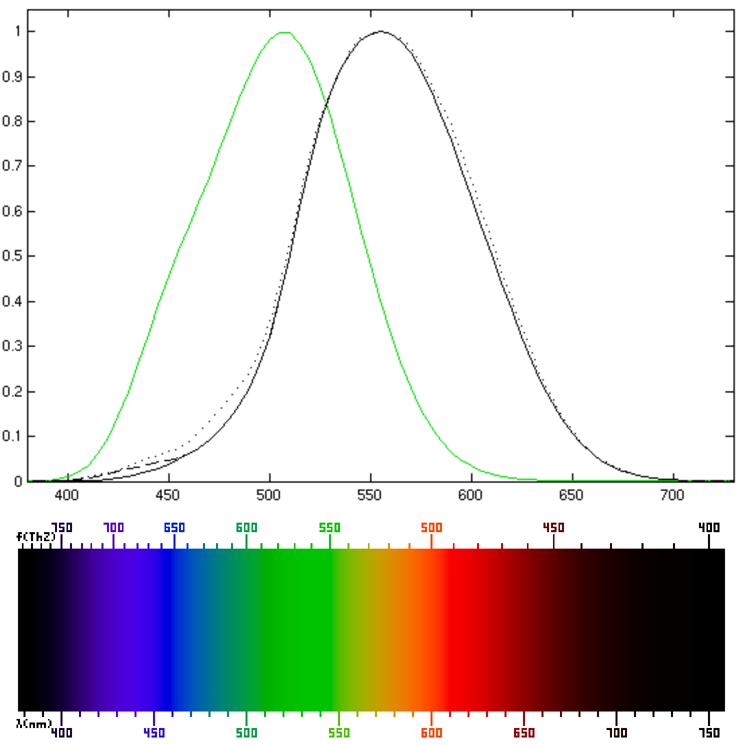

Figure 35. Luminosity function[31]. The green curve represents color vision (generated by the cone cells of the retina in brighter light) and the black curve represents rod cell-based vision in low light.

ii. A lumen is defined relative to a **candela** (cd), which is equivalent to the amount of light that an ordinary wax candle emits. Mathematically (don't panic, we'll explain…), 1 lm = 1cd • sr, where cd is a candela and sr is a **steradians**, which is defined by the solid angle generated from the center of a sphere with lines connecting the extremities of an area on the surface of the sphere equal to the square of the radius. A lumen, then, is the amount of light per second in a solid angle of one steradian. Whew! That's a complicated explanation…how about a picture that describes it?

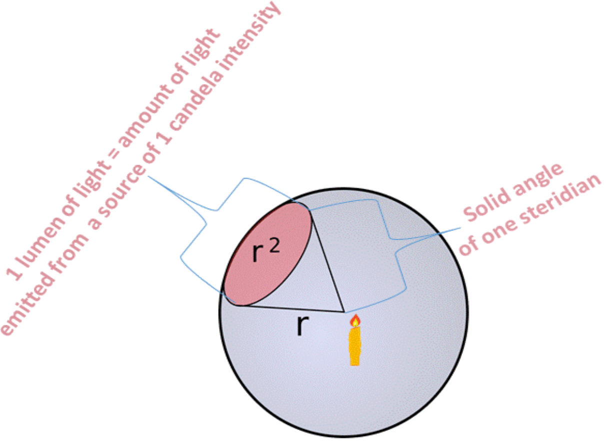

Figure 36. A steradian is defined graphically: A sphere with radius *r* and a region on the surface with an area (A) of r^2. The **solid angle** is = [A/r^2], which equals 1 steradian (sr) – (drawn by the author specifically for this publication)

iii. A sphere (which represents the three-dimensional path that light will take when it is emitted from a single source) has a solid angle of 4p, which equals 12.57 lumen (1 lm = 1cd • 4p sr). If an opaque object (like the ground upon which the candle in Figure 37 is sitting) blocks some of the 1 candela light source, then the light is "concentrated" onto a smaller area.

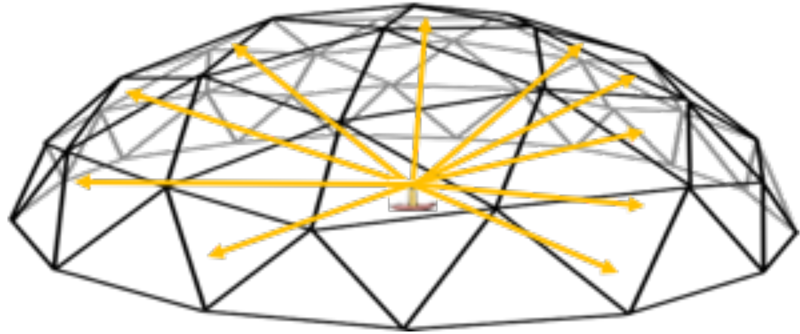

Figure 37. A wax candle (with an output of 1 candela) distributes the light evenly over the region not blocked by the opaque surface (drawn by the author specifically for this publication).