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.

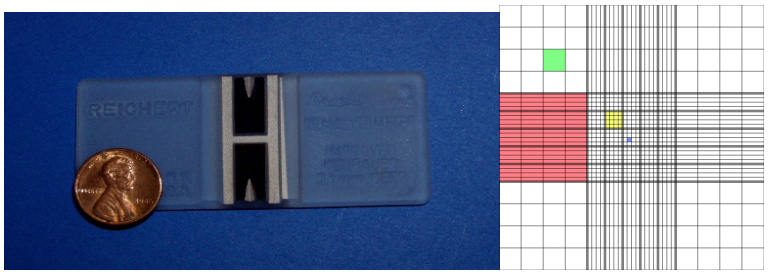

Figure 31. Hemocytometer used for algal cell counts. (Left) Hemocytometer slide with penny as a size reference. (Right) Schematic showing embedded grid on hemocytometer slide that helps with accurate counting.

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.

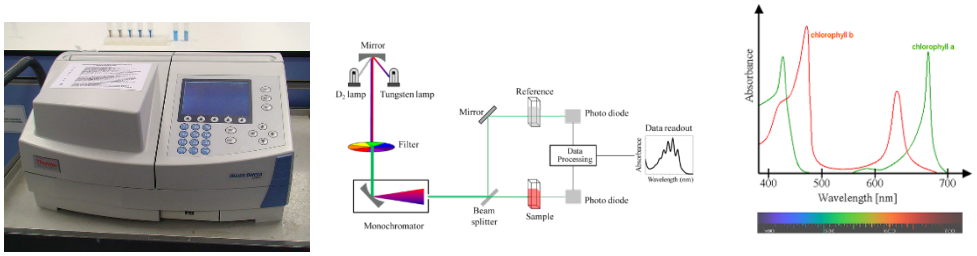

Figure 32. Spectrophotometry. (Left) Photograph of a typical, benchtop, laboratory-based spectrophotometer. (Middle) Schematic of spectrophotometer functionality, from light generation to wavelength selection to light detection and conversion of raw data into an analyzable signal. (Right) Chlorophyll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

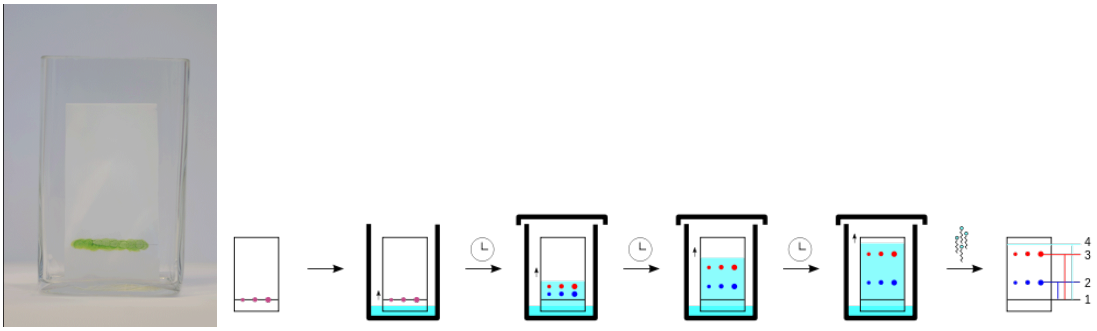

Figure 33. Thin-Layer Chromatography setup. (Left) TLC tank and plate. (Right) Schematic of TLC separation principles.

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.