Boyce Thompson Institute
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Algae to Energy: Optimizing Systems

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Algae to Energy Systems Lab
Experiments in Growth Optimization

Overview

The concept of utilizing fast-growing, high-lipid content microalgae to produce renewable liquid transportation fuels – specifically biodiesel – is not new; having first garnered attention in 1960. Investments by industrial and government sectors into "green energy" alternatives to fossil fuels over the last decade greatly advanced research and technological development of sustainable, land-based biofuel crops. However, at present, the development of novel microalgae biotechnologies is required to realize and evaluate the scaled-up, full-potential of microalgal energy systems – future technologic leaps likely conceived or discovered, years from now, by present day STEM students. The latter students – our future scientists and engineers – must be capable of interdisciplinary studies into fundamental biology, bioengineering, chemistry as well as logistic and life cycle processing. Thus, in grade 7-12 STEM classrooms, why not explore and, in doing so, impart a systems-thinking approach to address the question: "how can we sustainably produce diesel from algae in the future?" This workshop introduces teachers to bioenergy-related STEM careers, current hurdles in the algae-to-biofuel pipeline, and invites them to engage their students in addressing the complex microbiology, engineering and design, and socioeconomic challenges associated with producing biofuel from microalgae These challenges include, but not limited to, optimizing algal growth and lipid production, downstream environmental impacts, and improving production (input/output) efficiencies. Thus, teachers, and students, will learn about algae biology and how to design simple classroom photobioreactors with water bottles. Students will then be asked to design a series of experiments that manipulate algae growth requirements to determine the optimal methods for growing algae. Scientific inquiry, engineering, math and collaboration will be essential for photobioreactor success.

Grade Level

This project is designed for students in grades 9-12 in introductory to advanced biology, environmental science and engineering courses and can be modified for student in grades 7-16.

Time Frame

(Five to six, 45-minute class periods are required for this lab; additional time in class and out of class may be needed)

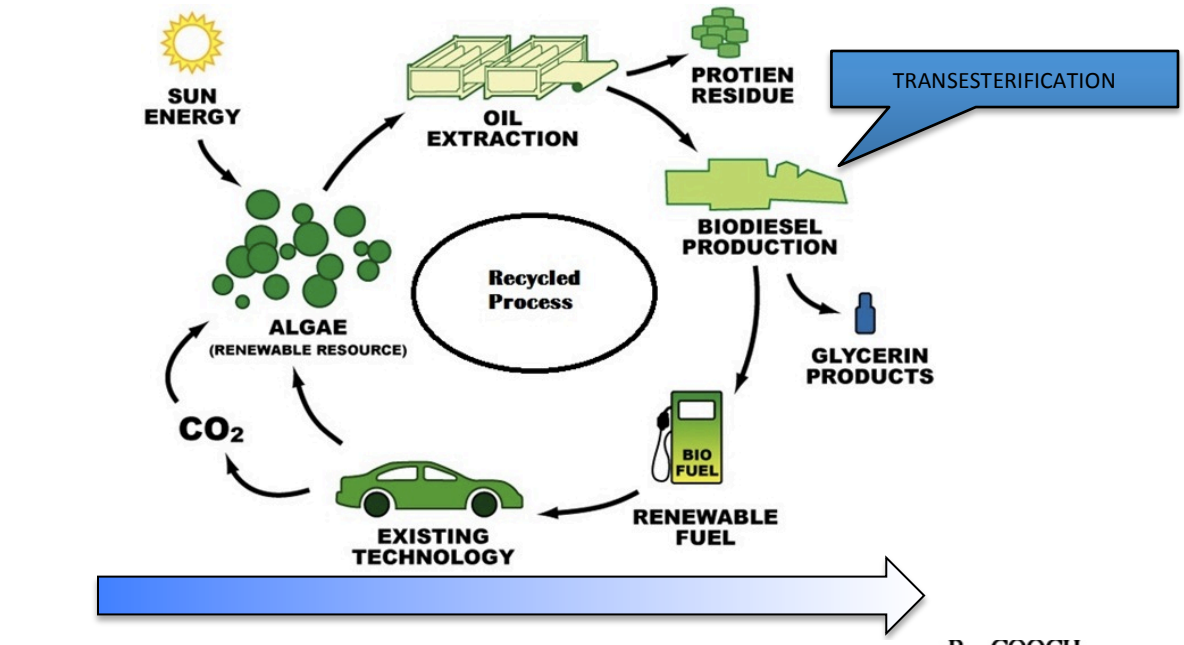
- 45 minutes to introduce topic
- 1 class period to design experiments
- 1 class period to assemble bioreactors
- 3-5 days of growth
- 1 class period to collect data
- 1 class period to analyze and discuss data, and wrap up

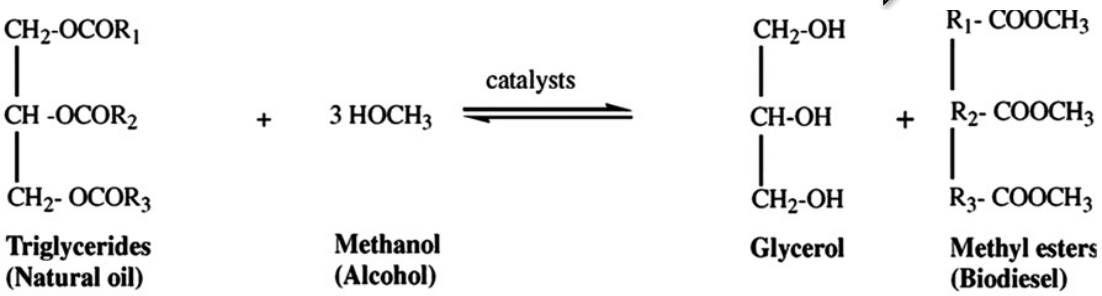
Background

Biofuels are defined as chemically-processed liquid fuel derived from living things, like animal fat or plant matter (1,2). In the last decade, research dedicated to **biofuels**, and bioenergy production systems, has increased remarkably in part due to the U.S. Energy Independence and Security Act of 2007, which requires a 20% benchmark reduction in gasoline consumption by 2017 (3). Title II of said act stipulates that the U.S. must increase its overall biofuel production to 36 billion gallons by calendar year 2022, of which 21 billion gallons must be produced from non-cornstarch products. However, ninety-five percent (95%) of first-generation biofuel feedstock crops – such as corn, rapeseed, palm, soybean, and sugarcane – are also produced for human consumption (2,3,4,5). This may create a host of potential problems whereby land and market for individual food and fuel crops could be in competition with each other. Thus, first-generation biofuel crops, alone, hold little potential to sustainably produce biofuel on the necessary scale to reach future benchmarks (2).

Algae are photosynthetic non-vascular plants found mostly in aquatic environments. Some are microscopic, and others are macroscopic. Like vascular plants, microscopic algae (syn. microalgae), produce and store lipids in the form of TriAcylGlycerols (TAGs). TAGs derived from algae can purified and converted to **biodiesel** (Figures 1 and 2) via a simple **transesterfication** reaction in the presence of methanol and an acid or base.

With increasing interest in biodiesel as an alternative to petroleum-based diesel, scientists are looking to microalgae. Algae can be cultivated en mass in open ponds called raceway ponds or in artificial, enclosed lighted environments called **photobioreactors** (PBRs). PBRs offer several advantages over raceway ponds: 1) they reduce the likelihood of contamination; 2) PBRs offer better control and optimization of conditions for algal growth (pH, light, temperature, etc); and, 3) permit higher cell yields and, hence, greater net yield biomass. However, PBRs can require more energy to operate than open pond systems, depending on the design.





Fatty acid	No. of carbon : No. of double bond	Fatty acid chain
Palmitic	16:0	R = - $(CH_2)_{14} - CH_3$
Stearic	18:0	R = - $(CH_2)_{16} - CH_3$
Oleic	18:1	R = - $(CH_2)_7$ CH=CH $(CH_2)_7CH_3$
Linoleic	18:2	R=-$(CH_2)_7$CH=CH-CH_2-CH=CH $(CH_2)_4CH_3$
Linolenic	18:3	R= - $(CH_2)_7$ CH=CH-CH_2-CH=CH-CH_2-CH=CH-CH_2-CH

Fig. 1. (ABOVE) General inputs and outputs within algae-to-biodiesel pipeline (Courtesy of mechanicalengineeringblog.com). (BOTTOM) Transesterfication of oil to biodiesel (see literature cited, 6); R_X are hydrocarbon groups.

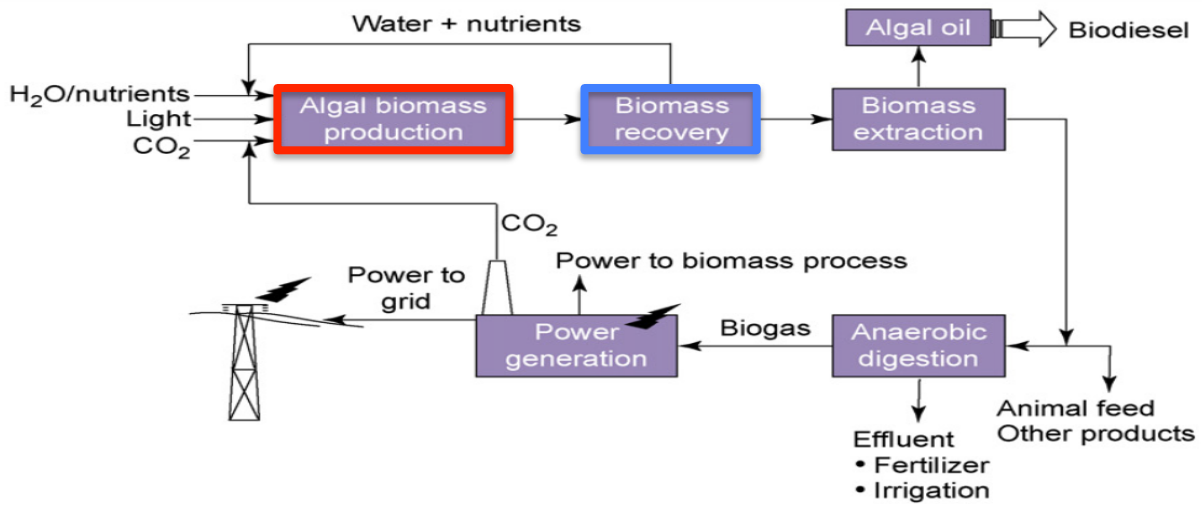


Figure 2. Schematic representation of the conceptual "algae to biodiesel pipeline" (Figure 1 *in* [7]). The focus of the classroom photobioreactor laboratory – highlighted in RED – is to optimize algae growth, and hence, overall biomass recovery (BLUE). Water, inorganic nutrients, carbon dioxide (CO_2), and light are provided to algal cultures, and cells within the liquid media are separated from the water and nutrient in the biomass recovery phase. The latter nutrients and water are captured and returned (recycled) for use in the growth of subsequent generations of algae. From recovered algal biomass, TAGs (oils) are extracted and separated for conversion to biodiesel and other bioproducts.

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Key Scientific Vocabulary

Algae
Photosynthetic plant-like organisms known as protists. They contain chlorophyll, are fast growing, and can live in fresh, salt, and waste water. Some types of algae, like Chlorella, are unicellular and microscopic, while others, like seaweed, are multicellular and macroscopic.

Biotechnology
Manipulation and development of biological organisms, their processes, systems and components to make useful products, and/or understand basic biological processes, often by means of genetic engineering.

Biodiesel
A biodegradable transportation fuel that can be used in diesel engines. Biodiesel, a fatty acid methyl ester, is produced through transesterfication of oils or fats from plants or animals.

Energy Return on Investment
Ratio of the amount of energy gained from a system to the amount of energy put into the production. An energy return on investment of less than one (when energy out is divided by energy in) the system would be considered not sustainable

Photobioreactor
An enclosed vessel used to grow algae where sunlight, water, carbon dioxide and nutrients are regulated. This system promotes the growth of high concentrations of algae and high oil yields that can then be converted into biodiesel and other products.

Photosynthesis
The chemical process by which organisms convert carbon dioxide into sugars, using the energy from sunlight.

Protists
Eukaryotic organisms, often unicellular and microscopic, sharing certain characteristics with animals, plants and/or fungi.

Raceway Pond
An open system where algae is grown in large ponds outside that are mixed. Algae concentrations in these systems are typically not as high as in photobioreactors, but the energy required to maintain the system is typically lower than the energy required to maintain photobioreactors.

Strain
A variety of species that is relatively uniform genetically because of continued inbreeding and artificial selection. Certain characters appear in successive generations as a result of inbreeding or self-fertilization, creating varieties within a single species that share similar, but unique genetic backgrounds.

Sustainability
The economic, environmental, and social stability of a practice, when considering meeting the needs of present and future generations. This is sometimes evaluated in a cradle to grave analysis.

Transesterification
The main reaction for converting oil to biodiesel, a chemical process by which lipid molecules are broken and rearranged using catalysis.

Yield
The amount of ending material derived during a process.

Learning Objectives

Pre-Lab Objectives: *Students will…*

- Review the concept of photosynthesis, global climate change, and key vocabulary for the lab.
- Discuss the importance of research and development in the area of renewable resources, specifically biofuel, when taking the availability of natural resources and climate change into account (Next Generation Science Standards [NGSS]: HS ESS3-1).
- Research and list the characteristics that affect algae growth.
- Identify, examine, and compare the benefits and disadvantages of growing algae in photobioreactors and raceway ponds.
- Students will compile possible questions (hypotheses) about microalgae transformation to biofuels, in list form or via flow diagram, which they would like to investigate through the construction and use of a photobioreactor.
- Independent variables should be limited to the scope of the laboratory (i.e., variables affecting the algae growth); however, the downstream consequences or implications of the data collected, should be addressed.
- Given what they know of plant biology (photosynthesis and plant growth), the students should also justify their hypotheses as well as identify the weakness(es) or limitation(s) of their hypotheses.

Lab Objectives: *Students will...*

- Construct and carry out an experiment that manipulates one or more variables involved in growing algae (carbon dioxide [CO_2], light, nutrients, etc.).
- Explain how their photobioreactor and algae growth is a model for photosynthesis (HS-LS1-5).
- Observe, analyze, graph, and summarize the results of their experiment.
- Discuss the results of their experiments in order to draft a recommendation of the optimal growing conditions for microalga *Chlorella protothecoides.*
- Explain how making alterations to optimize the photobioreactors and other existing energy technologies could reduce human impacts on natural systems (HS-ESS3-4, HS-LS2-7).
- Propose an area for future research (HS-LS2-7).

Post-Lab Objectives: *Students will…*

- Analyze the results of their research and place them in the context of the broader field of biofuel production.
- Compare the production and use of petroleum derived diesel and algal biodiesel and their effects on the biosphere, atmosphere, hydrosphere, and geosphere (HS-LS2-5).

- Discuss the importance of biofuel research when considering the challenges of locating facilities for growing algae, maintaining cultures, extracting the lipids for the creation of biodiesel, and transporting the fuel (HS-ESS3-1).

Definition of NGSS addressed above

1. HS-LS1-5: Use a model to illustrate how photosynthesis transforms light energy into stored chemical energy.
2. HS-LS2-5: Develop a model to illustrate the role of photosynthesis and cellular respiration in the cycling of carbon among the biosphere, atmosphere, hydrosphere, and geosphere.
3. HS-LS2-7: Design, evaluate, and refine a solution for reducing the impacts of human activities on the environment and biodiversity.
4. HS-ESS3-1: Construct an explanation based on evidence for how the availability of natural resources, occurrence of natural hazards, and changes in climate have influenced human activity.
5. HS-ESS3-4: Evaluate or refine a technological solution that reduces impacts of human activities on natural systems.

Laboratory Preparation

Proposed Timeline

1. *One month prior to the laboratory*. – Contact the BTI Education and Outreach Program (Teaching Laboratory Coordinator) for a photobioreactor kit and for live cultures of *Chlorella protothecoides*. The kit comes prepared for 8 groups of 4 students (materials for 1, 4-bottle bioreactor system per group) and includes airline tubing, valves, airstones, sterile cotton balls and toothpicks, hemocytometers, and air pump.

2. *One week prior to laboratory.* – If planning to utilize an additional species of alga in the experimental design, such as *Chlamydomonas reinhardtii*, live cultures can be order from Carolina Biological Supply Company© (Item no. 152040) to arrive within 2-3 business days. Additional and different alga species can also be purchased from the same company.

3. If you choose, construct photobioreactor demonstration bottle to increase culture volume and/or test classroom growing conditions/experimental variables. Don't forget to utilize aseptic technique whenever possible, and, soak (1h) and sterilize (boil 20 min.) your airstones before turning on your air.

4. *Day 1.* - Wednesday or Thursday. Review pre-lab materials with students and brainstorm questions they might like to examine with the photobioreactors. Use the Designing Your Algae To Energy Experiment section in order to help students determine what to test in each bottle. Guide them in designing a method to examine a chosen variable, while keeping other variables constant. Before the first lab, prepare materials for each group and set up stations for the lab the next day. Drill holes in the water bottle caps and cover holes with masking tape to store overnight. Soak the airstones in a beaker of bottled water.

5. *Day 2*. - Friday. Students construct their photobioreactor in groups to prepare the bottles for the system. Students take initial reading and record the data on their data sheets and in a class excel document titled Algae Photobioreactor Lab Data Tracker (provided).

6. *Day 3–7*. - Monday, Wednesday, Friday (20 min/day). Students record observations and collect measurement data.

7. *Day 8.* - Wrap up the lab by having students analyze the data, create graphs, draw conclusions, complete the lab, possibly present their findings, and have a final class discussion.

Planning Checklist

- ☐ Collect all items on the materials list
- ☐ Purchase algae culture online (Carolina Biological), or BTI if needed
- ☐ Buy at least 30, clear-plastic water bottles with screw cap lids: 1 for the teacher pilot photobioreactor, 1 for soaking and boiling the airstones prior to constructing the reactors, enough for the student photobioreactors
- ☐ Build a pilot photobioreactor a week in advance, to test several experiments and to culture (bulk) enough algae to inoculate more than 24 photobioreactors. This will also allow you to ensure that your classroom growing conditions support algae growth before beginning the full experiment.

Designing Your Algae to Energy Experiments

Independent Variables

Encourage students to ask questions about optimizing algae growth. Help them develop their own questions – and interests – and experimental design that permits them to examine one or more independent variables. Here is a list of suggestions to help you lead the discussion.

Consider using the following variables below to help your class organize their experiments.

1. Growth requirements

   Students can experiment with what algae needs to grow by tracking the growth of algae (cell concentration) across treatment combinations including differing light (photoperiod; presence/absence), nutrients (+/- urea and/or aquarium salt), and CO_2 (airflow) regimes. This can be a starting point that can lead into another round of experimentation that seeks to optimize the setup and produce more algae than the recommended conditions. Test groups could perform a simple, 2x2 factorial design varying only light and nutrients, consisting of 4 light-nutrient combinations: 1) no light (-) with nutrients (+); 2) no light (-) minus nutrients (-); 3) light (+) with nutrients (+); and, 4) light (+) minus nutrients (-). Depending on how many replicates you wish to include, you could also vary airflow across the bioreactors (e.g., no air versus complete airflow).

2. Algae species

   Students can compare the growth rates of both the C*hlorella* and *Chlamydomonas* (Carolina item # 131738) species, in order to determine which they would choose for algae production if they were opening a new biodiesel company. *Chlamydomonas* is another fresh water green algae, but grows much slower than *Chlorella*. This variable may be paired with another to accommodate four test groups (e.g., *Chlamydomonas* + 24 h. light, *Chlamydomonas* + 12 h. light, *Chlorella* + 24 h. light, *Chlorella* + 12 h. light).

- Examples of follow-up questions:
  - Which species preforms the best under which conditions?
  - Are there differences in growth among species-light combinations?
  - Given these data, what does this tell you about each species?
  - Which alga, and associated conditions, would you choose if you were charged with producing algae for biomass/bioenergy production? Why?
  - What treatment or combination of treatments yielded the greatest algal biomass (cells/mL, 500 nm reading)?

3. Growing media

   Nutrient medias can be altered to test the effect on algae growth. Miracle-Gro, and fish food can be mixed into the water, or water could be mixed with potting soil and filtered through a coffee filter before putting into the water bottles. You could also test fertilizers that vary in their percent nitrogen, phosphorous, and potassium **(N, P, K)** to see which elements most strongly affect algae growth.

4. Light

   Try different forms of light like halide, incandescent, fluorescent, LED lights, and sunlight. (*NOTE: We have found that fluorescent lamps work best)* Distance from the light source could also be manipulated. Light timers could be used to experiment with different light regimes (24 h light, 12 h. light, 2h. light). Bottles and their lights may need to be shielded from each other and the overhead lights to keep conditions consistent. Individual bottles can be wrapped in heavy-duty aluminum foil. Students could also experiment with blocking different wavelengths of light by covering their bottles with different colors of cellophane (clear-as a control, red, blue, green, yellow).

5. Carbon dioxide (CO_2)

   CO_2 can be added to the airstream to see how that affects growth. This can be done by mixing yeast, water, and sugar together in a 1L bottle. Bore, or drill, a hole in the cap, and connect the "CO_2 generator" to the air supply with a length of airline tubing and a "T" connector. This could also be done by collecting CO_2 from carbonated water. The increased CO_2 may mix with the water to form carbonic acid, potentially lowering the pH. This may influence the growth rate.

6. Competition

   Instead of following a controlled experiment, students could work in groups of two with one bottle, or as teams of 4 with two bottles to compete for the greatest algae yield (as determined by the teacher). **NOTE**: *If measuring algae growth via a spectrophotometer, students must take preliminary measurements across growth media / treatment as initial baseline measurements by growth media may have differing optical densities.*

Data Collection

Determine which kind of data collection suits your needs and capabilities. Remember that although student data tables are created to accommodate seven days of data collection, you can choose to take data every other day, or even just twice — with one reading on the first day and one on the last day you choose to run the experiment.

1. pH Testing

   No matter which data collection method you choose, you may also want to have students take a pH reading each day. This can fluctuate and may affect the growth of the algae, causing a confounding variable in the experiment. It is possible to increase the pH of the water or nutrient solution by dissolving a little baking soda in water, but the effects on algae growth may be variable. A pilot test measuring the associated change in pH relative to the amount of baking soda (mg/mL) can be done.

2. Photography

   Have students photograph their bottles each day, keeping in mind that it must be taken as consistently as possible (same light conditions, same distance, same angle). Students can store their pictures over time, crop them into small squares on the computer, and line them all up to visualize the progression. Different bottles can also be compared this way.

3. Paint color swatches

   Procure green paint swatches from a local hardware or paint store that contains a wide array of green colors. Have students hold the paint chips up to their bottles and record the number of the paint most closely resembling their bottle. At the end of the experiment, have students compare the paint colors from the first to the last day. Did it change a lot? In what bottles did the paint color stay almost the same?

4. Spectrophotometer

   Using a spectrophotometer to collect data is one of the quickest and easiest way to collect quantitative measurements of algae growth. In a spectrophotometer a light at a specific wavelength is emitted that measures the amount of the reflection. The denser a sample becomes, the greater the resulting optical density. In the literature, a wide range of wavelengths have been used to collect data on green algae. We suggest using 500 nm because there is an equation listed in the lab that will then allow students to convert their OD_{500} into the number of cells per mL. Be sure students **always mix** their photobioreactor bottles for 30 seconds before withdrawing their sample and to use plain distilled water to zero the instrument before testing their samples. Keep in mind that once samples have an optical density that is greater than 1.00, it is more accurate to dilute the sample. Thus, instead of measuring 1 mL of the algae sample you would measure a mixture of 0.5 mL algae sample and 0.5 mL water in the spectrophotometer. You would then multiply the resulting optical density

by two, to calculate the true optical density of the sample. If you enter your optical density data into the Algae Photobioreactor Lab Data Tracker spreadsheet, it will automatically calculate averages and standard deviations for the four conditions and display them on a graph.

5. Hemocytometer

   In conjunction with a compound light microscope, a hemocytometer could also be used to count and calculate the average number of algae cells per mL. The protocol for counting algae with a hemocytometer can be found at the end of this manual.

Algae Cultures

1. Chlorella *species*

   Recommended due to fast growth rate. Algae samples of *Chlorella* can be purchased from Carolina Biological (www.carolina.com, $7.50 + shipping, item #152069). These may take a day to process but will then be shipped overnight or within two business days. Upon receiving the sample, unscrew cap until it is slightly ajar to allow for air movement, and place culture near a light source. Samples come in about 30 mL allocations, however we suggest having at least 15 mL of an algae culture to inoculate each photobioreactor. This species is fast growing and requires high light to grow, meaning it will grow with 200 to 400 ft candles of fluorescent light. However, algae may grow faster with more direct and constant light. **Use your sample to create a pilot photobioreactor within a few days of receiving the culture.**

2. Chlamydomonas *species.*

   Liquid cultures of *Chlamydomonas* (www.carolina.com, $7.50 + shipping, item #131738) can also be purchased to give students the experience of examining a species of green algae that swims with its flagella. These may be used in the photobioreactors with the same recommended media but will grow much slower, with little to no visible growth within a week.

Materials and Methods

BTI Will Provide: (per class of 32 students)

- Sterile Toothpicks
- Sterile cotton balls
- 32 air stones
- 4 packs of aquarium tubing
- 4 air pumps
- 4 four-way gang valves
- 12 T-valves
- Aquarium salt
- Urea
- Glucose
- 8 Transfer pipets
- 1 compressed air can
- 8 hemocytometers
- culture of Chlorella protothecoides

You will need to provide:

- Chlorox® disinfectant wipes – *OR* – Paper towels and a store-bought liquid disinfectant (e.g.., Lysol® All-Purpose Cleaner, 32 fl. oz., trigger-bottle) containing the microbicide alkyl dimethyl benzyl ammonium chloride
- Power drill with 13/64 inch drill bit
- Ruler
- Scissors
- Permanent marker
- 30 or more 500 mL clear plastic unopened water bottles
- 100 mL graduated cylinder
- Balance (capable of measurement to 0.1 g)
- Weigh boats
- Light bank or fluorescent lamps
- Aluminum foil, baking soda, pH paper (optional – depends on experiment variables)
- Bleach

Safety Information

Listen to all laboratory instructions and wash your hands prior to and after working with algal photobioreactors. Review MSDS sheets for any chemicals with which you are using. **PLEASE NOTE:** *Upon completion of the laboratory, the algae cultures should be properly disposed of by adding 4.7 mL, undiluted bleach per bioreactor, swirl gently to mix, and let stand for 10 min. before pouring down the drain.*

Construction of the Photobioreactor

1. Before starting, select an appropriate workstation (e.g., benchtop or desk). Preferrably, to avoid possible contamination of the photobioreactor during or following construction, the workstation should be removed from windows, doors, and/or overhead airconditioning and heating vents that permit airflow – and associated airborne microbes (e.g., fungi and bacteria) – over the worksurface.
2. The chosen workarea should be approximately 4 ft^2 (0.4 m^2) and void of general clutter.
3. Wipe down the work surface thoroughly using a disinfectant wipe or the liquid disinfectant and paper towels. Discard used cleaning materials to trash.
4. Prepare a "clean zone." Once the worksurface is clean (sanitized), arrange four, unused disinfectant wipes (2x2) on the center of the worksurface (see below). Alternatively, unfold 4 pieces of paper towel and moisten with the liquid disinfectant – enough to make the towels stick to the work surface. All material and tools (e.g., scissors) entering "the clean zone," illustrated below, must be wiped down with "fresh wipes" or liquid disinfectant.

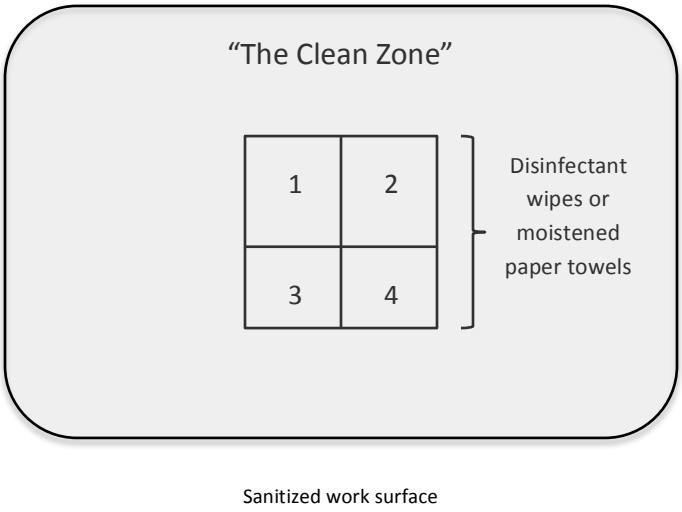


Fig 1. Illustation of a "Clean Zone" prepared for constructing the photobioreactor

5. Remove all paper/plastic labeling or decals from the clear plastic water bottle and, then wipe down the bottle with a "fresh wipe" or liquid disinfectant, making certain to clean the bottle cap and neck. Place bottle into your "clean zone"

6. Using permanent marker, label the neck of the water bottle with the date, assigned treatment, bottle number, and your names/initials (for example: 3/14/14, No Urea #2, JD [Jan Doe])
7. Using a 100 mL graduated cylinder, decant 70 mL from the water bottle; cap and seal immediately after decanting. Leave the remaining 430mL of water in the bottle.
8. Using a scale, weigh boat, and scoopula measure out the growth consistuents (nutrients) you wish to add to the photobioreactor. We suggest:
    a. **0.2 g aquarium salt (final concentration= 4.3 x 10^4 g/mL) —** Aquarium salts provides algae with the metals and ions needed for sustained growth.
    b. **0.8 g urea (final concentration= 1.7 x 10^3 g/mL) —**Urea is a form of nitrogen; providing a nitrogen-rich environment necessary for the growth of algae.
9. Carefully fold the the weight boat on itself and add growth constituents to the water bottle, making sure NOT to touch the bottleneck with the weigh boat.
10. Cap and seal immediately, and shake the nutrient-water solution for at least 2 min. until the constituents have completely dissolved. Set aside and in "clean zone."
11. Wipe down (surface sanitize) tubing for the airline and vent, and using separate wipes, the drill and drill bit. Place the tubing onto the moistened disinfectant wipes in your "clean zone."
12. Cut-to-length (Figure 1, A):
    a. 1, 2 ft piece of aquarium tubing (Aeration)
    b. 1, 3 in. piece of aquarium tubing (Vent)
        i. Wipe down each length of tubing and place in "clean zone"
13. Prepare the vent. (Figure 1, A and B). Pinch and remove a small piece of cotton, and using a sterile toothpick, force (stuff) the cotton approximately 1 in. into the tubing.
14. Drill two holes (diameter= 13/64 in.) into the cap of the bottle. Don't worry about shavings falling into the nutrient-water solution. (Skip this step if teacher is pre-drilling the bottles)
15. Take the tip of the aeration tube and forcefully insert the tubing through one of the previously-made holes in the bottlecap and feed approximately 1.5 ft of tubing through the cap and attach a sterile airstone to the tubing inside the bottle (Figure 1, C).
16. Carefully wipe the length tubing between the cap and airstone with a "fresh wipe," making sure keep the tubing and airstone off the work surface and away from conacting anything in the work area.
17. Carefully insert airstone-airline and seal the bottlecap. Strip out of the bottle any slack in the airline. The airstone should be gently touching the bottom-center of the bottle.
18. Insert vent tubing into the remaining hole and position the vent at a depth of no more than ¼ in. into bottle.
19. Quickly open the bottle, and using a sterile seriological or sterile transfer pipet, transfer 1 mL (2.5 x 10^3 cells) from the alga stock culture to the bottle – AVOID touching the pipet tip to the bottle's lip or neck.

20. Attach the free-end of the airline tubing to one of the spigots on the four-way gang valve or T-valve
21. Connect the four-way gang valve to the air pump with a 6 inch piece of airline tubing. Plug in the air pump and adjust the gang valve so that it appears that each bottle is getting equal air.
22. Place the photobioreactors in a well lit area (e.g. under two, equi-spaced lamps).
23. Follow your teachers instructions on what data to collect from your bottle, and how that is to be done. Before taking a reading, you should always swirl your sample for at least 30 seconds to ensure that all algae is mixed thoroughly throughout your sample, so your results are more consistent. Take an initial reading from your bottle and record it in your data table and in the class data table.
24. Over the next 7 days, evaluate and compare the estimated effect(s) of the individual treatments and/or treatment combinations on algae growth.

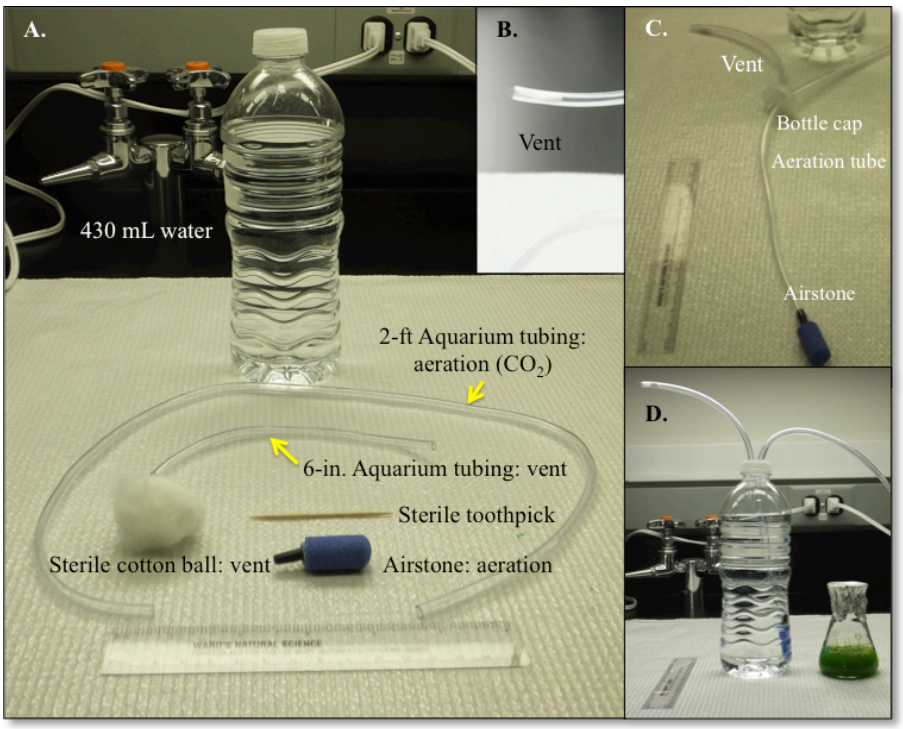


Figure 1. Construction basics for an algal bioreactor: (A) materials per reactor; (B) vent; (C) assembled components – vent, bottle cap, and aeration tubing with attached airstone; and, (D) bioreactor upon completion, ready for algae.

Figure 2 . General setup of a 4-bottle bioreactor system, varying only light regime(24:24 h, light:dark) across reactors.

Post-Lab Discussion

What are two ways that scientists are currently producing algae?

- Closed systems- photobioreactors
- Open systems- raceway ponds

What are the growing requirements for algae in these different systems?

- Access to sunlight, CO_2, phosphorous, nitrogen, water (salt, fresh, or waste water depending on the species of algae)

Do you think producing algae in this way is sustainable? What must occur for something to be sustainable?

Economical, socially acceptable, environmentally stable (triple bottom line)

- If the price of petroleum derived diesel fuel continues to increase, then even a cost intensive process may be more economical
- Consumers drive the market and the more knowledge people have about biofuels, the more they may choose to buy it
- The overall energy required to grow, process, and transport algal biodiesel must be lower than the amount of energy derived from the fuel. The total amount of CO_2 and other greenhouse gases the process produces must be lower or equal to the amount of carbon sequestered during the growing of algae.

How might science play a role in increasing the sustainability of algae for biodiesel?

- Research algae to find out what the best growing conditions are, how to increase their lipid production so we can increase our biodiesel yield, look for algae strains that are more algae strains or strains that require less nutrients

Based on your experiences in this lab, what are some of the hurdles that scientists might face when growing algae?

- *Collection of algae at the bottom of the tubes, not enough CO_2, contamination, water loss, nutrient loss, not enough light, growing time is too long, too small of yield for the amount of space, etc.*

Think about the inputs and outputs to your system. In what ways have the reduced the required inputs? In what ways have they optimized their outputs? What challenges to sustainability might there still be?

- Optional viewing of TED Talk entitled *Jonathan Trent: Energy from floating algae pods* which examines Jonathan Trent's research on algae and his ideas for overcoming some of the hurdles of algae production. Available at: http://www.ted.com/talks/jonathan_trent_energy_from_floating_algae_pods.html

People often say, "necessity is the mother of invention". How might that relate to biofuels, fossil fuels, and climate change?

Algae to Energy Pre-Lab Resources

Pre-Lab Resources and Discussion Questions

This suite of resources can be explored by the class or in interactive groups that then share the information they gathered from their particular source. These resources will give students background information necessary to jumpstart discussion.

Articles

Sustainable Development of Algal Biofuels in the United States (4 pages)
http://link.springer.com/content/pdf/10.1007%2Fs10811-009-9446-5.pdf

- Committee on the Sustainable Development of Algal Biofuels. (2012). *Report in brief: Sustainable development of algal biofuels in the United States*. The National Academy of Sciences

Algae Emerges as a Potential Fuel Source (2 pages)
http://www.nytimes.com/2007/12/02/us/02algae.html?_r=0

- The Associated Press (2007). *Algae emerges as a potential fuel source.* New York Times.

NPR radio program

Algae as car fuel: Possible, but not sustainable? (4 min)

- http://www.npr.org/2012/10/25/163606540/algae-as-car-fuel-possible-but-not-sustainable

Video

Energy 101: Algae-to-Fuels (3 min)

- http://energy.gov/articles/energy-101-algae-fuel

Interactive Web Resource

Nova scienceNOW: From Pond Scum to Power (5 min to run through all the sections)

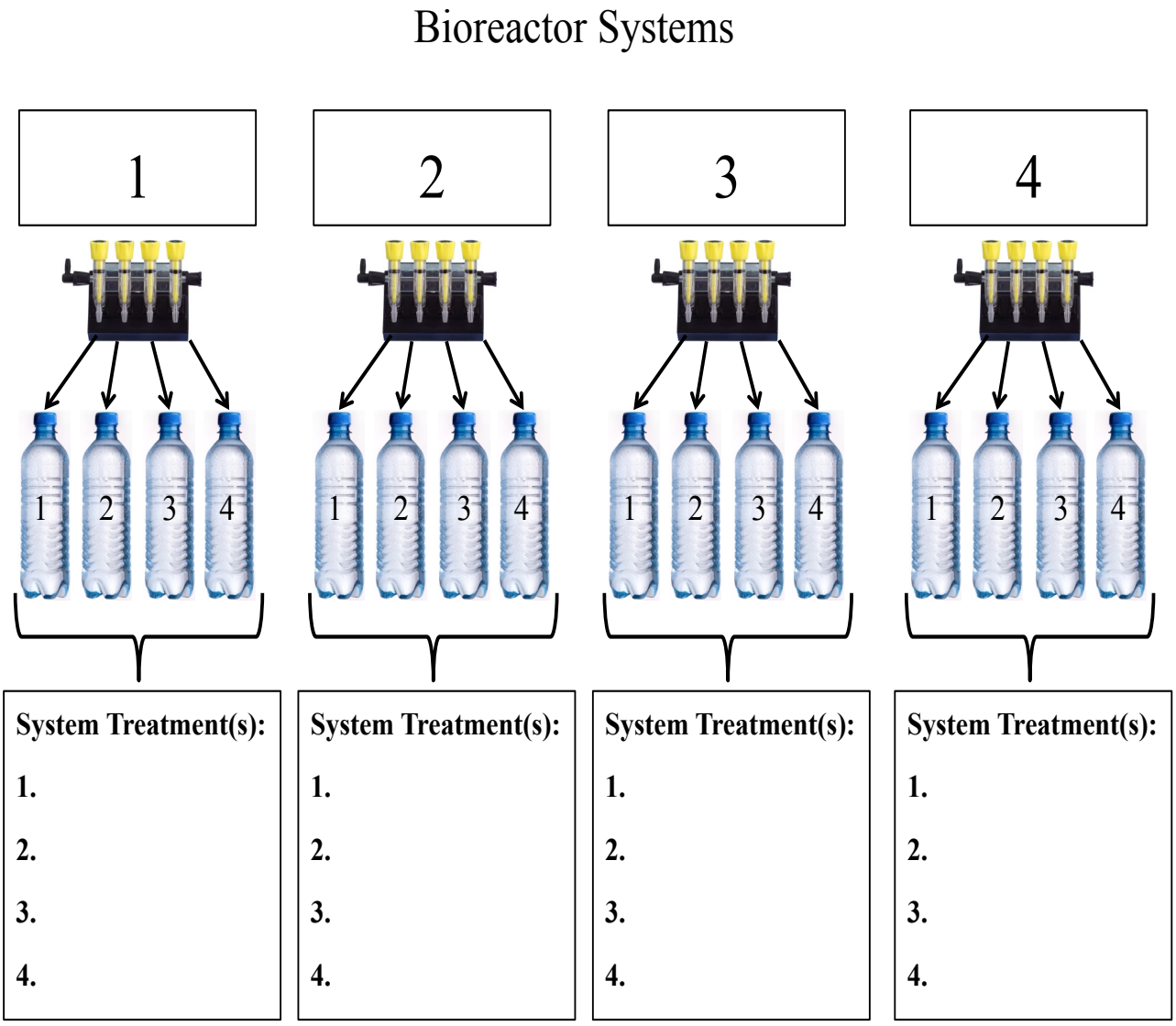
- http://www.pbs.org/wgbh/nova/tech/algae-biodiesel.html

Developing Research Questions

Imagine your laboratory has just received grant funding to conduct research on the optimal growing conditions for *Chlorella*, a freshwater green algae species. Formulate at least two *testable* questions that you could use to drive your research. Questions can be recorded on the top of the student lab, and discussed as a class.

Designing the Inquiry Experiment

Consider using the diagram below to help your class organize their experiments.



Algae to Energy Systems Lab - Student Background

Think about how you get to school, the store, the movie theatre, and your friend's house. Do you take a car, ride the bus, bike, or walk? We often find ourselves driving from place-to-place because it's easy and fast. But with a nation of drivers, these miles add up. Currently, the US transportation sector contributes 28% of our greenhouse gas emissions and 95.4% of US transportation is fueled by non-renewable energy sources. But what would happen if we could develop a more **sustainable** solution to our energy needs? We are now turning to biofuels and other renewable energy sources to provide such sustainable alternatives that reduce our dependence on fossil fuels (e.g., petroleum and oil). Believe it or not, microalgae may hold the potential to reduce our nation's consumption of fossil fuel resources, and greenhouse gas emissions.

Algae grow in the ocean, lakes, ponds, water fountains, birdbaths, fish tanks, and even puddles. Although algae are **protists**, they share a distant **common ancestor** (Figure 1), with and are similar to plants, as they both utilize **photosynthesis** to convert carbon dioxide into sugars using water and energy from the sun. There are more that 14,000 known species of green algae with great genetic variation within and among species. This variation is key to the development of algal-based renewable energy.

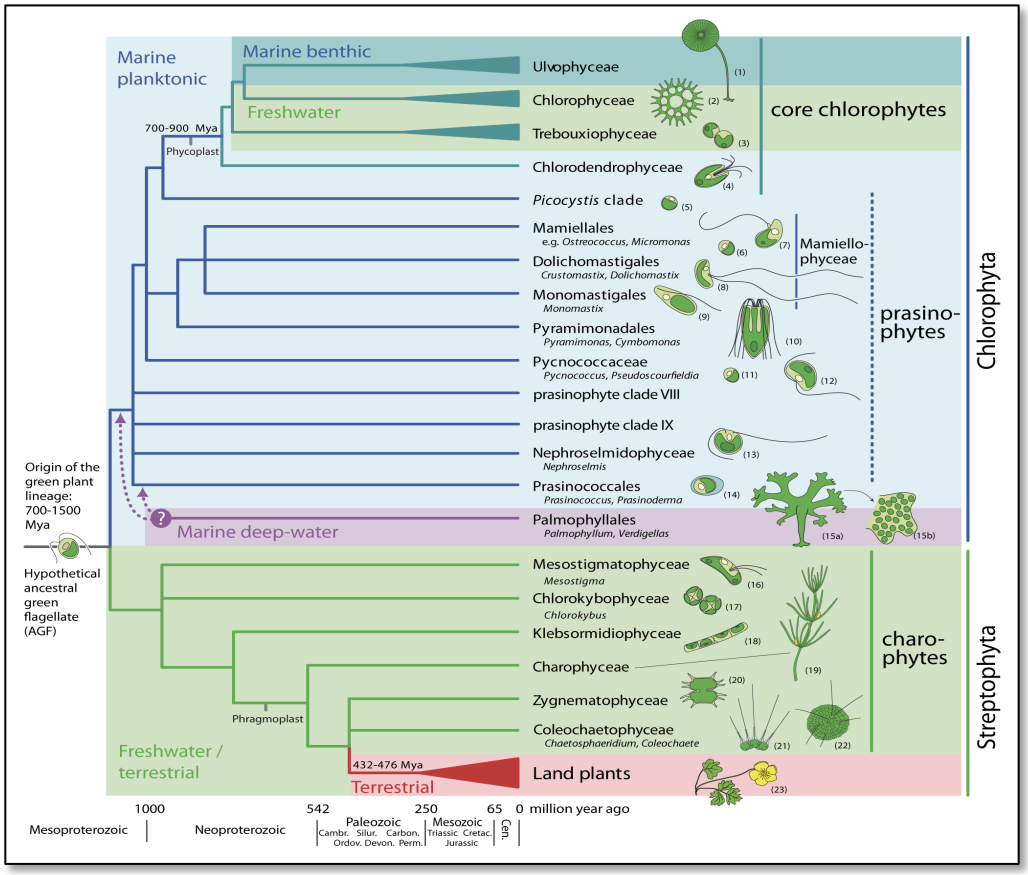


Figure 1. Deep phylogeny of freshwater and marine, green algae. (Figure 1 *in* Leliaert et al [2011], BioEssays 33: 683-692).

Algae store some of the sugar produced in photosynthesis as lipids. These lipids can then be removed from the cell and converted to **biodiesel** through a process known as **transesterfication** (Figure 2). Biodiesel has many of the same qualities of petroleum-based diesel and can be used in existing diesel engines with few modifications.

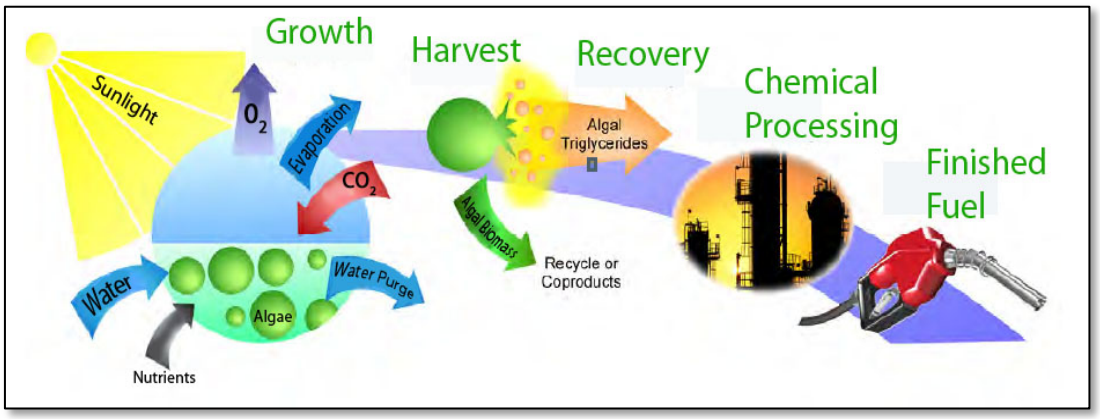


Figure 2. The process of converting algae to biofuel (National Academy of Science [2012]. Sustainable development of Algal Biofuels in the United States, National Academic Press, Washington, D.C.).

Microalgae require sunlight, carbon dioxide (CO_2), nitrogen, phosphorus, and a significant amount of water to grow. However, they do not require agricultural land space to grow, meaning they can be grown on land that is marginal and/or unsuitable for conventional agriculture. Algal production systems, such as **raceway ponds** and **photobioreactors**, can be constructed in deserts to obtain lots of sunlight, along coastlines to be near water sources, and/or next to factories or coal-fired power plants to harvest and incorporate waste CO_2 – a greenhouse gas – into the "algal pipeline" that would otherwise be released into the atmosphere (Figure 3).

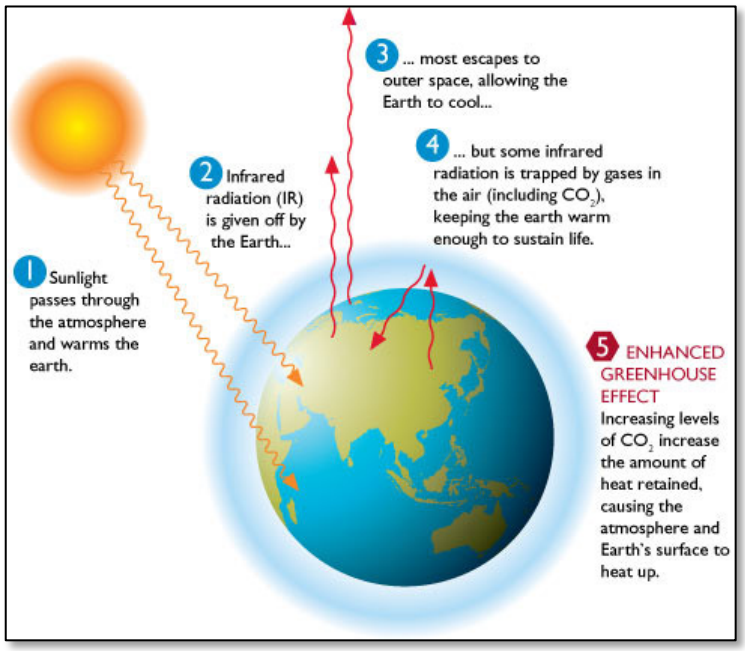


Figure 3. The greenhouse effect: the role of carbon dioxide (CO_2) and other greenhouse gases (Courtesy of CO_2 Cooperative Research Centre; Barton, Australia)

Unlike corn, soybeans, and cane sugar, algae production does not require food crop production. Moreover, regarding their culture and production, they have several advantages over competing, biofuel crops (Table 1).

Characteristic	Advantages
Rapid growth rate	Outgrow competing species; reduced culture area
High Oil content	Value of biomass increases with increasing oil content.
Growth in extreme environment	Reduced risk to contaminating bacteria or competing algae
Large cell size, colonial or filamentous morphology	Easy harvest and process
Wide tolerance of environmental conditions	Can readily control culture conditions
CO_2 tolerance and uptake	CO_2 sequestration and use of waste CO_2
Tolerance of shear force	Cheaper pumping and mixing methods
Tolerance of contaminants	Potential growth in polluted water
No excretion of autoinhibitors	No growth inhibition at high densities (exponential growth dependent upon culture conditions and time).

Table 1. Characteristics and associated advantages of microalgae grown for biofuel (Griffiths and Harrison [2009], Journal of Applied Phycology 21:493-507)

Scientists, chemists, and engineers are currently working on ways to optimize algae growth to make algae production more sustainable – see Figure 4, below, for a brief stroll along a proposed "algae-to-biofuel pipeline." For example, algae are now being grown in saltwater and wastewater and the nutrients are being recycled. Even with these improvements, a gallon of algal biodiesel is still very expensive. To lower the cost of production, the byproducts are being sold to make protein-rich animal feed, fertilizer, and biogas that can then be used to produce a source of energy.

Although these benefits are enticing, there are still many hurdles for scientists and engineers to overcome before algal biofuel can compete on the market with petroleum-based fuel. The **energy return on investment** must increase, meaning the amount of energy we gain from algae production must be higher than the amount of energy needed to produce the algae, or the system will not be profitable or environmentally sound. Scientists are currently working toward the sustainable production of algal biodiesel by researching many algal species and varieties within species known as **strains**, and their optimal growing conditions. They are working to improve algal strains so they will produce more oil and require lower levels of nutrients. BTI scientists are currently using **biotechnology** to develop algae that grows rapidly and produces more oils in less time. There is no perfect solution to our energy needs, but with some improvements, algal biofuel may be one important piece of the puzzle.

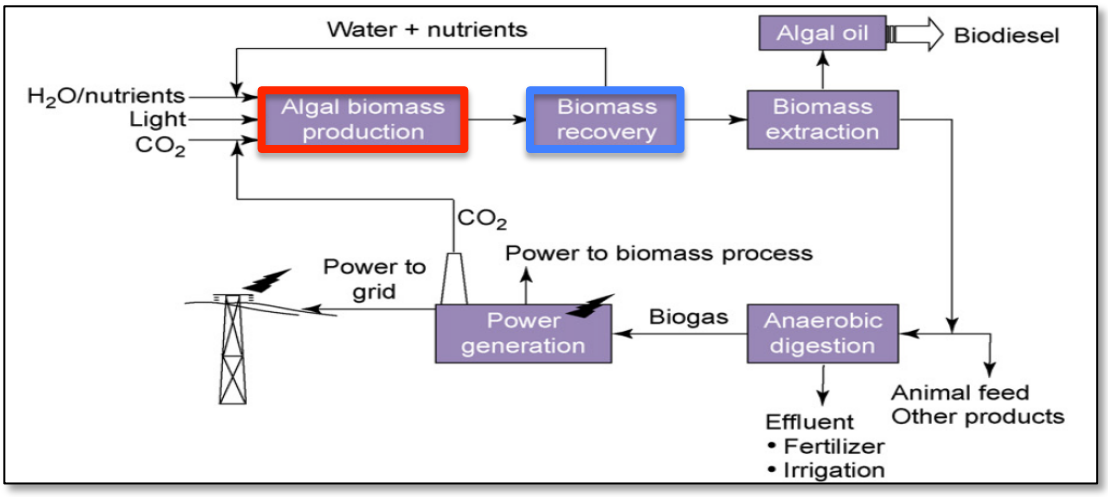


Figure 4. Schematic representation of the conceptual "algae to biodiesel pipeline" (Figure 1 in Chisti [2007], Trends in Biotechnology 26:126-131). The focus of your photobioreactor laboratory – highlighted in RED – is to optimize alga growth, and hence, overall biomass recovery (BLUE). Water, inorganic nutrients, carbon dioxide (CO_2), and light are provided to algal cultures, and cells within the liquid media are separated from the water and nutrient in the biomass recovery phase. The latter nutrients and water are then captured and returned (recycled) for use in the growth of subsequent generations of algae. From recovered algal biomass, TAGs (oils) are extracted and separated for conversion to biodiesel and other bioproducts.

Key Scientific Vocabulary

Algae
Photosynthetic plant-like organisms known as protists. They contain chlorophyll, are fast growing, and can live in fresh, salt, and waste water. Some types of algae, like Chlorella, are unicellular and microscopic, while others, like seaweed, are multicellular and macroscopic.

Biotechnology
Manipulation and development of biological organisms, their processes, systems and components to make useful products, and/or understand basic biological processes, often by means of genetic engineering.

Biodiesel
A biodegradable transportation fuel that can be used in diesel engines. Biodiesel, a fatty acid methyl ester, is produced through transesterfication of oils or fats from plants or animals.

Energy Return on Investment
Ratio of the amount of energy gained from a system to the amount of energy put into the production. An energy return on investment of less than one (when energy out is divided by energy in) the system would be considered not sustainable

Photobioreactor
An enclosed vessel used to grow algae where sunlight, water, carbon dioxide and nutrients are regulated. This system promotes the growth of high concentrations of algae and high oil yields that can then be converted into biodiesel and other products.

Photosynthesis
The chemical process by which organisms convert carbon dioxide into sugars, using the energy from sunlight.

Protists
Eukaryotic organisms, often unicellular and microscopic, sharing certain characteristics with animals, plants and/or fungi.

Raceway Pond
An open system where algae is grown in large ponds outside that are mixed. Algae concentrations in these systems are typically not as high as in photobioreactors, but the energy required to maintain the system is typically lower than the energy required to maintain photobioreactors.

Strain
A variety of species that is relatively uniform genetically because of continued inbreeding and artificial selection. Certain characters appear in successive generations as a result of inbreeding or self-fertilization, creating varieties within a single species that share similar, but unique genetic backgrounds.

Sustainability
The economic, environmental, and social stability of a practice, when considering meeting the needs of present and future generations. This is sometimes evaluated in a cradle to grave analysis.

Transesterification
The main reaction for converting oil to biodiesel, a chemical process by which lipid molecules are broken and rearranged using catalysis.

Yield
The amount of ending material derived during a process.

Algae to Energy Systems Student Lab

Your Challenge

Imagine your laboratory has just received grant funding to conduct research on the optimal growing conditions for Chlorella, a freshwater green algae species. Formulate at least two testable questions that you could use to drive your research.

What variable could you manipulate to examine one of your questions? Could you examine this variable at multiple levels?

Independent Variable:

What could you measure to examine the effects of your independent variable?

Dependent Variable:

Create a hypothesis that links your independent and dependent variable together in the context of algae: "If independent variable, then dependent variable."

Example: If we increase hours of light the algae receives, then the optical density will increase.

Materials and Methods

BTI Will Provide: (per class of 32 students, 8 groups of 4)

- Sterile Toothpicks
- Sterile cotton balls
- 32 air stones
- 4 packs of aquarium tubing
- 4 air pumps
- 4 four-way gang valves
- 12 T-valves
- Aquarium salt
- Urea
- Glucose
- 8 Transfer pipets
- 1 compressed air can
- 8 hemocytometers
- culture of Chlorella protothecoides

You will need to provide:

- Chlorox® disinfectant wipes – *OR* – Paper towels and a store-bought liquid disinfectant (e.g.., Lysol® All-Purpose Cleaner, 32 fl. oz., trigger-bottle) containing the microbicide alkyl dimethyl benzyl ammonium chloride
- Power drill with 13/64 inch drill bit
- Ruler
- Scissors
- Permanent marker
- 30 or more 500 mL clear plastic unopened water bottles
- 100 mL graduated cylinder
- Balance (capable of measurement to 0.1 g)
- Weigh boats
- Light bank or fluorescent lamps
- Aluminum foil, baking soda, pH paper (optional – depends on experiment variables)
- Bleach

Safety Information

Listen to all laboratory instructions and wash your hands prior to and after working with algal photobioreactors. Review MSDS sheets for any chemicals with which you are using. **PLEASE NOTE:** *Upon completion of the laboratory, the algae cultures should be properly disposed of by adding 4.7 mL, undiluted bleach per bioreactor, swirl gently to mix, and let stand for 10 min. before pouring down the drain.*

Construction of the Photobioreactor

1. Before starting, select an appropriate workstation (e.g., benchtop or desk). Preferrably, to avoid possible contamination of the photobioreactor during or following construction, the workstation should be removed from windows, doors, and/or overhead airconditioning and heating vents that permit airflow – and associated airborne microbes (e.g., fungi and bacteria) – over the worksurface.
2. The chosen workarea should be approximately 4 ft^2 (0.4 m^2) and void of general clutter.
3. Wipe down the work surface thoroughly using a disinfectant wipe or the liquid disinfectant and paper towels. Discard used cleaning materials to trash.
4. Prepare a "clean zone." Once the worksurface is clean (sanitized), arrange four, unused disinfectant wipes (2x2) on the center of the worksurface (see below). Alternatively, unfold 4 pieces of paper towel and moisten with the liquid disinfectant – enough to make the towels stick to the work surface. All material and tools (e.g., scissors) entering "the clean zone," illustrated below, must be wiped down with "fresh wipes" or liquid disinfectant.

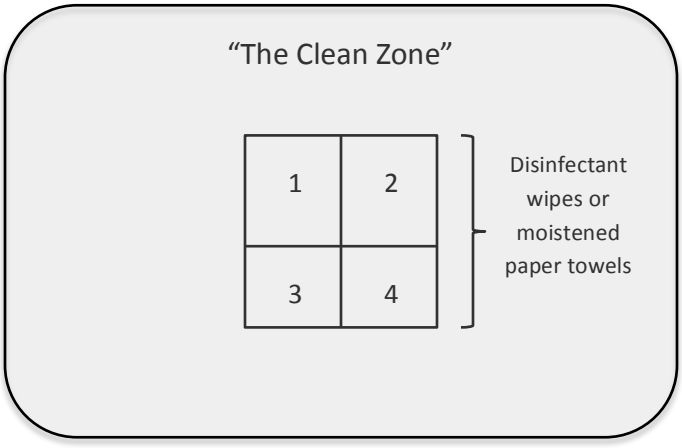


Fig 1. Illustation of a "Clean Zone" prepared for constructing the photobioreactor

5. Remove all paper/plastic labeling or decals from the clear plastic water bottle and, then wipe down the bottle with a "fresh wipe" or liquid disinfectant, making certain to clean the bottle cap and neck. Place bottle into your "clean zone"
6. Using permanent marker, label the neck of the water bottle with the date, assigned treatment, bottle number, and your names/initials (for example: 3/14/14, No Urea #2, JD [Jan Doe])
7. Using a 100 mL graduated cylinder, decant 70 mL from the water bottle; cap and seal immediately after decanting. Leave the remaining 430mL of water in the bottle.
8. Using a scale, weigh boat, and scoopula measure out the growth consistuents (nutrients) you wish to add to the photobioreactor. We suggest:
    a. **0.2 g aquarium salt (final concentration= 4.3×10^4 g/mL) —** Aquarium salts provides algae with the metals and ions needed for sustained growth.
    b. **0.8 g urea (final concentration= 1.7×10^3 g/mL) —**Urea is a form of nitrogen; providing a nitrogen-rich environment necessary for the growth of algae.
9. Carefully fold the the weight boat on itself and add growth constituents to the water bottle, making sure NOT to touch the bottleneck with the weigh boat.
10. Cap and seal immediately, and shake the nutrient-water solution for at least 2 min. until the constituents have completely dissolved. Set aside and in "clean zone."
11. Wipe down (surface sanitize) tubing for the airline and vent, and using separate wipes, the drill and drill bit. Place the tubing onto the moistened disinfectant wipes in your "clean zone."
12. Cut-to-length (Figure 1, A):
    a. 1, 2 ft piece of aquarium tubing (Aeration)
    b. 1, 3 in. piece of aquarium tubing (Vent)
        i. Wipe down each length of tubing and place in "clean zone"
13. Prepare the vent. (Figure 1, A and B). Pinch and remove a small piece of cotton, and using a sterile toothpick, force (stuff) the cotton approximately 1 in. into the tubing.
14. Drill two holes (diameter= 13/64 in.) into the cap of the bottle. Don't worry about shavings falling into the nutrient-water solution. (Skip this step if teacher is pre-drilling the bottles)
15. Take the tip of the aeration tube and forcefully insert the tubing through one of the previously-made holes in the bottlecap and feed approximately 1.5 ft of tubing through the cap and attach a sterile airstone to the tubing inside the bottle (Figure 1, C).
16. Carefully wipe the length tubing between the cap and airstone with a "fresh wipe," making sure keep the tubing and airstone off the work surface and away from conacting anything in the work area.
17. Carefully insert airstone-airline and seal the bottlecap. Strip out of the bottle any slack in the airline. The airstone should be gently touching the bottom-center of the bottle.

18. Insert vent tubing into the remaining hole and position the vent at a depth of no more than ¼ in. into bottle.
19. Quickly open the bottle, and using a sterile seriological or sterile transfer pipet, transfer 1 mL (2.5 x 10^3 cells) from the alga stock culture to the bottle – AVOID touching the pipet tip to the bottle's lip or neck.
20. Attach the free-end of the airline tubing to one of the spigots on the four-way gang valve or T-valve
21. Connect the four-way gang valve to the air pump with a 6 inch piece of airline tubing. Plug in the air pump and adjust the gang valve so that it appears that each bottle is getting equal air.
22. Place the photobioreactors in a well lit area (e.g. under two, equi-spaced lamps).
23. Follow your teachers instructions on what data to collect from your bottle, and how that is to be done. Before taking a reading, you should always swirl your sample for at least 30 seconds to ensure that all algae is mixed thoroughly throughout your sample, so your results are more consistent. Take an initial reading from your bottle and record it in your data table and in the class data table.
24. Over the next 7 days, evaluate and compare the estimated effect(s) of the individual treatments and/or treatment combinations on algae growth.

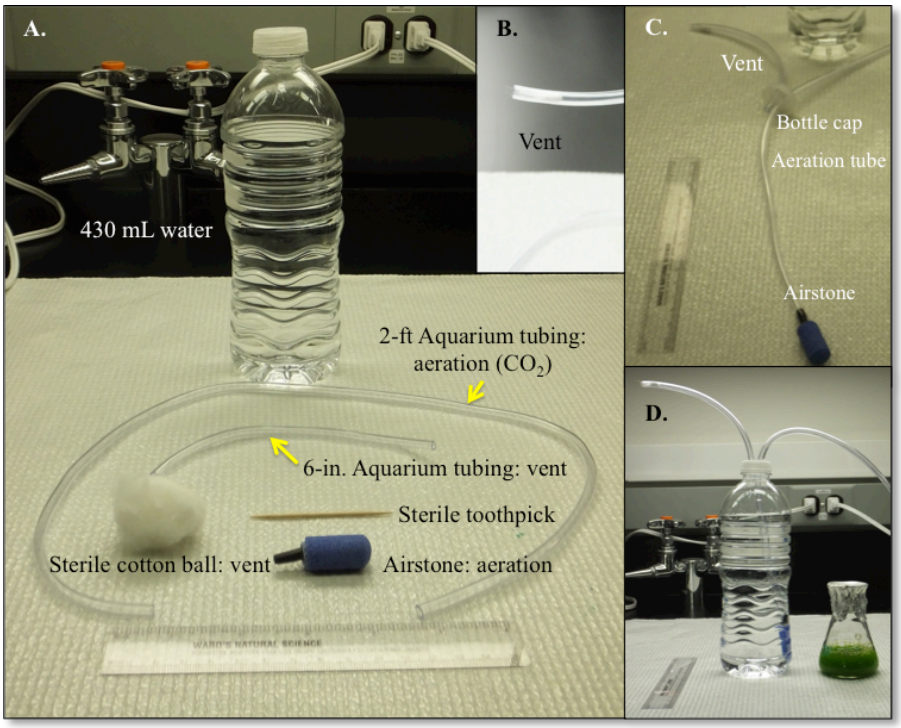


Figure 1. Construction basics for an algal bioreactor: (A) materials per reactor; (B) vent; (C) assembled components – vent, bottle cap, and aeration tubing with attached airstone; and, (D) bioreactor upon completion, ready for algae.

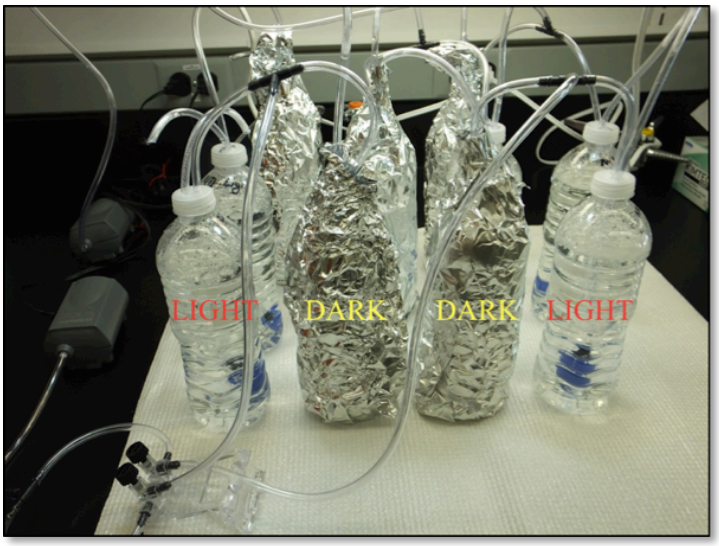


Figure 2 . General setup of a 4-bottle bioreactor system, varying only light regime(24:24 h, light:dark) across reactors.

Observations & Data Collection

Condition	Bottle	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Notes:
A=	1								
	2								
	3								
	4								
B=	1								
	2								
	3								
	4								
C=	1								
	2								
	3								
	4								
D=	1								
	2								
	3								
	4								

Questions

How many algal cells did you have on day 1 for bottle number _____?

How many algal cells did you have by day 7 for bottle number _____?

Graph & Analyze

Create a graph that displays your results.

Do you notice any trends in your data? Which condition supported the greatest growth? Which supported the least growth? Why might this be?

How much variability was there among the bottles in the same condition? What may have led to variability among the replicate bottles? How might this influence your understanding of your results?

If you were to perform this lab again, what changes would you make to explore your independent variable more fully? Propose and idea for future research.

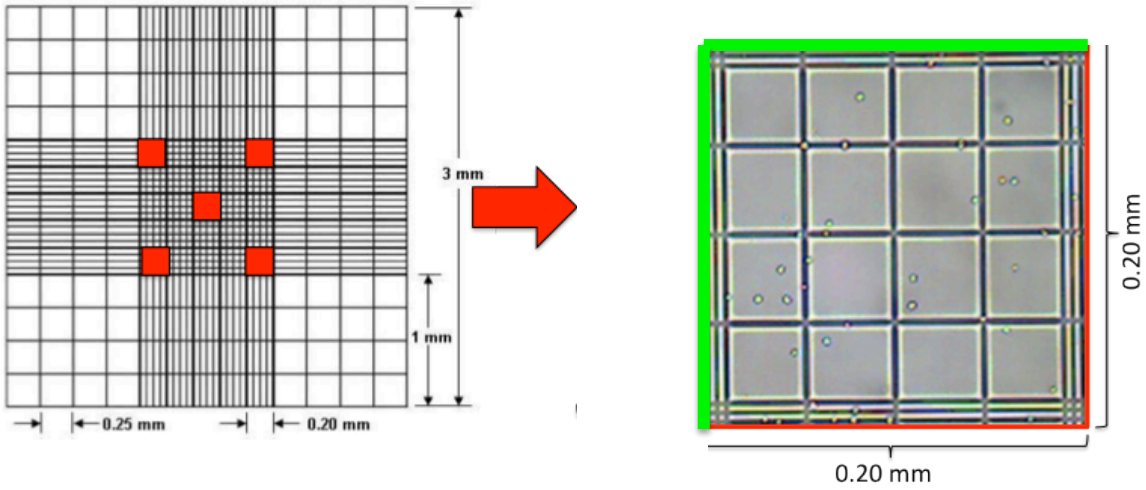
Conclusions

Based on the results of your research, what recommendations could you make to a company asking you about the growing requirements of the green algae Chlorella? Provide evidence to support your claim.

How might this information impact the company's ability to make biodiesel sustainably when thinking about the environmental or economic impacts of production?

Algae to Energy - Using and Re-using a Hemocytometer to Count Algae Cells

1) Prepare your sample by shaking your photobioreactor for at least 30 seconds and use a transfer pipet to remove 1 ml of algae. Add 10 ul of algae sample to slot A.
2) Count the cells. Using a compound microscope, count the number of cells in a square. Which size square you choose will depend on the density of your sample. If the sample contains a high density of cells, you will want to choose one of the smaller squares. If the sample contains a low density of cells, you will want to choose one of the larger squares. For the Algae to Energy lab, you will probably want to look at the small squares within the center square, highlighted in red, below. Choose 5 squares and count the number of cells in each square. If cells are on the border of the square, count only the cells touching the top and left sides of the square, as shown in green, below. Do not count the cells on the right and bottom sides of the square (shown in red)



3) Find the average cells/square. Add the number of cells from each square together and divide by 5 to get the average number of cells per square.
4) Calculate cell density.

$$\frac{Average\ \#\ of\ cells\ per\ square \times Dilution\ Factor\ (if\ any)}{Volume\ of\ the\ square} = Cell\ Density$$

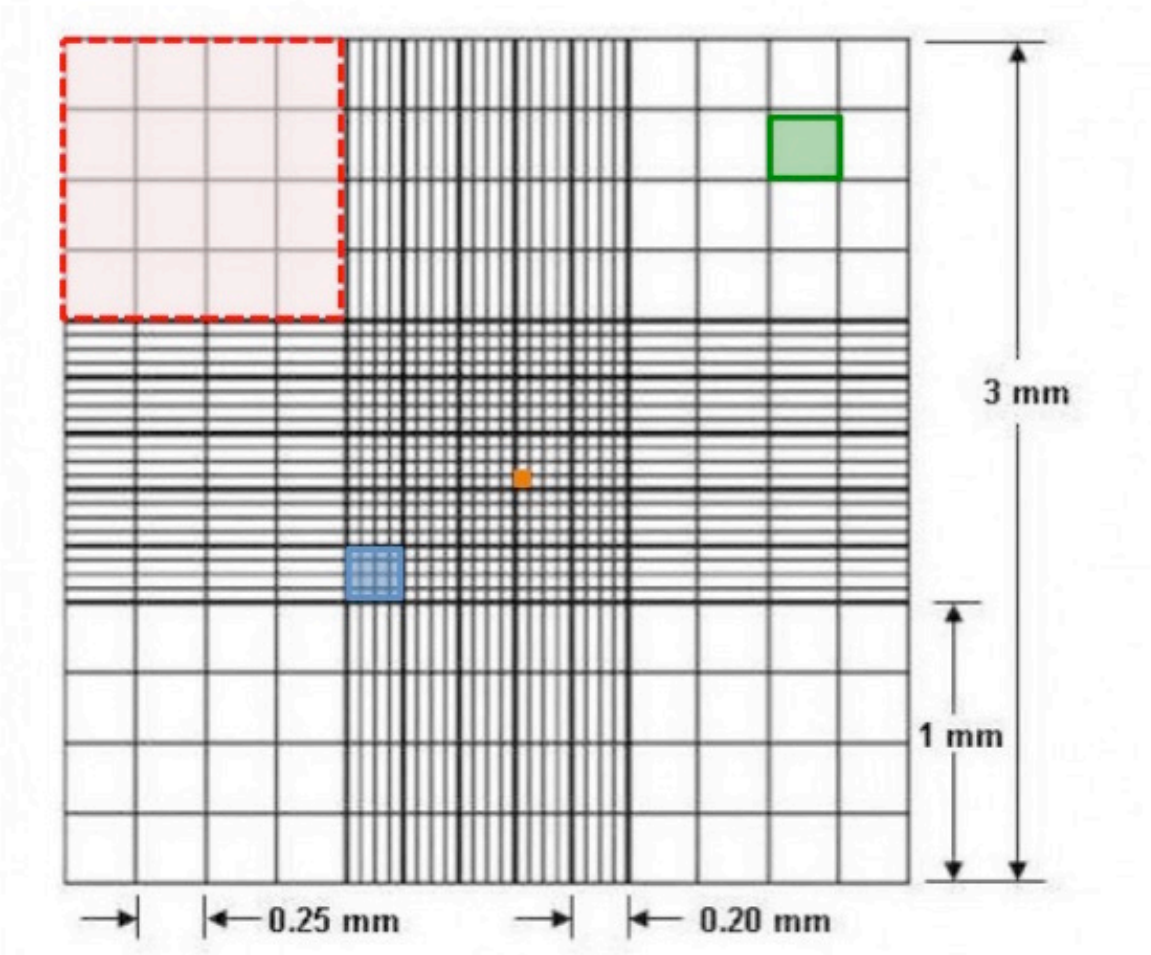
In this example, the average cells per square is 27. *If your sample was diluted, you will need to multiply this number by your dilution factor.*
If you are counting the small center squares, shown above in red, the volume of the square is 0.000004 ml.

$$Example: \frac{27\ cells}{0.000004\ ml} = 6{,}750{,}000\ cells/ml$$

Refer to the table on the next page for the volume of other squares.

This table, courtesy of hemocytometer.org, will help you calculate the volume per square that you are counting. Colors refer to the diagram, below. (http://www.hemocytometer.org/2013/04/11/hemocytometer-square-size/)

Unit	Width	Area	Volume (mm3)	Volume (mL)	#	
chamber	3 mm	9 mm2	0.9 mm3	0.0009 mL	2	per hemocytometer
Square (red)	1 mm	1 mm2	0.1 mm3	0.0001 mL	9	per chamber
Small square (green)	0.25 mm	0.0625 mm2	0.00625 mm3	0.00000625 mL	16	per corner square
Smaller square (blue)	0.2 mm	0.04 mm2	0.004 mm3	0.000004 mL	25	per central square
Smallest square (orange)	0.05 mm	0.0025 mm2	0.00025 mm3	0.00000025 mL	16	per smaller square



5) Clean out the hemocytometer for reuse. Though labeled as disposable, these hemocytometers can be cleaned and reused multiple times. Once the sample has been counted, use compressed air to expel the sample from the chamber by making a quick burst of air into the overflow space. Add distilled water to the A slot and expel it with the compressed air. Repeat this process three more times. Check your hemocytometer under the microscope to confirm that it is clean before reusing.
    a. The cleaning process can be seen here: https://www.youtube.com/watch?v=69T755-J33A (cleaning begins at the 4:15 mark)