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Reimagining an old photosynthesis classic: Quantifying the effects of different factors on photosynthetic rates using the dye bromothymol blue

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Here we present a quantitative approach to study the effect of various factors on photosynthetic rates of aquatic plants using bromothymol blue. We can measure the absorbance by the dye at 615 nm as an indication of the acidity due to CO_2 concentration in the solution, which relates to the rate of photosynthesis by the aquatic plant in the solution. We provide general instructions for using this dye to measure photosynthetic rates, as well as specific instructions for an experiment to study how photosynthetic rates are affected by the herbicide DCMU (3-(3,4-Dichlorophenyl)-1,1-dimethylurea), which blocks electron transport at the quinones in PSII. This example connects electron transport to CO_2 fixation and the change in the chemistry of the solution as measured through light absorption by the dye. This system can be used to study the effects of various factors on photosynthesis, which can be treated as inquiry-based or as guided-inquiry.

Keywords: photosynthesis, electron transport, herbicides, inquiry-based learning, bromothymol blue

Introduction

Addition of CO_2 to a solution leads to production of carbonic acid, which can be reversed when CO_2 is removed from the solution through a process such as photosynthesis. Bromothymol blue changes color based on changes in the pH, from yellow in acidic solutions to blue in alkaline solutions. Because of this, bromothymol blue has traditionally been used to demonstrate visually photosynthetic activity as the yellow solution turns blue in the presence of plants and light, indicating removal of CO_2 from the solution through photosynthesis. Absorption of light at 615 nm by the dye is high under alkaline conditions and low under acidic conditions (Abdel Rehim et al. 1987, Qi et al. 2003). Therefore, we can measure the change in absorbance by the dye at 615 nm as an indication of change in acidity due to changes in CO_2 concentration in the solution, including subtle differences in pH that may not result in color differences detectable by the naked eye (Figure 1). This allows us to use this system to quantitatively measure photosynthetic rates in a short period of time, which makes this a feasible way to study the effects of different factors on photosynthesis quickly during a standard 3-hour lab period.

Depending on the goals of the course and the objectives for the lab exercise, one can present a standard method to use this system to measure the CO_2 concentration in the solution (Appendix A), allowing the students to ask their own specific questions and design their own experiments. Alternatively, one can present a specific question to the students and use this system to answer it. In the example provided here, students are given a question on the mode of action of the herbicide DCMU in killing plants. As this herbicide is an inhibitor of a specific step in the electron transport chain in the thylakoids, the study of the effect of DCMU on photosynthesis brings

together the practical aspects of the herbicide use in agribusiness with the basics of the understanding of the relationship between electron transport and the photosynthetic carbon reduction (PCR) = the Calvin Benson Basham Cycle, and its effects on gas exchange by the leaf, and consequences for pH of the aquatic solution in a closed system.

Given the quantitative nature of the data collected by the students, this lab exercise will allow the students to use their data analysis skills to calculate descriptive statistics and present their data graphically. In addition, the data lend themselves to analysis by a standard t-test, if that type of data analysis is appropriate for the course level.

Preparation time for the lab is minimal. The solutions can be made ahead of time and kept at room temperature. A bicycle CO_2 cartridge (10-25 g) can be used as the CO_2 delivery system instead of using straws to blow in CO_2 by breath or using a CO_2 tank in the lab. Each cartridge contains enough CO_2 for multiple lab sections. Any fast-growing aquatic plant can be used – we have tried both *Elodea* (when it was commercially available in New York; it no longer is) and *Ceratophyllum*.

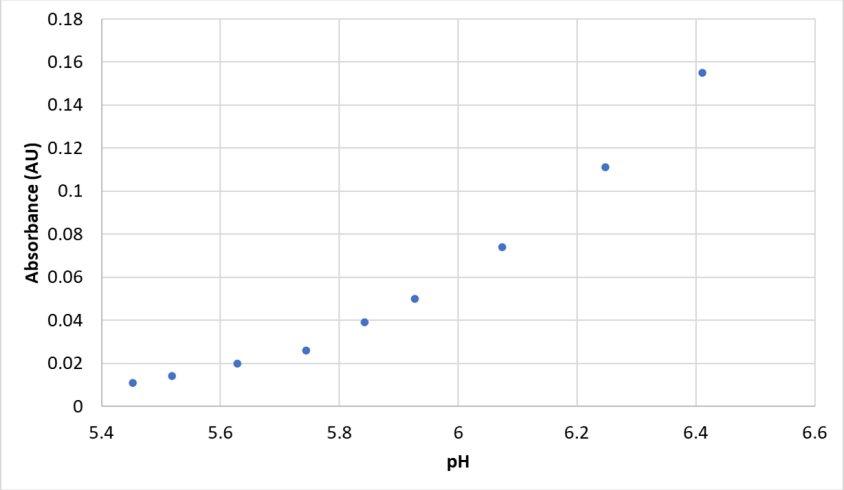


Figure 1. The relationship between pH and Absorbance at 615 nm for 0.002% bromothymol blue solution in the range typically measured in the experiment to measure rates of photosynthesis by aquatic plants such as *Ceratophyllum demersum*.

Student Outline

Objectives

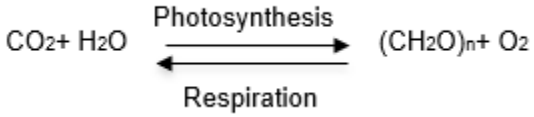
Design an experiment to investigate how rates of photosynthesis are affected by various factors
Collect, analyze, and present the data from such an experiment
Explain the connection between gas exchange in the leaf, carbon fixation, and electron transport
Discuss the limitations of such an experiment and suggest future steps based on the results obtained
Explain the difference between net and gross photosynthesis
Describe how and why bromothymol blue can be used to measure rates of photosynthesis

Introduction

Photosynthesis involves a series of photochemical and biochemical reactions that capture the energy from photons of light and store it in the organic bonds of simple sugars. The reactions in photosynthesis occur in multiple stages: a) the photochemical reactions (also called the light reactions) in the thylakoid membranes capture light energy, use it to transfer electrons through the electron transport chain to synthesize NADPH and to establish a proton gradient, which is then used to synthesize ATP, while generating oxygen molecules as a byproduct, and b) the biochemical reactions (also called the light-independent reactions) in the photosynthetic carbon reduction (PCR) cycle in the stroma use the ATP and NADPH from the photochemical reactions to reduce CO_2 to make 3-Carbon sugar molecules, which are the building blocks for more complex sugars and other compounds.

The sugars produced through photosynthesis can be broken down through the process of respiration to

release energy for other energy-requiring cellular activities. In Eukaryotic organisms including plants, aerobic respiration is carried out in mitochondria. The basic point of the complex reactions in respiration and photosynthesis can be summarized as follows (note that the reaction as written is not balanced):



The process of photosynthesis in chloroplasts uses CO_2 and releases O_2 while the process of aerobic respiration in mitochondria uses O_2 and releases CO_2. The plants you will study today have both mitochondria and chloroplasts and so photosynthesis and respiration may be occurring simultaneously within any given leaf of the plant. Thus, both processes will be affecting the relative concentrations of CO_2 and O_2 in the intercellular spaces in the mesophyll layer of a leaf.

In photosynthetic tissues, CO_2 consumption by chloroplasts (gross photosynthesis) during photosynthesis is counteracted by CO_2 production by mitochondria during respiration. The net amount is referred to as "net photosynthesis". What happens to net photosynthesis if:

a) The rate of respiration in the mitochondria is lower than the rate of gross photosynthesis in the chloroplasts of the leaf?
b) The rate of respiration is higher than the rate of gross photosynthesis?
c) The rate of respiration is equal to the rate of gross photosynthesis?

In an aquatic system, addition of CO_2 to the solution leads to production of carbonic acid, and thus acidification of the medium. Conversely, use of CO_2 through photosynthesis reduces the acidity of the medium. We can take advantage of this change in acidity to measure rates of photosynthesis with dyes that change color due to changes in pH. One such dye is bromothymol blue, which changes color from blue under alkaline conditions to yellow under acidic conditions. Depending on the magnitude of the change in pH, the color change may be intense enough to be visible to the naked eye. The color change can also be measured quantitatively using a spectrophotometer. Absorption of light at 615 nm by the dye is high under alkaline conditions and low under acidic conditions. Therefore, we can measure the change in absorbance at 615 nm as an indication of change in acidity of the solution, which in turn relates to the CO_2 concentration in the solution. This technique allows us to measure subtle differences in pH that may not result in color differences detectable by the naked eye.

We can also use this system to study the effects of various chemicals on photosynthesis. DCMU (3-(3,4-Dichlorophenyl)-1,1-dimethylurea) is one such chemical that has been used as an herbicide because it blocks electron transfer between the quinones in PSII (Metz et al. 1986, Taiz and Zeiger 2010). How will DCMU affect electron transport? How will it affect carbon fixation? How will its effect be detectable through the use of the dye bromothymol blue?

In this experiment, you will use bromothymol blue as an indicator dye to measure rates of photosynthesis in an aquatic plant, *Ceratophyllum demersum*. You will also study the effect of DCMU on the rate of photosynthesis in these plants. You will analyze the class data to write a short report on your findings.

Methods and Data Collection

Materials

- Bromothymol blue stock solution (0.04%)
- DCMU 5.0 mM stock solution in 50% acetone
- 50% acetone
- Distilled water
- *Ceratophyllum demersum*
- Weighing paper
- Paper towels
- Electronic Balance
- 15-mL tubes with caps
- Test tube racks

- 200-mL Beakers
- Graduated cylinders
- Erlenmeyer flasks
- Lamps
- Large beaker or aquarium with water as heat sink
- Spectrophotometer and 1.5 mL semi-micro cuvettes
- Spring water

Investigation: The effect of DCMU on the rate of photosynthesis

What are the class hypotheses (null and alternative) with regards to the effects of DCMU on the rate of net photosynthesis in *C. demersum* leaves? What is the justification for your hypothesis based on the mode of action of DCMU? What is the class prediction for the experiment based on the procedure below? Include in your report.

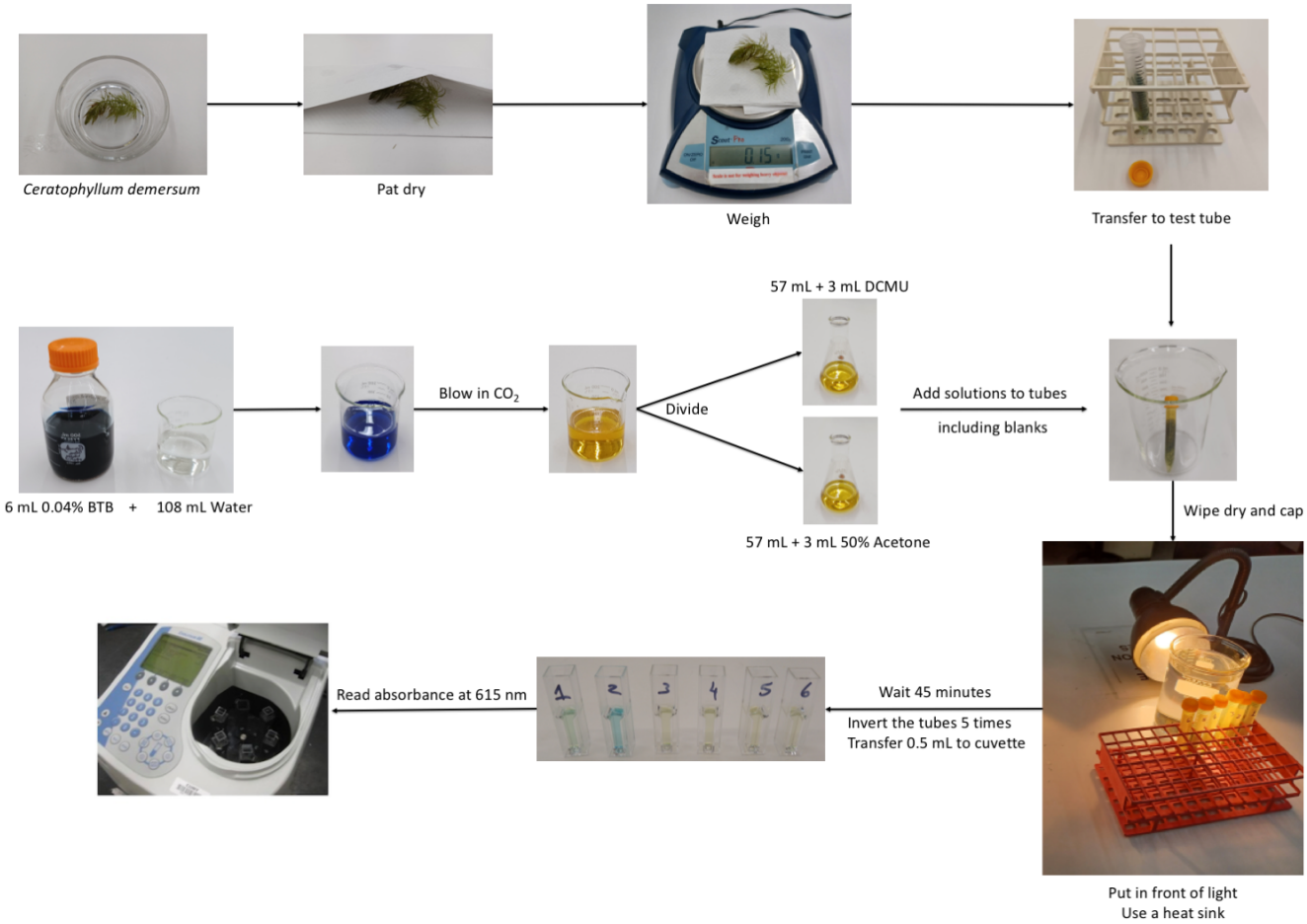


Figure 2. The procedure for measuring rates of photosynthesis in *C. demersum* in the presence or absence of DCMU using bromothymol blue as an indicator dye.

Procedure (also shown in Figure 2)

1. Take four sprigs of *C. demersum* (about 3 inches long each). Pat dry with a paper towel. Place a piece of weighing paper on the balance and tare. Weigh each piece of *C. demersum*. Place in tubes 1, 2, 4, and 5. Keep track of which piece is in which tube. Record the weights in the Table 1.
2. Take one 200-mL beaker. Add 108 mL of water and 6 mL of bromothymol blue stock solution.
3. Using the CO_2 set up in the lab, blow in enough CO_2 to turn the solution yellow.
4. Pour 57 mL of the yellow solution into each of the two Erlenmeyer flasks.

5. Label one flask “Bromothymol Blue”, the other “Bromothymol Blue + DCMU”.
6. To the first flask add 3 mL of 50% acetone. To the second flask add 3 mL of the DCMU stock solution. Swirl the flasks to mix well.
7. Add enough bromothymol blue solution to reach the top of each 15-mL tubes #1-3 including the one with no plant: #3 = blank for the “Bromothymol Blue” solution. It is ok to overflow a little. You do not want any air in the tube. It is best to hold one 15-mL tube in an empty beaker as you pour in the solution, so that any overflowing solution is captured in the beaker. Cap the tubes and wipe dry. Place in the test tube rack.
8. Repeat with “Bromothymol Blue + DCMU” for tubes #4-6. Note: tube #6 = blank for the “Bromothymol Blue + DCMU” solution.
9. Place the tubes in front of the lamp. Use a large beaker or a fish tank filled with water as a heat sink, unless you are using a lamp that does not generate much heat. Turn on the lamp. Start the timer.
10. After 45 minutes invert the tubes 5 times and then use a micropipettor with the appropriate tip to transfer 0.5 mL of the solution from each tube into a spectrophotometer cuvette. Label the cuvettes accordingly.
11. Measure the absorbance at 615 nm, using the appropriate blanks for the different solutions. Record the absorbance values for time t = 45 in Table 1.
12. Empty the solution from each tube and cuvette into the waste container.
13. Rinse the plants with distilled water several times and place in a container with spring water.

Table 1. Group Data: Measured absorbance values at 615 nm and rates of photosynthesis of *C. demersum* subjected to DCMU or the solvent acetone.

Tube #	*C. demersum* weight (g)	Solution used	Absorbance at t = 45 (AU)	Photosynthetic rate (AU/hr.g)
1		Bromothymol Blue + Acetone		
2				
4		Bromothymol Blue + DCMU		
5				

Data Analysis

1. Calculate the photosynthetic rates by dividing the measured absorbance by the time period. Correct for the size of the plant used by dividing the rate by the weight of the plant in each tube.
2. Combine your group data with the rest of class data to use in the data analysis.
3. Make a column graph of the means ± standard deviations for the class data for the calculated rates for the two treatments using the class data. Include in your report.
4. Use the class data to carry out a t-test to determine whether the effect of the DCMU compared to the control was statistically significant. Include in your report.
5. Do the class data support or reject the class hypothesis? How confident are you? What does this tell you about the effect of this herbicide? Include in your report.

Cited References

Metz, JG, Pakrasi, HB, Seibert, M, Arntzen, CJ. 1986. Evidence for a dual function of the herbicide-binding D1 protein in Photosystem II. FEBS Letters 205(2):269-274.

Taiz, L, Zeiger, E. 2010. Plant physiology. Fifth edition. Sunderland (MA): Sinauer Associates, Inc.

Materials

The supplies list below is for a lab of 24 students divided into 6 groups of 4 each based on the student handout provided.

Supplies	Number / Amount
Bromothymol blue stock solution (0.04%)	6 mL per group, 36 mL per lab
DCMU 5.0 mM stock solution in 50% acetone	3 mL per group, 18 mL per lab
50% acetone	3 mL per group, 18 mL per lab
Ceratophyllum demersum	at least 4 sprigs (3” long) per group, 24 per lab
Electronic Balance (to 0.01 g)	1 per group would be ideal, but groups can share
15-mL tubes with caps	6 per group, 36 per lab
Test tube racks	1 per group, 6 per lab
200-mL beakers	2 per group, 12 per lab
100-mL graduated cylinders	1 per group, 6 per lab
10-mL graduated cylinders	1 per group, 6 per lab
100-mL Erlenmeyer flasks	2 per group, 12 per lab
75 or 100 W lamps	At least 1 per group (2 would be better) 6 per lab (12 would be better)
Large beaker or aquarium with water as heat sink if using lamps that generate a lot of heat	1 large beaker per lamp, or if using an aquarium as a heat sink, then multiple lamps can be put behind one
Spectrophotometer	1 per group would be ideal, but groups can share
1.5-mL spec cuvettes	6 per group, 36 per lab
P1000 Micropipettors with tips	1 per group, 6 per lab
Permanent markers	1 per group, 6 per lab
CO_2 delivery set up (see Figure 3) OR straws	CO_2 delivery set up: 1 per lab; Straws: at least one per group, 6 per lab
Distilled water	110 mL per group, 660 mL per lab
Waste container for 250 mL of liquid waste	1 per group, 6 per lab
Weighing paper	
Paper towels	
Gloves	
Spring water for the aquatic plants	

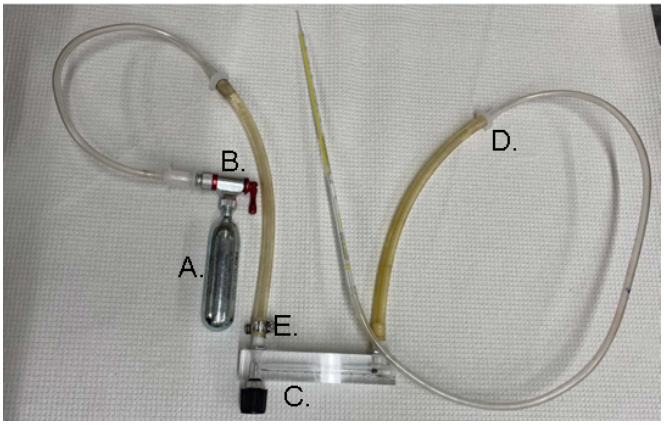


Figure 3. CO_2 delivery set up using a 16g CO_2 cartridge. A. CO_2 cartridge, B. CO_2 cartridge valve, C. gas flow meter, D. airline tube, and E. clamp rings.

A. CO_2 Cartridge: $13
https://www.amazon.com/Pro-Bike-Tool-Threaded-Cartridges/dp/B076YVFGKC/ref=sr_1_1_sspa?crid=2P2IPIZV583MG&keywords=co2%2Bcartridges&qid=1656092911&sprefix=CO2%2Bcart%2Caps%2C283&sr=8-1-spons&spLa=ZW5jcnlwdGVkUXVhbGlmaWVyPUEyTUpaNzgwWDNJR1BFJmVuY3J5cHRlZElkPUEwNDE1NTI1M0JIVzE0VkZJUkVUMyZlbmNyeXB0ZWRBZElkPUEwMDY2MDQxMTZYVjYzOUNOV0hWTCZ3aWRnZXROYW1lPXNwX2F0ZiZhY3Rpb249Y2xpY2tSZWRpcmVjdCZkb05vdExvZ0NsaWNrPXRydWU&th=1

B. CO_2 Cartridge Valve: $20-$25
https://www.amazon.com/gp/product/B076DBP2JZ/ref=crt_ewc_img_srh_1?ie=UTF8&smid=AL3PM2CMR6UEA&th=1

C. Gas Flow Meter: $20
https://www.amazon.com/dp/B07DNW6JHX/ref=cm_sw_r_oth_api_i_R8MEGM8NZJWWN1NF9MZ9?_encoding=UTF8&psc=1

D. Airline Tubes: $5-$15
Depending on the size needed. We used ½" and 3/4" tubes with tube connectors. These items can also be obtained on Amazon or from any fish/pet store.

E. Clamp rings

Notes for the Instructor

To add CO_2 to the solution, students can use straws and blow CO_2 from their breath into the solution. If you have access to a CO_2 tank and regulators, you can set up a CO_2 delivery system that works for you. We rigged up a system relatively cheaply using CO_2 cartridges that are used by bicyclists (Figure 3). Each 16g cartridge can be used for more than 3 liters of bromothymol blue solution.

Any fast-growing aquatic plant that grows in high light can be used. If *Elodea* is available in your state, then that is an option. As *Elodea* is no longer commercially available in certain states like New York, we are using *Ceratophyllum demersum*, which is available from lab supply companies such as Carolina Biological, or pet stores that sell aquatic plants for fish tanks.

You can have the students measure the absorbance of the solutions at time 0 and at time 45 minutes (using distilled water to zero the spec) to see any changes over time even in the absence of plants, which have to be taken into account. You can also have the students use water to zero the spec and measure the absorbance of the "blank" tubes and then have the students manually subtract those values with an explanation of why that must happen. Also, if you or the students make a pH-versus-dye absorbance curve such as Figure 1 in the introduction, then you

can compare these absorbance values for the blank tubes and those with plants to those on the pH/absorbance curve to see that they fall in the range where a change in the pH corresponds to a change in absorbance and thus validating the method.

This lab exercise can be run as guided inquiry asking the students to come up with their own questions. Usually the possibilities include i) different wavelengths of light depending on light filters or light bulbs available, ii) shade cloths and different light intensities, iii) different temperatures, as the set up can be placed inside a fridge, for example, and iv) various "pollutants" to which the students can subject the plants by soaking them ahead of time. Examples of the pollutants include the following: a) de-icers and anti-icers, b) industrial waste salts, c) salinization, and d) agricultural pollution.

Cited References

Abdel Rehim, F, Said, FIA, Abdel-Azim, A, El-Dessouky, MM, Youssef, N. 1987. Use of bromothymol blue solutions as a spectrophotometric dosimeter. International Journal of Radiation Applications and Instrumentation. Part C. Radiation Physics and Chemistry 30(3):209-214.

Qi, Z, Matsuda, N, Santos, J, Itoh, K, Takatsu, A, Kato, K. 2003. A study of molecular adsorption of bromothymol blue by optical waveguide spectroscopy. Langmuir 19(2):214-217.

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About the Authors

Fardad Firooznia received his BS in Biology from Yale University and his PhD in plant physiology from Cornell University. He has been an instructor at The City College of New York (CCNY) since 2010, where he teaches large introductory biology courses as well as upper-level seminar courses in ethnobotany, ecophysiology, and plant physiology.

Jhunior Morillo received his BS in Biology and MS in Entomology and Ecology from the City College of New York (CCNY). After receiving his MS, Jhunior was hired as a Senior Laboratory Technician at CCNY, where he continues to work very closely with students and professors. He is currently responsible for preparing various biology laboratories.

Appendix A

General procedure for measuring rates of photosynthesis using bromothymol blue as an indicator

This is the basic procedure for measuring rates of photosynthesis using this system without any additional inhibitors, etc. Students can be provided with the basic procedure, which they can modify depending on the experiment they are designing.

1. Take five sprigs of *C. demersum*. Pat dry with a paper towel. Place a piece of weighing paper on the balance and tare. Weigh each piece of *C. demersum*. Place in tubes 1-5. Keep track of which piece is in which tube. Record the weights.
2. Take one 200-mL beaker. Make 100 mL of 0.002% Bromothymol blue solution using the 0.04% stock solution provided:
   ___ mL 0.04% stock solution + ___ mL distilled water
3. Using the CO_2 set up in the lab, blow in enough CO_2 to turn the solution yellow.

Note: Steps 4 and 5 are used if you want to check the change in absorbance over time in the absence of any plants. Otherwise, skip.

4. Using a micropipettor with the correct tip, transfer 0.5 mL of each bromothymol blue solution to a spectrophotometer cuvette.
5. Use distilled water as the blank. Measure the absorbance at 615 nm. Record the absorbance values for time t=0 min.
6. Add enough bromothymol blue solution from the beaker to reach the top of each 15-mL tubes 1-5 and 6 (no plant). Cap the tubes, wipe dry, and place in the rack.
7. Place the tubes in front of the lamp. Use a large beaker filled with water or a fish tank filled with water as a heat sink. Turn on the lamp. Start the timer.
8. After 45 minutes invert the tubes 5 times and then use a micropipettor with the appropriate tip to transfer 0.5 mL of the solution from each tube into a spectrophotometer cuvette. Label the cuvettes accordingly.
9. Use distilled water as the blank. Measure the absorbance at 615 nm. Record the absorbance values for time t=45 min.
10. Empty the solution from each tube into the waste container.
11. The plants can be rinsed in distilled water, kept in spring water for a day, and used again in future labs.

Appendix B

Sample Data

Table 2. Measured absorbance values at 615 nm and rates of photosynthesis of *C. demersum* subjected to DCMU or the solvent acetone. In this example, the specs were zeroed with distilled water, and the absorbance by the tubes without plants (the "blank" tubes) was measured and then subtracted from the measurements for tubes with plants.

Tube #	*C. demersum* weight (g)	Solution used	Absorbance at t = 45 (AU)	Absorbance corrected for blank (AU)	Net Photosynthetic rate (AU/hr.g)
1	0.47	Bromothymol Blue + Acetone	0.078	0.041	0.1163
2	0.73		0.081	0.044	0.0804
3	1.24		0.117	0.08	0.086
4	0.76		0.089	0.052	0.0912
5	0.76		0.119	0.082	0.1439
6	Blank		0.037		
7	1.55	Bromothymol Blue + DCMU	0.035	-0.001	-0.0009
8	0.38		0.037	0.001	0.0035
9	0.46		0.039	0.003	0.0087
10	0.46		0.043	0.007	0.0203
11	0.76		0.039	0.003	0.0053
12	Blank		0.036		

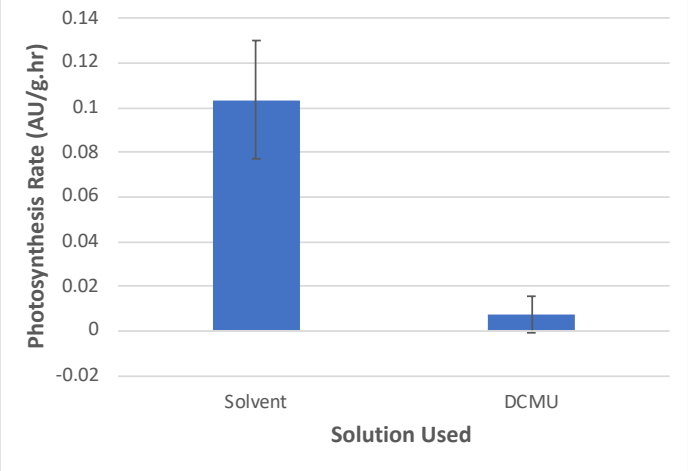

Figure 4. The effect of DCMU on the rate of photosynthesis in *C. demersum*. Columns represent means, and error bars represent standard deviations. Sample size was 5 for each treatment. The means are statistically significantly different at 95% confidence level.

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