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Using Algae in Undergraduate STEM Education: A Flexible Inquiry-Based Investigation at Scale

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Algae are a diverse group of organisms that influence biogeochemical cycles, feed millions of people, and inspire myriad entrepreneurial ventures. In the classroom, algae are truly an incredible teaching tool that open inroads towards learning in diverse inter-related topics central to any biology curriculum. We have developed a laboratory investigation intended for high school and introductory undergraduate education using consumer water bottles, basic equipment and relatively affordable supplies. In this lab, students integrate various biological concepts and applications through inquiry-based, student-driven research. Teams will apply the basics of photosynthesis and the interplay between two fundamental metabolic modes: cellular respiration and photosynthesis. A number of independent variables such as salinity, nutrients, light, and species are alterable for a myriad of multivariate experimental designs. Students partaking in this lab will gain experience in basic microbiology and plant science skills such as sterile technique, cell counting, and pigment extraction.

Keywords: algae, photobioreactor, photosynthesis, microbiology, inquiry-based, STEM

Introduction

Algal biology and related concepts are uniquely suited for teaching an array of basic concepts in biology, where students in introductory courses struggle to find purchase. In this way, student-led investigations into algal growth and diversity offer an exciting tool for increasing student engagement and enhancing learning outcomes. Yet the design and implementation of large-scale laboratory investigations focused on algae is challenging, even when wisdom and resources are readily available.

To address this problem, we designed an inquiry-based laboratory exercise where students conduct an algae growth experiment using consumer water bottles as photobioreactors (PBRs). Students add sterile salts, nutrients, and experimental treatments, then fit the bottles with a bubbler to introduce air and mix the cultures. In the process, students apply the basics of photosynthesis, learn basic microbiology skills like sterile technique, and design then execute an experiment of their own conception.

After designing treatment groups, constructing and inoculating PBRs, student cultures grow for a week, and this process is repeated to allow for replication. Multiple rounds of growth could conceivably be completed in the course of a semester.

While this investigation was designed with biofuels in mind, the material herein is flexible and is pertinent in many contexts. Any application focused on generating biomass is within the scope of this framework and myriad modifications can be made to this investigation. This investigation could easily be adapted with a more prescribed curriculum as an introductory plant biology exercise or for high-school classrooms.

Given that research in algal biology is so easily connected to concepts in both applied and fundamental biology, students with diverse interests can identify with the learning experience of this lab. We implemented this investigation in a large undergraduate course (>1000 students) and it has become a student favorite, often leading to a desire for further research experience.

Manuscripts published by undergraduate students who completed this investigation can be found online in the *Journal of Introductory Biology Investigation*

(https://undergradsciencejournals.okstate.edu/index.php/jibi).

Student Outline

Objectives

- Understand and apply the basics of photosynthesis
- Use sterile technique and other microbiology skills
- Design and execute an algal growth experiment
- Describe multivariate effects on algal growth

Introduction

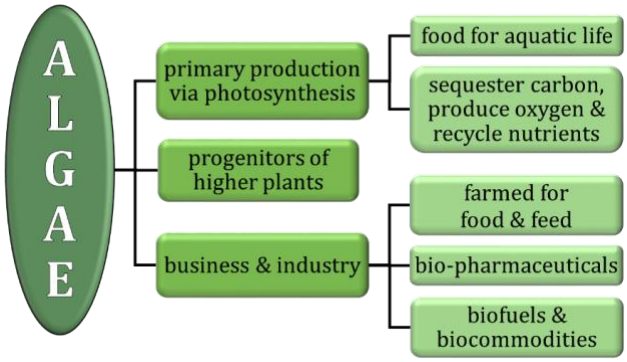

Figure 1. The biological and commercial significance of algae.

Algae are a wildly diverse group of eukaryotic organisms (meaning they have a nucleus) commonly divided into three lineages: green algae, red algae and glaucophytes. These three lineages arose from distinct endosymbiotic events where a eukaryotic cell engulfed a cyanobacterium (a prokaryotic photosynthetic cell), thereby gaining the ability to photosynthesize. Wow! While these lineages exhibit different ecophysiological properties, all algae share a set of basic requirements for their growth and division; for example, light, carbon, nitrogen, phosphorus, and other micronutrients like Si, Mg, Fe and vitamins all play an important role in algal biology.

Through millions of years of evolutionary trial and error, single-celled microalgae eventually gave rise to more complex multi-cellular organisms called macroalgae. This group can take many forms, like the common Caribbean seaweed *Sargassum*, or algae that build the 'forests of the ocean' – kelp, in the order *Laminariales*. Notably, green algae are the ancestral progenitors of the higher plants we see here on land today! All of that evolutionary development made algae highly effective photosynthetic machines that exhibit some of the highest photosynthetic efficiencies ever recorded. Algae are responsible for much of the carbon sequestration, oxygen production, and habitat formation our ecosystems rely on – just like the Rainforests!

This investigation will focus on microalgae, who have received considerable attention in recent years for their potential in producing renewable biofuels, sustainable protein for food and livestock feed, and many other biocommodities. One common theme in algal biofuel research is the concept of nitrogen starvation, to which algae respond by storing up starch and lipid for better times. Yet nitrogen is necessary for growth, so this method is a double-edged sword – it increases starch and lipid content but decreases growth rate. This trade-off is the focus of a many algal biofuel studies. If we can understand this response, we may be able to produce affordable biofuel.

While the physiology and ecology of a number of algal species are well-studied, there are still plenty of exciting basic-science questions in ecophysiology worthy of investigation. What's more, there are many newly discovered strains of algae whose physiology remains relatively un-studied. In particular, the species *Picochlorum* has received considerable attention as of late due to its rapid growth, biomass composition, and broad salinity tolerance (Henley 2004).

Now it is your turn to break new ground in algal research and development – what will *you* discover?!

Methods and Data Collection

Your instructor will provide some background knowledge and a basic outline of the tasks this investigation involves. Once you have completed some background research on algal physiology and ecology you can proceed with forming your hypothesis, designing your experiment, and building your photobioreactors.

Part A: Designing Your Experiment

The first step in your algae growth experiment is to choose a topic about the growth physiology of algae to investigate and form a hypothesis. Keep it simple and only change one independent variable related to your research question. You can measure multiple dependent variables to better analyze and understand growth physiology. You will work in groups to construct a number photobioreactors (PBRs) using bottles of water. At least two of your bottles will serve as control groups, the other bottles will be your treatment groups. Treatments should be organized in duplicate or as levels of an individual variable (low, medium, high). A number of independent variables can be manipulated in this investigation including nutrients (N & P), salinity, inoculation density, and carbon source.

In order to simplify the setup and design, your instructor will provide a pre-made growth media (GM). This will come in concentrated form labelled as 100xGM; thus, you will dilute this 100x to yield standard 1xGM in your PBRs with a base level of nitrogen, phosphorus and bicarbonate, as outlined in Table 1. The final nitrate concentration (NO_3) after adding the 100xGM is 200 µM, and the final phosphate concentration (PO_4) is 40 µM; refer to Table 3 for details. Experimental nitrate concentrations may vary between 200 µM and 2000 µM, and phosphate concentrations may vary between 40 µM and 400 µM;

refer to Table 3 for further details. Consider the commonly used ecological terms oligotrophic, mesotrophic, and eutrophic, which correspond to nitrogen and phosphorus concentrations of approximately 0-1 µM, 2-10 µM, and 11-1000 µM, respectively.

Bicarbonate increases the concentration of dissolved CO_2, thereby providing carbon for photosynthesis. However, bicarbonate will change the culture pH, as dissolved CO_2 takes the form of carbonic acid - this is why the oceans are acidifying with increased atmospheric CO_2! Refer to Table 3 for more information on other carbon sources. If your algae is a halophilic (salt loving) or marine species, salt should be added to all cultures unless low salinity is your independent variable. The 100xGM contains sea salt (or aquarium salt) which contains a mixture of electrolytes (Mg, Ca, K) but is mostly NaCl. The salinity of your PBR will be 0.2% after you add the 100xGM, and sea water has a nominal salinity of 3.5%; you will use NaCl to alter gross salinity with a baseline of 2% (Table 2). If you alter Mg, Ca, or K, pay attention to chemical formulas e.g. *K*NO_3. In regard to novel experimental additives like food coloring, miracle grow, energy drinks (Fig. 2), or powdered milk etc. - start small! The dry weight of your cultures will reach a maximum of ~2 g/L, so you want to add <0.1 g of any additive.

Table 1. Final media contents after the addition of 100x GM.

	Final%	Final
Sea Salt	0.2	-
KH_2PO_4	-	39.7 µM
KNO_3	-	197.8 µM
$NaHCO_3$	-	1.0 mM

Table 2. Salinity adjustment guide.

Salinity	g of NaCl to each bottle (~400 mL)
1.0%	4 g
2.0%	8 g
3.5%	14 g
5.0%	20 g

Table 3. Use this as a guide for altering nutrient levels. You may use these amounts when filling out Table 5 below.

Treatment	Stock	Add to each bottle
Nitrate - KNO_3	1 M	80 µL
Nitrate is the most common inorganic nitrogen source in the environment. Adding 80 µL to a PBR (400 mL) will double (NO_3) from 200 to 400 µM.		
Urea – $CO(NH_2)_2$	1 M	40 µL
Urea is an organic nitrogen source with 2 nitrogen atoms per molecule. Thus, adding 40 µL of urea to a PBR is equivalent to adding 80 µL of KNO_3.		
Phosphate – NaH_2PO_4	0.5 M	32 µL
Phosphate is needed for the DNA backbone and metabolic function (ATP). Adding 32 µL to a PBR (400 mL) will double (PO_4) from 40 to 80 µM.		
Bicarbonate - ($NaHCO_3$)	0.5 M	2 - 8 mL
Bicarbonate is an inorganic carbon source that mimics increased atmospheric CO_2.		
Glucose – $C_6H_{12}O_6$	-	0.010 – 0.100 g
Vinegar – $C_2H_3O_2$-	10% v/v	10 µL – 1 mL
Glucose and acetic acid are organic carbon sources. These may be added if investigating heterotrophic or mixotrophic growth. Acetic acid → acetyl-CoA.		

Figure 2. Start small when adding novel treatments. Additives should not be so concentrated as to color the culture. A) Healthy, green PBR cultures after one week of growth; B) cultures with a large amount of miracle grow C) cultures with a large amount of energy drink.

Part B: Building Your Water-bottle Photobioreactors

Table 4 gives stepwise instructions for constructing and inoculating your PBRs. Refer to Tables 1, 2, and 3 for details regarding the design of your experimental treatments. Be sure to record all additions to your PBRs and treatment

groups in Table 5. Cell count, dry weight, and chlorophyll content data may be gathered after inoculation for an initial data point today in lab and at the beginning of lab the following week for the final data point.

Table 4. Recommended inoculation protocol for control bottles.

1. Decant water from the bottle until ~400 mL remain.
2. Add 4 mL of **100x growth media** with a sterile transfer pipet.
3. Add 2 mL of **0.5 M bicarbonate** with a sterile transfer pipet.
4. Add 8 g of salt and firmly cap the bottle and shake.
5. Now add 4 mL of **inoculum** with a sterile transfer pipet.
6. Sterilize the assembled tubing and fasten it to your bottle.

You now have an assembled and inoculated **PBR**!

Table 5. Use this table to document additions to your treatment groups.
Suggested amounts are provided but these values may change according to the investigation.

		Control Bottle 1	Control Bottle 2	Treatment Bottle 1	Treatment Bottle 2
Total volume (mL)		~ 400	~ 400	~ 400	~ 400
Inoculum volume (mL)		4			
Growth Media Treatments	100x GM (mL)	4	4	4	4
	Treatment 1: e.g. phosphorus	None added			
	Treatment 2: e.g. nitrogen	None added			
	Treatment 3: e.g. salt	None added			

Part C: Growing Your Cultures

Your instructor will inform you of options for your growth environment, i.e. light, temperature and time. Briefly, your cultures should be grown at room temperature (25°C), under moderately high white light, for one week. Place your cultures in their intended spot and hook up the air supply to the inlet tube of your PBRs. The bubbling rate should be high enough to stir your cultures, but not so high that foam forms at the top of the cultures or liquid is pushed out of the venting tube. If the bubbling rate is too high, you might have a mess on your hands or worse, you will lose your data. As a rule of thumb, the bubbling rate should be faster than one bubble per second, but slower than you can count with your eye (10 bubbles/second).

Part C: Gathering Your Data

Three main dependent variables may be investigated: cell count, dry weight, and chlorophyll content. You should record observations of at least two of these measurements with cell count being the most informative. Typical cell densities range from 100,000 to 100,000,000 cells/mL, where the latter looks 'pea soup green.' Dry weight is a simple measure that provides an estimation of the total of biomass in the culture by filtering, drying and weighing a known volume of culture. The quantification of chlorophyll content is performed via solvent extraction and UV-Vis spectrophotometry - this is the most accurate measure. However, there are more convenient methods for chlorophyll quantification – ask your instructor for details. Protocols for dry weight and chlorophyll quantification are provided in Appendix A.

Part D: Data Analysis & Discussion

The final but most important step in your algae growth experiment is analyzing and interpreting the data!

Data analysis is relatively simple, but interpretation can be nuanced. First count your cells to compare cell growth and division between bottles; cell count is usually expressed in millions of cells per milliliter (or cells mL^{-1}). Next measure your chlorophyll content, which is often given in units of mg L^{-1}, which is equal to µg mL^{-1}. If you divide the chlorophyll content in µg mL^{-1} by your cell count in cells mL^{-1}, the result is the amount of chlorophyll in each cell, or the cell specific chlorophyll content. This may change in ways that are not intuitive.

Say for instance you place a culture under low light and a culture under high light with the same amount of nutrient. You might predict that the culture under low light would yield a *lower* cell specific chlorophyll content, because the cells are supplied with less light for photosynthesis. Yet it is likely the case that your culture under lower light will results in a *higher* cell specific chlorophyll content, because each cell is trying harder to capture light for photosynthesis – and that is chlorophyll's job. You can also compare the dry weight of your cultures to calculate the total biomass yield from each of your treatments – which treatment grew more stuff?!

Go forth and discover, but remember that an alga you are not! You will need to think outside of your human-sized box to understand algae. These little creatures have been working on photosynthesis since Precambrian times, so you have some catching up to do!

Figure 3. Images of growth shelving and PBRs during cultivation for this investigation at Oklahoma State.

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Henley WJ, Hironaka JL, Guillou L, Buchheim MA, Buchheim JA, Fawley MW, Fawley KP. 2004. Phylogenetic analysis of the 'Nannochloris-like'algae and diagnoses of Picochlorum oklahomensis gen. et sp. nov. (Trebouxiophyceae, Chlorophyta). Phycologia. 43(6):641-652.

Ritchie RJ. 2008. Universal chlorophyll equations for estimating chlorophylls a, b, c, and d and total chlorophylls in natural assemblages of photosynthetic organisms using acetone, methanol, or ethanol solvents. Photosynthetica, *46*(1):115-126.

Materials

Necessary supplies needed to conduct this investigation include: water bottles, cotton, aquarium pumps and tubing, gang valves, chemicals for growth media, a balance (0.0001 g), sterile transfer pipettes, microscope and a hemocytometer. Supplies required for chlorophyll and dry weight analysis include: micropipettes, microcentrifuge and tubes, spectrometer and cuvettes, acetone or methanol, vacuum filter apparatus, glass fiber filters, and a drying oven.

The use of a handheld fluorometer/turbidimeter available from Turner Designs has been especially helpful and in gathering rapid and reliable measures of chlorophyll content as well as turbidity, which can be used to estimate biomass density <https://www.turnerdesigns.com>.

Improvements like the use of bottle-top dispensers available from (VWR Cat. No. 470134-946) are student-user friendly, cut down on waste, and prevent last-minute in-lab issues, which simplifies the planning process and streamline operations during lab. Access to advanced equipment such as a chlorophyll fluorometer, flow cytometer, or elemental analyzer will greatly enhance the analytical complexity of this investigation and expand opportunities for student discovery.

Notes for the Instructor

The difficulty in conducting this lab lies in the logistics of producing stock solutions and growing large volumes of dense, healthy inoculum in a sterile fashion ahead of time. A basic workflow of the required preparations is outlined in Figure 4. Detailed information regarding media recipes, procedures for growing stock cultures, and notes on preparing inoculum for students can be found in the Appendix B; see detailed recipes for full growth media in Appendix C. We recommend partnering with an expert for the design, planning, and preparation of this investigation.

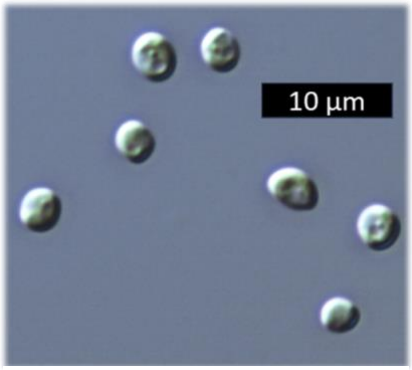

Figure 5. DIC image of *P. oklahomense.*

We have had success growing *Picochlorum oklahomense*, a halophile with broad salinity tolerance (Henley 2004). *Picochlorum* exhibits a number of beneficial characteristics for this lab such as low settling rate, hardy growth, and flexible physiology. What's more, using saline media mitigates contamination - an issue that cannot be over-emphasized as healthy inoculum is necessary for success. Some well-known strains of algae (e.g. *Nannochloropsis*, *Dunaliella*, *Chlorella*) may be purchased from Carolina Biological, but not *Picochlorum*. This species is relatively new to the field and can be purchased from various algae culture collections.

The media recipes included in the student handout are designed such that control PBRs will yield a satisfactory level of growth for student experiments; however, media for stock growing stock cultures for inoculum will be more complex and include a trace metals, vitamin and iron solution. Attention to detail in regard to micronutrients like iron and vitamins is important for growing healthy stock cultures, but not necessary for

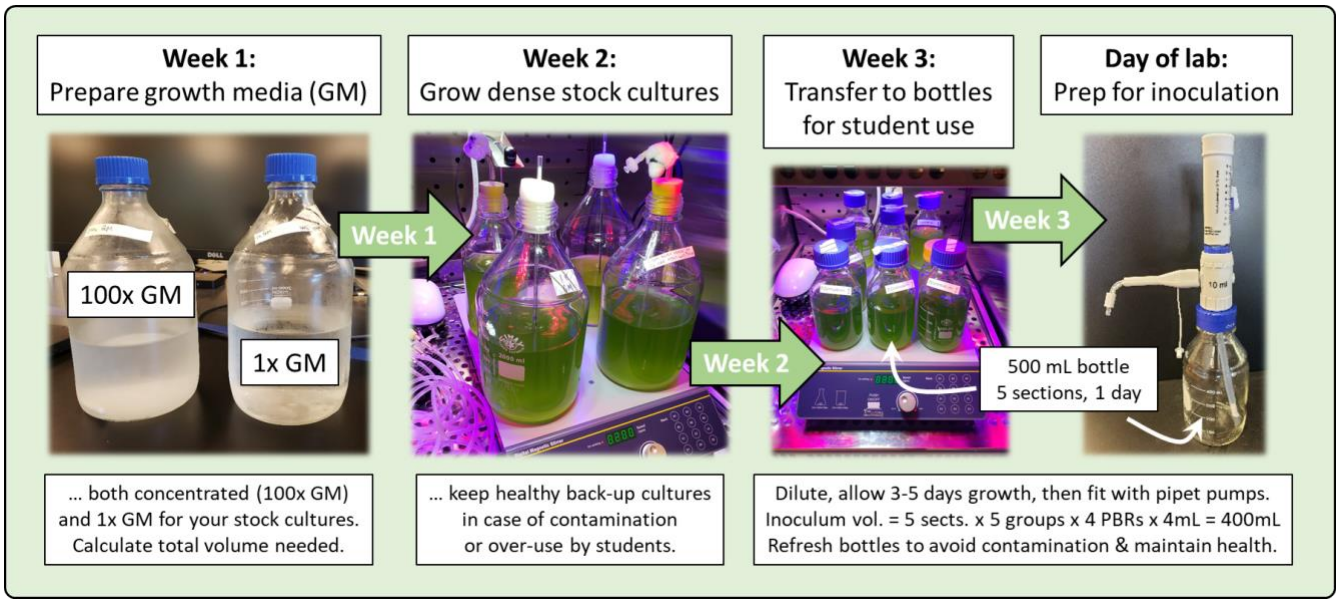

Figure 4. Follow this general workflow for weekly laboratory preparations.

student cultures – if you prepare your stock cultures with sufficient micronutrients they will make it into the students' PBRs. If you wish to alter the species grown, care should be taken in re-designing media contents and growth conditions.

Due to time, space, and material constraints, we settled on 4 bottles per group of 2-4 students. Ideally, groups setup their experiment with two control bottles and two treatment bottles; they will either repeat the exact treatments during weeks one and two, or change the treatment groups by doubling, tripling, etc. their particular treatment. The issue here is that cultures used to inoculate are never the same and inoculation volumes may vary from week to week. While this is not ideal, it is the reality of large scale inquiry-based investigations.

Your growth setup can be as simple as shelves and fluorescent tubes available at your local hardware store, along with shoe-box sized plastic boxes that fit eight PBRs. With standard shelving (ca. 2'x4'), 10-12 shoeboxes can fit on one shelf and accommodate up to 24 groups with 4 PBRs each – this may be altered to fit your particular needs.

Students will inevitably want to alter the light and temperature their cultures are grown at, but it is particularly difficult to offer options for these variables. Altering temperature requires a climate-controlled room or incubator large enough to accommodate your student's PBRs; otherwise grow them at room temperature (25°C). Altering light *quantity* is easily achieved with the use of window screens or by changing the distance between the lights and cultures. However, altering light quality is difficult unless you purchase LED arrays with white/blue/red bulbs that are individually controllable; care must be taken to alter only *quality* and not *quantity*, think about the number of photons provided. Otherwise, grow your cultures under moderately high white light supplied by white fluorescent bulbs ideally intended for plant growth. LED arrays intended for commercial applications can be exceedingly bright and dangerous to the naked eye, not to mention too bright for some algal cultures.

Your cultures may be grown for 5-10 days, but one week is convenient for class schedules. Growing cultures for two weeks will result in late-stationary phase cultures; this is not a problem but should be taken into account when analyzing results. Growing cultures for longer than two weeks may result in contamination or cell death.

The bubbling rate should be high enough to stir your cultures, but not so high that foam forms at the top of the cultures or liquid is pushed out of the venting tube. If the bubbling rate is too high, you might have a mess on your hands may results in the loss of data. As a rule of thumb, the bubbling rate should be faster than one bubble per second, but slower than you can count with your eye (10/second). Some evaporation may occur, high bubbling rate will exacerbate this.

Note: This lab was developed by the Boyce Thompson Institute for Plant Research, then adapted and improved for use in large scale undergraduate labs at Oklahoma State University [Shackleton 2015].

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About the Authors

Dylan T. Franks graduated in May of 2020 with a Ph.D. in Plant Science from the Department of Plant Biology, Ecology, and Evolution at Oklahoma State University; he has since formed Arrowhead Algae, a small business focused on producing algae for animal feed and other biocommodities.

Donald P. French is a professor in the Department of Integrative Biology at Oklahoma State University focused on the impact of technology and methods of instruction on student attitude and performance. John F. Stewart is a postdoc researcher in the Department of Integrative Biology focused on designing biology classroom activities that facilitate authentic research experiences; he is also responsible for managing the Life Science Freshman Research Scholar program which facilitates freshman entering faculty laboratories to conduct life science research. Moria G. Harmon is a lecturer in the Department of Integrative Biology responsible for managing the

functions of the BIOL 1114 lab and lecture; she works tirelessly towards student success.

Appendix A: Procedures

Dry Weight Procedure

Materials:

- Culture samples (10-25 mL)
- Vacuum flask, tubing, & filter apparatus
- Glass fiber filters (934-AH, GF/C or GF/F)
- 10 mL pipette or 25 ml graduated cylinder
- Deionized (DI) water, squirt bottle, forceps, aluminum foil, and a sharpie
- Balance (0.0001 g)

1. Using a marker, label one filter per sample with dots (1=●, 2=●●). Pre-dry filters at 80°C for at least 10 minutes.
2. Cool the filters and record the **pre-weight** to the tenth of a mg (0.0001 g).
3. Place the filter in the apparatus and turn on the vacuum. The filter apparatus should be secured at all times.
4. Swirl the culture sample to fully homogenize and withdraw a known volume.
5. Slowly dispense the culture sample onto the center of the filter and rinse with 5 mL of DI water.
6. Carefully remove the filter, place it in a labelled foil envelope.
7. Place labelled envelopes in a drying oven set to 80°C for at least 30 minutes.
8. Cool the filters and record the **total-weight** to the tenth of a mg (0.0001 g).

Dry weight = total-weight – pre-weight.

Chlorophyll Extraction & Quantification Procedure

Materials:

- Culture samples (1-3 mL)
- P1000 micropipette
- Microcentrifuge and tubes
- Spectrometer and 1 mL cuvettes
- 100% acetone or methanol

Extraction:

1. Pellet a 1 mL aliquot of culture at 10,000xg for 5 minutes.
2. Carefully remove 900 µL of the supernatant without removing any cells.
3. Re-suspend the pellet by adding 900 µL of acetone or methanol and vortex.
4. Incubate at room temperature and in the dark for at least 1 hour and vortex.
5. Pellet cell debris at 10,000xg for 5 minutes. (You should see no green in your cell pellet, it should be white.)
6. Transfer the extract to a fresh tube. Keep the extract out of direct light and store in the freezer.

Quantification:

7. Fill the cuvette with acetone, place it in the spectrometer, and blank the spectrometer.
8. Empty the cuvette and transfer the extract.
9. Read the sample making sure to record the absorbance at the appropriate wavelengths.
10. Rinse the cuvette and repeat for all samples.

The equations for calculating chlorophyll content in acetone are as follows:

$$\text{Chl a} = -0.300\,(A_{630} - A_{750}) - 1.754\,(A_{647} - A_{750}) + 11.91\,(A_{664} - A_{750})$$

$$\text{Total Chl} = 22.08\,(A_{630} - A_{750}) - 10.24\,(A_{647} - A_{750}) + 5.422\,(A_{664} - A_{750})$$

The equations for calculating chlorophyll content in methanol are as follows:

$$\text{Chl a} = -0.324\,(A_{630} - A_{750}) - 6.415\,(A_{647} - A_{750}) + 16.44\,(A_{664} - A_{750})$$

$$\text{Total Chl} = 27.96\,(A_{630} - A_{750}) - 12.92\,(A_{647} - A_{750}) + 1.002\,(A_{664} - A_{750})$$

This equation gives units = mg L^{-1} = µg mL^{-1}.
$A_{\#\#\#}$ = Absorption at ### wavelength, e.g. A_{647} = absorption at 647nm.
Adapted from Ritchie, 2008 *Photosynthetica*, *46*(1), pp.115-126.

Appendix B: Laboratory Prep Procedures

Instructor Workflow

Follow this general workflow for lab preparations:

PREP	Week 1	Make growth media, dry cycle bottles, start a stock culture
	Week 2	Scale-up stock culture, check shelving & lighting setup, plan
	Week 3	Prep nutrient stocks, tubing setups & scale-up stock cultures
LAB	Week 1	Aliquot inoculum & media into bottles, fit with dispensers
	Week 2	Prep culture & media as above, prepare for data gathering
	Week 3	Prepare for data gathering & analysis, clean-up

100x Growth Media (GM) Stock Mixing Procedure

Materials:

- 2 L pitcher
- 2 L bottle and a large stir bar
- Hot plate w/ stirring
- Aquarium salt
- KH_2PO_4 - Potassium Phosphate Monobasic
- KNO_3 - Potassium Nitrate
- NaHCO3 – Bicarbonate
- Tris buffer (pH ~8)
- Weigh boats and spatula

➢ 100x GM stock concentration of salt is very high, so it is difficult to fully dissolve all of the solute.

1. Carefully slide a stir bar into a 2 L bottle.
2. Add about 1800 mL of DI water to the bottle.
3. Place the bottle on the hot plate, set the heat to medium and stirring to high.
4. Heat the water but **do not boil** (~10 min) and turn **OFF** the heat.
5. Weigh aquarium salt, KH_2PO_4 and KNO_3 and add them to the bottle.
6. Bring contents to **2000 mL** with DI water in the 2 L pitcher (careful - **HOT**!!!).
7. Transfer the solution back to the 2 L flask (careful - **HOT**!!!).
8. Insert a foam stopper in the flask and cover the lid with a piece of aluminum foil and autoclave tape.
9. Clearly label the bottle with “100X Growth Media" and the date.
10. Autoclave the 100x stock at 121°C for at least 30 minutes.

Recipe for 2 L (2000 mL) of 100x GM Stock

	MW	g to add	[Final]	
Aq Salt	-	200.0	10.0	%
KH_2PO_4	136.086	1.080	3.97	mM
KNO_3	101.103	4.00	197.8	mM
Bicarbonate	84.007	0.840	5.0	mM

1x GM Mixing Protocol

Materials:

- 2000 mL pitcher
- 25 & 250 mL graduated cylinder
- 100x GM stock
- 2 L bottle and large stir bar
- Neoprene stopper fitted with glass pipets

1. Add 20 mL of 100x GM stock with a graduated cylinder to the 2 L pitcher.
2. Add 40 g of salt to obtain a final salinity of 2%.
3. Bring the solution to 2000 mL with DI water.
4. Carefully slide a stir bar into a 2 L bottle and transfer the solution.
5. Carefully insert a neoprene stopper fitted with glass pipets.
6. Cover the lid with a sturdy piece of aluminum foil and autoclave tape.
7. Clearly label the bottle with "Growth Media" and the date.
8. Autoclave the media at 121°C for at least 30 minutes.

Post autoclaving nutrient additions (add to 2000mL)

Ingredient	Amount
Vitamins	1 mL
Thiosulfate	2 mL
Antibiotics	2 mL (if necessary)
See Media SOPs for recipes for these solutions	

Stock Culture Inoculation & Inoculum Prep Procedure

Materials:

- Culture for inoculation
- Autoclaved bottle with media and large stir bar
- Sterile 250 mL flask
- 70% Ethanol (EtOH)

- ➢ Be as sterile as possible, as these cultures will provide students with inoculum.
- ➢ If these cultures are contaminated it will affect the quality of the investigation.

1. Obtain a stock culture and a 2 L bottle of sterile media.
2. Sterilize hood, hands and instruments with 70% EtOH.
3. Place the cooled media bottle in the hood.
4. Use a sterile flask to transfer ~200 mL of inoculum into the 2L media bottle.
5. Replace the bottle-top assembly (Fig 6).
6. Place the bottles under your lighting and hook up the air.

Figure 6. Examples of bottle-top assemblies. Neoprene stoppers (left) are re-usable and durable, but require drilling holes and cutting flint glass tubing to size. Disposable foam stoppers (right) can be autoclaved and re-used ~10 times until the foam degrades; these can be cut with a sterile razor blade and fitted with a 1mL serological pipet for air supply.

Appendix C: Media and Solutions

Growth Media Recipes

Stock Solutions:

Media component	FW (g/mol)	1° Stock Recipe	1° Stock Molarity	Stock 2% AS Molarity	Minimal 2% AS Molarity
NaCl	58.44	-	-	342.0 mM	" "
$MgSO_4$ (anh.)	120.366	29.8 g/250 mL	0.9903 M	1.980 mM	" "
KCl	74.551	15.0 g/250 mL	0.8048 M	1.610 mM	" "
$CaCl_2$ (anh.)	110.98	5.55 g/250 mL	0.2000 M	400.0 µM	" "
$NaNO_3$	84.945	25.0 g/250 mL	1.1765 M	11.77 mM	600.0 µM
NH_4Cl	53.491	6.675 g/250 mL	0.4991 M	499.2 µM	-
KH_2PO_4	136.086	1.25 g/250 mL	0.4991 M	367.4 µM	80.0 µM
Thiosulfate	112.128	1.25 g/250 mL	0.1056 M	44.59 µM	" "
$NaHCO_3$	84.007	10.5 g/250 mL	0.0446 M	5.00 mM	" "
Tris Base	121.14	100 g/1000 mL (titrated to pH 8.0)	0.8255 M	8.25 mM	" "

Media component: FeEDTA		FW (g/mol)	1° Stock Recipe	1° Stock Molarity	Stock 2% AS Molarity
$FeCl_3 \cdot 6H_2O$		270.32	0.162 g/200 mL	0.0029 M	8.99 µM
Prepare by adding 0.162 g of $FeCl_3$ to Solution A and mix with Solution B. Titrate to pH 7.5 with NaOH.					
Solution A	0.1 N HCl	36.46	862 µL/100 mL	0.100 M	-
Solution B	$Na_2EDTA \cdot 2H_2O$	372.24	2.5 g /100 mL	0.0336 M	100.7 µM

Vitamin Mix	FW (g/mol)	1° Stock Recipe	2° Stock Recipe /250 mL	Base Molarity (0.5 mL of 2° Stock/L)
Thiamine (B_1)	265.35	-	50 mg	0.377 µM
Biotin (B_7)	244.31	10 mg/10 mL	250 µL of 1° Stock	2.047 µM
Cyanocobalamin (B_{12})	1355.38	10 mg/10 mL	250 µL of 1° Stock	0.369 µM

Trace Metals Mix	FW (g/mol)	1° Stock Recipe	2° Stock Recipe/250 mL	Base Molarity (2 mL of 2°Stock/L)
H_3BO_3	61.8338	4.283 g dissolved in 1.25 mL conc. HCl		554.13 µm
$CoCl_2 \cdot 6H_2O$	237.9315	121.5 mg/20 mL	0.25 mL of 1°Stock	51.06 nM
$MnCl_2 \cdot 4H_2O$	197.9057	43.2 mg/20 mL	0.25 mL of 1°Stock	21.83 nM
$ZnSO_4 \cdot 7H_2O$	287.5525	664.5 mg/20 mL	2.5 mL of 1°Stock	2.311 µM
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1236.013	311.9 mg/20 mL	2.5 mL of 1°Stock	252.34 nM
H_2SeO_3	128.97	64.5 mg/100 mL	25.0 µL of 1°Stock	1.00 nM
Prepare by adding the above components to ~100 mL of deionized water, then bring to 250 mL. Do not titrate.				

Antibiotic (AB) Mix	FW (g/mol)	1° Stock Recipe	Base Molarity (1 mL of 1° Stock/L)
Ampicillin Sodium Salt	371.39	12.5 g/250 mL or 50 mg/mL	134.63 µM (50 µg/mL)
Streptomycin Sulfate	1457.58	6.25 g/250 mL or 50 mg/mL	34.3 µM (50 µg/mL)

Artificial Seawater (AS):

- This sheet is adapted from Artificial Saltwater recipes for algal growth at any salinity and allows for any media component to be altered as needed.
- These recipes are optimized either for stock cultures or for minimal media, which has much lower [N] & [P] to allow for modifications.
- Salt proportions: NaCl and other salts change in proportion according to %AS. However, fresh water still contains a basal level of Mg, K and Ca.

  *Note: anhydrous forms change MW!
- Tris buffer should be titrated to pH 8.0. Tris buffer prevents large swings in pH due to photosynthesis and ensures that carbon remains biologically available.
- Bicarbonate is added to supply inorganic carbon. Review bicarbonate dynamics in your textbook for more detailed info.
- Chelated iron (FeEDTA) is an essential nutrient. Pre-made solutions are available for purchase.
- Algae need their B vitamins, although the requirements are species specific. Little is known about *Picochlorum* in this regard.
- Thiosulfate is used as an antioxidant and sulfate source; it is not clear if it is essential.
- If needed, antibiotics may be added to specific cultures in order to 'passage' the culture through a selective media, thereby allowing the algae to out-compete the bacteria before subsequent dilution of the culture.

Growth Media Salts	**0%**	**1%**	**2%**	**3.5% AS**
	… for a total volume of 1000 mL.			
NaCl	-	10 g	20 g	35 g
$MgSO_4$ (anh.)	1 mL	1 mL	2 mL	3.5 mL
KCl	1 mL	1 mL	2 mL	3.5 mL
$CaCl_2$ (anh.)	1 mL	1 mL	2 mL	3.5 mL

Growth Media Recipe	**Stock 2% AS**	**Minimal 2% AS**
	…for a total vol. of 1000 mL.	
NaCl	20 g	20 g
$MgSO_4$ (anh.)	2 mL	2 mL
KCl	2 mL	2 mL
$CaCl_2$ (anh.)	2 mL	2 mL
$NaNO_3$	10 mL	0.51 mL*
NH_4Cl	1 mL	-
KH_2PO_4	10 mL	2.18 mL**
Trace Metals	2 mL	2 mL
FeEDTA	3 mL	3 mL
Tris Buffer	10 mL	if necessary
…add the nutrients below after autoclaving with a sterile syringe filter		
Thiosulfate	1 mL	1 mL
$NaHCO_3$	10 mL	10 mL
Vitamins	0.5 mL	0.5 mL

Calculating Nutrient Concentration: $C_1V_1 = C_2V_2$					
$NaNO_3$*	1.1765 mM	X mL	=	600 µM	1000 mL
		X	=	0.5099 mL	
KH_2PO_4**	0.0367 mM	X mL	=	80 µM	1000 mL
		X	=	2.1798 mL	

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