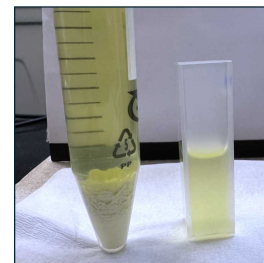


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Quantification of Coral Chlorophyll Using Shimadzu UV-VIS 2450 Spectrophotometer

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Coral Bleaching RCN pro...



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We use this protocol and it's working

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Abstract

Most corals living in shallow, tropical waters have a symbiotic relationship with unicellular dinoflagellate algae belonging to the family *Symbiodiniaceae*. These algal cells are located within membrane-bound vacuoles in gastrodermal cells in the oral layer of the host coral and thus, are referred to as *endosymbiotic*. These endosymbiotic algae can photosynthetically fix carbon due to the presence of chlorophyll *a* and *c*₂ photopigments within their chloroplast organelles. The overall photosynthetic capacity of corals is affected by the concentration of total chlorophyll pigments per endosymbiotic algal cell and the density of endosymbiotic algal cells in the coral host. When corals are exposed to stressful environmental conditions, including prolonged periods of elevated seawater temperature or high ultraviolet radiation, the symbiotic relationship between corals and algae is disrupted. During this process, known as **coral bleaching**, the concentration of total chlorophyll pigments per endosymbiotic algal cell can decrease (i.e., photopigment degradation) and/or the density of endosymbiotic algal cells in the coral host can decrease (i.e., algal loss) (e.g. Porter *et al.* 1989; Lesser 1997; Grottoli *et al.* 2006). Both processes result in the coral colony appearing pale or white (i.e., bleached), and photosynthesis is reduced. Chlorophyll concentration is the second most commonly used metric for assessing coral bleaching status after *Symbiodiniaceae* density (McLachlan *et al.* 2020).

This protocol outlines a method of quantifying the concentration of total chlorophyll pigments (i.e., chlorophyll *a* and *c*₂) per unit of biomass (i.e., micrograms of chlorophyll per gram dry weight) of Scleractinian coral samples that have been ground into a homogenous paste consisting of aragonite skeleton, coral host tissue, and endosymbiotic *Symbiodiniaceae* cells. This procedure employs a double solvent extraction (using 100% acetone) and uses a Shimadzu UV-VIS 2450 spectrophotometer to measure the photopigment absorbance at three wavelengths of light (630 nm, 663 nm, and 750 nm). **If you are using a different make and model of spectrophotometer, please fork this protocol and replace the "Measuring Chlorophyll absorbance" steps with instructions appropriate to the spectrophotometer you are using.** The concentration of chlorophyll *a* and *c*₂ pigments are calculated using the equations from Jeffrey & Humphrey (1975) for dinoflagellates using 100% acetone solvent.

There are nine steps to this protocol that are completed over five days:

- 1) Preparation of ground coral samples
- 2) Day 1: First acetone extraction
- 3) Day 2: Measuring Chlorophyll absorbance from first extraction
- 4) Day 2: Second acetone extraction
- 5) Day 3: Measuring chlorophyll absorbance from second extraction
- 6) Day 3: Filter and dry the chlorophyll samples
- 7) Day 4: Weigh dried pans then combust the dried samples
- 8) Day 5: Weigh burnt pans
- 9) Calculations

This protocol was written by Dr. Rowan McLachlan and was reviewed by co-authors (Dr. Andréa Grottoli and Dr. Rebecca Vega Thurber).

Acknowledgments



I would like to thank Dr. Kimberly H. Halsey (Oregon State University) for kindly allowing me access to her UV-Vis Spectrophotometer and Vaishnavi Padaki (Oregon State University) for training me to use this instrument and facilitating access to the laboratory.



Materials

Reusable materials:

Lab coat
Safety goggles
Mortar and pestles (preferably ceramic, but metal, glass, or Pyrex will also work)
Small silicone or rubber spatula
Metal spatula
Forceps
Electronic pipet controller
1000 µl capacity pipette
Test tube rack
Water-resistant marker pen
500 ml Erlenmeyer flask with side arm (part of vacuum rig)
250 ml Borosilicate funnel (47 mm diameter)
Borosilicate frit base (47 mm diameter)
Rubber stopper with a hole for frit-support (part of vacuum rig)
Metal clamp for filtration assembly
Quartz cuvettes (x2)
Squirt bottles (x2, one for acetone, one for water)

Disposable materials:

Nitrile Gloves
Aluminum foil
ToughSpots™ labels
10ml glass serological pipet
Aluminum weighing pans (one per sample)
15 ml polypropylene centrifuge tubes (two per sample)
200–1000 µl pipette tips
Disposable glass test tubes
Kim wipes™
Lens paper
GF/F filters (45mm diameter, 0.7 µm pore size, one per sample)

Equipment:

Freezer (-80 °C)
Refrigerator (4°C)
Fume hood
Weighing balance accurate to 4 decimal places
Vortex
Refrigerated swinging bucket centrifuge (non- refrigerated and fixed-rotor type centrifuges will also work)
UV-VIS 2450™ spectrophotometer (other makes and models will also work, however this protocol describes how to use this specific type)

Drying oven
Muffle furnace
Vacuum pump

Chemicals:

Phosphate-free soap (e.g. Liquinox™)
Ultra-pure water
Acetone, 100%, ACS reagent grade

Software:

UV Probe
Microsoft Excel

Safety warnings

- ! This procedure uses hazardous chemicals:
- Complete your institution's chemical safety training before working with acetone.
 - Read the Safety Data Sheets (SDS) forms for 100% acetone.
 - Use powder-free nitrile exam gloves throughout the procedure.
 - Wear a lab coat and safety glasses throughout the procedure.
 - Dispose of all chemical waste in appropriately labeled containers.

Before start

Washing glassware

- All glassware should be washed using phosphate-free soap (e.g. Liquinox) and scrubbed with bottle brushes. Glassware should then be rinsed in tap water, before being rinsed three times in three separate baths of deionized ultra-pure water.

Baking aluminum pans and filters

- Bake all GF/F filters and aluminum weighing pans in a muffle furnace at 450°C for two hours. These items do not need to fully cool overnight before removing but can be removed shortly after the baking cycle ends. Caution as items may be very hot and may cause burns to skin.

Preparation of ground coral samples

- 1 Grind frozen coral fragments.** Remove a coral fragment from the $-80\text{ }^{\circ}\text{C}$ freezer and place it within the mortar (Fig. 1A). Next, using the pestle, break the fragment into smaller $\sim 1\text{cm}^3$ pieces (Fig. 1B). Place one hand over the top of the mortar to prevent pieces of coral from being ejected. Continue to crush the frozen coral pieces into smaller (Fig. 1C) and smaller (Fig. 1D) pieces. Next using circular movements grind the coral pieces into a homogenous paste until the mixture resembles hummus (Fig. 1E). Finally, use the pestle to gather all of the coral paste together at the base of the mortar (Fig. 1F). Try to grind the coral from the start (Fig. 1A) to finish (Fig. 1F) as quickly as possible (i.e., in no more than 5 minutes) to prevent the sample from completely defrosting and potentially degrading.

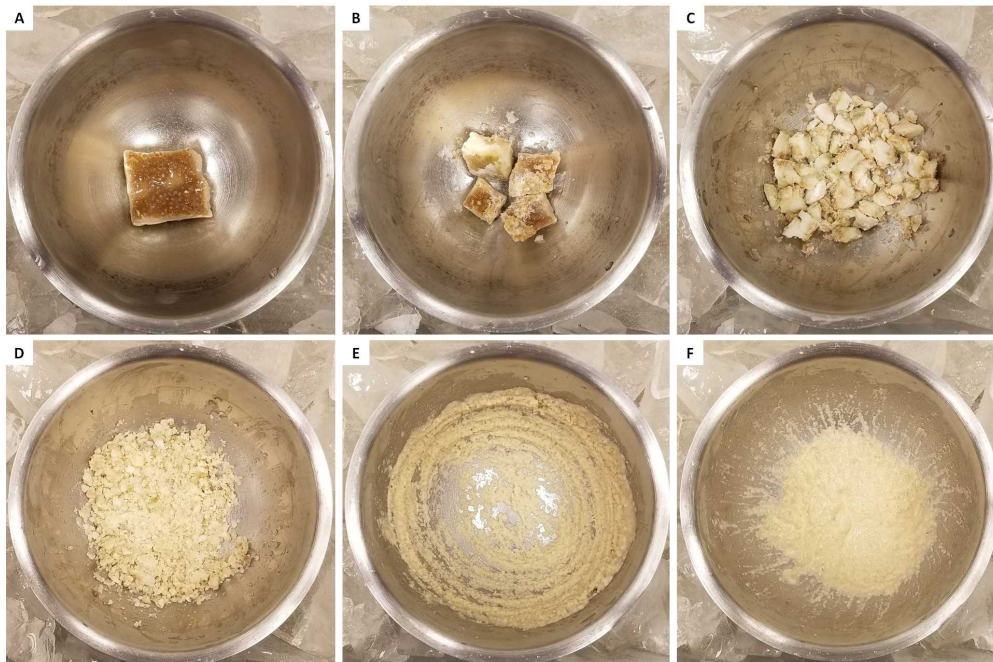


Fig. 1. Sequential photos showing the inside of the mortar during the process of grinding (A) the coral fragment into (F) a homogenous paste.

- 2 Weigh and partition ground coral paste for chlorophyll analysis.** Place a pre-baked aluminum weighing pan on the balance and tare (i.e., zero). Remove the pan. Using a small rubber or silicone spatula, transfer all the ground coral material from the mortar into the aluminum pan. Place the pan on the balance and record the *total wet weight* of the ground coral fragment (in grams). Using a metal spatula, measure 2 g of the wet paste from the pan into a pre-labeled 15 ml polypropylene centrifuge tube, henceforth referred to as "tube A" (Fig. 2). It is important to label the tube on the cap using sticky labels such as ToughSpotsTM (Fig. 2A) as acetone vapor and drips will

quickly remove permanent marker written labels. Record the exact weight of the paste removed as the *wet weight of the subsample*. Cap and return tube A (Fig. 2B, C) to the -80 °C freezer as soon as possible. Allow samples to refreeze fully before moving on to the next step. The remaining ground coral material in the pan not allocated for chlorophyll analysis can be transferred to a separate container (e.g., 5–50 ml Eppendorf tube or cryovial) and archived in a -80 °C freezer for other future analyses (e.g., biomass, protein, lipid, etc.).

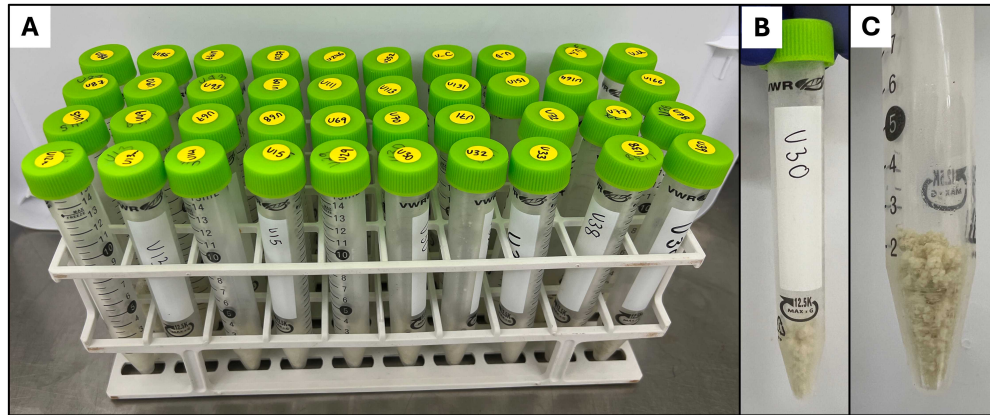


Fig. 2. A) Pre-labelled 15 ml centrifuge tubes with ToughSpots™ labels on the cap. B) Photo of tube A containing the 2 g ground coral subsample to be used for chlorophyll analysis. C) Close-up photo of the ground coral subsample.

- 3 **Foil wrap centrifuge tubes.** Chlorophyll pigments are easily degraded by light. Therefore, the acetone extraction step must be completed in total darkness. To achieve this, all sample tubes need to be wrapped in aluminum foil. Prepare squares of aluminum foil approximately 13 cm². Remove sample tubes from the -80 °C freezer and carefully wrap each tube (Fig. 3A–D). Ensure at least two layers of foil around each tube for maximum effectiveness. Remove excess foil at the base of the tube by twisting and pinching (Fig. 3C, D) or cutting with scissors.

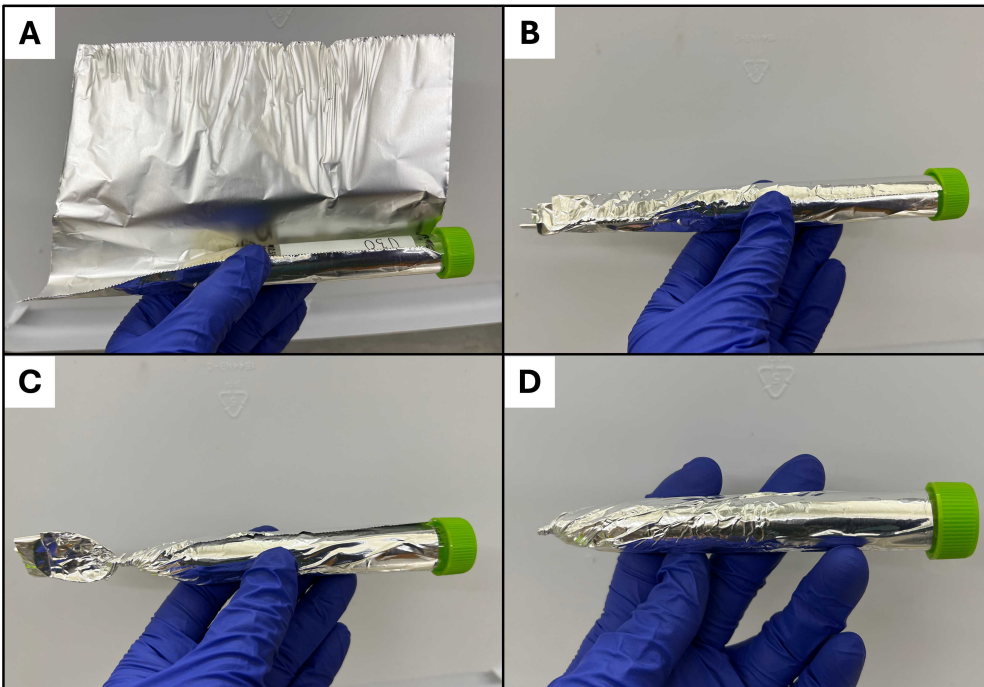


Fig. 3. A–D. Steps for wrapping tubes in aluminum foil to block light.

- 4 **Store.** Return the now-foil-wrapped tubes (Fig. 4) to the -80 °C freezer until you are ready to start the acetone extraction steps.



Fig. 4. Rack of 15ml centrifuge tubes wrapped in aluminum foil.

Day 1: First acetone extraction

- 5 **Add 100% acetone to sample tubes.** Remove sample tubes from the freezer and place them inside a fume hood along with the other supplies needed (Fig. 5). Attach a 10ml glass serological pipet to an electronic pipet controller. Note: it is important to use glass instead of polystyrene pipets as they are not compatible with acetone and will dissolve. Add 10 ml of 100% acetone (reagent grade/ACS) to each sample tube and cap tightly.



Fig. 5. Supplies needed for adding acetone to ground coral sample tubes. This step must be conducted inside a fume hood.

- 6 **Vortex samples.** After adding acetone and tightly capping the sample tubes, vortex for 30 seconds each (Fig. 6). Remove tubes from the aluminum foil sleeve briefly and check that the ground material is thoroughly mixed and is not stuck to the inner sides of the tube. If the material is stuck to the tube wall, use a clean metal spatula to scrape the coral material free and re-vortex. Return all tubes to their foil sleeves



Fig. 6. Vortex sample tubes to ensure that the ground coral material mixes thoroughly with the acetone.

- 7 **Refrigerate samples.** After vortexing, refrigerate samples for 24 hours at 4°C.

Day 2: Measuring Chlorophyll absorbance from first extraction

- 8 **Turn on the spectrophotometer.** This protocol used the SHIMADZU UV-VIS 2450 spectrophotometer (Fig. 7A). The power switch is located on the left side of the instrument (Fig. 7B). Turn on the spectrophotometer approximately 30 minutes before using it to allow the bulbs to warm up.



Fig. 7. A) SHIMADZU UV-VIS 2450 spectrophotometer and B) labeled diagram showing the location of power switch (image source: UV-VIS 2450 manual).

- 9 **Clean the spectrophotometer bulbs.** Open the sliding door of the cuvette chamber and gently clean the four bulbs (Fig. 8A) using lens paper (Fig. 8B). Note: Do not use Kimwipes to clean the bulbs.

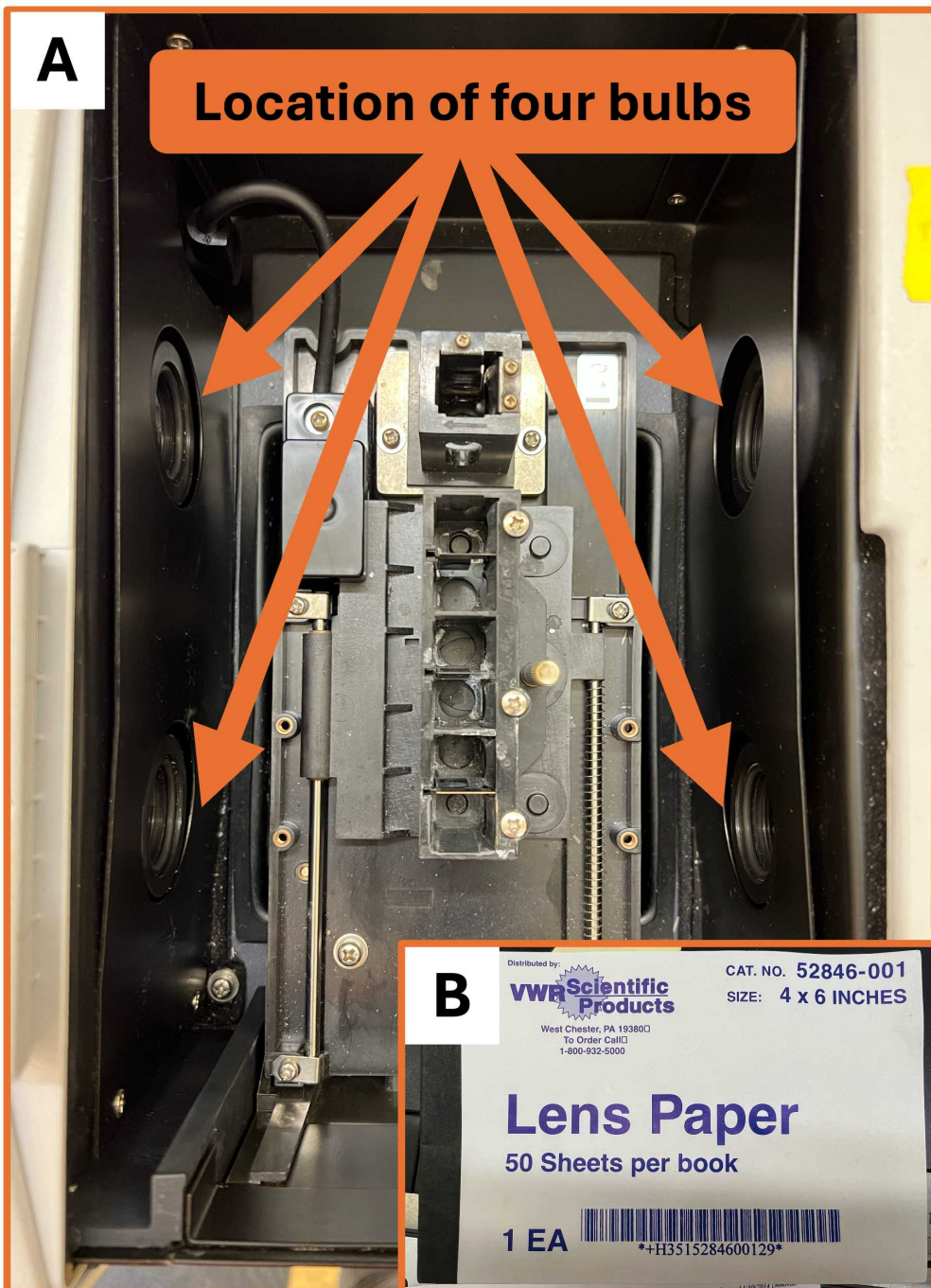


Fig. 8. Image showing the location of the four bulbs within the cuvette chamber of the UV-VIS 2450 spectrophotometer, and B) example of lens paper product that is suitable for cleaning the bulbs.

- 10 **Connect the UV-VIS instrument to the UV Probe software.** Open the UV Probe software by double-clicking the icon on the desktop (Fig. 9A). Click on the Connect button (Fig. 9B). This will initiate a series of performance checks and will take approximately 10 minutes to complete. When this is complete, click "OK".



Fig. 9. UV Probe software icons to A) open UV Probe software and B) connect the software to the UV-VIS spectrophotometer.

- 11 **Edit spectrum acquisition parameters.** To set up your acquisition parameters, click on *Edit* → *Method*. Click on the *Attachments* tab (Fig. 10A). Be sure the correct cell holder is selected. Select "None" if you are using the standard 2-cell holder. For this protocol, we used the 6-cell holder (Fig. 8A). Next, click on the *Measurement* tab (Fig. 10B). Set the wavelength range to 750–600 nm and the sampling interval to 1.0 nm (Fig. 10B). Next, click on the *Instrument Parameters* tab (Fig. 10C) and change the slit width to 0.5 nm. When done setting up the method, click the "OK" button located at the bottom right of the dialog box (Fig. 10A–C).

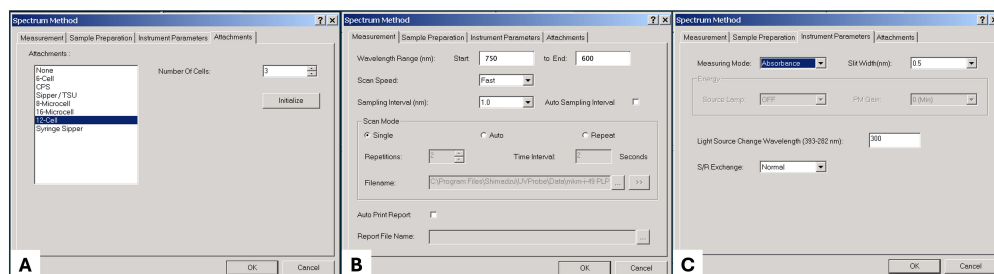


Fig. 10. Dialog box of the *Spectrum Methods* within the UV-Probe software showing the A) "Attachments" tab, B) "Measurement" tab, and C) "Instrument Parameters" tab.

- 12 **Clean the cuvettes.** Clean two quartz cuvettes (square cells with optical length of 10 mm) using three rinses with ultrapure deionized water followed by one rinse with 100% acetone. For each rinse step, fill the cuvette with ~2 ml of water/acetone, cap it with a plastic lid (or cover the open end with your thumb - note: make sure you are wearing gloves), invert the cuvette several times, and then pour out the rinse liquid into a waste container. Dry the outside of the cuvette using a Kimwipe. Inspect the cuvette to ensure the inside and outside are clean and free of residue or lint.
- 13 **Load blank cuvettes into the spectrophotometer.** Using a 1000 μ L pipet, fill each cuvette with 2 ml of 100% acetone. Place one cuvette in the front compartment and one

cuvette in the back compartment. Make sure the two polished windows of the cuvette are parallel to the light beam direction. The back compartment is the cell holder for the reference beam and the front holder is the cell holder for the sample beam (Fig. 11 A–B). Close the sliding chamber door.

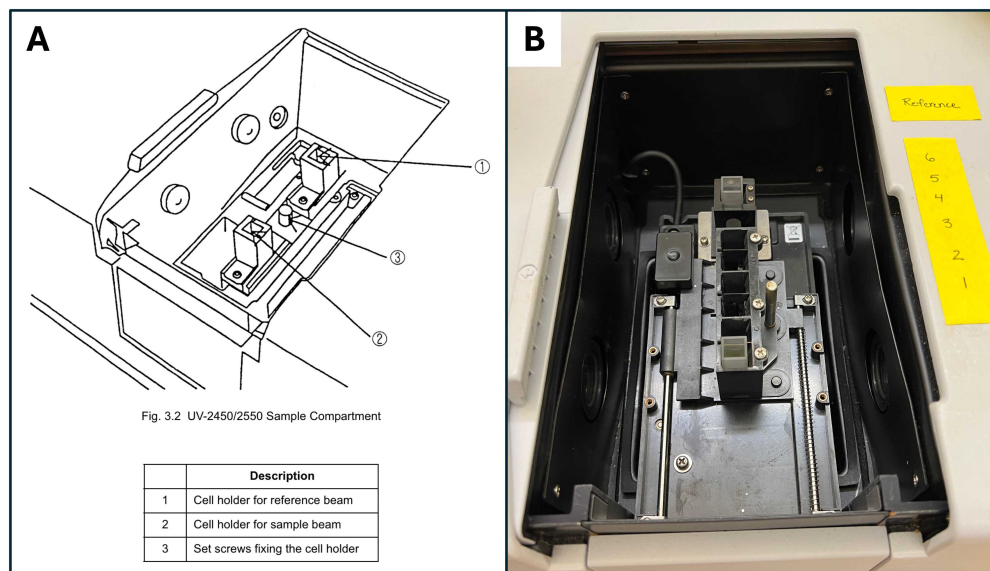


Fig. 11. A) Diagram showing the location of the two cell holders (image source UV-VIS 2450 manual) and B) the inside of the UV-VIS 2450 spectrophotometer showing the acetone-filled cuvettes within the holders.

- 14 **Set the baseline.** After loading the acetone cuvettes into the holders, click the "Autozero" button (Fig. 12A). Next, click the "Baseline" button (Fig. 12B) to zero over the entire wavelength range. This may take a few minutes. Once complete, open the sliding chamber door and remove the cuvette from the frontmost cell holder. Dispose of the acetone in a waste container and clean the cuvette using the methods described in step #12.



Fig. 12. UV Probe software icons for A) auto zero and B) setting the baseline.

- 15 **Centrifuge samples.** After the 24-hour extraction period is complete, remove tube A from the refrigerator and centrifuge for 10 minutes at a relative centrifugal force of 3100

x g (which equals 4000 rpm in the Eppendorf 5810R centrifuge with rotor A-4 81 which was used in this protocol, Fig. 13). Afterward, remove tubes from the centrifuge rotor holder with extreme care so as not to mix the particulate matter (ground coral) with the supernatant (extracted chlorophyll pigments). Keep tubes inside the aluminum foil sleeves at all times. Carefully place the sample tube rack next to the UV-VIS spectrophotometer.



Fig. 13. Eppendorf Centrifuge 5810 R used in this protocol.

- 16 **Load the sample cuvette with the chlorophyll supernatant.** Carefully remove the sample tube from the rack and the foil sleeve. Using a 1000 μ L pipet, fill each cuvette with 2 ml of the supernatant containing the extracted chlorophyll pigments (Fig. 14). Take care not to accidentally resuspend the ground coral particulate matter as this will affect the absorbance reading. It is also important that the extracted supernatant is at room temperature before loading the cuvette. This is important to prevent condensation from accumulating on the outer walls of the cuvette, which will interfere with the absorbance reading.

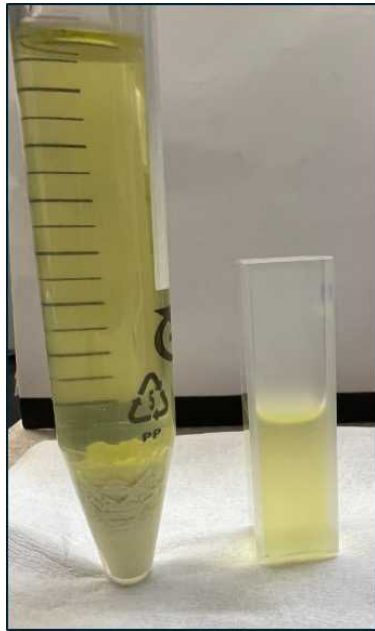


Fig. 14. Left: Sample tube containing the ground coral material and the acetone solvent within which the chlorophyll pigments have been extracted. Right: quartz cuvette filled with 2 ml of the extracted chlorophyll supernatant.

- 17 **Measure chlorophyll absorbance.** Load the sample cuvette into the frontmost holder and close the sliding chamber door. Ensure that the two frosted sides of the cuvette are perpendicular to the beam of light from the bulbs. Click on the Spectrum mode icon (Fig. 15A) and click "Start" (Fig. 15B). The UV-VIS spectrophotometer will now measure the absorbance of your sample across the wavelength range of 750–600nm. It takes about three minutes per sample.

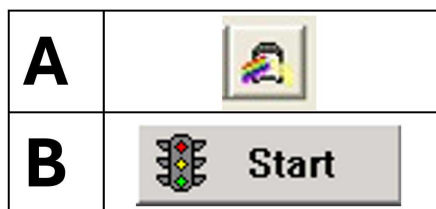


Fig. 15. UV Probe software icon to A) start Spectrum Mode and B) to start the acquisition.

- 18 **Save raw absorbance data.** When the acquisition is complete you will be asked for a filename. To view the numerical raw data, click the "Data Print" icon (Fig. 16). Highlight the data. Copy and paste the data into Excel. You can additionally save your spectrum data from within the UV Probe software by clicking on *File* → *Save*. To clear your

spectrum and acquire new data, click on *File* → *Properties*. Select your file and then click on the "Delete" button. Ensure you have saved your file before clicking delete or all data will be lost.



Fig. 16. UV Probe software "Data Print" icon to view raw data.

- 19 **Pour the 2ml cuvette subsample back into tube A.** After reading the absorbance of the sample, and saving the data, it is time to remove the sample cuvette. Open the sliding chamber door and remove the sample cuvette from the spectrophotometer. Pour the 2 ml chlorophyll subsample back into the original sample tube (i.e., tube A) and cap.
- 20 **Repeat steps for other samples.** To prepare the spectrophotometer for the next sample, return to the Spectrum Mode screen by pressing the icon (Fig. 16). Clean the sample cuvette using the methods described in step #12. Repeat steps #16-19 for the remaining samples.
- 21 **Turn off the UV-VIS.** When you have finished acquiring all your spectra and have saved all of your data, remove both the reference and sample cuvettes. Click the "Disconnect" button (Fig. 17). Exit the UV Probe software and turn off the UV-VIS using the power button (Fig. 7C).



Fig. 17. UV Probe software icon to disconnect the UV Probe software from the UV-VIS spectrophotometer.

Day 2: Second acetone extraction

- 22 **Note on double vs. single chlorophyll extraction.** The concentration of chlorophyll pigments in your coral samples is dependent upon a variety of factors including coral and algal genotype, species, collection location, collection depth, and specimen size, to name a few. It is recommended that you conduct a test on a subset of your samples to determine if a double extraction is needed in order to extract 100% of the chlorophyll pigments from your sample (thus accurately measuring total chlorophyll). If no additional chlorophyll pigments are extracted during the second extraction, then it is likely that a single extraction procedure will be sufficient. The methods for conducting a double extraction are outlined below.



- 23 **Separate supernatants from particulate matter.** After measuring the chlorophyll absorbance from the first extraction, it is time to prepare the samples for the second extraction. Centrifuge all sample tube A's again using the methods described in step #15. Using a 10 ml glass serological pipet attached to an electronic pipet controller, remove the supernatant without removing any of the particulate matter. Transfer the supernatant to a new, pre-labeled 15 ml polypropylene centrifuge tube - henceforth referred to as "tube B". Store tube B in a refrigerator for now. At this point, you should have two 15ml centrifuge tubes per sample: tube A which now contains the ground coral particulate matter and no liquid, and tube B which contains the supernatant from extraction #1.
- 24 **Add new acetone for the second extraction.** Add 10 ml of fresh 100% acetone to sample tube A, vortex, and refrigerate using the methods described in steps #5, 6 and 7.

Day 3: Measuring chlorophyll absorbance from second extraction

- 25 Repeat steps #8-21 on tube A.

Day 3: Filter and dry the chlorophyll samples

- 26 **Assemble the filtration equipment.** Assemble the vacuum-filtration system assembly: funnel (250 ml), glass fritted base (47 mm diameter), GF/F filter, metal clamp, rubber stopper, disposable test tube, side-arm Erlenmeyer flask (Fig. 18). Note: by placing a foam ring inside the side-arm Erlenmeyer flask, this supports the test tube, and prevents it from falling over or breaking.

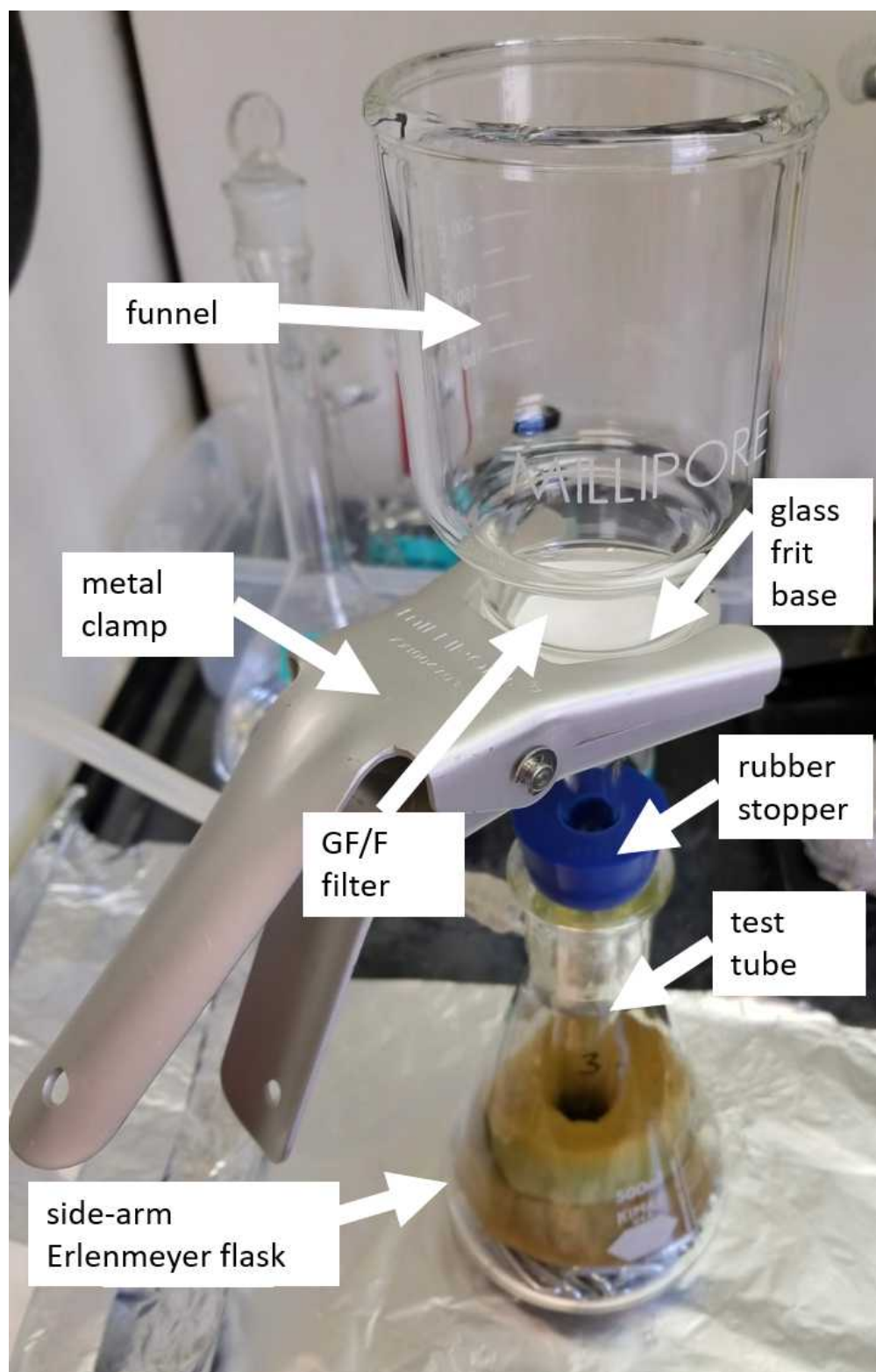


Fig. 18. Components of the vacuum assembly. The side-arm Erlenmeyer flask is connected to an electronic vacuum pump (not shown in the image above). The GF/F filter is placed between the funnel and the glass frit base, held together with the metal clamp. The glass base is connected to the side-arm Erlenmeyer flask using the blue rubber stopper. Make sure to place a test tube inside the flask before pouring the acetone through the filtration assembly.

- 27 **Filter samples.** Turn on the vacuum pump and pour the contents of both tubes A and B through the glass vacuum filtration system fitted with a pre-burned GF/F filter, and a disposable glass test tube as shown in Figure 18 above. Use a metal spatula to scrape the funnel walls, to ensure all particulate matter passes onto the GF/F filter. Turn off the vacuum. Carefully unclamp the filtration system. Lift the glass funnel, and hold it over a pre-labeled aluminum weighing pan. Use the spatula and remove any material stuck on the funnel into the pan. Using forceps or a spatula, gently remove the GF/F filter from the glass-frit, and place this inside the same weighing pan. Disassemble the filtration system, and dispose of the acetone collected in the glass test tube. Reassemble the filtration system with a clean test tube, frit-support, GF/F filter, and funnel. Continue and repeat for all samples.
- 28 **Dry pans.** Transfer aluminum pans (now containing the GF/F filters and particulate organic matter) into a 60 °C drying oven and dry them for 24 hours. Include an extra empty pan + new GF/F filter to serve as the control.

Day 4: Weigh dried pans then combust the dried samples

- 29 **Remove pans from oven.** After samples have been drying for 24 hours, remove pans from the oven and allow them to cool for 10 minutes on the lab bench.
- 30 **Weigh dried pans.** Weigh each dried pan on a balance. All pans are weighed in triplicate. Enter these values into excel and calculate the average of the three "Pan + Filter + Dry Residue Weights".
- 31 **Transfer pans to a muffle furnace.** After weighing the dried pans, place them into a muffle furnace. Burn the dried samples at 450 °C for 5 hours.

Day 5: Weigh burnt pans

- 32 **Remove pans from muffle furnace.** The next day, remove pans from the muffle furnace and allow them to cool for 5 minutes before weighing (if still warm).
- 33 **Weigh burnt pans.** Weigh each burnt pan on a balance. All pans are weighted in triplicate. Enter these values into excel and calculate the average of the three "Pan + Filter + Burnt Residue Weights".

Calculations

- 34 **Calculate the AFDW.** The ash-free dry weight (AFDW) of the coral sample is calculated using the following equations:

[1] Δ Coral weight (g) = average weight dry coral pan (g) – average weight burnt coral pan (g)

[2] Δ Control weight (g) = average weight dry control pan (g) – average weight burnt control pan (g)

[3] AFDW (g) = Δ Coral weight (g) – Δ Control weight (g)

- 35 **Calculate the chlorophyll concentration.** The concentration of chlorophyll a and c2 pigments is calculated using the following equations from Jeffrey & Humphrey (1975) for dinoflagellates using 100% acetone solvent. Note the symbol E_x denotes the extinction value at wavelength x.

[1] [Chlorophyll a] = $[11.43 (E_{663} - E_{750})] - [0.64 (E_{630} - E_{750})]$

[2] [Chlorophyll c₂] = $[27.09 (E_{630} - E_{750})] - [3.63 (E_{663} - E_{750})]$

[3] [Total Chlorophyll]_{1st extraction} = [Chlorophyll a]_{1st} + [Chlorophyll c₂]_{1st}

[4] [Total Chlorophyll]_{2nd extraction} = [Chlorophyll a]_{2nd} + [Chlorophyll c₂]_{2nd}

[5] Total Chlorophyll in ground coral subsample = [Total Chlorophyll]_{1st extraction} + [Total Chlorophyll]_{2nd extraction}

- 36 **Normalize the chlorophyll concentration to AFDW.** The following calculation is used to normalize the chlorophyll concentration to the tissue biomass of the coral subsample.

[1] Total Chlorophyll per unit biomass ($\mu\text{g gdw}^{-1}$) = Total Chlorophyll in ground coral subsample (ug) $\div$ AFDW (g)



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