Author:	C. Wall
Site:	Dr. Ruth Gates' Laboratory Hawai'i Institute of Marine Biology University of Hawai'i
Protocol:	**Coral tissue lipid analysis**
Detailed Title:	Lipid analysis in coral or algae tissue using separatory funnels
Original Date:	01/2018
Additional Authors:	L. Rodrigues, A. Grottoli, et. al
Last Updated:	20190109
Updated By:	CW
Notes:	* adapted from Grottoli Lab protocol

Overview

In Scleractinian corals, the majority of photosynthetically fixed carbon produced by the endosymbiotic zooxanthellae is transferred as glucose/glycerol to the coral host and stored in tissue lipids. Lipids are used to support metabolic functions and are incorporated into mucus secretions and gametes. The following lipid extraction procedure has been modified from several published methods (Harland et al. 1991; Harland et al 1993; Grottoli-Everett and Kuffner 1995). The procedure has also been adapted to allow for future analyses of lipid classes and stable isotopes on the extracted lipid sample. Coral cores, branch fragments may be ground in this protocol, or a lyophilized (freeze-dried) tissue slurry can be used. Volumes of sample/tissue, solvents, and KCl are important. Do not deviate from ratios used here.

Equipment and Chemicals List:

Reusable equipment required to extract 9 lipid samples and 1 blank:

Sonicator or sonication bath
Balance (0.0001 g)
Heating Plate
Drying Oven (60°C)
Muffle Furnace (450°C)
Cork Borer and Sharpener – *for coral core collection in mounding corals*
Calipers – *for SA of fragments if grinding*
Retort Stands (2) with Rings (10)
Faucet-attached Vacuum Pump with Erlenmeyer Flasks
Nitrogen Filter and Drying Rig
60 ml Separatory Funnels (10)
2-oz Mortars and Pestles (10 sets)
50 ml Erlenmeyer Flasks (10)
20 ml Glass Pipettes for chemical dispensing (3)

Disposable equipment required to extract 9 lipid samples and 1 blank:

Nitrile Gloves
Glass Test Tubes (10)

Pasteur Pipettes (10)
Glass Fiber Filters (GF/F) (10)
Aluminum Pans (10)
Large and Small Amber Vials (10 each)
Aluminum Foil
Teflon Tape

Chemicals required per sample:
Chloroform ACS grade – 32 ml
Methanol ACS grade – 10 ml
0.88% Potassium chloride (KCl) – 16 ml
UHP grade 5.0 Nitrogen (Order from BOC gases) – *important for downstream lipid analysis*

Lab Safety:
This procedure uses hazardous chemicals (chloroform and methanol):
1. Read the MSDS forms for each chemical in the procedure.
2. Use nitrile gloves throughout the procedure. If chloroform spills on the gloves, remove gloves immediately and discard, wash hands, and use new gloves.
3. Wear a lab coat and safety glasses throughout the procedure.
4. Wear ear protection while operating the sonicator.
5. Dispose of all chemical waste in appropriately labeled containers.

Preliminary Steps:
1. Burn at 450°C for at least 2 hours all of the glassware used in the procedure. This includes: 60-ml separatory funnels (remove stopcocks and plastic fittings), glass Pasteur pipettes, test tubes, 50-ml Erlenmeyer flasks, amber vials. The mortar and pestles are the only glassware that should not be burned, as they will crack.

2. Label each Erlenmeyer flask with the ID number of each sample first, and then weigh them. Handle with gloves only.

3. Burn at 450°C for at least 2 hours the aluminum foil pans and glass fiber filters, and sheets of aluminum foil. Label the bottom of each pan with the ID number of each sample first, place one filter inside each pans and weigh them together. Handle with gloves only.

4. Mortar and pestles, spatulas, and any other equipment that can not be burned should be rinsed in chloroform and allowed to dry in the fume hood prior to use.

5. Prepare at least 300 ml of a 2:1 (v/v) chloroform:methanol solution (200 ml of chloroform and 100 ml of methanol). This volume is enough to extract lipids from 9 samples + 1 blank.

6. Prepare at least 160 ml of a 0.88% potassium chloride solution with Milli-Q water (8.8 g of KCl with 1 liter of water). This volume is enough to extract lipids from 9 samples + 1 blank.

Note: For both the chl:meth solution and the KCl solution, if you are running many samples over several days, it is possible to make up larger quantities of each solution and store them (in the flammable cabinet of the fume hood). The solutions should be stirred each day prior to use.

7. Set-up two retort stands with 5 rings per stand. The rings should be covered with plastic tubing, so that the funnels do not fall through. Assemble the separatory funnels with their plastic fittings and stopcocks. The separatory funnels sit in each ring.

8. Set-up the nitrogen drying rig: Use UHP 5.0 nitrogen gas and connect to a nitrogen filter/regulator. Splice gas coming out of the filter with chloroform-rinsed silicon tubing and attach the final end of each piece of tubing with a glass Pasteur pipette to reach each sample. Small regulators can be placed on each line of the nylon tubing, these are available at aquarium stores. Place the samples on a heating plate during the drying process (no hotter than 60°C, do not boil sample). The entire set-up must be placed in the fume hood.

Procedure:

1. For each complete lipid run (total of 10 sets of equipment), one should be run as a blank: follow the exact procedure outlined below, but without a coral sample.

2. *Approach a*: Remove a piece of the coral tip (approximately 1cm^3) from the sample. Measure and record the length and diameter with calipers.

   Approach b: Use a cork borer to remove a small piece of coral from the sample, providing a known surface area. In either case (a,b), measure and record the wet weight (to the nearest 0.0001g)—this is good data collection practice but not directly used in lipid biomass calculation.

   Approach c: Freeze dry a known volume of isolated and homogenized coral tissue (from blastate slurry extracted from airbrush/waterpik), generally ca. 1-3 ml of tissue is sufficient; 15 ml falcon tubes work well for this. Grind the dried tissue powder with a spatula, weigh and place in 20 ml scintillation vials.

3. *In apprach (a,b):* Grind the whole coral (tissue plus skeleton) in a darkened 2-oz mortar. The mortars must be completely covered on their outsides with black electrical tape, as the lipid extraction must occur in the dark.

4. *In method (a,b*): Add 5 ml of the chl:meth solution while grinding. Add an additional 15 ml of chl:meth and stir. Cover the mortar with a pre-burned, labeled piece of aluminum foil.

   * *For method c*: Wrap scintillation in aluminum foil (label vial in marker and lids with marker+tape! Labels are prone to come off) add the 20 ml total volume of chl:meth to scintillation vials, using a spatula to disturb the powder encase clumps exist. Rinse spatula with 100% chloroform between each sample.

5. Sonicate the mortar/scintillation vial and contents for 5 minutes. (Sonic bath should be placed inside the fume hood if using a bath.)

6. Set aside and allow the extraction to occur for 1 hour in darkness—if available, put samples in a flammable safe refrigerator for extraction period.

7. Using a spatula, scrape the sample (ground coral + chl:meth) out of the mortar/vial and filter through a pre-burned glass fiber filter (GF/F) with faucet-attached vacuum pump into a glass test tube. Rinse out mortar with 5 ml of chl:meth to remove any remaining sample. Rinse filter with 5ml of chl:meth to ensure all lipids pass through the filter.

8. Dry filters plus coral skeleton (in the drying oven) in pre-burned aluminum pans at 60°C for 24 hours. Weigh. Burn filters plus dried coral skeleton (in the muffle furnace) at 450°C for 6 hours. Weigh. The difference between these weights is the ash free dry weight of the sample.

9. Transfer the chl:meth solvent from the glass test tube to a separatory funnel and record color. Color is not used in analysis but is seen as a relative indication of symbiont abundance.

10. Add 11 ml of 0.88% KCl gently along the wall of the separatory funnel. Gently invert funnel 3 times, remove stopcock after each time to release pressure. Let funnel stand until a clear separation is observed. Drain organic phase (bottom yellow phase) into a pre-burned glass test tube and cover with a piece of pre-burned aluminum foil to avoid evaporation. Do not shake the separatory funnel as it will cause an emulsion and the organic and aqueous phases will not separate.

11. Add 5 ml of 100% chloroform to the aqueous phase (opaque/white in color, left in funnel), invert funnel 3 times, allow to separate. Drain organic phase (bottom, clear/ uncolored) into same test tube.

12. Discard aqueous phase (left in funnel) in appropriate waste container.

13. Rinse the inside of the separatory funnel with 1-2 ml of 100% chloroform, drain into the waste container.

14. Pour organic phase from the test tube back into the chloroform-washed separatory funnel. Add 5 ml of 0.88% KCl, invert 3 times and allow to separate. This removes any water-soluble compounds that are still remaining in the sample.

15. Add a few squirts of 100% methanol to the bottom of the pre-weighed Erlenmeyer flasks. Drain organic phase (cloudy and yellow in color – the cloudiness results from traces of water left in the sample) from the separatory funnel into the Erlenmeyer flasks. The methanol in the bottom of the flasks “clings” to the traces of water and the sample turns clear (is no longer cloudy, but remains colored).

16. Dry under UHP grade 5.0 nitrogen. Turn on tank regulator to the lowest flow rate. Fit Pasteur pipettes to the ends of the spliced tubing. One pipette is used to direct the nitrogen to

each sample. While drying, place samples on a heating plate turned on low heat until a constant weight is attained. Record weight. This is the total lipid dry weight.

17. Resuspend with 5 ml of chloroform. Transfer 3 ml to larger amber vials and 2 ml to smaller amber vials for storage in the –80ºC freezer. Wrap the outside of the cap with Teflon tape. These samples can be used for future lipid class and stable isotope analyses.

18. Correct all samples weights against the weights determined from the blank run. For example, if the blank ash-free dry tissue weight is 0.0001g, subtract this value from each of the sample ash-free dry tissue weights.

References:

Grottoli-Everett AG, Kuffner IB (1995) Uneven bleaching within colonies of the Hawaiian coral *Montipora verrucosa*. In: Gulko D, Jokiel PL (eds) Ultraviolet Radiation and Coral Reefs. HIMB Technical Report, pp 115-120.

Harland AD, Fixter LM, Spencer Davies P, Anderson RA (1991) Distribution of lipids between the zooxanthellae and animal compartment in the symbiotic sea anemone *Anemonia viridis*: wax esters, triglycerides, and fatty acids. Marine Biology 110: 13-19.

Harland AD, Navarro JC, Spencer Davies P, Fixter LM (1993) Lipids of some Caribbean and Red Sea corals: Total lipid, wax esters, triglycerides and fatty acids. Marine Biology 117: 113-117.

Schoepf, V., A. G. Grottoli, M. E. Warner, and others. 2013. Coral energy reserves and calcification in a high-CO2 world at two temperatures. PLoS One **8**: e75049.

Wall, C. B., R. A. B. Mason, W. R. Ellis, R. Cunning, and R. D. Gates. 2017. Elevated pCO2 affects tissue biomass composition, but not calcification, in a reef coral under two light regimes. R Soc Open Sci **4**: 170683.

Sample Data Sheet and Calculations:

A	B	C	D	E	F	G	H
Sample ID	Length (cm)	Diameter (cm)	Wet Weight (g)	B. Pan + B. Filter (g)	B. Pan + B. Filter + Dry Residue (g)	B. Pan + B. Filter + B. Residue (g)	Dry Tissue Weight (g)
Calcul-ations							= F - G
Ex. 1	1.81	0.79	1.4011	4.5632	4.8005	4.7542	0.0463

I	J	K	L	M	N	O
Sample ID	Erlenmeyer Flask (g)	Flask + Dry Lipid (g)	Dry Lipid Weight (g)	Total Biomass (g)	Lipid/ Biomass (g/gdw)	Comments (Eg. Lipid extract color)
Calcul-ations			= K - J	= H + L	= L/M	
Ex. 1	15.6391	15.6590	0.0199	0.0662	0.3006	Dark Yellow