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Protocol:	Stable isotope preparation and processing	
Detailed Title:	Stable isotope preparation and processing protocol for coral, symbiont algae, and SPOM	
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Additional Authors:	Contributions from literature and A. Grottoli Lab at OSU	
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Notes:		

Overview:

General procedure for the processing of coral for stable isotope analysis ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$). Coral and *Symbiodinium* tissue is removed from skeleton with an artist's airbrush and filtered seawater (0.2 μm). Host and symbionts are separated by centrifugation, and freeze dried

General materials:

- air brush and air cylinder
- 1 × 50 mL falcon tubes for each coral sampled
- falcon tubes (2 × 15 mL tubes for each sample= 1 for host, 1 for symbiont)
- microcentrifuge tubes (2 × 1.5 mL tubes for each sample= 1 for host, 1 for symbiont)
- filtered seawater (millipore, 0.2 μm)
- 25 mm sintered glass filter
- pre-burned aluminum foil
- tin boats (Costech Analytical: 9 x 10mm, part # 041080), and silver if acidifying
- muffle furnace (> 450°C)
- drying oven (60°C)
- freeze drier
- 20 μm mesh
- vacuum pump
- side arm flask

Chemicals:

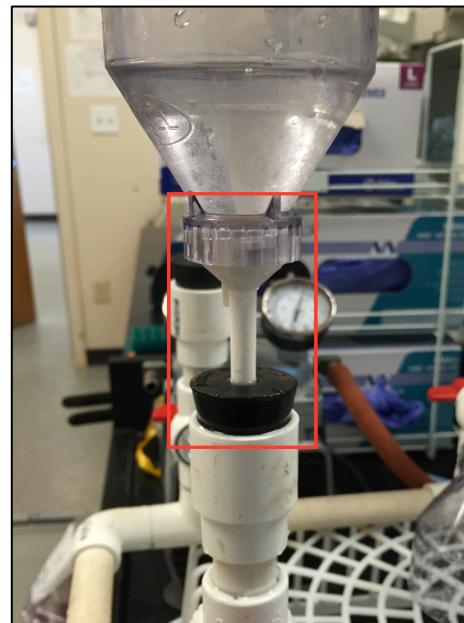
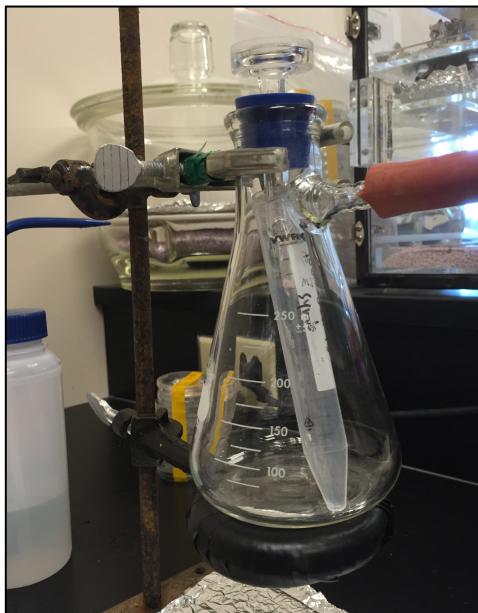
- ethanol (70 - 100%) in squirt bottle
- millique or DI water (ddH₂O)

Coral and symbiont fraction preparation (general and broad purpose)

1. Remove coral tissue from skeleton with air brush using filtered seawater (0.2 µm). Collect blastate into a plastic bag and transfer blastate to a 50 mL falcon tube on ice; wash the plastic bag with ~2 mL FSW and add to falcon tube with blastate.
2. Homogenize blastate with a tissue homogenizer, briefly (~ 10 sec); rinse homogenizer with FSW and add to blastate. Note final blastate volume. Wash the homogenizer with ethanol followed by DI between samples.

Host-symbiont tissue separation

3. Remove an aliquot of tissue blastate and place in new 15 mL falcon tube. Depends on volume of blastate (5 mL is sufficient for most samples).
4. Filter the fraction to be used in isotope analysis through nylon mesh (20 µm pore size: Millepore Nyon Net Filters Ref #NY2004700) to remove residual carbonate from the skeleton and large tissue chunks from samples. ([Maier et al., 2010](#))
5. Keep pump on low pressure (≤ -0.2 Bar)
6. Place 15 mL falcon tube into sidearm flask and filter sample directly into tube



Figures (left) falcon tube inside a side-arm flask, connected to Gast-pump. Not the use of a ring flask-holder (base), retort stand, and clamps at neck of flask. **(right)** the Pall Corp. plastic filter holder, can replace glass VWR fritted glass filter base.

**** update**** if fritted-glass filter base is clogged or old, this can slow filtration. Can attempt to do burn debris out (600°C), or avoid use of filter base. In case of the latter, use the Pall Corp. plastic funnel and filter holder with the plastic filter base and put the Millepore filter directly on the plastic base. Rinse between samples with ethanol and ddH₂O.

7. After filtering the blastate, the symbiont cells need to be separated from host tissues
 8. If a sonication bath or tissue sonicator present, can use this to help break apart host-symbiont cells
 - a. Sonication bath: keep samples on ice, and have water bath a slushy of water + ice. Sonicate for 10 – 15min.
- ** update**** we no longer have a sonicator in our lab. See Ku'ulei if needing.
9. Label 2nd centrifuge tube as “host fraction”; first tube should say “iso” or “symb”.
 10. Centrifuge the complete tissue blastate at 1500 – 2000 × g for 3 – 5 min to separate host/symbionts.
 - a. **In GATES LAB centrifuge**, this is 4000 rpm = ~ 2000 × g. Don’t trust me, calculate it for yourself.
 - b. After centrifuging, check supernatant under microscope to see if symbiont cells present. Complete separation is likely never 100%, but several rounds of centrifugation can make this as “good as possible”.
 11. Remove host fraction (supernatant) and place into separate falcon tube (15 mL). Wash symbiont pellet with FSW to remove host fraction residue on top of pellet (white, fluffy, mucus-fat layer). 1500 × g may pellet can also lead to host (white or creamy in color). This layer should be scraped off with a spatula or aspirated off with pipet. Add this to supernatant host fraction.
 - a. *Symbiont pellet*: Rinse and wash with 2 mL FSW at 1500 × g. Should require 1 – 2 runs on centrifuge of 3 – 5 min.
 - b. *Host fraction*: Should be ~ 5 – 6 mL at this stage. Centrifuge at ~ 7500 × g or 8000 rpm in CORE LAB. This will centrifuge most/all the host fraction material out of the supernatant.
 - i. After this, discard supernatant, add 2 – 3 ml of FSW to pellet, and centrifuge at ~ 2000 × g for 5 min.
 - ii. Discard supernatant, and rinse with 1 ml ddH₂O. This is needed to remove salts prior to isotope analysis
 - iii. *Alternatively, remove supernatant at (i), and using <2ml ddH₂O, rinse the pellet debris down to bottom of falcon tube. Then pipet all material into a microcentrifuge tube to be spun in step (12).
 12. Once host and symbiont fractions are rinsed and in ddH₂O, add each to microcentrifuge tubes. Centrifuge at top speed on benchtop centrifuge (13,000 × g) for 1 min; discard supernatant.
 13. Pellets (host and symbiont) frozen at -80°C in labeled microcentrifuge tubes
 14. Lyophilize samples for 3h or more.
 15. Take samples to isotope lab, weigh out < 0.5 – 1 mg on microbalance and pack in tin capsules.

Isotope sample preparation

Preparation procedures (24 hrs before sample collection)

1. Combust GF/F filters (25mm, 0.7um pore size), aluminum foil at 450-500°C for 4-6 hours in muffle furnace.
2. After 4-6 hours in furnace, let filters and materials cool and weigh filters individually.
3. Place a rectangle of aluminum foil in small, plastic petri-dish or filter holder. Dish should have a sticker on it stating arbitrary #, mass of burned filter, host or symbiont (some info will be added after filtering).
4. Weigh filter and place on aluminum foil, folding foil over the filter to create a "sleeve". Leave space atop filter, you don't want to smother it...
5. Note sample ID on bottom of plastic dish and weight. The sample ID here will correspond to a biological sample.

Filtration and sample collection (host or symbiont biomass on filter)

6. Filter an aliquot of host or symbiont suspension onto the GF/F filter by very, minimal vacuum filtration, and wash the filter with MQ or DI water to remove salts (~0.5 mL).
7. Filtrate volume will vary, and may be ~2 – 4 mL (depends on concentration. The filtration will be slow as the filter is loaded. This is expected.
8. If doing carbon and nitrogen isotope analysis, this can be done on the same filter, BUT if acidifying filter to remove DIC (skeleton, seawater) then carbon and nitrogen will have to be analyzed on separate filters.
9. Place the filter on aluminum "pillow-sleeve" on the plastic dish with label.
10. Place the filter and dish pan in drying oven at 60°C overnight. Once dry, measure filter to obtain dry mass of sample. Samples must be completely dried and free of salts.
11. C:N host/symb range from 5.0 – 8.0. Optimum sample dried mass will be ~1 mg.
 - a. For Carbon: 200-600 ug tissue optimal
 - b. For Nitrogen: 20 ug tissue minimum
 - c. If filters >1mg, then can cut filter down to reach appropriate dry mass. More mass is always better!

* to obtain correct amount of organic material, trial dilutions may be needed
12. If acidifying filter: [use silver boats instead of tin boats]. Using a glass pipette or syringe, add 0.1 HCl onto filter drop-wise to remove skeletal CaCO₃ and seawater DIC. May require 5 mL of acid.
13. Filters should sit overnight before being dried and weighed. If doing this step, filters need to be processed within 48 hrs. Otherwise, filters can be stored for future analysis—although not packed into boats until acidification has occurred.
14. Carefully pack sample into tin/silver boats in labeled 96-well plates, using combusted metal forceps to assist in packing and compressing filters. All material (filter press, forceps) need to be rinsed with 100% EtOH between samples. Final samples should look like a rectangular brick.

ALTERNATIVE TO ACIDIFICATION: Detailed in (Maier et al., 2010)

1. Can compare acidified and non-acidified gauge filtered samples
2. Pass filter slurry through 20 um mesh gauge to remove skeletal debris
 - *gauze filtered supernatant:*
 - centrifuge to separate host/symbiont tissue
 - decant supernatant (host), wash symbiont fraction twice with ~ 3 mL FSW
 - filter tissue onto pre-combusted, pre-weighed GF/F filters
 - wash filter with MQ/DI water and dry at 60°C for 24 hrs and weigh again
 - pack filters into tin boats and place in labeled 96-well plate

Isotope ($\delta^{13}\text{C}$ or $\delta^{15}\text{N}$) analysis on seawater (liquid)

1. Collect ~ 30-50 mL of seawater in falcon tube or scintillation vial leaving no head space at the top (use conical caps on scintillation vials)
2. Add HgCl₂ to sample and seal with parafilm and ensuring air tight
3. Store at 4°C until processing for ^{13}C (*note this is a liquid sample)

Isotope ($\delta^{13}\text{C}$ or $\delta^{15}\text{N}$) analysis on seawater (filtered)

1. Filter seawater or fraction of seawater onto GF/F filter (>1 L)
2. Best to use a Pall Corp 200 mL plastic filter cup + 25mm disk with filter holder
3. The more mass on filter the better (can always subsample)
4. Rinse filter with DI, place onto burned foil and dry at 60°C

Isotope analysis ($\delta^{13}\text{C}$ or $\delta^{15}\text{N}$) of particulate organic material (POM)

Coral mucus:

1. Take water sample of known volume and filter onto pre-combusted, weighed GF/F
2. Water sample may need to be ~200 mL depending on coral mucus release (see Tremblay et al. 2012; acidification may be necessary)

Sediment SPOM:

1. weigh out sediment sample (5 – 8 mg, depending on type...8mg KBay OK)
2. pack into silver pan and acidify with drops of 0.1 HCl until no more bubbling
3. leave in drying oven (unpacked) for 12-24 h; pack cups once dry

Isotope analysis ($\delta^{13}\text{C}$) of coral skeletal material

1. Wash coral skeleton with 6% bleach and let sit overnight; rinse with DI water and dry at 60°C (Muscatine et al. 2005) ** Grottoli (pers. comm.) warns against this **

2. 100-200 um of skeletal material removed from apical tip of coral by diamond-tipped Dremmel tool, to give ~300 ug skeletal material
 - * the amount of material should be chosen according to yearly growth rates and sampling periods that reflect exposure to treatment or conditions
3. grind skeletal material to a fine powder, and subsample ~80 ug of material for analysis of isotope composition
4. pack sample into tin boats, and will be acidified under 100% ortho-phosphoric acid in automated carbonate device (depending on processing lab specifications)

References

Grottoli *et al.*, 2004. Lipids and stable carbon isotopes in two species of Hawaiian corals, *Porites compressa* and *Montipora verrucosa*, following a bleaching event. *Mar Biology* 145:621-631

Maier, *et al.* 2010. Stable isotopes reveal limitations in C and N assimilation in the Caribbean reef corals *Madracis auretenra*, *M. carmabi* and *M. formosa*. MEPS 412:103-112

Muscatine, *et al.*, 2005. Stable isotopes (D13C and D15N) of organic matrix from coral skeleton. *PNAS* 102:1525-1530.

Tremblay *et al.*, 2012. Autotrophic carbon budget in coral tissue: a new 13C-based model of photosynthates translocation. *J Exper Biol* 215:1384-1393.