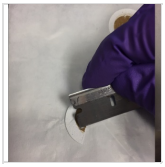

Jul 09, 2020

Airbrushed Coral Sample Preparation for Organic Stable Carbon and Nitrogen Isotope Analyses

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ABSTRACT

This method for separating coral tissues from algal endosymbiont (Symbiodiniaceae) for stable isotope analysis is modified from previously published methods (Hughes et al. 2010). There are three parts to preparing coral samples for stable carbon and nitrogen isotope analysis: 1) airbrush to remove coral tissue and algal cells from skeleton and store at -80 °C until ready to separate, 2) separate the coral tissue from the algal cells through centrifugation and filtering, and 3) dry and pack separated tissues into tin capsules for analysis in a stable isotope ratio mass spectrometer.

This method was modified from Hughes et al. (2010) by James Price with the assistance of Alex Smith and Kerri Dobson and with the guidance of Andréa Grottoli at The Ohio State University.

Literature Cited

Hughes A, Grottoli A, Pease T, Matsui Y (2010) Acquisition and assimilation of carbon in non-bleached and bleached corals. Mar Ecol Prog Ser 420:91–101. doi: 10.3354/meps08866

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KEYWORDS

Stable Isotopes, Heterotrophy, Coral Tissue, Symbiodiniaceae

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MATERIALS TEXT

Legend

[1] for preparation of the coral slurry via airbrush

[2] for separation of coral host tissue from algal endosymbionts

[3] for preparing samples prior to analysis

Reusable materials:

·Airbrush [1]

- 250 ml beaker[2]
- 50 ml beaker[2]
- 25 mm glass frit [2]
- Silicone stopper with 1 cm hole [2]
- 10 mL glass funnel [2]
- 25 mm spring clamp [2]
- Erlenmeyer side-arm vacuum flask [2]
- Mesh filter setup (i.e., a 15 ml polypropylene centrifuge tube with the bottom cut-off and a hole in the lid) [2]
- Six-port vacuum setup (alternatively an Erlenmeyer vacuum flask) [2]
- Water faucet vacuum assembly [2]
- Tweezers [2,3]
- Plastic box/argon gas setup [3]
- Folding block [3]
- Aluminum foil [3]
- Microcentrifuge tube rack [3]
- 96-well polypropylene plate[3]

Disposable materials:

- Nitrile gloves [1,2,3]
- Sterile bags [1]
- 15 ml polypropylene centrifuge tubes [1,2]
- 50 ml polypropylene centrifuge tubes [1,2]
- 5 cm x 5 cm nylon mesh (20 micron) squares (2 per sample) [2]
- Glass fiber filter (GF/F; 0.7 µm) [2]
- Aluminum weighing pans [2]
- Tissue wipes [2]
- Glass Pasteur pipettes (5 ¾") [2,3]
- 70% Isopropyl alcohol wipes [3]
- Razor blade [3]
- Weighing paper [3]
- Costech Analytical 9 mm x 10 mm tin capsules [3]
- Costech Analytical 3.5 mm x 5 mm tin capsules [3]

Equipment:

- -80 °C freezer [1,3]
- Tissue Tearor or other mechanical tissue homogenizer[2]
- Sonicator with probe [2]
- Weighing balance accurate to 4 decimal places [2]
- Vortex [2]
- Centrifuge with capacity for 15 ml polypropylene centrifuge tubes [2]
- Vacuum pump [2]
- Drying oven [3]
- Heat plate [3]

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Chemicals:

- Sodium Chloride [2]
- 10% Hydrochloric Acid [2]
- Argon gas (99.998% pre-purified) [3]

SAFETY WARNINGS

Lab Safety

This procedure uses hazardous chemicals:

- Complete your institutions chemical safety training before working with hydrochloric acid and argon gas.
- Read the MSDS forms for each chemical in the procedure.
- 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 STARTING

Washing glassware

§All glassware should be washed using phosphate-free soap (e.g. Liquinox) and scrubbed with clean, non-rusted bottle brushes. Glassware should then be rinsed in tap water, before being rinsed three times in three separate baths of 0.22 µm filtered ultrapure water.

Baking glassware & other supplies

§Load glassware into a muffle furnace and bake overnight at 450 °C for approximately four hours. Allow to cool completely in the oven overnight before removing to prevent cracking of the glass.

§Upon removal from muffle furnace, all glassware must be handled using gloves to prevent lipid contamination from skin.

§Bake all GF/F filters (25mm diameter, 0.7 µm pore size, one per sample), aluminum weighing pans (one per sample), Pasteur pipets 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.**

Labeling polypropylene tubes and aluminum pans

§Label 15 ml and 50 ml polypropylene tubes for each sample:

oTube A (15 ml polypropylene centrifuge tube): Label with Sample ID, “Whole Slurry”, and Date

oTube B (15 ml polypropylene centrifuge tube): Label with Sample ID, “Algal Fraction”, and Date

oTube C (15 ml polypropylene centrifuge tube): Label with Sample ID, “Host Fraction”, and Date

oTube D (50 ml polypropylene centrifuge tube): Label with Sample ID

Preparation of Coral Slurry

1 **Prepare for airbrushing.** Fill airbrush reservoir with 15 ml of 0.22 µm filtered ultrapure water (See Fig. 1 for the type of airbrush used). With nitrile gloves on, place coral fragment (surface area of the fragment should be approximately 2–3 cm^2) in a new clean bag.

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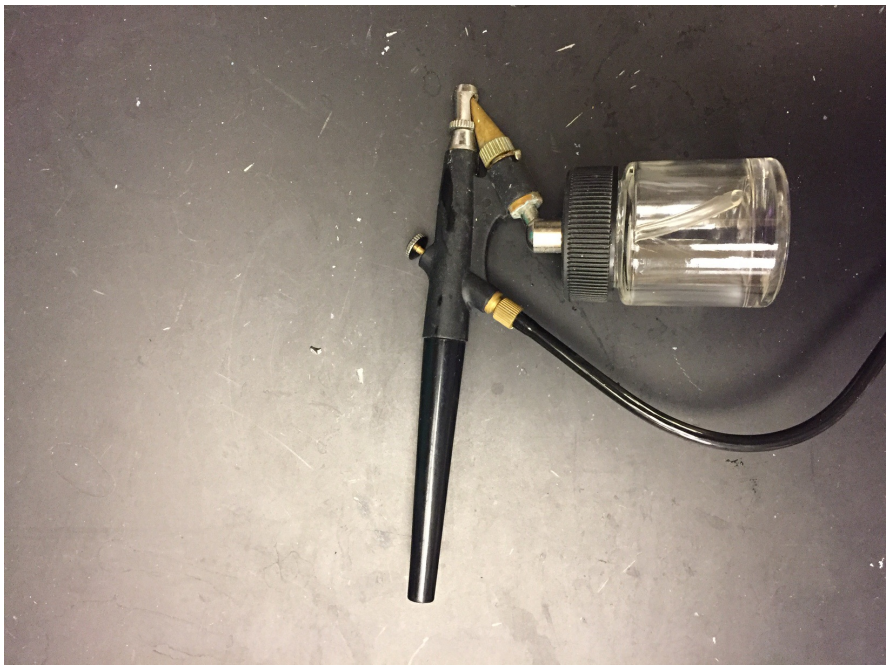

Figure 1 The airbrush and water reservoir setup used for removing tissue from coral skeletons.

2 **Airbrush the coral fragment.** Hold the coral fragment from outside the bag, such that the bag remains open for the airbrush, but you do not have to directly handle the fragment. Airbrush all surfaces of the coral fragment that have tissue. Ensure that you are airbrushing downward into to the bag, rotating the coral as necessary. You should not need to use all the water in the airbrush reservoir, depending on the size and porosity of the coral fragment. Note that depending on the coral species, the removal of tissue may be less noticeable. For instance, the deeper tissues of Poritid corals will be more difficult to remove via airbrush vs. the thinner and more easily removed tissues of Pocilloporid corals.

3 **Store coral slurry.** When all tissue has been removed from the coral skeleton (or as much as possible), open the pre-labeled 15 ml polypropylene centrifuge tube (Tube A) and carefully pour the slurry from the bag into the tube. Then, briefly spray the airbrush into the bag to rinse any remaining material into the tube. Leave at least 1 ml of space in Tube A to accommodate any expansion during freezing. Once the slurry had been transferred, store the 15 ml polypropylene centrifuge Tube A upright at -80 °C until ready to proceed to the separation stage.

Separation of Host Tissue from Algal Endosymbiont Cells

4 **Defrost and subsample the coral slurry.** When ready to proceed with separating the coral host from the endosymbiotic algal cells, defrost six samples of coral slurry (Tube A) in a room temperature water bath for ~5 minutes. While the samples are defrosting, prepare a 35 ppt sodium chloride solution (3.5% NaCl). Add 1.4 g of NaCl to 40 ml of 0.22 µm filtered ultrapure water and stir until dissolved, and set aside, covered until step #7. Next, fill two 250 ml beakers and one 50 ml polypropylene centrifuge tube with 0.22 µm filtered ultrapure water (see Fig. 2). Clean the mechanical tissue homogenizer by submerging the metal probe in the water and turning it on in each 250 ml beakers of ultrapure water for 10 seconds, followed by the 50 ml polypropylene centrifuge tube. ***Replace the 0.22 µm filtered ultrapure water in the 50 ml polypropylene centrifuge tube between each sample***. Once the samples are defrosted, turn on the cleaned mechanical tissue homogenizer and homogenize the coral slurry in the 15 ml conical tube for 10 seconds, avoiding contact between the homogenizer blade and the side of the tube. This ensures that the slurry is homogenous prior to subsampling. Using a Pasteur pipette, transfer 3 ml of coral slurry into Tube B. Store remaining original coral slurry at -80 °C. Repeat for the next five Tube A slurry samples. Note that a subsample of a greater volume can be used if the original coral slurry is found to be too dilute to obtain a measurable algal cell fraction for stable carbon and nitrogen isotope analyses (an ideal amount is approximately 500 µg carbon, 50 µg nitrogen).

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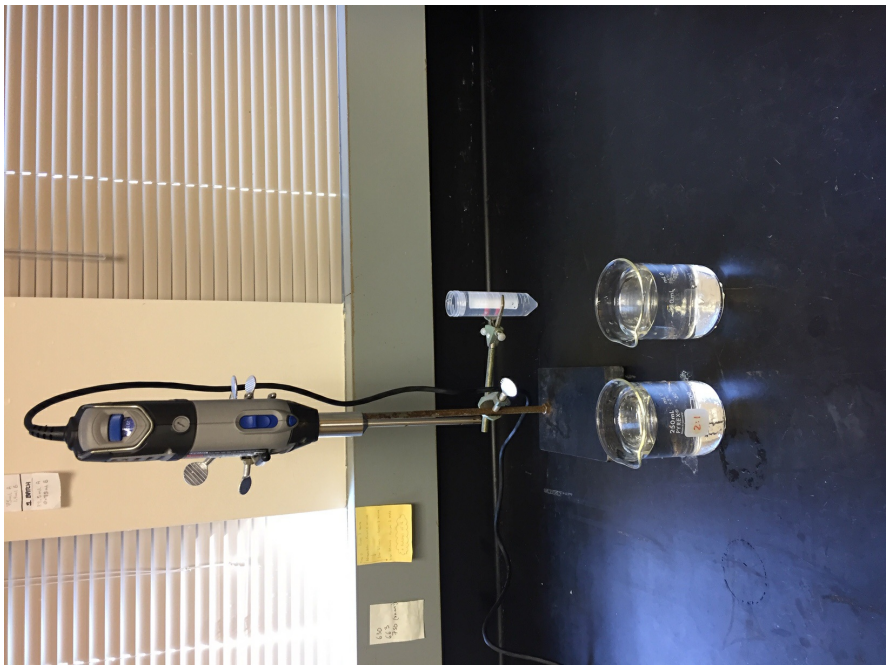

Figure 2 The mechanical tissue homogenizer and the two 250 ml beakers and 50 ml polypropylene centrifuge tube of 0.22 µm filtered ultrapure water used for cleaning between samples.

5 **Homogenize the subsample of coral slurry.** Proceeding with the 3 ml subsample of coral slurry (Tube B), homogenize the subsample with the cleaned tissue tearor for 50 seconds, ensuring to avoid bumping the blade of the tissue tearor directly into side of the tube (Fig. 3A). Next, move the subsample to the sonicator probe, such that the probe is approximately 2 cm below the meniscus of the subsampled slurry (Fig. 3B). Sonicate for 60 seconds (20% amplitude, 1 second pulses). Clean the sonicator by spraying the probe with 0.22 µm filtered ultrapure water and wiping down with a clean tissue wipe and proceed to the next sample. Repeat for all remaining 5 samples.

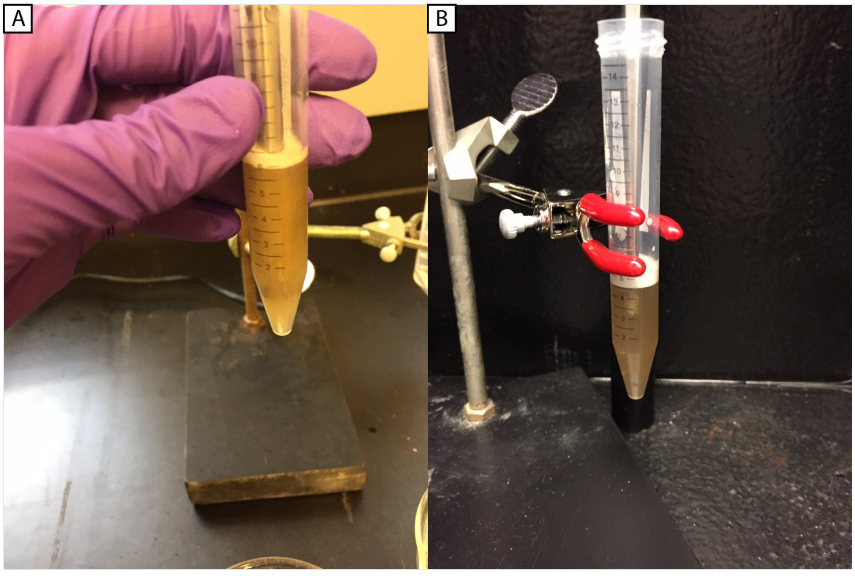

Figure 3 A) The mechanical tissue homogenizer within the coral slurry sample. B) The sonicator probe within the coral slurry sample, approximately 2 cm below the meniscus of the slurry.

Isolation of host fraction. For all samples, centrifuge the 3 ml subsample of coral slurry in Tube B at 500 x *g* for 2.5

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6 minutes. When the centrifuge cycle is complete, carefully remove all 6 samples from the centrifuge and use a glass Pasteur pipette to transfer the supernatant (avoiding the algal endosymbiont pellet) to each sample's respective Tube C. The host fraction in Tube C can be stored at -80°C until ready to start packing tin capsules for stable isotope analysis. It is not necessary to get every last drop of supernatant as this method does not quantify the amount of host fraction, only the ratios of ^{13}C:^{12}C and ^{15}N:^{14}N of the host fraction. In addition, avoidance of the algal pellet minimizes contaminating the host fraction with algal cells.

7 **Isolate algal fraction.** For all samples, add approximately 3 ml of previously made 35 ppt NaCl solution to each algal endosymbiont pellet in Tubes B. Vortex for 10 seconds and centrifuge at 400 x *g* for 2 minutes. When centrifuge cycle is complete, carefully pipette off the supernatant (avoiding the algal endosymbiont pellet) and discard. Repeat this step again to fully remove any remaining host fraction tissue from the algal pellet of each Tube B. You should be left with a small pellet consisting of almost entirely algal cells and some skeleton in the very tip of Tube B.

8 **Filter through nylon mesh to remove large non-algal material.** Prepare the mesh filter assembly (Fig. 4A) and set aside one unlabeled 50 ml polypropylene tube as the "rinse tube". This rinse tube can be reused as often as necessary because it does not contact any portion of the coral sample. Place your rinse tube into the side-arm vacuum flask. Place two pieces of clean 5 cm x 5 cm nylon mesh (20 µm pore size– the two mesh pieces should be offset at 45° of each other to ensure efficient filtration) in the mesh filter assembly and insert into the side-arm vacuum flask so that it is directly above the empty 50 ml rinse tube (Fig. 4B&C). Turn on the vacuum pump and use a Pasteur pipette to rinse the mesh with 3 ml of ultrapure water, removing any dust or particulates. Turn off the vacuum pump and discard the water from the rinse tube.

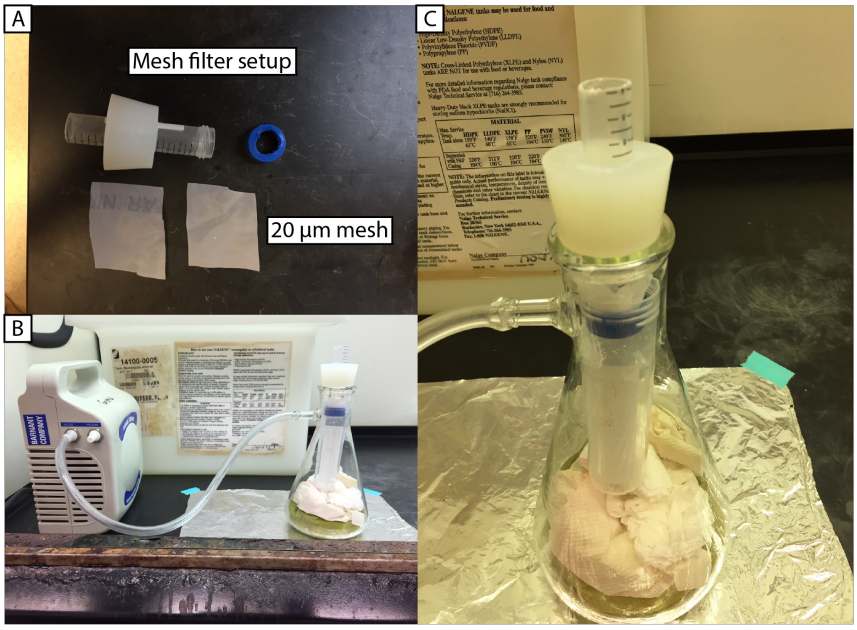

Figure 4 Photo of A) the materials used in the mesh filter setup, B) the assembled mesh filter setup in the side arm vacuum flask attached to a vacuum pump, and C) a close-up view of the assembled mesh filter setup in the side arm vacuum flask.

8.1 Next, replace the rinse tube with Tube D for the sample in question.

Add 5 ml of ultrapure water to the algal pellet in Tube B. Cap and vortex for 10 seconds to ensure algal pellet is resuspended. Turn on the vacuum (house vacuum, vacuum pump, or water faucet vacuum assembly are all suitable vacuum sources). Using a glass Pasteur pipette, transfer the contents of tube B into the mesh filter assembly, and filter the algal fraction through the nitex mesh. Rinse the mesh with at least 10 ml of ultrapure water or until it looks like no more algal cells are able to be filtered through the mesh (some algal cells will often remain on the mesh because they are trapped within mucus, tissue, etc.). The algal cells should have almost entirely filtered through the mesh and into the 50 ml tube below. It is not critical to capture every single algal cell as only the ratios of ^{13}C:^{12}C and ^{15}N:^{14}N

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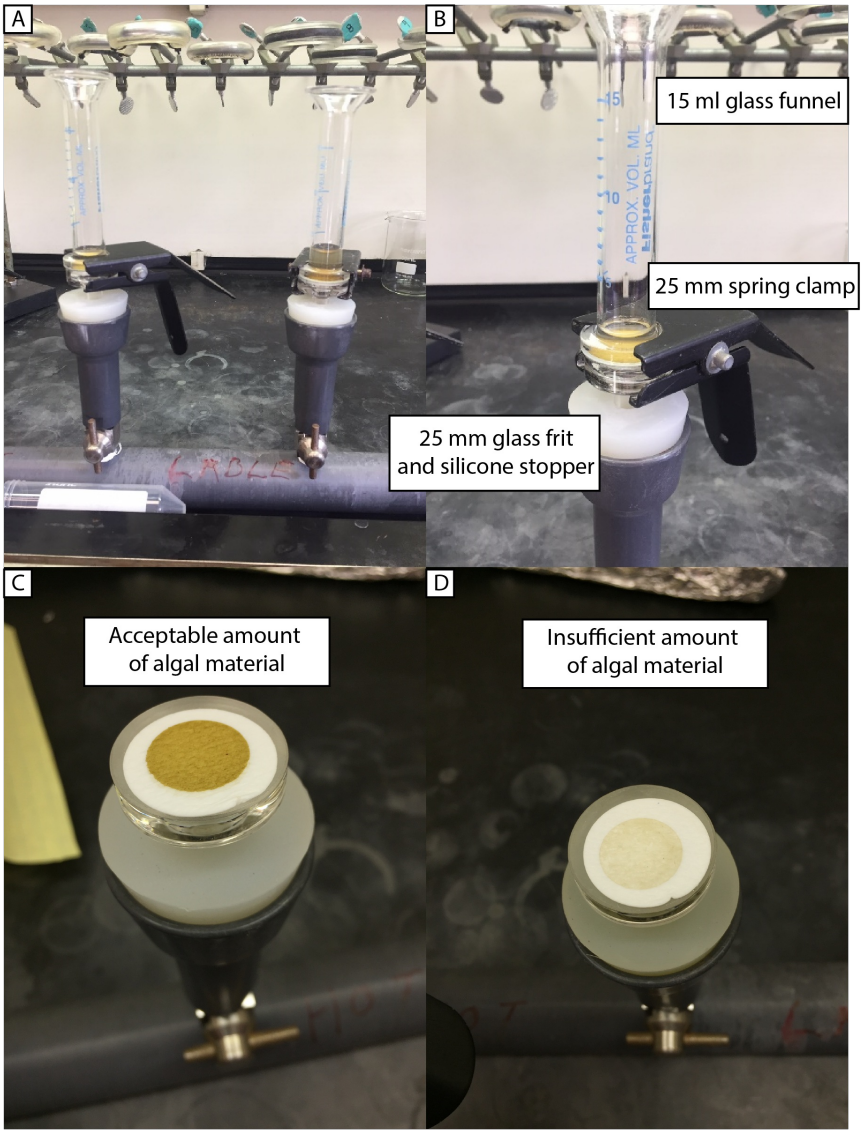
are measured using this method, not the amount of algal material in the sample. This filtering process helps to remove any lingering animal or cnematocyst cells from the algal fraction of the sample. Remove Tube D from the vacuum flask, cap the tube, and store in the refrigerator at 4 °C until finished filtering all six samples in this batch. Discard and replace the used mesh, clean the filtering apparatus in an ultrapure water bath, and repeat the process (assembly, rinsing, filtering) for each sample.

9 **Load the algal fraction on the glass fiber filter.** Prepare the glass fiber filter assembly. Place a glass fiber filter (GF/F) on the glass frit surface of the filter assembly and clip a 15 ml funnel on top. Up to six glass fiber filter assemblies can be used at one time with the six-port vacuum setup or one at a time if you only have a single filtration setup. Turn on the vacuum for the first filter assembly. Vortex the sample in Tube D for 10 seconds. Using a Pasteur pipette, transfer all of the algal fraction from Tube D to the glass fiber filter in the open port (Fig. 5A&B). A dark green/brown layer should begin to form on the glass fiber filter as sample drains through (Fig. 5C&D). If the filter starts to clog, increase the vacuum strength by opening the vacuum valve more and/or by placing your hand over the top of the funnel opening to enhance the vacuum draw. Once the algal fraction has been entirely filtered, close the vacuum valve or turn off the vacuum directly for the sample. Repeat for the whole batch of samples.



10 **Acidify the algal fraction to remove skeletal material.** When all samples have been filtered through the GF/F, turn off the vacuum. Once the vacuum is stopped, make sure to break the seal on the silicone stopper to release any

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residual suction. Then, use a glass Pasteur pipette to place four drops of 10% HCl on the algal endosymbionts that are now isolated on the glass fiber filter. Let the 10% HCl sit for 30 seconds and then reopen that sample to vacuum. Using a new glass Pasteur pipette, rinse the 10% HCl through the glass fiber filter with 5 ml of ultrapure water. Repeat for all samples. Turn off the vacuum and break the vacuum seal on the silicone stopper again.

11 **Dry the algal fraction.** Once the 10% HCl has been rinsed through the glass fiber filter, find the corresponding pre-baked and labeled aluminum pan. Using clean tweezers (wiped with 70% isopropyl alcohol between samples), transfer the filter with the algal fraction to the aluminum pan. Place the pan into a drying oven to dry at 60 °C overnight. Repeat for all samples.

Prepare Samples for Analysis

12 **Prepare the host fraction.** Using a room temperature water bath, defrost the host fraction samples (in Tube C) that you will be able to process that day (typically 20 – 30 samples in 8 hours). While thawing, prepare the clear plastic box and drying setup in the fume hood and connect argon gas. Once thawed, vortex the host fraction in Tube C for 10 seconds and then centrifuge at 2,000 x *g* for 5 minutes to pellet any remaining algal cells from the host supernatant.

13 **Prepare folding station.** While centrifuge runs, clean tabletop and then tape clean aluminum foil to the table where you will be folding samples into the tin capsules. Using forceps, prepare 9 mm x 10 mm tin capsule doublets for each sample, such that one capsule fits inside another (to form a doublet, see Fig. 6). The outer capsule will be flared out to catch any overflowing liquid. Weigh the doublet and then place it in every other opening on the microcentrifuge tube rack making sure to record its location.

Figure 6 An example of a tin capsule doublet, where one 9 mm x 10 mm tin capsule is placed within another to avoid the liquid host fraction from leaking out during the drying process.

14 **Dry down the host fraction.** Turn on hot plate that is inside the plastic box and set to approximately 90 °C. Once centrifuge cycle from step 1 is complete, use a Pasteur pipette to transfer approximately 0.25 ml of supernatant into one of the previously weighed 9 mm x 10 mm tin capsule doublets (Fig. 7A). Add the coral sample ID to the data sheet of the microcentrifuge rack position and doublet weight. Repeat for all samples. Let the host fraction dry down inside the plastic box. Place the doublet tins underneath the glass pipettes blowing argon gas to limit exposure to air (Fig. 7B). If there are more samples than glass pipettes blowing argon gas, be sure to rotate the samples every 15 – 20 minutes. Once dry, weigh the doublet tins again. You need approximately 1.5 mg of dried coral host tissue to get an ideal sample of 500 µg of C and 50 µg of N for reliable isotopic analysis.

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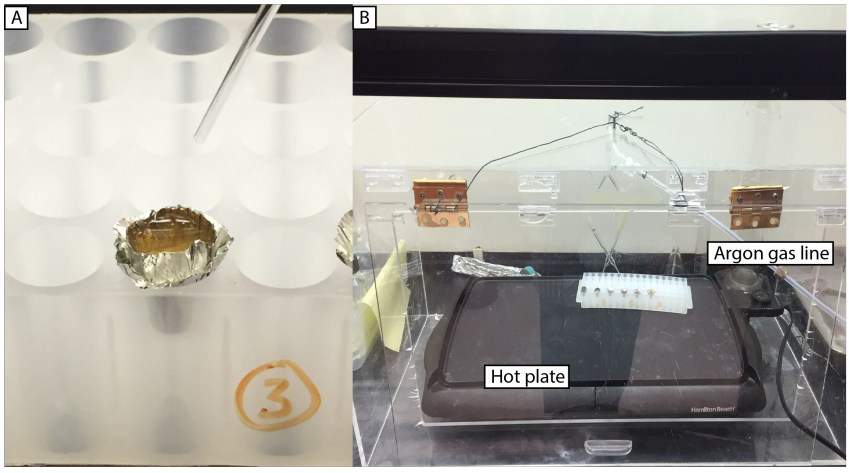

Figure 7 A) The liquid host fraction in a tin capsule doublet under a stream of argon gas and B) the entire plastic box/argon gas setup with a microcentrifuge rack holding six samples.

15 **Package and fold the host fraction tin capsule doublets.** Once the host fractions have completely dried (approximately 2 hours) and been confirmed to contain approximately 1.5 mg of dried tissue, they can be folded. Clean the folding setup (aluminum foil, tweezers, and folding block, see Fig. 8A) with a 70% isopropyl alcohol swab between each sample. Using two pairs of clean forceps, press the inner tin capsule closed and fold it over itself on the folding block. Then, flatten the outer tin around it (Fig. 8B). Once flattened, fold the capsules so that they form a “Z” shape, then flatten again (Fig. 8C). Fold into another “Z” shape in the opposite direction, then flatten again (Fig. 8D). Compress the capsule as small as possible to remove all air (a possible contaminant for $\delta^{15}N$) and to avoid sharp corners that may catch on the elemental analyzer wheel during analysis. Once compressed, place the encapsulated sample into a 96-well polypropylene plate for storage (Fig. 8E). Make sure to note which sample is in which well on the plate (i.e. A1, A2, etc.). Store in a desiccator cabinet. If storage longer than one week is required, store at -80 °C until ready to analyze.

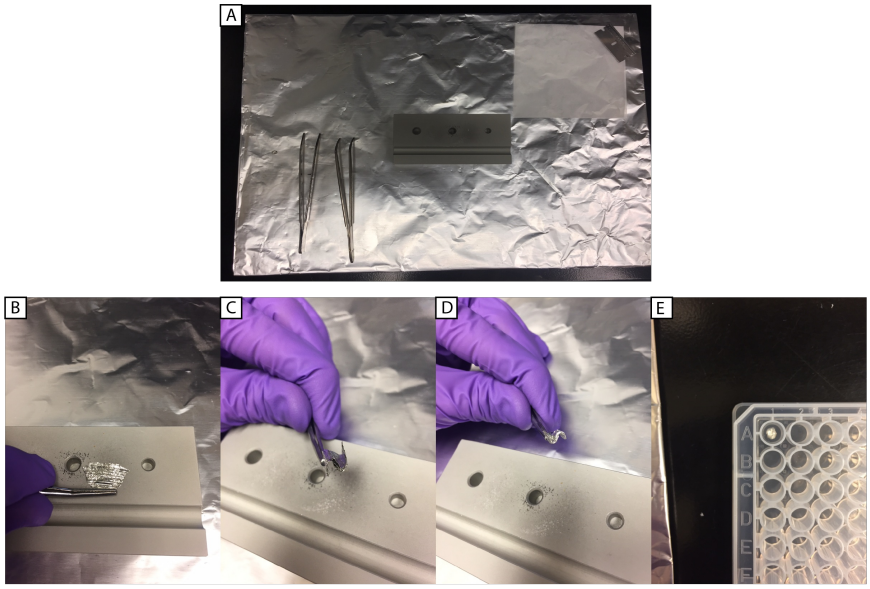

Figure 8 A) The supplies needed for folding the tin capsules (Note: the razor blade and weighing paper are only necessary for the algal fraction). B-E) A demonstration of the folding technique used, which begins by flattening the tin capsule that contains the sample, folding it into a "Z" shape and flattening again, then folding into a "Z" shape in the opposite direction, and finally compressing the tin as small as possible without ripping the capsule.

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16 **Package and fold the algal fraction tin capsules.** Clean the folding setup (aluminum foil, tweezers, razor blade, and folding block) with an alcohol swab between each sample. Place the dried algal fraction (currently on a glass fiber filter) on a piece of weighing paper. Place one 3.5 mm x 5 mm tin capsule in the folding block and gently flare out the edges. Use the razor blade to cut the filter in half. Then, using the tweezers, hold the edge of the filter and slowly scrape the dried algae off the top of the filter with the razor at a 45° angle (Fig. 9A&B). Scrape lightly to avoid ripping the filter (scraping the filter optimizes the performance of the elemental analyzer and minimizes the buildup of melted filter glass in the combustion column). Pick up the filter scrapings with the tweezers and place them in the tin capsule in the folding block (Fig. 9C). Repeat with the other half of the filter. When the filter scrapings are all in the tin capsule, close the top of the tin using tweezers and then flatten the capsule. Then, as you did with the host fraction, fold the capsules so that they form a "Z" shape, then flatten again (Fig. 8B-E). Fold into another "Z" shape in the opposite direction, then flatten again. Compress the capsule as small as possible to avoid sharp corners, and then place into a 96-well plate for storage. Make sure to note which sample is in which well on the plate (i.e. A1, A2, etc.). Store in a desiccator cabinet. If storage longer than one week is required, store at -80°C until ready to analyze.

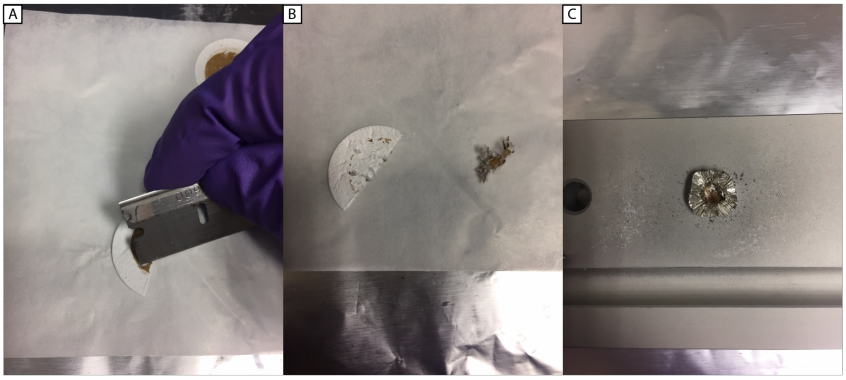

Figure 9 A) A demonstration of how to scrape the algal material from the glass fiber filter, and B) what the scrapings look like when complete. C) An image of the flared tin capsule in the folding block filled with algal scrapings from the glass fiber filter.

17 **Isotopic analyses**. Compressed tin capsules of both the algal and host fractions are now ready to be loaded into an elemental analyzer that is connected to a stable isotope ratio mass spectrometer for isotopic analyses of $\delta^{13}C$ and $\delta^{15}N$. In order to report the error associated with sample preparation, 10% of all samples should be run in duplicate. In order to report potential contamination associated with sample preparation, a blank sample (all steps but with 0.22 µm filtered ultrapure water only) should be run periodically as well.

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