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Stable Isotope Protocols: Sampling and Sample Processing

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The protocols below are designed to provide the information needed by researchers or managers to conduct natural abundance stable isotopic analyses of marsh food sources (suspended particulate organic matter [SPOM], vascular plants, benthic microalgae [BMI], benthic macroalgae) and sediments, as well as common invertebrate and vertebrate consumers (snails, mussels, crabs, macroinfauna and fish). A list of supplies required to carry out the protocols is given in Table A-1.

A. Primary producers—phytoplankton, benthic algae, vascular plants.

A.1 Suspended Particulate Organic Matter

Collections of suspended particulate organic matter (SPOM) are obtained for estimating the isotopic signature of phytoplankton. Water samples of at least several liters should be collected with a clean container and processed as soon as possible. The amount of water required will depend on the chlorophyll content of the water; the goal is to obtain a filter with rich gold to green color. In eutrophic waters characteristic of bays and marsh channels, several hundred ml may suffice. In more oligotrophic waters, up to 10 l may be required. Zooplankton and macrodetritus can be removed from the sample by pre-filtering water through 100-200 μ m mesh. It is also advisable to conduct total chlorophyll and organic matter analyses (Standard methods) of the water sample, which will aid in the interpretation of the isotope data. After pre-filtering, the water sample should be filtered through an ashed (450°C x 4 h) 47 mm glass fiber filter (GF/F) at low pressure (5 in. Hg vacuum). Samples for C isotope analysis should be treated with 1N HCl to remove carbonates, while samples for N and S isotope analysis should be rinsed thoroughly with distilled water. Often C and N isotope samples are performed on the same sample, and the effect of acid on the N isotope values can be checked by analyzing acidified and unacidified samples. In our experience, a brief rinse with 1N HCL, followed by a distilled water rinse, seldom affects the N isotope value of SPOM. Acid-fuming samples for C isotope analysis is an alternative approach to removing carbonates from SPOM samples, and in low carbonate environments may be preferred. After filtering and rinsing, samples should be prepared for isotope analysis or storage. (See Notes on Storage at end). Samples may be frozen, dried at low temperature (60°C) or in a freeze-dryer, and stored in a dessicator, prior to analysis.

A.2 Benthic microalgae

Benthic microalgae (BMI) can be collected for isotope analysis using a variety of techniques. Investigators should consider the nature of the algal community and logistical constraints when selecting their approach, and consider trying several alternatives. After

filtering and rinsing, samples should be prepared for isotope analysis or storage. (See Notes on Storage at end).

A2.1 Density centrifugation with Ludox (colloidal Si)

Collect surface sediment (top 0.3-0.5 cm, or depth of algal mat). Homogenize the sediment by stirring. Transfer 15 ml of sediment to 15 ml of distilled water in a 50 ml centrifuge tube. Stir, shake vigorously, and centrifuge at speed 6 for 5 min. Decant supernatant and repeat. Add 20 ml Ludox, shake vigorously. Add 5 ml DI water carefully to the top, trying not to mix with the Ludox solution. Centrifuge at speed 3 for 5 min, using a swinging bucket rotor. Pipette the golden brown layer of microalgae at the top (if present) and filter it through a 53 μm -mesh Nitex (to remove large detritus particles and meiofauna) onto a combusted (450°C x 4 h) GF/F or AH filter (experiment to see which works better). Examine the filter under a dissecting microscope, removing contaminant animals and detritus. Process enough sediment to produce 2 samples minimum; at least one C/N and one S sample. Examine the filter under a dissecting microscope and rinse with HCl 1N and DI for C and N analysis. For S analysis, rinse only with DI water. Filters may be dried at low temperature (60°C), (or frozen). For isotope analysis, scrape the algal material with a spatula and put it into a pre-weighed tin boat.

Note: exact volumes of water/ludox, and centrifugation times may change with different sediment and algal mat types

A2.2 Vertical migration technique

***In situ* collection of benthic microalgae.**

In this protocol, sediments are not removed from the marsh surface and the “incubation period” is 1-3 hours in the field. In addition to nitex screen, ashed Si or sand, and filtered seawater in a spray bottle, you will need to prepare a device to shade the nitex screen. We have used “rings”, made of either PVC pipe or styrofoam (for wreath crafting). The ring should be 1-3 inches high and 8-12 inches in diameter. Fiberglass screen is glued or otherwise fixed to the top surface (we, have used rubber bands to hold screen onto PVC sections). This technique works best in muddy sediments, as they do not lose water as much as sandy sediments during low tide. Arrive at the selected site as soon as sediment is exposed during falling low tide. Collections made on rising low tide will not be as successful. Sprinkle Si/sand on sediment in area that matches rings or another shading device. Lay wetted nitex carefully over Si/sand, smoothing out any air bubbles with spatula. Sprinkle additional Si/sand on top. Spritz (spray) with filtered seawater or DI water to insure good contact with underlying sediment and to prevent/minimize dehydration. Cover with the ring and fiberglass screen. Sometimes spritzing during the incubation is necessary. Prior to submergence of the next high tide, harvest the microalgae by carefully pulling off sediment and transferring the nitex (folded to a cone shape) to a clean plastic cup. Store in a cooler for transport back to the lab, and remove the microalgae from the nitex screen, and process the filters as described below.

Lab culture of algal mats

Set up an incubation area under fluorescent (grow) lights. Lights should be approx. 12-18" from surface of sediment. Use putty knives to collect sections of algal

mats or surface sediments from marsh or creek bed surface. Where mats are cohesive, section deeply enough to collect the entire mat (0.5 to 1 cm deep). Disturb the surface as little as possible. Shallow plastic trays are appropriate containers for sediment samples. Distribute the samples in trays so that, as much as possible, there is a continuous flat surface (visualize a tray of brownies after cutting). Return the trays to the lab. Spritz thoroughly with filtered seawater. Gently smooth out surface depressions, cracks or hills where possible. Remove pieces of detritus or live plants with tweezers. Sprinkle with ~1 mm of ashed, acid-rinsed Si (or similarly prepared beach sand). Place wetted nitex screen (63µm mesh) over Si, then sprinkle about 1-3 mm of Si on top of the nitex. Wet (spritz) thoroughly. You may need to experiment to find the optimal Si coating depth. Cover samples with saran wrap to prevent desiccation; leave under lab fluorescent lights overnight. Spray the cultures before you leave in the evening, and first thing in the morning. Harvest the cultures approx. 18-24 hrs after collection. Remove the nitex screen to a v-shaped tray, and rinse down with filtered seawater (FSW). (Alternatively, wash the nitex screen in a cup filled with water). Collect rinse water in a plastic cup. Additional rinses with DI water may be done. Often Si/sand is rinsed into the cup. Sometimes the nitex must be scraped with a spatula to remove algae. After rinsing algae into a beaker, stir thoroughly, and then decant the supernatant to an ashed GF/F filter. Apply a gentle vacuum. Remove filter and examine under a dissecting scope, removing inorganic and organic debris and animals with tweezers. If the sample is for C-N, return to filter tower and rinse with HCl. If for S, rinse thoroughly with DI water. Filters for S analysis should be clogged, C-N filters may be lighter. Record appearance of filters and treatment in notebook. Place filters in drying oven, wrapped lightly in aluminum foil, or freeze.

Bulk mat samples

This procedure is only possible when a thick, cohesive algal mat is present. To obtain a bulk mat sample, remove the surface microalgal layer from the sediment. Wash in filtered seawater (hold it with tweezers and shake/swish it in a pyrex dish.) Use the dissecting scope to pick out as much detritus, mineral matter, and animals as possible. Bear in mind that black particles or sediments may be pyrite (FeS_2) and should be removed from the sample for S analysis. Save on ashed filter or ashed scintillation vial.

General

Composition of all BMI samples should be qualitatively assessed by microscope examination. We recommend investigators use a dissecting microscope to examine filtered samples, and that subsamples of the community be examined with a compound microscope. The following categories are representative of those that we typically assign BMI samples: Diatom-dominated (D); cyanobacteria-dominated (CB); macroalgae-dominated (MA); and mixed community with no clear dominant taxa (X).

A.3 Benthic Macroalgae

Collect fresh macroalgae in a labeled plastic bag. Keep cool until return to the lab. Benthic macroalgal samples should be examined under a dissecting microscope and cleaned of sediments and epibiota prior to processing. It is often very difficult to get a clean sample of some benthic macroalgae, particularly those that form mats and/or are epiphytized by microalgae. After cleaning, the samples should be rinsed in DI water, and

prepared for analysis. If necessary, rinse and rub material in 5% HCl for about 1 min to dissolve any shell material, then rinse again in DI water to remove acid. Dry algae at about 40°-45° C in labeled aluminum foil dishes/open pouches (don't set samples on bottom of oven, it gets too hot). Grind dried samples with a mortar and pestle (use scissors or a clean coffee grinder if very fibrous). Clean grinder, scissors or mortar & pestle with methanol between samples, allow to air dry. Place the sample in a numbered glass vials that has been combusted (500°C overnight). Store in a desiccator or freezer if long-term storage is required. Load into preweighed tin boats (Costech) or silver boats (1 mg sample) for analysis. Keep covered and clean, do not touch with hands, use special bent forceps.

A.4 Vascular Plants

Collect vascular plants in the field and keep material cool (or frozen) until processing. Living green blades, belowground biomass, and detrital samples of vascular plants should all be considered for separate isotopic analyses. These types of material should not be combined. All tissue needs to be examined under a dissecting microscope and cleaned of epibiota, then rinsed thoroughly in distilled water, with gentle agitation or scraping. After rinsing, samples should be prepared for isotope analysis or storage as described above for benthic macroalgae. (See Notes on Storage at end).

A.5 Sediments

Remove sediments from the cm layer of interest (upper 4 cm is standard). Samples need not be quantitative; collect 5-10 g. Dry in an oven at 60°C, grind into fine powder with a mortar and pestle. Put about 2 mg in a double tin cup and acidify with PtCl₂ or HCL to remove carbonates. Let dry prior to analysis.

B. Consumers

B.1 Epifauna (snails, crabs, mussels)

Collect samples in labeled Ziploc or whirlpac bags. Add filtered seawater to cover epifauna and let sit 24 hours to evacuate gut. Rinse in DI water then freeze at -20° C until ready to analyze. Crack shell and remove muscle tissue. Rinse tissue in DI water and put in a numbered vial. Record data on data sheet. Dry samples in drying oven at 35 - 40° C overnight and store in a desiccator or freezer. Samples in vials should be ground into a fine, homogeneous powder using a combusted mortar and pestle. (Clean with methanol between samples) Ground samples may be placed back in the numbered vial or into preweighed tin boats (0.4 to 1.0 mg) and stored in a desiccator or freezer.

B.2 Infauna

Sample infauna in small cores or with a scoop to designated depth in the sediment (upper 2-6 cm is standard). Keep cores cool, and covered (in the dark) until used. Mist with filtered seawater in the lab to keep moist. Sieve sediments through a desired mesh (we use a 300 µm mesh) with filtered seawater. If sediments don't sieve well (e.g., detritus-filled sediments) sort the sample unsieved, taking small amounts. Sort sediments using methanol-cleaned forceps. Remove macrofauna and then place without sediment into labeled petri dishes containing filtered seawater. Label each dish with relevant

information (e.g., date, site, treatment, vertical fraction). Let infauna sit for several hours or overnight to evacuate guts. Then, rinse specimens individually in DI water, shake off water and place in preweighed tin cups (if small) or combusted vials (if large). Be sure forceps are dipped in methanol and air dried before and after handling each specimen. For small animals, place multiple individuals of the same species in one boat to make up 0.5-2 mg of dry weight. Record contents (taxon and number) of each vial or boat on isotope record sheet. Be sure to include collection date, site, location, treatment, vertical fraction etc.. Place tin boats or vials in drying oven at 35 - 40° C overnight (on a shelf, not the bottom). Store vials and tray in desiccator or freezer (freeze if long-term storage is needed) until analysis.

B.3 Fish

Collect fish from creek channels or the open bay using timed dip net searches. Keep cool or freeze at -20° C until ready to analyze. Record the species and length then remove heads, stomachs, tails and scales. Put the remaining tissue (fillet) in numbered combusted vial. Dissection is completed using a scalpel and probe under a dissecting microscope to insure only muscle tissue is analyzed. If the fish is extremely small (≤ 1.5 cm), remove head and stomach and place the rest of the fish in a numbered combusted vial. Record data on data sheet. Dry vials in drying oven at 35 - 40° C overnight and store in desiccator or freezer. Samples in vials should be ground into a fine, homogeneous powder using a combusted mortar and pestle. Ground samples may be placed back in the numbered combusted vial and stored in a desiccator or freezer. Put a small amount of the powdered fish (1 mg) in a pre-weighed tin boat.

C. General Preparation for benthic macroalgae, vascular plants, epifauna, infauna, and fish.

Tissue may be stored dried at room temperature, or frozen. Tissue must be dried and ground to a fine powder prior to isotope analysis. Low-temperature drying (<65 °C) has been demonstrated to have little effect on C and N isotope composition of organic material. Freeze-drying may also be used, but has been reported to slightly alter the isotopic signature of material. Dried tissue should be ground into as fine a powder as possible, to insure good homogenization and complete combustion. Depending on the sample type, powdering may be accomplished with a mortar and pestle or coffee grinder. Fibrous tissue, such as vascular plant stems or fish tissue, may require a commercial grinding device, such as a Wiley mill or WigLBUG. Dried and powdered samples will be placed into preweighed tin boats (appropriate for elemental analyzer-check with isotope lab first) or combusted glass vials. Preweigh tin boats (record position number in trays of 98 cups). To combust vials, cover them with foil and combust at 500°C overnight in a muffle furnace. Place smaller vials in a pyrex crystallizing dish if needed. Once cool, wrap lab tape around each vial and number. Combust (500°C overnight) all glassware that contacts specimens, clean with methanol all utensils that can't be combusted (forceps, spoons, plastic dishes).

Acidification

Acidification is recommended for samples subject to $\delta^{13}\text{C}$ analysis. Weigh the boat + sample and record the weight on the data sheet. Place this boat into another boat. Double boating is required prior to acidification! Add 12 μl (one drop) of 10% PtCl_2 solution to remove carbonates and place in a fume hood overnight. If still bubbling, repeat above until all carbonate has been removed (no more bubbling). Close both boats to ship samples for isotope analysis. Obtain PtCl_2 from Fisher Scientific. Use 25 ml of the acid in 250 ml of 1 N HCl to make a 10 % solution. Keep the solution in a labeled dark glass bottle in the refrigerator.

Analysis

$\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analyses are conducted on single individuals or for small macrofauna, on several individuals combined. Prior to combustion, samples may be acidified with 10% PtCl_2 to remove carbonates. Contract with a university or private isotope lab to obtain data; specifics on analysis will vary with the lab. Investigators should verify number of replicate analyses, quality control procedures, turnaround time, and amount of material (μg or μmol C, N and S) required for the mass spectrometer before choosing a vendor.

Stable isotope data are expressed in part per thousand (‰) deviation from international standards using the following equation:

$$\delta X = (R_{\text{sample}} / R_{\text{standard}} - 1) \times 1000$$

Where $X = {}^{13}\text{C}$ or ${}^{15}\text{N}$, and R = ratio of heavy/light isotope content (${}^{13}\text{C}/{}^{12}\text{C}$ or ${}^{15}\text{N}/{}^{14}\text{N}$). Working standards, sucrose and ammonium sulfate, are ($\delta^{13}\text{C} = -23.83\text{‰}$ vs. VPDB, $\delta^{15}\text{N} = +1.33\text{‰}$ vs. air N_2).

Table A-1. Supplies required for natural abundance stable isotope analyses of wetland suspended particulate matter [SPM], benthic microalgae [BMI], benthic macroalgae [BMA], vascular plants [vp], fish and invertebrates. vm = vertical migration method, dc=density centrifugation method.

<u>Supplies:</u>	<u>Available from:</u>	<u>Protocol:</u>
Ashed 47 mm GFF filters	General scientific suppliers	SPM, BMI vm
Ashed 47 mm AH (GFF, GFC)	General scientific suppliers	BMI dc
Filter forceps	General scientific suppliers	SPM, BMI all
Nitex screen (100-200 mm mesh)	Aquatic Ecosystems; AREA	SPM
Filtration apparatus	General scientific suppliers	SPM, BMI all
1 N HCL	General scientific suppliers	SPM, BMI all
Squeeze bottles	General scientific suppliers	SPM, BMI all
Ludox HS-40	Fisher; Sigma	BMI dc
50 ml centrifuge tubes polyprop.	General scientific suppliers	BMI dc
Si (can substitute cleaned sand)	Fisher; Sigma	BMI vm
Window screen, fiberglass	Hardware store	BMI vm-field
Putty knife (4-8 cm)	Hardware store	BMI vm, dc
“Rings”-PVC >8” diameter	Hardware store	BMI vm-field
Rings-craft	Craft or hardware store	BMI vm-field
Shallow trays c. 10x14x3”	General suppliers, hardware store	BMI vm-lab
Petri dish, small shallow dish	General scientific suppliers	BMI dc
Spatula spoon, stainless steel	General scientific suppliers	
Nitex screen (63, 55 mm mesh)	Aquatic Ecosystems; AREA	BMI vm
Nitex screen (55 mm mesh)	Aquatic Ecosystems; AREA	BMI dc
Insect (featherweight) forceps	Fine Science Tools; Ben Meadows	BMI all
Tin boats	Costech	All
Glass vials	General scientific suppliers	All
5 ml pipette	General scientific suppliers	BMI dc
Centrifuge, swinging bucket	General scientific suppliers	BMI dc
Ziploc bags (1pt, 1 qt)	Grocery store	VP
Dissection stereomicroscope	General scientific suppliers	All
Compound microscope	General scientific suppliers	SPM, BMI opt
Latex or vinyl gloves	General scientific suppliers	All
Combusted glassware (vials covered with foil)		BMA
Mortar & pestle	Scientific Suppliers	BMA
Wig-L-Bug tissue grinder	Crystal Labs	VP, Fauna
Spray bottle	Hardware store	BMI vm
12-16 oz plastic cups	Scientific supply Co.	BMI vm
Aluminum foil, heavy duty	Grocery Store	All
Methanol	Chemical Co. or Univ. Storehouse	BMA, VP
Distilled/Deionized water		All
Dip net or throw net	Memphis Net and Twine	Fish
PtCl ₂	Fisher Scientific	All
1N HCl	Scientific supply Co.	Plants
Scalpel	Scientific supply Co.	Fish, lg inverts
Drying oven	Scientific Supply Co.	All
Dessicator	Scientific Supply Co.	All
-20°C Freezer	Home appliance Co.	All
Muffle furnace	Scientific supply Co.	All
Table Top Centrifuge	Scientific supply co.	BMI ludox