

PROTOCOL

PREPARATION OF MUSCLE TISSUE FROM JUVENILE SPOTTED SEATROUT (*Cynoscion nebulosus*) FOR $\delta^{13}\text{C}$ AND $\delta^{15}\text{N}$ STABLE ISOTOPE ANALYSIS USING ISOTOPE RATIO MASS SPECTROMETRY (IRMS)

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INTRODUCTION

The muscle tissue of juvenile seatrout is used for stable isotope analysis ($\delta^{13}\text{C}$ & $\delta^{15}\text{N}$). These isotopes are used to determine the trophic position of each individual fish. The $\delta^{13}\text{C}$ is used to determine the ultimate source of carbon in the system and also track the flow of energy within a system. The $\delta^{15}\text{N}$ is used to determine trophic position within a system, and aids in assigning a trophic level to individuals. If the separation is good, i.e. significant differences between zones/locations/etc., isotope signatures may be used to discriminate/classify fish to individual nursery seagrass beds.

The following steps are followed to remove the tissue needed for analysis, store and prepare samples for introduction in IRMS (Isotope Ratio Mass Spectrometry).

EQUIPMENT

Quart Ziploc bags	5	Ziploc or Hefty One-Zip
Aluminum foil	1 box	Aluminum foil box
Class 100 latex gloves	2 pair	VWR brand / VWR 40101-Size code
Kim Wipe E-XL Wipers	1 Box	Kimberly Clark / VWR 21905-0
Sharpie X-Fine tipped black permanent marker	2	Boise Office Solutions N213801
Plastic Ruler 30cm length	1	
Glass Petri dish	1	
Stainless steel scapel	1	VWR 25607-947
Stainless steel tweezers	2	VWR 82027-408 or 82027-386
Glass scintillation vials 20 ml	1 per fish	VWR 66022-081
Polystyrene caps for scintillation vials	1 per fish	VWR 66024-200
Tin Capsules 3.5 x 5 mm (every 10 th or 12 th fish needs 3)	1 per fish	VWR 101189-546
TimeMed Labeling Trap	1 roll	VWR 36429-107 (aqua)

PREPARATION OF LAB WARE

It is important to ash all vials in an insulated high temperature oven used to store muscle tissue because “ashing” removes any residual carbon that may be found within the vials which could contaminate the samples. The high temperature oven is located in the Stable Isotope lab in the Chemistry building at ODU. Prepare at least one 20 ml scintillation for sample you intend to analyze. Place each vial in a 1 or 2 l beaker, but do not place the caps on the vials. Cover the beaker with aluminum foil and place it on a shelf within the oven. After “ashing” is completed, remove vials from the beaker and store them in a Ziploc bag to avoid airborne contamination.

TISSUE REMOVAL

The following steps illustrate the methods used to remove muscle tissue from the frozen fish and store the tissue prior to analysis. Each fish should have a unique identification number from the project that it was collected for. This number should be used for all subsequent data collected from this fish.

1. Remove an individual fish from the freezer and take it to your lab space where you will be removing the muscle tissue.
2. If no biological data has been recorded, measure lengths and weight:
 - a. Lengths:
 - Total: The length from the tip of the snout to the tip of the tail fin. May not always be possible because some fish are missing their tails, or tail fins are broken.
 - Standard: The length from the tip of the snout to the caudal peduncle.
 - Fork: The length from the tip of the snout to the fork in the tail fin rays.
 - b. Weight: Take the total weight in grams using a precision scale, with resolution to at least 0.001 g.
3. Place the fish on a glass Petri dish under a dissecting microscope to remove muscle tissue.
4. Make an incision with a clean scalpel from the lateral line to the dorsal fin just behind the operculum. Make a second incision from the operculum to the caudal fin just above the lateral line. In larger juvenile fish, it may also be necessary to make a third incision along the dorsal fin from the operculum to the caudal fin.
5. Peel the skin away from the incised areas using a scalpel and forceps to expose the dorsal muscle tissue.
6. Remove the exposed muscle tissue using a scalpel and forceps while being careful not to include any skin or bone fragments from the vertebrate.
7. Place the muscle tissue in a labeled and ashed 20ml scintillation vial and store the vial in a freezer until it can be freeze-dried.
8. Clean the Petri dish, scalpel and forceps with tap water to remove any tissue that may cross-contaminate subsequent samples and dry with a Kimwipe.

FREEZE DRYING

Use a freeze drier to desiccate all samples prior to homogenization. The freeze drier is located in the Stable Isotope lab in the Chemistry building at ODU.

1. Load the vials onto trays located in the freeze drier. Each tray can hold approximately 50-60 samples.

2. Seal the freeze drier and leave the samples overnight to dry. For the size of our muscle tissue samples, 10-12 hrs in the freeze drier is ample to completely desiccate the tissue.

SAMPLE PREPARATION

The following steps illustrate the methods used to prepare the freeze dried samples for analysis in an IRMS (Isotope Ratio Mass Spectrometer). All preparation should be done in your normal lab space and prepped samples can be stored at room temperature until ready for analysis. Some stable isotope facilities will every 10th to 12th sample to be run in triplicate to control for within sample variance.

1. Grind the muscle tissue in the scintillation vial to a fine and uniform powder after freeze drying. The powder must be uniform to ensure homogeneity of the sample. This can be difficult given the fibrous nature of muscle therefore one must grind the tissue as much as possible. If the sample is not homogenous avoid using the fibrous pieces in the analysis, i.e. try to retrieve the finely grounded material for the analysis.

If samples are being run at the Stable Isotope laboratory located in the Chemistry building at ODU, then the following applies:

- 2a. The powdered tissue is weighted into tin boats; you need a sample weight of approximately 0.5 mg for IRMS to give proper readings. The samples are weighted on an analytical balance with a resolution of at least 0.00001 grams.
- 3a. After proper amount of sample is placed within each boat, it is pinched closed and stored in labeled cell wells.
- 4a. The labeled cell wells are transported to the IRMS in the Chemistry department. If an autosampler is attached to IRMS the samples are placed within it based on the order found on the cell well. If not, then samples are individually placed into the instrument also based on order found on the cell wells.

If samples are being shipped to another facility for analysis, we used the Isotope lab (COIL) located at Cornell University, the following applies:

- 2b. Randomly order the finely ground tissue samples for analysis. The samples, in the scintillation vials, are thoroughly packed for transport to isotope facility via courier service. The vials are packed to avoid any movement that may cause them to break and are amply cushioned to also avoid breakage.
- 3b. Download and fill out the necessary shipping files which are specific for each facility. Make copies of these files and properly store.
- 4b. Email the facility manager the spreadsheet you used for the generation of the sample order. This aids them in assuring samples are run in the order specified.