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Protocol for Experimental Design and PPL Solid Phase Extraction for Chemical Exudates Released by Marine Macrophytes in Preparation for LC-MS/MS Analysis

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We use this protocol and it's working

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Abstract

It is increasingly clear that small molecules, or metabolites, are key in understanding ecological and evolutionary processes from elemental cycling to microbial symbioses. However, extracting molecules from marine systems remains a challenge. Here, we build off previous work (see Thukral et al., 2022) that provides methods for isolating dissolved organic matter (DOM) from bulk seawater using Solid-Phase Extraction (SPE). We extend this approach by providing both direct and indirect approaches to extract the chemical exudates released by marine macrophytes and their associated microbiomes using the seagrass, *Zostera marina*, as our model. In addition to embedding pictures on technical setups and sample preparation for liquid chromatography tandem mass spectrometry (LC-MS/MS), we also provide tips on experimental design for planning field and lab-based experiments. Together, this protocol aims to centralize methods related to (i) planning and implementing experiments and (ii) acquiring the data that identifies the small molecules released by key marine foundation species and their microbial symbionts.

Materials

Materials for Indirect Porewater Sampling via Rhizon Samplers:

Rhizosphere Research Products Female Luer 10cm Membrane length (Article no. 19.21.01F)

Materials for Direct Porewater Sampling via Milking, Equipment:

1 or 2 thermometers to check that the solute is at the correct temperature

Waterproof tape to label magenta boxes

Waterproof markers to label tubes

A liquid nitrogen dry shipper/dewar to freeze samples post-milking

2 spray bottles for keeping leaves moistened during milking process

Squirt bottles for washing off rhizosphere sediment into conical tubes

Homemade magenta box floats - see Image 2

Large beaker for rinsing the sediment off of the plants

50 mL sterile conical tubes for seedlings

50 mL conical tube floats - Sigma Millipore CAT# R7526-5EA - see Image 3

Magenta boxes for adult plants, big enough to hold 20 mL of solution - plantmedia SKU# 30930007-1

15 mL sterile conical tubes for collecting rhizosphere sediment and roots/rhizome tissue

Whirlpaks to collect leaves/shoot

Sterile 20 mL syringes to filter sterilize exudates - Fisher Sci #14-955-460

0.22 μ m/30 mm Acrodisc syringe filters for exudates - Fisher Sci #50-206-3074

5 mL sterile screw cap tubes to collect filtered exudates, 4 tubes per sample exudate - Sigma Millipore AXYSCT5MLS

Sterile scalpels to separate the leaves from the roots & rhizomes

Photo set up to take images of the plants after the milking process

Materials for Direct Porewater Sampling via Milking, Reagents:

Filter sterilized Instant Ocean (IO). Depending on the size of the experiment, expect to need ~5 or more liters of IO

Materials for Direct Porewater Sampling via Milking, Additional Equipment for Water Bath Setup:

Seedling Heat Mat with Digital Thermostat Controller (optional for controlled heating water baths; example: Luxbird LB-HT02)

Two plastic bins

2, Cardboard sheets

2, Tin foil sheets

Water to fill tub

General Materials for SPE:

Agilent SE Vacuum Manifold (cat no. 12234100)

HDPE vacuum carboy (cat no. 2226-0050PK) for flow-through collection

Bulkheads for carboy cap (cat no. 6149-0001PK)

3/8" i.d. Rubber vacuum tubing (Flinn Scientific, cat no. AP8279) OR

1/2" i.d. Rubber vacuum tubing (Flinn Scientific, cat no. AP8280) (depending on size of bulkhead fittings)



50mL Polypropylene Falcon Tubes (Fisher Scientific cat no. 14-432-22)

10µL Polypropylene pipette tips (whichever brand is compatible with available pipettes)

200µL Polypropylene pipette tips (whichever brand is compatible with available pipettes)

5mL Polypropylene pipette tips (whichever brand is compatible with available pipettes)

p10 pipette (whichever brand is compatible with tips)

p200 pipette (whichever brand is compatible with tips)

p5000 pipette (whichever brand is compatible with tips)

SPE Cartridges:

Agilent Bond Elut PPL cartridges, 200mg, 3mL (cat no. 12105005)

Agilent Bond Elut Adapters for 3mL cartridges (cat no. 12131001)

Reagents:

LC-MS grade 100% MeOH (ThermoFisher Scientific, cat no. 047192.K7)

Trace metals grade HCl (Fisher Scientific, cat no. A5058-P500)

LC-MS grade H₂O (Fisher Scientific, cat no. W64)

Before start

This general procedure is written for smaller volumes (50mL or less). For larger volumes, see Thukral et al., 2022.

To prevent contamination, avoid materials that can leach into your samples such as: silicone coatings and PEG contaminants.



Indirect Pore Water Sampling: Rhizon Sampling of plant (non-destructive)

- 1 Use a 10cm long Rhizon sampler attached to a syringe to pull porewater from seagrass rhizosphere, pulling from a depth that maximizes root area.
When planning this type of experiment, plan to have 2-3 no-plant controls (pot with bare sediment, or outside the meadow if field sampling) to understand metabolite differences across control and experimental groups.
The depth you insert the Rhizon sampler may vary based on meadow or mesocosm set up.
Tip: you can place a wooden block or PVC tiles within syringes to lock vacuums in place.

- 1.1 Draw porewater for  00:15:00 , replacing syringes as needed

15m

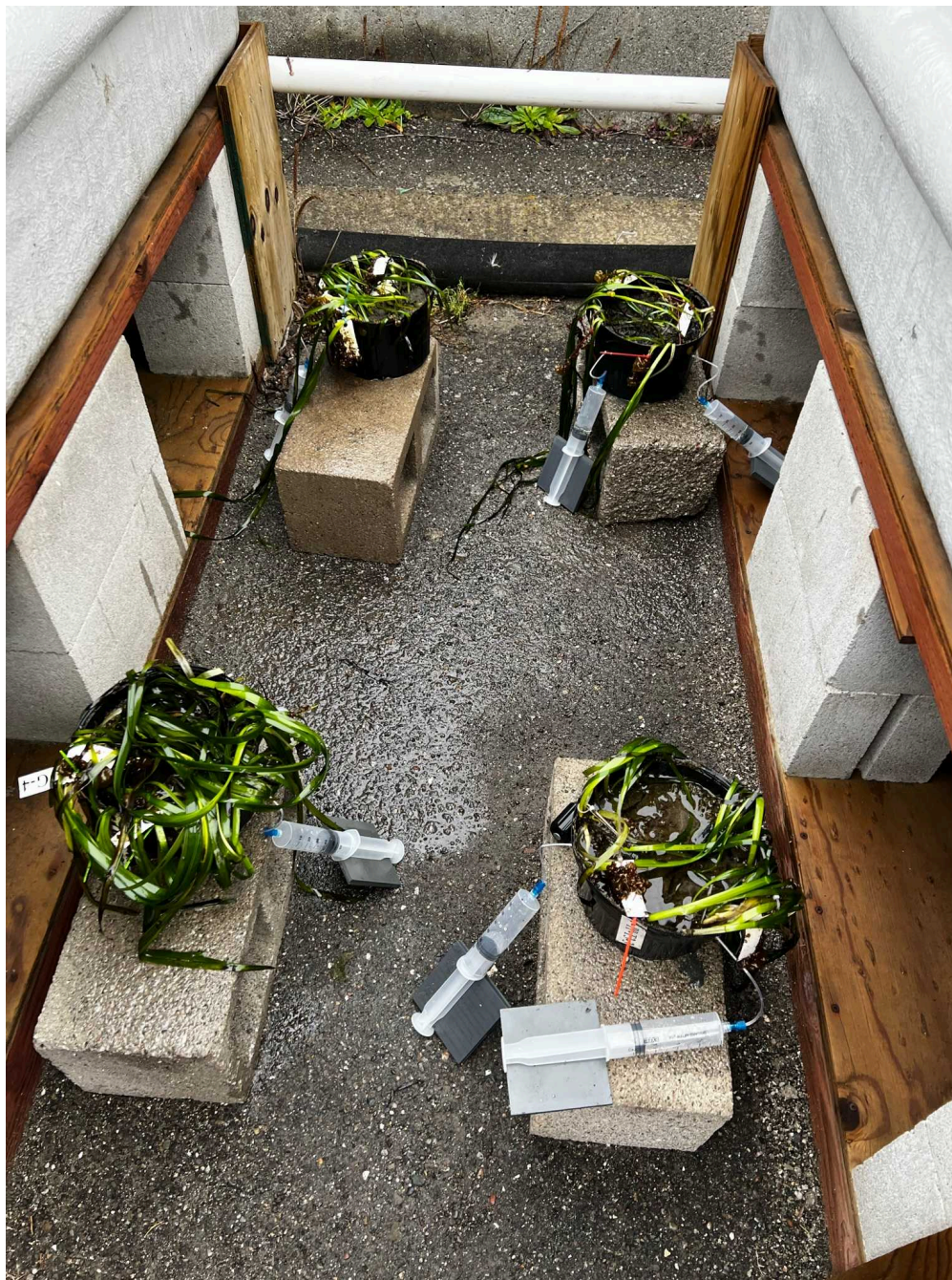


Image 1. Sampling seagrass rhizosphere porewater from pots in mesocosm using Rhizon sippers.

- 1.2 Transfer collected porewater in sterile 50mL Falcon tubes and store at -20°C until SPE.

Direct Porewater Sampling: Milking of Plant (destructive)

4h

- 2 When planning this type of experiment, plan to have 2-3 no plant controls for each control and treatment group. These controls will be important to understand metabolite differences across all control and experimental groups.
- 3 Materials Prep:
 - 3.1 Prior to starting the milking procedure, label all necessary material. Each sample being milked should have:
 - a Magenta box or 50mL conical tube per plant
 - 1, 15 mL conical tube for rhizosphere sediment
 - 1, 15 mL conical tube for roots/rhizome tissue
 - 4, 5 mL screw cap tubes for sterilized root exudates
 - 1 Whirlpak for leaves/shoots
 - 3.2 For the 50 mL conical foam floats, label each slot with however many seedling samples are to be milked for exudates

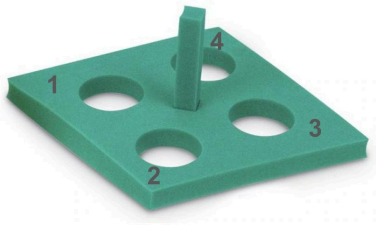


Image 2. Example of numbered floats to help keep track of samples in case something tips, or other mishaps occur.



Image 3. Magenta box floats that were made from kitchen sponges and pieces attached using a glue gun.

- 3.3 Fill spray and squirt bottles with sterile Instant Ocean (IO)
- 3.4 Pre-wet the magenta box floats so that it is easier to fit onto the magenta box

4 Experimental Procedure I: Milking the roots for exudates

- 4.1 Fill each magenta box or conical tube by measuring out  20 mL sterile IO.

Tip: a bottle top dispenser (such as Varispenser 2 - Eppendorf Catalog No. 4966000053) would be useful at this stage.

- 4.2 Assemble the magenta boxes or 50 mL conical tubes into their respective float devices and place them in the tank so that the IO in the milking chambers reaches the tank temperature. Measure the temperature of the tank and of the magenta boxes/50 mL conical tubes using thermometers.



Image 4. Example of how the milking chambers (magenta boxes and 50 mL conical tubes) look in their respective floats in the tank. Thank you to Sogin Lab member Diane-Marie Branche-Smith for the photo.

- 4.3 Gently pull the plant from its sediment and tap/shake until only the closely adhering sediment is left on the roots/rhizome.
- 4.4 Using a squirt bottle containing sterile IO, rinse adhering sediment into designated 15 mL conical tubes.
- a. Once IO is nearing 10-12 mL, stick roots/rhizomes in the tube and gently cap the top of the tube with your thumb (plant leaves are hanging outside of the tube), and shake the tube to remove more of the adhering sediment. The aim is to have ~2-5 "mL" of sediment in the tube.
- 4.5 Once the adhering sediment is collected, use a beaker filled with sterile IO to finish rinsing the plant of its sediment.



- 4.6 Place the plant in its designated milking chamber with roots submerged in the sterile IO. Allow the leaves to hang over the magenta boxes or upright in the conical tubes.
- 4.7 Allow the roots to be milked for  03:00:00 . During this time, it is important to spray the leaves with IO to prevent drying out/plant stress. For the first  00:20:00 , keep an eye on how long it takes for the plants to start looking a little dry so that appropriate time can be taken to ensure the leaves are moistened during the milking process.
- 5 Experimental Procedure II: Root exudates and tissue collection
- 5.1 When the milking period is over, remove the milking chamber(s) from the tank(s) and remove the plant. These steps also apply to the no plant control chambers.
- 5.2 Assemble the 20 mL syringe and filter
- 5.3 Remove the plunger and pour the  20 mL of root exudates into the syringe and replace the plunger
- 5.4 Filter sterilize  5 mL of the root exudates into all four 5 mL screw cap tubes
- 5.5 Freeze the root exudates, and all other samples, in the liquid nitrogen shipper/dewar
- 5.6 For tissue collection:
Take a photo of the plant. Example shown in Images 5 and 6.
Separate the leaves from the roots/rhizome using a scalpel.
Place leaves/shoots into their designated Whirlpak.
Place roots/rhizome into their designated 15mL conical tube.

3h 20m

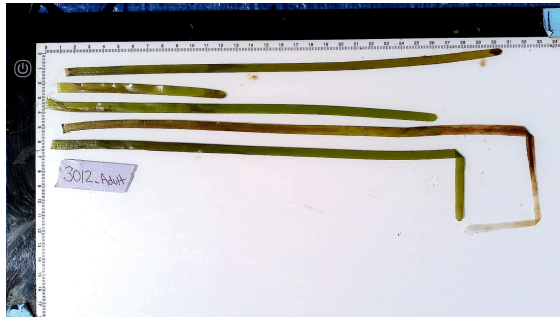


Image 5. Example photo of above ground tissue from adult plant.



Image 6. Example photo of below ground tissue from adult plant.

6 Experimental Procedure III: Modifications for Indoor Tank Set-Up

Note: For researchers that are growing *Z. marina* indoors or within a laboratory setting, these steps offer minor modifications of Protocols 2-5 for capturing milking exudates

6.1 Materials in addition to the standard procedure:

Water bath (for changing milking incubation temperatures)

Alternative: to construct your own water bath (per bath, see image 7 for construction) and Materials list

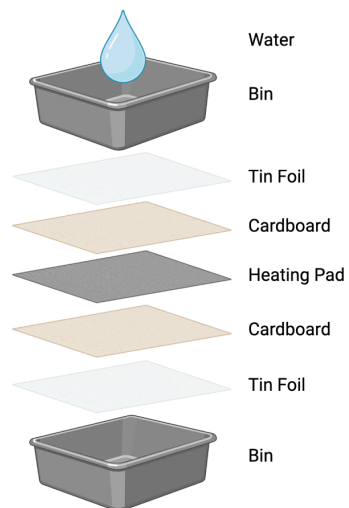


Image 7. Example of “Do-It-Yourself” construction of controlled heated water bath with seedling heating pad (made with BioRender). Be sure to insulate the heating pad between the cardboard and tin foil to avoid temperatures fluctuating due to air exchange.

6.2 Procedure: Follow instructions as described in Protocols 2-5.

Preparation of Materials I

7 Acid Washing Glassware

7.1 Rinse glassware with DI water

7.2 Wash equipment with DI water and Alconox

7.3 Submerge glassware in 0.1M HCl bath and let soak for  01:00:00

1h

7.4 Rinse with DI water 2x



7.5 Rinse with 100% Methanol

7.6 Air dry

Preparation of Materials II

8 Setting up tubing and rinsing materials.

8.1 Cut vacuum tubing to desired length with a precise, flat cut.

8.2 Rinse reservoirs and stopcocks with LCMS-grade MeOH followed by LCMS grade H₂O.
Note: You may Parafilm baths after rinsing materials for continued use of baths throughout sample processing.

8.3 Make 0.01M HCl (acidified H₂O) by aliquoting  1 L of LCMS-grade H₂O in acid-washed 1L glass bottle and add  1 mL of 37% LCMS-grade HCl.
Note: you may have to make more batches depending on sample size

8.4 Make a batch of 1M HCl (to acidify samples) by adding  91.67 mL of LCMS-grade H₂O then  8.33 mL of LCMS-grade HCl in acid-washed 100mL glass bottle.
Note: you may have to make more batches depending on sample size

Preparation of Materials III

9 Setting up SPE station.

9.1 Connect SPE station to an HDPE vacuum carboy as a solvent flow-through container, leaving one bulkhead fitting open to prevent a vacuum.

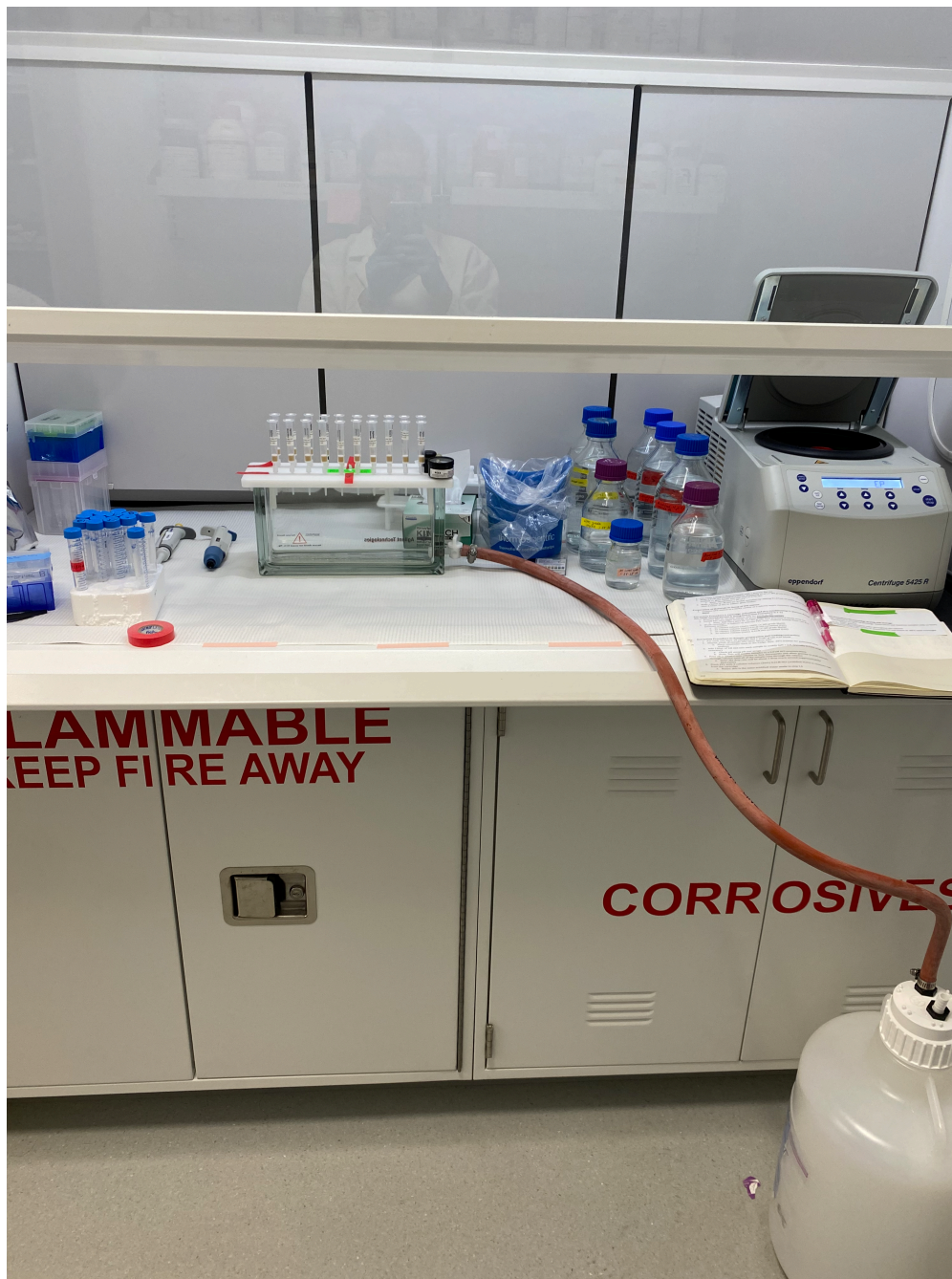


Image 2. SPE station set up: Agilent vacuum manifold connected to solvent waste carboy.

Extraction Procedure I

30m

10 Cartridge Activation and Pre-Conditioning.

Note: 1 person can do Extraction Procedure I while another is doing Extraction Procedure II.1-2.

10.1 Remove lids on vacuum station only for ports in use.

10.2 Install PPL cartridges for all samples.

Note: Add an extra port set up for an LCMS-grade H₂O procedural blank each use.

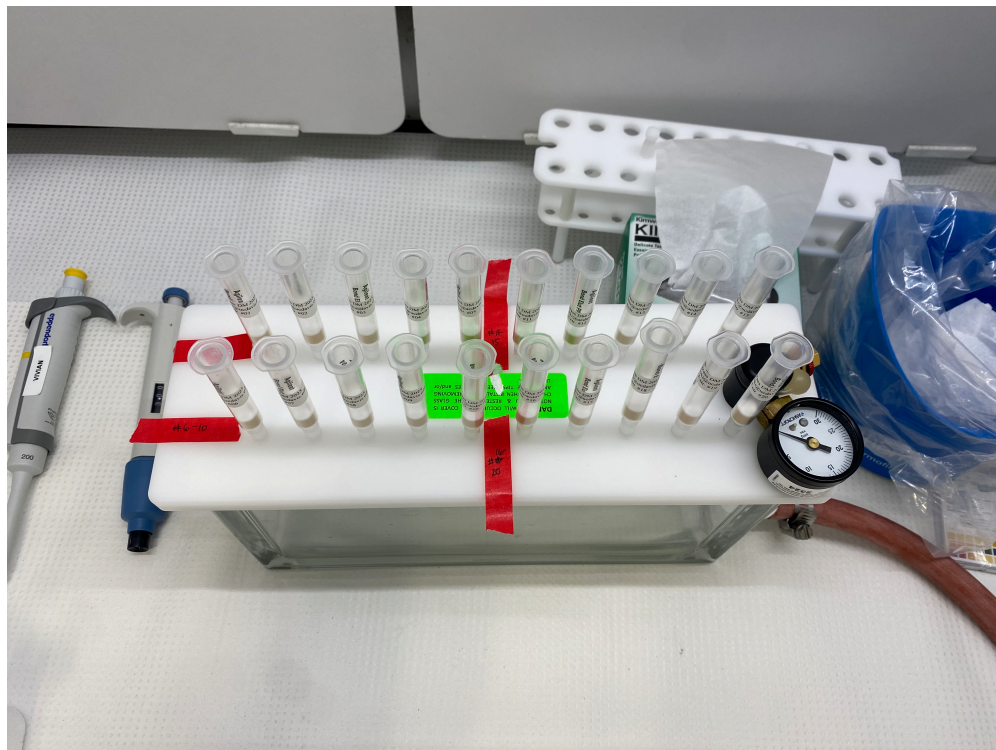


Image 3. SPE sample set up: Sample PPL columns with extra PPL column as blank in Agilent SPE vacuum manifold.

10.3 Wash cartridges as follows to activate PPL resin, without letting them run dry between washes:

- 3x column volume 100% LCMS grade MeOH
- 3x column volume 0.01M HCl (acidified H₂O)
- 3x column volume 100% LCMS-grade MeOH
- 3x column volume 0.01M HCl (acidified H₂O)

Extraction Procedure II

30m

11 Sample Preparation and Loading/Extraction.

- 11.1 Aliquot  15 mL of sample into clean, sterile Falcon tube.

Note: place remainder back in fridge, then -20°C.

- 11.2 Acidify samples to 0.01M by adding  150 µL of 1M HCl into each sample to acidify (pH = 2.0, typically 0.12% acid for seawater)

Note: Can check pH using pH test strips.

Note: This will acidify samples to a 0.01M HCl concentration.

Note: You will have to adjust calculations depending on total sample volume

- 11.3 Pour approximately ~3mL of samples into a reservoir at a time until all sample volume (~15mL total) has passed through the column via gravity (approximately 8mL/min)

- 11.4 Flush PPL with 2 column volumes 0.01M HCl (acidified H₂O) to remove salt from cartridge.

Extraction Procedure III

12 Cartridge Drying and Storage until Elution

- 12.1 If the PPL columns don't fit the vacuum concentrator rotor, remove the rotor and replace with two microtube floats that overlap. Dry the PPL columns in the vacuum concentrator overnight using the following settings: D-AQ with no heat indefinitely (infinity symbol)

Note: Ensure that the tips of the columns do not touch the surface of the vacuum concentrator.

Note: Alternatively, dry the samples under N₂ gas until dry



Image 4: Set up of microtube floats loaded with PPL columns in the vacuum concentrator for drying

- 12.2 Wrap cartridges individually in aluminum foil and freeze at -80°C (or -20°C if -80°C is unavailable) or proceed to elution.
Note: For field processed samples, without resources to dry, wrap samples in aluminum foil and freeze at -80°C until shipment on dry ice for drying and elution.

Protocol references

This protocol was modified from:

Martin, B., Gleeson, D., Statton, J., Siebers, A., Pauline, G., Ryan, M., and Kendrick, G. (2018). Low light availability alters root exudation and reduces putative beneficial microorganisms in seagrass roots. *Frontiers in Microbiology*, 8. <https://doi.org/10.3389/fmicb.2017.02667>

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Terrados, J., Williams, S.L. 1997. Leaf versus root nitrogen uptake by the surfgrass *Phyllospadixtorreyi*. *Marine Ecological Progress Series*, 149:267-277. doi:10.3354/meps149267

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