Plant & Soil Sciences
Townsend Hall, Rm. 152
531 S. College Avenue
Newark, DE 19716-2170
Phone: 302-831-2531

January 18, 2021

Editor, Journal of Visualized Experiments

Dear Editor:

I have uploaded a revised manuscript entitled "**A method to preserve wetland roots and rhizospheres for elemental imaging**" for consideration of publication in the *Journal of Visualized Experiments.*

This manuscript was peer reviewed (JoVE62227) and a revision was requested. We have carefully considered each comment by the peer reviewers and have made appropriate changes in the manuscript. Enclosed is a point-by-point response to reviewers and editors. We believe the peer review process has substantially improved this manuscript.

I hope you share our enthusiasm for this work and will consider it for publication in the *Journal of Visualized Experiments*.

Sincerely,

Angelia L. Seyfferth
Associate Professor of Soil Biogeochemistry
Dept. of Plant and Soil Sciences
University of Delaware

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have carefully checked and ensured there are no spelling or grammar issues.

2. Please provide an institutional email address for each author.

We have provided the missing information.

3. Please provide the complete address of the affiliations.

We have provided the missing information.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have removed personal pronouns throughout the manuscript.

5. Please define all abbreviations before use (ICP-MS, PPE)

We have defined abbreviations at first occurrence.

6. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials: e.g., Styrofoam, FixiForm, EpoTek, IsoMet, Loctite 404, Buehler PetroThin sectioner etc. We must maintain our scientific integrity and prevent the subsequent video from becoming a commercial advertisement.

We have removed all commercial language and referenced them in the Table of Materials

7. Please include a one line space between each protocol step and highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

We have added the one-line space and have highlighted the portion of the protocol needed for the video.

8. For units of time, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks (Lines: 89, 113, 119, 120).

We have made the appropriate abbreviations

9. Line 91: Please mention the volume of the liquid nitrogen used.

This will vary depending on the size of the foam cooler used and the size of the copper blocks. The critical point is that the blocks are submerged, which is detailed in the protocol.

10. Line 114: Please mention the volume of epoxy added.

This varies depending on the size of the block needed and the form used. As in above, the critical point is that it entirely covers the sample.

11. Line 126: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

We do not use a centrifuge in this protocol. The rpms are for the speed of the saw used. We have removed the speed as part of the generalization of the equipment used.

12. Line 128: Please mention in detail the sand cut process. Is the process manual or automated? How long is the sand cut performed?

We have added that the sanding is done manually and requires about 30 seconds of sanding at each grit

13. Figure 2: Please include the details of the steps required to image the roots using synchrotron XRF in the protocol section.

In our opinion, XRF is not part of the protocol. This protocol is regarding sample preparation for image analysis, and we have chosen to use synchrotron XRF imaging to do so, but the samples could be imaged in a variety of ways (e.g., light microscopy and photographs, LA-ICP-MS). We instead refer the reader to inquire with the synchrotron facility and beamline of interest to utilize time on the instrument.

14. Figure 3: Please define the white box in the Figure Legends.

The white box is now defined in the Figure 3 legend.

15. Please confirm the filming location: Newark DE or Upton NY. We can only film at one location.

The filming location is Newark, DE.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The present manuscript describes a protocol used to preserve root structure and rhizosphere chemistry of wetland plants through slam-freezing and freeze drying. This protocol could be effective in studying plant-soil relationships as it minimizes root damage and sample distortion. Also, the protocol offers the possibility to obtain multiple samples quickly and with minimal cost increases according to the authors.

Major Concerns:

The authors suggest that this method could be adopted for studies of plant-soil relationships in a wide variety of environments, but they only discuss its application for wetland plants. I would therefore suggest including some information on the potential benefits and limitations in using this technique for plants grown in other environments. In my opinion, it is not very clear if this method could be used to obtain further information from the samples, such as microbial biomass. Finally, this method is being compared to the flash-freezing technique, however there is no clear evidence of the greater efficacy of this protocol. Do you have any data and relative statistical analysis that would support your statements about the reduced root damage and sample distortion occurring with the described protocol compared to other techniques?

We thank the reviewer for this important feedback. The statement about the technique being transformative to a wide variety of environments was meant to include wetlands that are not rice paddies. We have changed this language to better articulate our intent in the introduction. We do not have any information about whether this technique could work in dryer/upland environments, so we will not speculate on that here. This protocol and elemental imaging is meant to help understand root soil relationships where the microbiome undoubtedly plays a role in the geochemistry (e.g., Fe

oxidation, Fe reduction) but this protocol is not intended to characterize the microbial biomass. The carbon in the epoxy resin would likely render such approximations inaccurate, and the imaging techniques mentioned are intended for elements heavier than nitrogen. We agree that this protocol is compared to the flash freezing techniques as an improvement to help preserve structural information in the roots. We have cited existing literature that shows the distortion caused by flash freezing, and evidence here and in one of our earlier papers on how this technique allows the structure of roots to be preserved. We have ensured that our statements are substantiated by evidence in the revised manuscript.

Minor Concerns:
SUMMARY
Comment #1: Line 13 I would suggest using species instead of organism.

We agree and have made the change.

INTRODUCTION
Comment #2: The authors first introduce similar techniques in the Discussion, while in my opinion this should be mentioned in the Introduction. I would like to know in the Introduction which protocols were previously available that this protocol could be replacing.

We agree and have added a paragraph on this in the introduction to make this clearer.

Comment #3: Line 39 instead of "various sizes" the authors could use "various sizes, function, diameter and structure".

We agree and have made the change.

PROTOCOL
Comment #4: Line 128 "with" is missing.

We agree and have made the change.

Comment #5: What is the suggested thickness of the sample to be placed between the two copper blocks?

We have added this information to section 1.1 and 2.2.

REPRESENTATIVE RESULTS:
Comment #6: Lines 151-152 Please rephrase by changing order "Of the 63 samples, 14 contained no roots, while 31 contained 1-3 roots and 18 contained more than 3 roots".

We have made the change.

Comment #7: Lines 152-154 The authors state that roots may be present in varying levels of quality. Why some roots are distorted, could we predict when distortion is going to happen and include that information in the protocol?

Unfortunately, roots grow in many different directions in flooded soil, even horizontally, and so this is nearly impossible to predict. The ease of this technique allows one to have several cubes from the same rhizosphere and inspect them prior to “wasting time” on samples that are not promising. We have made these advantages clearer in the text.

DISCUSSION

Comment #8: Could this protocol be used to study root exudation and microbial activity in the rhizosphere?

It depends on the exudate of interest. If the interest is in exudation of elements heavier than nitrogen (e.g., root exudation of As) then it is possible to visualize it but the direction is not clear with imaging alone. The epoxy contains carbon, and so the epoxy is likely to interfere with any C imaging that could be achievable with softer X-rays or SIMS.

Comment #9: Line 180 "inside and outside" could be replaced with "root and rhizosphere soil".

We agree and have replaced “inside and outside” with “root and rhizosphere soil in situ”

Reviewer #2:

Manuscript Summary:

The manuscript by Seyfferth et al. (A method to preserve wetland root rhizospheres for elemental images) presents an interesting method for sampling wetland soils while preserving spatial and chemical gradients in rhizosphere sections of the sample. In short, instead of using liquid nitrogen to freeze a sample, the authors employ copper blocks at liquid nitrogen temperatures where, presumably, the enhanced heat transfer through the copper facilitates more rapid freezing of the sample and fewer artifacts than LN cooling. The authors provide nice images showing the types of chemical structures that can be preserved on the root surfaces and convince the reader that this can be a viable method.

Major Concerns:

* The comparison to alternative methods (lines 177-180) appears a bit incomplete. Could the authors comment on the similarity (or presumably the improvements) of this technique to flash coring for instance? Also, the authors point to DGT as a way to examine root soil interactions but don't mention previous efforts using LA-ICP, LIBS, or NanoSIMS approaches (amongst others) that can make measurements of the rhizosphere and roots themselves.

We agree and have expanded the introduction and discussion sections to include some of the literature on this topic.

* It would be nice to highlight a bit more how this work differs from that done when analyzing plant or other tissues by using freezing on copper blocks. Is this simply the same approach but applied towards soil samples?

We appreciate this important feedback. Existing methods exist to examine root ultrastructure, but those are not typically combined with soil. The advantage of this method is that it allows for

preservation of both the roots and the soil in situ, rather than studying one or the other. In the revised text, we have tried to make this clearer.

Minor Concerns:

* In sections the writing is a bit unclear. For example, the first line of the abstract reads: "Roots are critically important for understanding how plants interact with their soil environment". This is a bit unclear as roots are the primary means by which plants actually interact with soils and the link to "understanding" is not evident. Do the authors mean something to the effect of "Understanding roots is critically important to assessing how plants interact with their soil environment"? A similar comment on lines 34-35 - do roots make understanding (as the authors seem to imply) or require understanding due to their importance?

We agree and have modified the sentences to make them clearer.

* Line 37: there is a jump here to "human nutrition" (presumably through arsenic mobilization in wetland soils?) where one could also make the case that plant nutrition is strong impacted by this rhizosphere processes (especially since the authors mention P) but this is not mentioned.

We agree and have revised this section accordingly.

* Lines 79-82: If I understand correct, the authors are sampling a plant from a wetland into a bucket then transporting back to the lab for actual freezing. Doesn't this run the risk of altering the redox gradients in the sample? For instance, in a waterlogged sample, when you pull it out of the Earth how does pore water not rapidly exchange leading to rapid changes in redox conditions at small spatial scales? Is there no feasible way to perform the actual freezing process in the field or must all steps be performed in the lab after harvesting and transporting the sample?

We appreciate this important feedback. We have actually used this method in the field, which is certainly possible in field locations where access to liquid nitrogen is nearby. However, in some very remote field areas, liquid nitrogen may not be available. In those cases, one would have to transport the wetland soil with plant roots intact. This can be done by essentially taking a large core and placing the core into gas impermeable containers. We have made this clearer in the revised text.

* Could the authors comment on the ability to perform this type of flash freezing in the field itself without first requiring sample removal from in situ conditions (e.g., by driving the cooled copper blocks into a wetland soil to freeze a sample in place)? Is the method dependent upon immerse the copper blocks in liquid nitrogen or could a similar effect be achieved using a Sterling or thermoelectric cooler in contact with the copper blocks?

We do not think it is possible to drive the cooled copper blocs into the soil itself, however one could use another source to cool the copper blocks as long as the temperature can get low enough with the other source. The plant would have to be excavated from its environment but not from the surrounding soil. In the revised text, we have tried to make this clearer, and we hope that the video will further shed light on exactly how this is done.