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TITLE:

A method to preserve wetland roots and rhizospheres for elemental imaging.

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KEYWORDS:

Slam-freezing; freeze-drying; speciation mapping; X-ray fluorescence; LA-ICP-MS; root plaque

SUMMARY:

We describe a protocol to sample, preserve, and section intact roots and the surrounding rhizosphere soil from wetland environments using rice (*Oryza sativa* L.) as a model species. Once preserved, the sample can be analyzed using elemental imaging techniques, such as synchrotron X-ray fluorescence (XRF) chemical speciation imaging.

ABSTRACT:

Roots extensively interact with their soil environment but visualizing such interactions between roots and the surrounding rhizosphere is challenging. The rhizosphere chemistry of wetland plants is particularly challenging to capture because of steep oxygen gradients from the roots to the bulk soil. Here a protocol is described that effectively preserves root structure and rhizosphere chemistry of wetland plants through slam-freezing and freeze drying. Slam-freezing, where the sample is frozen between copper blocks pre-cooled with liquid nitrogen, minimizes root damage and sample distortion that can occur with flash-freezing while still minimizing chemical speciation changes. While sample distortion is still possible, the ability to obtain multiple samples quickly and with minimal cost increases the potential to obtain satisfactory samples and optimizes imaging time. The data show that this method is successful in preserving reduced arsenic species in rice roots and rhizospheres associated with iron plaques. This method can be adopted for studies of plant-soil relationships in a wide variety of wetland environments that span concentration ranges from trace-element cycling to phytoremediation applications.

INTRODUCTION:

Roots and their rhizospheres are dynamic, heterogeneous, and critically important for understanding how plants obtain mineral nutrients and contaminants[1–3]. Roots are the primary

pathway by which nutrients (e.g., phosphorus) and contaminants (e.g., arsenic) move from soil to plants and thus understanding this process has implications for food quantity and quality, ecosystem functioning, and phytoremediation. However, roots are dynamic in space and time growing in response to nutrient acquisition needs and they often vary in function, diameter, and structure (e.g., lateral roots, adventitious roots, root hairs)[2]. Heterogeneity of root systems can be studied on spatial scales from cellular to ecosystem-level and on temporal scales from hourly to decadal. Thus, the dynamic and heterogeneous nature of roots and their surrounding soil, or rhizosphere, poses challenges for capturing rhizosphere chemistry over time. Despite this challenge, it is imperative to study roots in their soil environment to characterize this critical plant-soil relationship.

The rhizosphere chemistry of wetland plants is particularly challenging to investigate because of steep oxygen gradients that exist from bulk soil to the roots, which change in space and time. Because roots need oxygen to respire, wetland plants have adapted to the low oxygen conditions of wetland soils by creating aerenchyma[4, 5]. Aerenchyma are hollowed cortical tissues that extend from shoots to roots, allowing the diffusion of air through the plant into the roots. However, some of this air leaks into the rhizosphere in less suberized parts of the roots particularly near lateral root junctions, less mature root tips and elongation zones[6–9]. This radial oxygen loss creates an oxidized zone in the rhizosphere of wetland plants that affects rhizosphere (bio-geo)chemistry and is distinct from the reduced bulk soil[10–12]. To understand the fate and transport of nutrients and contaminants in wetland rhizospheres and roots, it is critical to preserve the chemically reduced bulk soil, the oxidized rhizosphere, and roots of wetland plants for analysis. However, because the bulk soil contains reduced soil constituents that are oxygen-sensitive, root and soil preservation methods must preserve root structures and minimize oxygen-sensitive reactions.

Methods exist to fix plant tissues and preserve the ultrastructure for imaging, but those methods cannot be applied to chemically preserve roots growing in wetland soil. For investigations where only the elemental distribution within plant cells is desired, plants are typically grown hydroponically and roots can be easily removed from solution, fixed under high-pressure freezing and freeze substitution and sectioned for a variety of imaging applications including high-resolution secondary ion mass spectrometry (nanoSIMS), electron microscopy, and synchrotron X-ray fluorescence (S-XRF) analysis[13–15]. To investigate Fe plaque on the outside of wetland roots, these hydroponic studies must artificially induce Fe plaque formation in solution[16], which does not accurately represent the heterogeneity of the distribution and mineral composition of Fe plaque formation and associated elements in situ[17–20]. Methods exist to preserve wetland soil and associated microorganisms with freeze-coring[21], but it is difficult to obtain roots with this technique. Current methods to visualize roots growing in soil and their rhizospheric chemistry consist of two primary measurement types: elemental fluxes and total elemental concentration (and speciation). The former is typically measured using diffusive gradients in thin films (DGT)[22–24], in which soil is placed into rhizoboxes to support plant growth in a laboratory setting and labile elements in the soil diffuse through a gel into a binding layer. This binding layer can then be imaged to quantify the labile elements of interest. This technique can successfully illustrate relationships between roots and the rhizosphere[24–27], but artefacts from root-bounding may exist

by growing plants in rhizoboxes, and information on the root interior is not captured with DGT. The latter involves sampling of the roots and rhizosphere, preserving the sample, and directly analyzing elemental distribution on a sample section. For this environmental sampling of wetland plant roots and their surrounding rhizosphere, careful sample handling is required to avoid artefacts from sample preparation.

Here a protocol is described that effectively preserves root structures and rhizosphere chemistry of wetland plants by slam-freezing and freeze drying. Flash-freezing can drastically slow down transformations of oxygen sensitive solutes but may damage roots and may cause mobilization when samples dry out. However, slam-freezing where the sample is frozen between copper blocks pre-cooled with liquid nitrogen minimizes root damage and sample distortion[28]. The preserved samples are then embedded in an epoxy resin that preserves As speciation[20, 29] and can be cut and polished for imaging of roots within their rhizosphere soil. The samples in this report were analyzed by S-XRF chemical speciation imaging after thin sectioning. However, other imaging techniques could also be used, including laser ablation-inductively coupled plasma mass spectrometry (LA-ICP-MS), particle induced x-ray emission (PIXE), secondary ion mass spectrometry (SIMS), and laser induced breakdown spectroscopy (LIBS) imaging.

PROTOCOL:

1. Preparation of slam-freezing equipment

1.1. Place two copper blocks (~5 cm x 5 cm x 15 cm) horizontally inside of a clean cooler capable of holding liquid nitrogen and pour enough liquid nitrogen to submerge the blocks. Once the bubbling subsides, place two spacers on top of one copper block at each end.

NOTE: The spacer height determines the height of the sample to be frozen; this example uses a 2 cm spacer to create cubes approximately 3 cm x 3 cm x 2 cm. The volume of the liquid nitrogen will depend on the cooler size. This example uses approximately 1 L for approximately 5 cubes in series.

CAUTION: Use proper personal protective equipment and ventilation as liquid nitrogen is a cryogen and an asphyxiant.

1.2. Using tongs and cryogenic gloves, stand up the other copper block on its end, to make retrieval easier when the sample is in place.

2. Sample collection and slam-freezing

2.1. Extract the desired plant and rhizosphere from the wet soil using a shovel and ensure that the dug hole is much larger than the desired root volume. Place the soil and plant into a container and place it on a benchtop.

NOTE: The entire potted soil and plant from a pot study can also be used.

2.2. Determine the desired soil location where roots are to be taken (i.e., depth and proximity to the shoot). Cut away excess soil using a steel blade, taking care not to disturb soil in the desired area. When the desired area is reached, cut a root "cube" approximately 3 cm x 3 cm x 2 cm and immediately place the cube between the two spacers on the horizontal copper block. Using cryogenic gloves, pick up the vertical copper block and place it on top of the spacers to slam-freeze the rhizosphere cube.

2.3. After bubbling subsides (~5 min), retrieve the slam-frozen rhizosphere cube from the copper blocks and wrap inside of a pre-labeled aluminum foil square. Mark the orientation of the block on the foil if desired. Place in a second container of liquid nitrogen until storage in a -80 °C freezer.

2.4. Repeat as needed to obtain the desired number of root cubes from the field site or the experiment. Ensure both copper blocks are given time to cool between samples.

3. Freeze-drying and embedding rhizosphere cubes

3.1. Prepare the freeze dryer according to the manufacturer's instructions. Take care to ensure it has obtained the proper vacuum pressure and temperature prior to removing samples from the -80 °C freezer.

3.2. When freeze dryer is ready to receive samples, place one frozen rhizosphere cube inside of a clean and acid-washed 50 mL tube and cover loosely with a clean disposable wipe. Secure the wipe with a rubber band. Repeat as needed to ensure one cube per tube.

NOTE: If the sample is too large for a tube, it can be placed directly into the freeze dryer vessel using the aluminum foil as a sample holder.

3.3. Place tubes containing samples in freeze dryer vessels and freeze dry for several days. The exact drying time will depend on soil properties.

NOTE: Store dried samples in the freeze dryer or a desiccator to avoid rehydration.

3.4. Use a steel blade to cut dried soil cubes to size so that they fit into the desired form (e.g., 25 mm diameter form is ideal for most applications). Label each form, place the soil cubes in the forms and place the forms inside a vacuum desiccator.

3.5. Prepare epoxy according to manufacturer's instructions. Ensure that the chosen epoxy is not contaminated with and does not cause speciation changes of desired elements [20, 29, 30].

3.6. Use a dropper to add epoxy to the form on one side of the soil, till it entirely covers the sample. The soil will darken in color as the epoxy wets the soil.

NOTE: Add the epoxy slowly to allow the air in the soil to escape.

3.7. Once forms are filled with epoxy, close the vacuum desiccator and turn on the vacuum. Depending on the amount of air trapped in the soil, more epoxy may need to be added to the forms periodically. Check the level of epoxy every 30-90 min for the first 1-4 h and add epoxy as needed.

3.8. Remove the sample from the form once the epoxy has hardened (~5 days).

4. Cutting and sectioning the rhizosphere cubes

4.1. Cut the sample using a diamond blade precision wet saw. Cut the samples in different locations if no roots are obtained in the previous cut.

4.2. Manually sand the cut samples with progressively finer sandpaper (e.g., 220, 500, 1000, and 1500 grit) on the cut side for ~30 s.

4.3. Perform surface imaging of the samples using techniques such as LA-ICP-MS.

NOTE: To prepare thin sections for S-XRF, either send the samples out to a company capable of preparing the thin sections (single or double side polishing) or follow the **steps 4.4 – 4.6** as described below.

4.4. Glue the desired sample side to a quartz slide using super glue and allow to cure overnight.

4.5. Using a thin sectioning machine, cut soil on slides to 2 mm thick and then grind to the desired thickness (typically 30 µm). The sample surface can be polished if desired.

4.6. Perform S-XRF imaging of the sections. Follow the appropriate steps at the desired synchrotron facility and beamline to apply for and utilize imaging time.

REPRESENTATIVE RESULTS:

This method allows for preservation of roots and chemical species in the roots and rhizosphere of wetland plants and into the bulk soil. In this work, the method was used to evaluate As speciation and co-localization with Fe and Mn oxides and plant nutrients in the rhizosphere of rice (*Oryza sativa* L.). Rice was grown at the RICE Facility at the University of Delaware where 30 rice paddy mesocosms (2 m x 2 m, 49 plants each) are used to grow rice under various soil and water management conditions with the goal of lowering As and Cd uptake into rice grain. This experiment provided 1470 individual plants from which rhizospheres could be sampled throughout the growing season.

Given a sufficient number of samples, thin sections were able to capture a variety of root morphologies. **Figure 1A** shows several root diameters present within the soil matrix as

transverse sections. However, some soil sections may contain few, if any, roots. In this work, 63 soil blocks were processed and cut once on the wet saw to determine which subset of samples were suitable for thin sectioning. Of the 63 samples, 14 contained no roots, 31 contained 1-3 roots and 18 contained more than 3 roots. Note that the roots may be present in varying levels of quality. **Figure 1B** shows a well-preserved root, a root distorted by the freeze-drying process, and a root that was pulled out during the thin sectioning process.

Root thin sections were analyzed using S-XRF to map the location of elements of interest. **Figure 2B** shows a root transverse section with a lateral root in longitudinal section. **Figure 2C** shows this root section analyzed by XRF with a tricolor plot of Fe, Mn, and As. The Fe is present in the soil and surrounding the root in the Fe plaque, and Fe plaque is also visible on the light micrograph images. Manganese is uniquely present in the cortex of the lateral root, but also co-locates with Fe in some areas in the Fe plaque, appearing as a green-blue hue. Arsenic was mostly present in the vasculature of the lateral root, merging into the vasculature of the primary root. Chemical speciation imaging separated the various As species of interest by taking repeated XRF maps at multiple incident beam energies and using linear combination fitting to standard As XANES spectra. The As speciation maps are shown in **Figure 2D** and show variability in the localization of As species. **Figure 2E** shows the same data as a tricolor plot. The tricolor plot shows arsenite and arsenite glutathione closely associated in the vasculature, while arsenate is primarily located on the exterior of the root associated with Fe plaque.

FIGURE LEGENDS:

Figure 1 – Rice root thin sections showing a variety of methodological outcomes in a silty loam soil (scale bars are 0.5 mm). **A**) Numerous different root diameter cross-sections are evident (in white boxes). **B**) Roots may be damaged during the process. The root in the white box has remained intact and circular, while the root in the orange box has been compressed during the freeze-drying process. The red box shows where a root was pulled out during the thin sectioning process.

Figure 2 – Transverse section of a rice root with a longitudinal section of a lateral root in a silty loam soil. A) Soil section showing several root transverse sections in white boxes. White arrows denote longitudinal sections. Scale bar is 2 mm. **B**) Soil section showing root from upper left corner of panel A. White rectangle denotes area imaged by synchrotron XRF. Scale bar is 0.5 mm. **C**) Tricolor XRF image of arsenic (red), iron (green), and manganese (blue). The maximum scale of As, Fe, and Mn are in a ratio of 1:50:2.5. Scale bar is 100 μm. **D**) Arsenic XRF speciation maps for arsenite glutathione, arsenite, and arsenate, where warmer colors indicate higher concentrations of As. Scale bar is 100 μm. **E**) Tricolor plot of As species, where the maximum intensity is scaled to arsenite (red) = arsenate (green) = 0.5 arsenite glutathione (blue). Scale bar is 100 μm.

Figure 3 – Rice root thin section from a silty clay paddy soil. Numerous cracks in the soil have occurred during freeze-drying, but these cracks did not distort the lateral root longitudinal section, which is depicted by the white rectangle. Scale bar is 0.5 mm.

DISCUSSION:

This paper describes a protocol to obtain preserved bulk soil + rhizospheres of wetland plant roots using a slam-freezing technique that can be used for elemental imaging and/or chemical speciation mapping.

There are several benefits of this method over existing methods. First, this method allows the simultaneous investigation of roots and the surrounding rhizospheres. Methods currently exist to preserve and chemically image roots out of their soil environment by washing away the soil and preserving roots[31, 32] or by growing plants in artificial environments (e.g., rhizoboxes) and using DGT methods to examine root-soil interactions[24, 33, 34] but without the ability to observe the root itself. The method described here allows for the direct investigation of the root and surrounding rhizosphere soil in situ for observation of root-soil relationships. A similar technique has been used to examine in situ rice roots and surrounding rhizosphere but with plunging the sample into liquid nitrogen[11, 35, 36] rather than slam-freezing described here. The faster tissues are frozen, the less likely they are to form ice crystals[37]. Slam-freezing between pre-cooled copper blocks rapidly cools the sample and therefore minimizes the formation of ice crystals and subsequent plant tissue damage that can occur with flash-freezing using liquid nitrogen at ambient temperatures[11, 38]. Using the method described here, several samples can be taken from the same plant, multiple plants from a field, and/or the environment in a relatively short period of time. Once obtained, many samples can be freeze-dried, embedded in epoxy, and cut with a diamond blade wet saw with minimal cost. These samples can then be investigated with a light microscope to identify promising samples that can be directly imaged (e.g., LA-ICP-MS) or further processed for thin sectioning and sXRF imaging. One thin section can capture multiple roots of various sizes on the same slide, which helps to capture the heterogeneous rhizosphere and maximize imaging time on the instrument. This method also can be used to directly observe plant-soil relationships such as As sequestration in Fe plaques without disturbing the rhizosphere. Existing methods to induce Fe plaque formation on wetland roots like rice using hydroponic experiments[39–41] fail to capture the heterogeneity of Fe plaque in terms of root coverage and mineral composition that occurs in soil-grown plants[11, 18, 20, 42–44].

For the method to be successful, it is critical to follow a few key steps. First, ensure that the sample location and orientation selected addresses the desired question. Second, use an epoxy that is free of trace element contamination and has been shown to preserve chemical speciation of As elements of interest[20, 29, 30]. Third, add epoxy slowly and place the sample in epoxy under vacuum to facilitate epoxy wetting of the sample and removal of entrapped gas. Following these steps will provide a high-quality sample of bulk soil, rhizosphere, and roots that can be used for image analysis.

Several limitations of the method should be considered. First, drying the frozen sample on a freeze-dryer can cause deformation of the soil, which may affect roots. This is likely to be particularly challenging in soils with high clay content and thus propensity for collapse as clays dry. As an example, a rice rhizosphere sample obtained from a silty clay was prepared and cracking of the soil is apparent whereas the Fe plaque-coated rice root is unaffected (**Figure 3**).

[**Place Figure 3 here**]

The data show that this technique can successfully obtain micron-scale information in a silt loam (**Figures 2, 3**), and it is likely that the technique can be successful in coarser textured soils; however, soils with higher clay content might pose challenges and should be investigated further. Second, roots can be pulled out of the soil upon sectioning. This challenge is not unique to the protocol described in this paper but should be considered. Third, roots may not be present in every soil cube, so many samples need to be obtained and cut to capture the rhizosphere of the desired plant. Fourth, the preservation method requires liquid nitrogen, which might pose challenges for remote field studies. Here, the protocol has been successfully used in the field, which was less than 2 miles from a liquid nitrogen Dewar. However, if liquid nitrogen is not available within a short drive from a remote field site, several options exist to obtain the sample. This includes using another source to cool the copper blocks or excavating the entire plant and surrounding soil with a large PVC ring, placing this into gas-impermeable material, and transporting to the nearest liquid nitrogen source for preservation. For this, it is important to ensure that the plant shoot is not cut from its roots prior to obtaining the rhizosphere sample. If needed, the sample can also be placed under refrigeration and shipped overnight to the laboratory for preservation. Once received in the laboratory, sections can then be preserved using liquid nitrogen-cooled copper blocks. Finally, speciation changes are possible with any preservation method of wetland soils and rhizospheres. To avoid this, samples must be obtained and slam-frozen quickly or other measures taken as above to avoid exposure to oxygen. The edges of freeze-dried samples can then be shaved to avoid edges that may have had higher exposure to oxygen. The preservation of reduced arsenic species in the root and rhizosphere samples here (**Figure 1D, E**) and in previous work[28] suggests that this slam-freezing technique is able to preserve oxygen-sensitive chemical species if carefully performed.

This method can be used to address several key questions in rhizosphere science. These include applications related to studying nutrient and contaminant interactions in the rhizosphere that may include interactions of contaminants and nutrients with Fe or Mn plaques. The method allows for the study of temporal and spatial heterogeneity of plant-soil relationships and the examination of how root morphologies interact with elements in the rhizosphere in situ. It can be used in applications related to food security such as in understanding arsenic uptake by rice, nutrient dynamics in the rhizosphere, or applications related to phytoremedation such as metal(loid) uptake into wetland plants.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

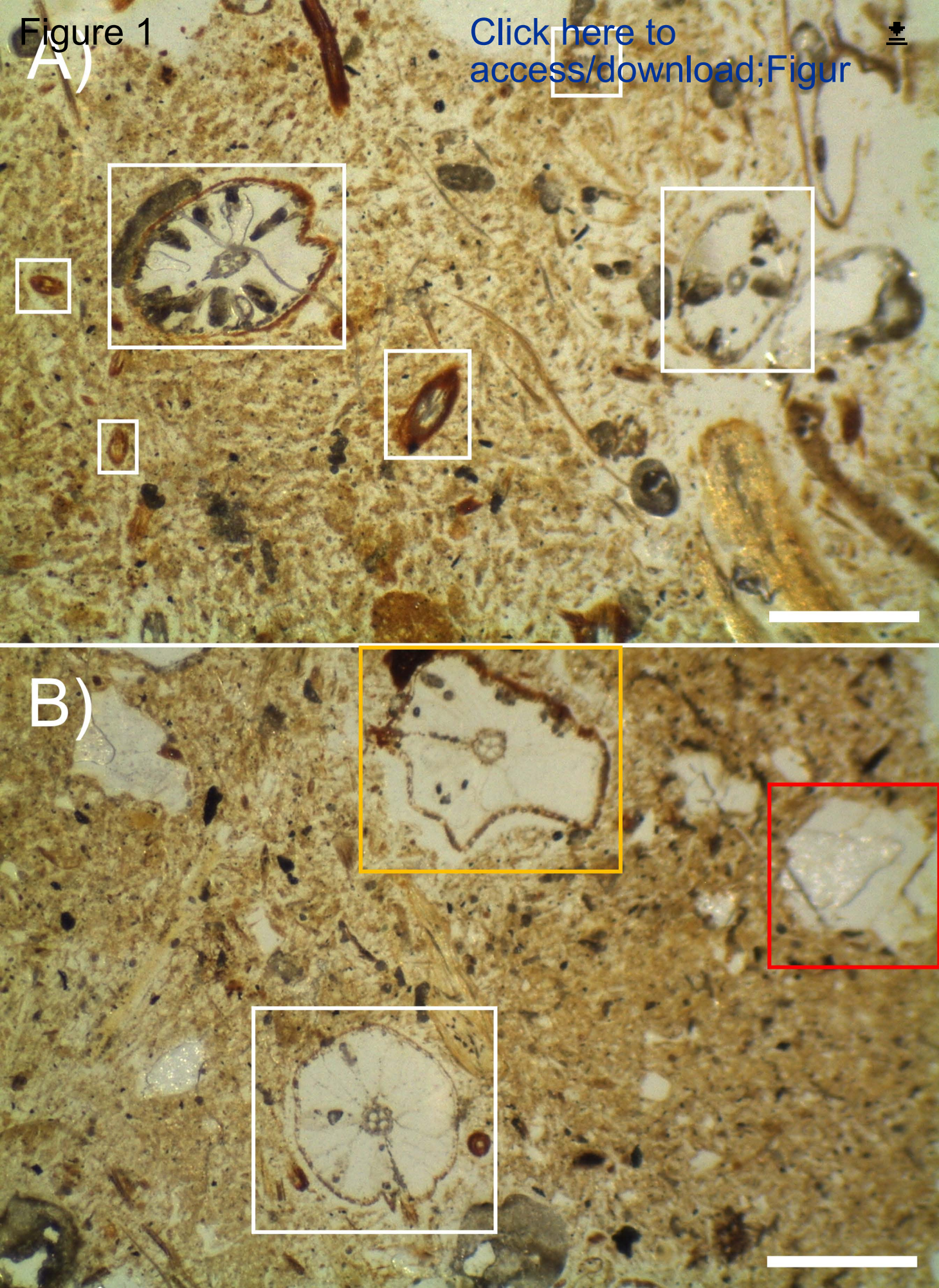


Figure 2

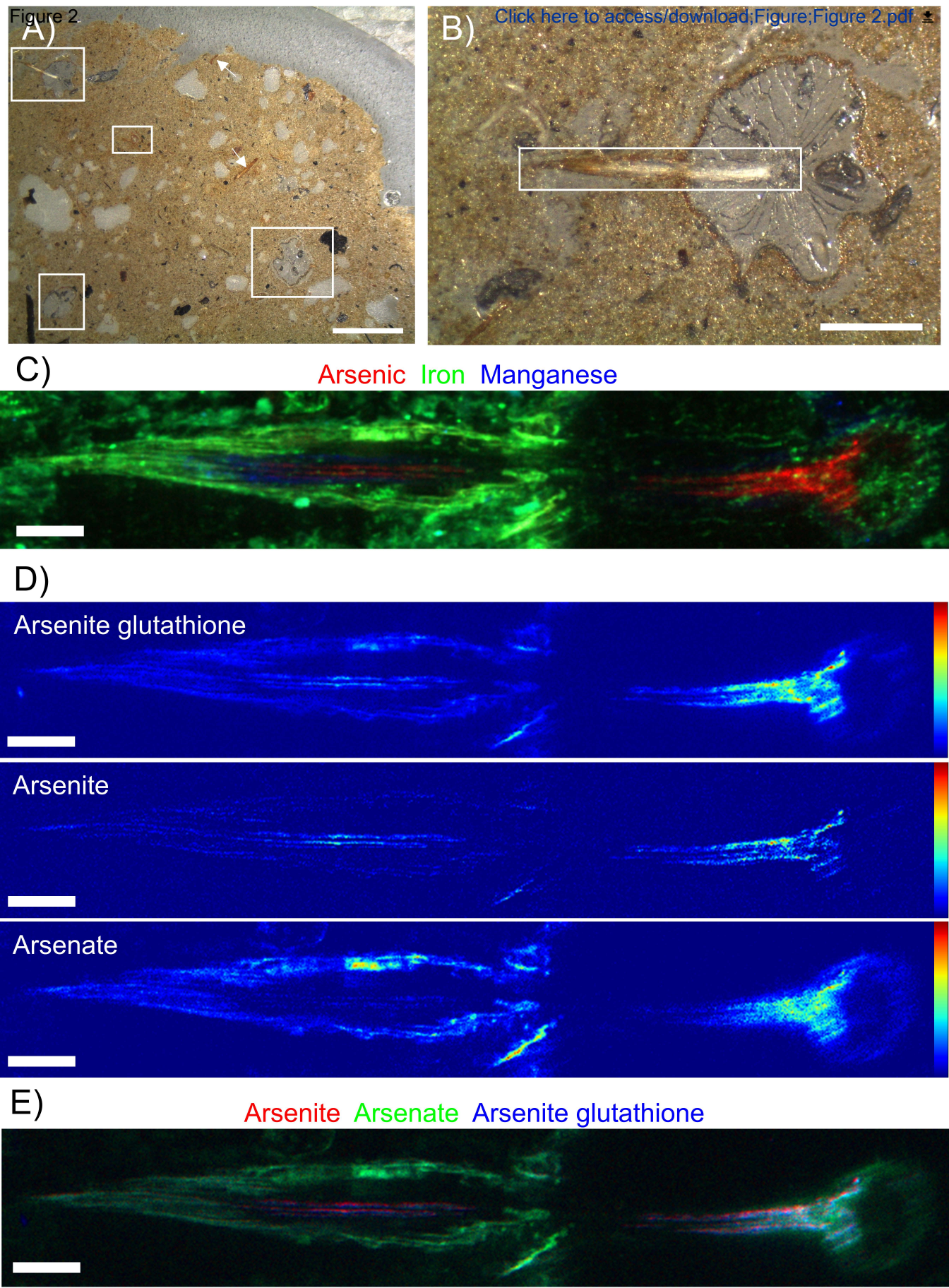
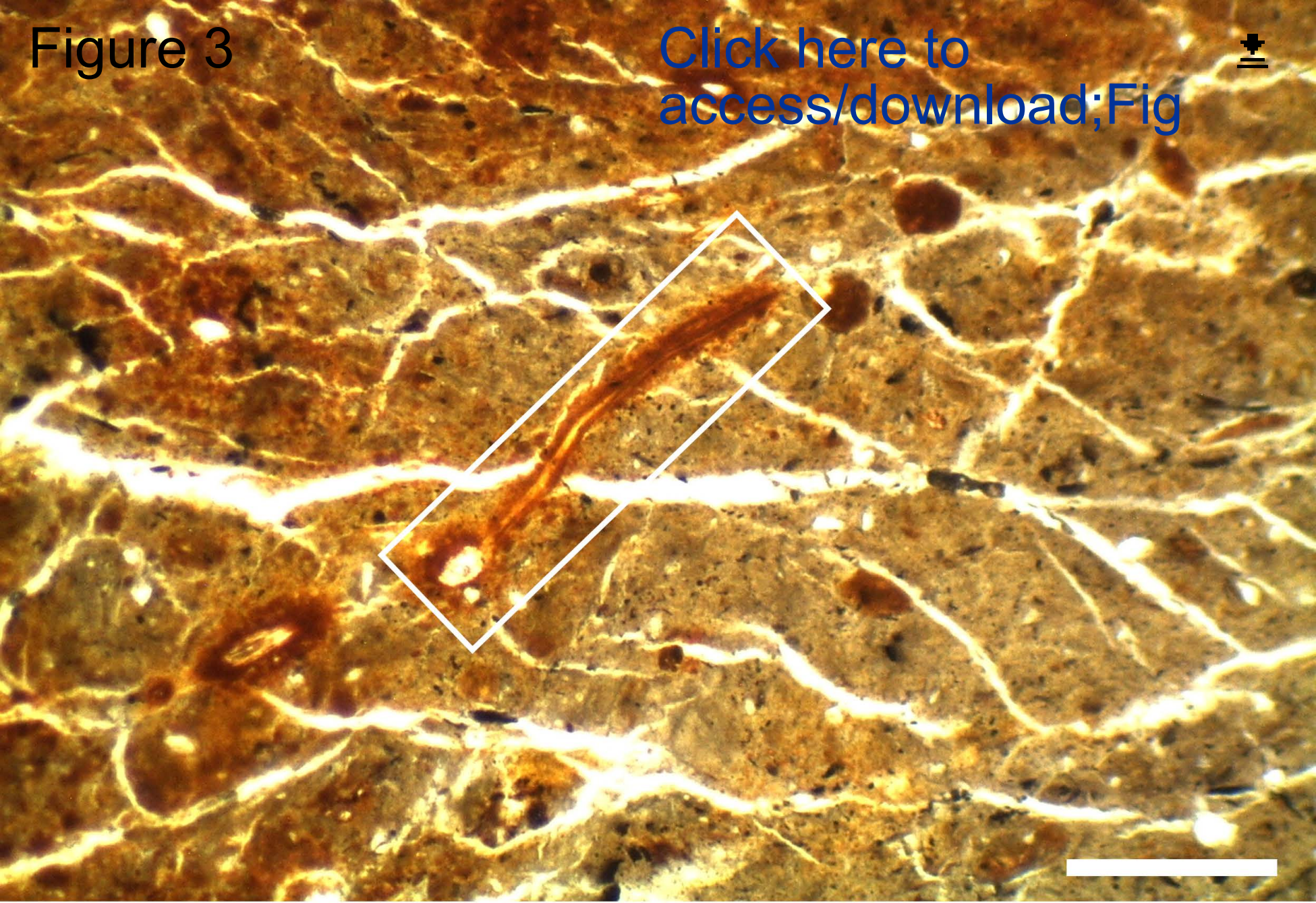


Figure 3

Click here to access/download;Fig



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Copper blocks	McMaster Carr	89275K42	
Diamond blade	Buehler	15 LC, 102 mm x 0.3 mm	operation speed: 225 rpm
Epoxy forms	Struers	40300085	FixiForm
Epoxy	Epotek	301-2FL	
Superglue	Loctite	404	
Thin sectioning machine	Buehler	PetroThin	
Wet saw	Buehler	IsoMet 1000	

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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.