**Editorial comments:**  
Changes to be made by the Author(s) regarding the written manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have carefully proofread the article and corrected all grammatical and spelling errors.

2. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

We added the following statement of goals to the introduction:

“The overall goal of this method is to provide a single throughput protocol for analyzing metabolites, lipids, and microbial proteins from a single sample thereby creating a fully paired dataset for building metabolic networks while constraining analytical errors.”

3. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

We converted rpms to centrifugal force throughout the protocol where applicable (i.e. lines 224, 282, 287, & 354).

4. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:

5. 1.2: Please specify roughly how much soil/peat is needed for the protocol.

We added the following text to allow users to determine the sample size needed for the protocol:

“Approximately 45 mg C is needed for this protocol. For peat (typically 45% C), 100 mg of dried peat are required. Larger amounts of sample may be needed for low organic samples like mineral or forested uplands soils depending on the C content.”

6. 1.3: What is used to grind the samples?

We clarified that one can use a high-speed ball mill to grind the samples in the bolded text as follows:

“When ready to analyze the samples, freeze dry to constant weight, then grind the samples **in a high-speed ball mill** **using stainless steel grinding balls** to homogenize and break up any aggregates.”

7. 1.5: Is the sample shaken in a shaker?

We clarified that a shaker table may be used for this step:

“Add 1 mL of distilled, degassed water (H2O) to each sample, cap the vials and shake for 2 h **on a shaker table.”**

8. 1.6: Centrifuge for how long?

We clarified that the samples should be centrifuged for 30 minutes.

9. 1.8: Please describe how to separate the two solvent layers. Are the two layers labeled?

The two layers are not labeled per se, but the chloroform-containing fraction is denser than the methanol containing fraction so it will be the lower layer. We clarified this in the instructions and added text to suggest using a separation funnel or careful pipetting to separate the two layers:

“Carefully separate the two resulting solvent layers, **which will be visually distinguishable**, for separate analysis by FTICR-MS by using a **separation funnel or simply remove the top layer by careful pipetting. The chloroform-containing fraction will be on the bottom while the methanol-containing fraction is less dense and will be on the top.”**

10. 1.9: There are two chloroform extracts from step 1.8, correct? Are they both diluted and analyzed? Please also specify the dilution factor.

There is one chloroform extract and one methanol extract generated during [now] step 1.7. We clarified this as noted in the previous response. We added a dilution factor (2:1) and a note that the methanol fraction does not need to be diluted further in methanol.

11. 2.1: Please provide the composition of tuning solution. If it is purchased, please cite the Table of Materials. Please describe the calibration process. For instance, what volume of tuning solution is needed?

This is a commercially available tuning solution. We added a reference to the table of materials noting that the tuning solution can be purchased from Agilent and added details about volume of solution needed for the tuning.

12. 2.2: How is the Suwannee River Fulvic Acid standard prepared? Is it dissolved in methanol? Please describe how to tune and optimize.

We added text to describe preparing SRFA samples as follows:

“Prepare the Suwannee River Fulvic Acid standard (Table of Materials) by diluting 1 mg mL-1 in milliQ filtered water then diluting the resulting solution to 20 ug mL-1 in methanol. Direct inject 23 uL of this final solution to the ESI source coupled to the FTICR spectrometer through a syringe pump set to a flow rate of 3.0 μL min-1. Set needle voltage to +4.4 kV, Q1 to 150 *m/z* and glass capillary at 180° C. Inspect resulting spectra using the DataAnalysis software package 4.2 (Bruker Daltonics) to confirm the quality of the data.”

13. 2.8-2.10: These steps can be combined into one. Please mention why water and methanol-extracted fractions are combined. Please specify how to dry the combined extract.

We clarified that the samples should be freeze dried. We combined steps 2.8-2.10 into a single step. We explained that combining the water and methanol fractions streamlines subsequent steps in the protocol but point the user to the Discussion where the two options are treated in more detail:

“Note: After analyzing by FTICR-MS, the decision can be made to either combine the water and methanol extracted fractions in order to streamline the remaining steps or the fractions can be kept separate throughout subsequent steps. The advantages and disadvantages of each approach are described at length in the Discussion. If doing so, combine the water and methanol-extracted fractions. Dry using a vacufuge/freeze dryer and save the remainder of the extracts for subsequent GC-MS (water, methanol or water + methanol), LC-MS (chloroform) and NMR (water + methanol) analysis.”

14. 4.1: This step seems to be repetitive to what mentioned in 2.8-2.10. (-97.6 °C)?

We revised the protocol to eliminate this repetitive instruction. We also clarified in the earlier step that a vacufuge-freeze dryer can be used to aid in drying down the methanol extracts.

15. 4.2: Please describe how to prepare blanks and FAME calibration samples.

We added additional text to the protocol to better describe blank and FAME preparation:

“Prepare blank control samples of HPLC-grade hexane in GC-MS autosampler vials22. Dissolve 100 mg fatty acid methyl esters mixture retention time standard (FAMEs: C8–C28) in 200 uL hexane.

To protect carbonyl groups, add 20 μL of 30 mg mL-1 methoxyamine hydrochloride in pyridine to each of the methanol extracts and water extracts (or combined methanol/water extracts if using) from step 2.5, blanks and calibration samples. Seal vials with caps.”

16. 6.2: Please describe how to deconvolute and align features.

We added additional information to the GC- MS data processing section:

Inspect all the data files to ensure that they were correctly captured. Pay attention to potential shifts with regards to internal standard retention times and intensities to confirm that that the data was captured consistently throughout the analysis.

Convert the vendor specific MS data format to a general MS format if required. Process raw data files using MetaboliteDetector calibrating retention indices based on the FAME internal standards. After aligning the retention times of all data files, continue with deconvolution and finally metabolite identification by matching retention indices and GC-MS spectra against the FiehnLib polar metabolite library23.

Cross-check remaining unidentified metabolites against the NIST14 GC-MS library using spectral matching. Validate identifications individually to eliminate false identifications and reduce deconvolution errors.

17. 7.2: Please specify the size of the NMR tube used. What volume of water extracts is used?

We clarified in the protocol that a 3 mm outer diameter (O.D.) NMR tube is used and that the sample volumes are typically in the range of 180 – 300 L:

Dilute the remainder of the water extracts by 10% (vol/vol) with a 5 mM 2,2-dimethyl-2-silapentane-5-sulfonate-d6 internal standard. Alternatively, you can combine water and methanol extracts from step 1 then resubstitute in water. By doing so however, you might lose some of the volatile compounds during the freeze-drying step. **Typical final sample volumes are in the range 180-300 L**

Transfer mixture into a **high-quality 3 mm outer diameter (O.D.) borosilicate** glass NMR tube.

18. 8.4: Please write the text in the imperative tense.

We revised to imperative tense.

19. 8.5: Please describe how this is done. We generally require that software steps be more explicitly explained ('click', 'select', etc.).

We added more detail on the software steps as follows:

“Upload the raw LC-MS/MS data files into LIQUID24 along with the target file (i.e. list of >25,000 lipid species) for the respective ionization mode (positive or negative). Process the raw file. Manually validate the resulting identifications by examining the MS/MS spectra for presence of diagnostic ions, if applicable, matching fragment ions (e.g., fatty acyl chains), isotopic separation, mass ppm error of the precursors, and the retention time. Export resulting list of confidently identified lipids as a .tsv file.”

20. 9.1, 9.5: Although a reference has been provided about the protocol, we still need more specific details for filming. For instance, what extract is washed? What is the ratio of extract/cold methanol? What volume of 100 mM NH4HCO3 and 8 M urea is added? What wavelengths are measured for protein concentration?

We added text to this section to clarify that the methanol-containing extract is to be washed in 20 times the volume of cold methanol. We indicated that the pellet should be redissolved in the NH4HCO3/Urea solution to a final protein concentration of 1mM (approximately 10-fold). Finally, we added that the absorbance measurements should be made at 562 nm as follows:

“Extract proteins according to the MPLEx protocol20 from the remainder of the methanol phase resulting from step 2.4, by washing the extract with 20 times the extract volume of additional cold (-20 °C) methanol.

Use a 1 mL/50 mg Phenomenex Strata C18-E (PN 8E-S001-DAK, Torrance, CA), to condition C18 solid phase extraction (SPE) columns with 3 mL MeOH, 2 mL of 0.1% trifluoroacetic acid (TFA) in water, followed by the addition of the extract from step 9.1 at a rate no greater than 1 mL/min. Following the addition of the sample, wash the column with 4 mL of 95:4.9:0.1 water:acetonitrile:TFA, then allow to go dry. Place a 1.5- mL collection tube under the SPE column, and elute the sample with 1.0 mL of 80:19.9:0.1 methanol:water:TFA.

Concentrate the extracts to 100 uL under vacufuge, then measure the protein concentration by bicinchoninic acid (BCA) colorimetric assay25at a wavelength of 562 nm.

Centrifuge extracts at 10,000 x g for 10 min at 4 °C. Discard the resulting supernatant and dry the remaining pellet under vacuum for 5 min. Resuspend the protein pellet in water to a final concentration of 0.1 ug peptide uL-1 Add dithiothreitol to a final concentration of 5 mM and incubate at 60 °C for 30 min. Dilute 10-fold with 100 mM NH4HCO3/8M urea solution and CaCl2 to a final concentration of 1 mM and incubate at 37 °C for 3 h.”

21. 9.9: This step does not have sufficient details to replicate. Please add more details.

We added additional details about the solid phase extraction process including the following text:

“Use a 1 mL/50 mg Phenomenex Strata C18-E (PN 8E-S001-DAK, Torrance, CA), to condition C18 solid phase extraction (SPE) columns with 3 mL MeOH, 2 mL of 0.1% trifluoroacetic acid (TFA) in water, followed by the addition of the extract from step 9.1 at a rate no greater than 1 mL/min. Following the addition of the sample, wash the column with 4 mL of 95:4.9:0.1 water:acetonitrile:TFA, then allow to go dry. Place a 1.5- mL collection tube under the SPE column, and elute the sample with 1.0 mL of 80:19.9:0.1 methanol:water:TFA.”

22. 9.10: Please specify the volume of water added.

We added text to indicate that water is added to a final peptide concentration of 0.1 ug mL-1.

23. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

We combined many of the protocol steps together reducing the total number of steps from 61 to 41.

24. Please include single-line spaces between all paragraphs, headings, steps, etc.

We added spaces between all of the paragraphs, headings and steps in the manuscript.

25. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted 2.75 pages of the essential steps of the protocol for filming.

26. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

We checked to make sure we highlighted only complete sentences that included an imperative action. No steps in our protocol involve anesthetization or euthanasia.

27. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We checked that steps are complete and details relevant for performing each step are included.

28. Please submit the figures as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). Please save any .ai files as a .pdf for submission but maintain .ai files for production purposes. If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300 dpi.

We submitted images as a .tif file in response to communications we had during the review process requesting .tif files.

29. Figure 1: Please define the error bars in the figure legend.

We added definition of error bars to the figure legend.

30. Figures 2 and 3: Please add a short title for each figure.

We added a title for each of these figures to the figure legend.

31. Please revise the table of the essential supplies, reagents, and equipment to include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

We have included this table.

**Reviewers' comments:**  
**Reviewer #1:**  
Manuscript Summary:  
Manuscript describes sequential, multimodal spectroscopic and spectrometric approach for the analysis of proteins, primary and secondary metabolites, and lipids within soil, a complex matrix. The technique is applied to only one ecosystem but is ready applied to others. It is generally well written although could adopt a more consistent "voice" throughout.  
  
Major Concerns:  
The starting point for extracts in LC-MS and Proteomics is not explicitly described. What earlier step created these extracts?

We clarified in the protocol that the extracts were generated during step 2.4.

Minor Concerns:  
-The minor concerns listed below are to increase comprehension of manuscript.  
-Consider including some description of the system you are studying in the abstract, perhaps as the last sentence. As currently written, the transition from the abstract to the introduction which immediately talks about the global importance of wetlands is quite abrupt.

We added a final sentence to the abstract to explain that we used the peat samples as a demonstration in this paper but that the method can be applied to other kinds of natural organic matter samples as well.

“Herein we present results of applying this method to natural organic matter samples collected from peatlands, however, the protocol is applicable to any natural organic matter sample (e.g. peat, forested soils, marine sediments, etc).”

We have added the following text to explain why we are interested in characterizing NOSC in peatland systems specifically.:

“Peatlands produce globally significant quantities of CO2 and CH46 via microbial respiration of naturally occurring organic matter. The NOSC of the organic carbon substrates determines the relative proportion of CO2:CH4 produced which is a critical parameter because of the higher radiative forcing of CH4 compared to CO211, but also because modeling efforts have identified this ratio as a critical parameter for estimating C flux in peatlands12. In the absence of terminal electron acceptors other than CO2, it can be shown by electron balance that organic C substrates with NOSC > 0 will produce CO2:CH4 > 1, organic C with NOSC = 0, produces CO2 and CH4 in equimolar ratio and organic C with NOSC < 1 will produce CO2:CH4 < 113.”

-Line 93 - consider replacing "directly related" to correlated.

We have made the suggested change.

-Line 123 - consider replacing "directly" to linearly

We have made the suggested change.

-Paragraph starting on line 127 continues until line 191. Can you identify natural breaks in the discussion? Perhaps a break between bulk and molecular techniques that occurs at line 137, and then breaks between discussion of each molecular technique?

We have made the suggested change to break this paragraph up into several smaller sections.

-In most cases it is easy to follow the flow of different subsample analyses in the protocol. However it is not carried thru the entire protocol. For example, it is unclear where the chloroform extracts come from for the LC-MS analysis. Earlier, in the description this was unambiguously indicated (e.g. step 1.6 on line 230). Can you do this consistently?

We added an indication of the step that generated the extracts in the protocol as requested:

**Liquid chromatography mass spectrometry (LC-MS) lipidomics analysis**

Dry the remaining chloroform extract **generated during step 2.4** under vacuum. Then rewet the dried organic layer with 200 μL methanol.

-Within the Representative results section the "voice" of the narrative changes from standard journal language to a more of oral one.  
-Line 381 Suggest rephrasing "We will compare these result to those from a permafrost bog and fen from northern Sweden…"

We have made the suggested change.

-Line 387 - proteomics shows what proteins are expressed. The potential of microbial community comes from the meta genome.

We meant to indicate that the proteins indicate potential in that they act as the catalysts for transformation, thus the proteins indicate what transformations are metabolically possible. While the transforms in the metabolites indicate which of these possible reactions are actually occurring. We have revised this in the text as follows:

“While the proteomics results indicate which proteins are expressed, the metabolic data shows which reactions are actually occurring.”

-Figure 2 - It would be helpful in the text to explicitly say what samples (depth horizons) are plotted in 2b and 2c. It is also difficult to discern the differences for the two depth from the VK plot itself. It might be helpful to have an additional table of the % of sugar, lipid, protein, etc. for the depths described. This would better support the arguments that are presented.

We added depths to the figure legend. We also added a panel to this figure containing a bar chart showing average amino acids, lipid classes, and sugars at the three measured depths.

-Lines 400-410 - I expected that a discussion of preservation of metabolites at depth due oxygen limitation and their NOSC values to be presented here. Is that not observed?

We do observe consumption of high NOSC compounds and preservation of low NOSC compounds in the peat and added the following text to the Representative Results section describing this phenomenon:

“As can be seen in Figure 2d sugars (NOSC = 0) and amino acids (0 < NOSC < 1) are consumed in the surface peat, while lipids (-2 < NOSC < -1) appear to accumulate with depth indicating. This is consistent with expectations based on NOSC values that higher NOSC compounds are more readily degraded while lower NOSC compounds persist in the highly anaerobic (i.e. TEA-limited) conditions of subsurface peat.”

-Line 465 - Is clinically supposed to be chemically?

No, we meant clinical pathogens. The idea being that environmental samples contaminated with medically-relevant microorganisms could be rendered safe by this protocol.

-Line 493 - similar to earlier comments - proteomics reveals the proteins and enzymes that are expressed. The potential comes from metagenome analysis.

Again, we meant to highlight that as catalysts of the reaction the enzymes do not provide the most direct evidence that a reaction is occurring or has occurred only that the microbial enzymes are present, that is what we meant by potential. By coupling that information with the metabolic analyses provides corroborative evidence that the reaction we think should occur has actually in fact taken place. We revised the text as follows:

“Coupling the proteomics analysis with the metabolic analyses provides compelling corroborative evidence that a reaction is actually occurring.”

-Line 503 - missing "of" but critical piece "of " the C cycling puzzle.

We added the missing word.  
  
**Reviewer #2:**  
The manuscript describes the pipeline for co-extraction and analyses of proteins and metabolites from environmental samples. Overall, the manuscript is well presented and details analyses that are becoming more utilized. I have some minor comments and suggestions below:  
  
L42 : Microbial composition is a bit vague here - and also incorrect. None of the analyses detailed here provide either information on the 'community composition', while the detected proteins only offer a partial view of microbial (cellular) composition - and this kind of analysis isn't discussed.

We revised the text to indicate specifically that we measure proteomics and not microbial composition.

L52: 'the system' is vague

We clarified this to “natural ecosystems”.

L112: 'also mediated'? The preceding paragraph described microbial degradation of OM. The whole sentence is somewhat confusing in the context of the earlier paragraphs.

It’s not clear to us what the reviewer finds confusing here. Thermodynamics ultimately limits decomposition. Microorganisms control rates through the production of enzymes that act as catalysts. We begin with the discussion of thermodynamic control to define the limits of what is possible. Then we discuss kinetic control to focus in on what is actualized in the system. We removed the word “also” and rearranged the indicated sentence in hopes that this would clarify our meaning.

L154: I would consider NMR to be reasonably sensitive - I think 'only metabolites in very high concentrations can be quantified' is too strong a statement. Perhaps some detection limits could be given here?

This is clearly subjective, so we revised the text to indicate that reliable quantification requires at least 1 uM concentration.

L181: Is metabolites meant here, rather than 'metabolomics'?

We changed this to metabolites.

L366: There are no details provided on how the mass spectra are matched to a database of predicated peptides. This is a critical step (databases selection or design) that should be at least discussed (perhaps in the discussion?)

We added the following text regarding the database matching:

“Convert RAW spectra files to mzML format using msConvert or ProteoWizard 3.0.10200 accepting all default parameters. Use the universal database search tool MSGFPlus v2017.01.13 to search resulting proteomes against a targeted protein database of protein sequences predicted from relevant metagenome assembled genomes. Append common contaminates (e.g. trypsin, keratin, albumin) and remove all exactly duplicated protein sequences to improve peptide-to-mass-spectrum match statistics. Evaluate the resulting MSGF Spectral Probability scores to determine which peptide-to-mass-spectrum match is best. Use the Q-Value from MSGFPlus to filter the entire data pile to 1% false discovery rate (FDR).”

We also added the following caveats on proteomics analysis to the discussion:

In this procedure for identifying proteomics, only fully tryptic peptides are searched, thus endogenous peptidase activity and in-source fragmentations will be missed. On the other hand, oxidized methionine may be considered as a post-translational modification for the peptide candidates as this modification commonly occurs during sampling processing and handling. Quantification of enzymes using peptide elution areas can be done, but was outside the scope of this project.

L383: Are these truly all enzymes? Or are some structural proteins or even proteins of unknown function?

These are enzymes for which Enzyme Commission numbers were able to be identified.

L389: Are these 67,040 unique metabolites? Or are some likely shared across measurement platforms?

67,040 unique metabolites. There is some overlap among techniques, but redundant compounds were not counted in this number.

L500: 'declining enzyme activity' isn't technically accurate here. Enzymes are at lower abundances at depth, but we don't have any information on their actual kinetics.

Point taken. We revised this to declining enzyme expression.

Figures 2 and 3 were of low quality, and so I wasn't able to effectively view or assess them.

We have uploaded revised high resolution tiff files of all of the figures in response to a request from the editor.