Phospholipid Fatty-acid Analysis (PLFA)

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Summary:

1. Prepare soil and glassware:
   Freeze, freeze-dry, grind, weigh into teflon tubes, hexane rinse the glassware

2. Extract fatty acids from whole soil
   DAY 1 (approx. 2-3 hours for 40 samples)
   a) Shake (1 hr)
   b) Centrifuge
   c) Decant to 30 ml tube
   d) Add $CHCl_3$ and H_20
   e) Separate phases for 18 hrs

   DAY 2 (approx. 3 hrs for 40 samples)
   f) Aspirate (remove) aqueous phase, **save $CHCl_3$ phase! (on bottom)**
      Optional: filter organic phase into glass container ~ 15 ml volume
   g) Dry down w/ N_2; store @ -20C

3. Transfer to clean tube
   DAY 3 (transfer is about 3-4 minutes a sample, dry down is about 45 minutes)
   a) Add four 0.5 ml washes of $CHCl_3$
   b) Dry down w/ N_2; store @ -20C
      If cloudy, add MeOH until is clear (~0.5ml) and dry down again
   c) Store w/ N_2 @ -20 C)

4. SPE cartridge: separate lipid classes
   DAY 4 (about 3/4 day for 40 samples)
   a) Transfer lipids to SPE cartridges
   b) add 5 ml $CHCl_3$ = neutral fraction
   c) add 5 ml acetone = glycolipid fraction
   d) add MeOH = Phospholipid fraction (polar lipids for PLFA)
   e) dry down w/ N_2, store @ -20C

5. Make Methyl-Esters
   DAY 5 (approx. 3/4 day for 40 samples)
   a) Redissolve dry lipid in 1 ml tol:MeOH (1:1) and 1 ml 0.2 M KOH
   b) Vortex, incubate at least 15 minutes 35C)
   c) Cool to room temp, add 2 ml hexane, 0.3 ml acetic acid, 2 ml H20, vortex 30 seconds
   d) Centrifuge to separate phases
   e) Transfer upper phase to 4 ml amber vial
   f) Re-extract lower phase as above 1 more time and save upper phase
   g) Dry down, store @ -20C

6. Transfer to GC vials (with 100µl liners)
   DAY 6 (approx. 3-4 minutes per sample)
   Use small aliquots of hexane to resuspend lipids with internal standard

THE CARDINAL SINS: **WATER, HEAT, LIGHT, OXYGEN**. ANY OF THESE WILL DESTROY YOUR FATTY ACIDS. *AVOID AT ALL COSTS!*
Also, use only glass, teflon or metal. Avoid plastics that are soluble in chloroform.

Step 1. Prepare soil and glassware:

Rinse all glassware in hexane.

I add approximately 2-3 ml hexane to tubes, and vortex them for a few seconds. I decant the hexane to another tube, and vortex. I do about 6 tubes with one aliquot of hexane, then I dump it and start with new hexane. This is to reduce the hexane used. It is not necessary to have new hexane for each piece of glassware.

Prepare the soil

- Freeze soil after collection
- Freeze-dry as soon as possible: soil can be sotred indefinitely freeze-dried, in a freezer.
- Grind soil in a mortar and pestle to homogenize and mix it.
- Weigh into teflon tubes

Step 2. Extract fatty acids from whole soil

DAY 1 (approx. 2-3 hours for 40 samples)

1. Weigh out 4 g of freeze-dried sample (Can use other amounts, but 3-4g at least is best for most soils.)
   - Weigh soil into 30 ml teflon centrifuge tubes (pre-rinsed with hexane), record exact mass.
   - Label the tube: use a solvent-resistant marker or a ballpoint pen on tape

2. **In fume hood**: add 3.6 ml P-Buffer, 4 ml $CHCl_3$ and 8 ml MeOH **in that order**. Need three re-pipettors, 5-10 ml capacity. Test the exact volume being dispensed with graduated cylinder!

   The solvent proportions and the order of addition are important, in order to separate have the organic and aqueous phases. If different soil amount than 4g is used, then be sure to maintain a 2:1:0.8 vol:vol ratio between MeOH:CHCl3:P-buffer. The method usually uses 1ml $CHCl_3$ for each 1g soil, or each 1mg cell material. (ie 4g soil needs 4 ml $CHCl_3$ in first extraction. The amount of soil used can vary. 4-5 g is good for mineral soil. Use less for highly organic soil. Also, organic soil may require more than 1x the amount of solvents. I used 1g 'soil' for Alaskan tundra, and 3x the amount of solvents in this step.

   If material is not freeze-dried prior, then you must adjust sample to a total water volume of the P-buffer listed below (i.e. soil water + added buffer = 4 ml), then add the chloroform and methanol.

g soil	ml P-Buffer* =(1.8*ml$CHCl_3$)/2	ml CHCl3	ml MeOH
1	0.9	1	2
2	1.8	2	4
3	2.7	3	6
4	3.6	4	8
5	4.5	5	10

*Note: we have simplified the P-buffer step. To avoid changing the volume of the repipettor after step 2, we combine the volume of P-buffer from step 6 with the volume from step 2 and divide in half. It works fine, even though it is not exactly the ratio specified above.

3. Shake for 1 hr: place the tubes horizontally for maximum shaking/disruption; ***keep dark!(Cover with aluminum foil)***

4. Centrifuge for 30 min at 2000 rpm, 25°C (or could do 10 min at 2500 rpm)
   Equalize weight of tubes!
   (Need a floor centrifuge with rotor head capable of handling 30ml tubes. Need as many spaces in it as possible, to do as many tubes as possible. This step is a bottleneck for processing large numbers of samples.)

5. In fume hood: ***keep as dark as possible!*** Decant the supernatent to 30 ml screw cap glass tubes (round bottom, teflon lined caps). Label the tube , not the cap, clearly, use a ballpoint pen or a solvent-resistant marker.

6. Add 3.6 ml P-buffer and 4 ml $CHCl_3$ to 40 ml screw cap glass tube containing sample. Approximately 25-30 ml total volume. (Or, add same amount of $CHCl_3$ as initially, and add an equal volume of P-buffer. This is the step that we changed, to avoid changing the repipettor setting.)

7. Shake the glass tube for 1 minute (cap tightly!), vent periodically.

8. Let stand overnight for separation in the dark (cover in aluminum foil), away from heat.

DAY 2 (approx. 3 hrs for 40 samples)

1. **In fume hood:** Aspirate the top layer off (approximately 2/3 of the way down) of the tubes after over-night separation. Use a vacuum aspirator. (side-arm flask connected to vacuum line in hood, with Tygon tubing and a glass disposable pipette—pipette sucks aqueous phase into the flask, via vacuum) Depending on OM content of the soil, the top layer may contain soil: aspirate as much of it as possible; may need to use a different piece of tubing for samples with lots of soil. **SAVE THE BOTTOM LAYER ($CHCl_3$ layer)!!**

   DO NOT LEAVE ANY OF THE AQUEOUS PHASE IN THE SAMPLE. (water molecules will attack fatty-acid double bonds)

   Note: The aqueous phase contains the LPS-OH fraction. If desired, use a separate clean pipette for each sample, and SAVE the aqueous phase for later manipulation.

2. Evaporate the $CHCl_3$ layer off with N_2. Keep lights off whenever possible. Is OK to use heat to speed the process, but DO NOT exceed 30°C.
   The gas should be ruffling the surface of the liquid, but not very vigorously. Watch the level of liquid and move the rack with tubes up as the liquid evaporates (or move the gas manifold downward); takes about 1 hour with heat.

Notes: N2 tanks run dry with alarming frequency. **Always have a back-up tank available,** and if you are going to run out, be sure to store the samples under N2 gas prior to the tank running dry.

If at any time after this step you need to store the samples: **you must FILL the tube with N_2 gas, prior to capping it off!!!** (Oxygen in the atmosphere will destroy the structure of the fatty acids.)

Step 3. Transfer to clean tube

DAY 3 (transfer is about 3-4 minutes a sample, dry down is about 45 minutes)
Resuspend lipids in 2ml $CHCl_3$, transferring to clean glass tube.

Specific procedure for transfer:

Need to have:

- 2 glass pipettes (0.5 ml capacity), with rubber pipette bulbs: label one 'CLEAN', and the other 'TRANSFER'
- Clean tube or other glass container filled with $CHCl_3$, labeled 'CLEAN'
- Clean tube or other glass container with $CHCl_3$, labelled 'RINSE'
- Container for $CHCl_3$ waste

Transfer:

1. Using TRANSFER pipette, draw 0.5 ml from RINSE container, squirt into waste beaker
2. repeat twice more to clean the transfer pipette
3. Using the CLEAN pipette, draw 0.5 ml from CLEAN container, and add it directly to the bottom of the sample tube. **Put the CLEAN pipette aside.** Keep tip from getting contaminated.
4. Swirl the tube to dissolve the fatty acid residue.
5. Using TRANSFER pipette, remove the liquid from sample tube, and transfer to a new sample tube. Don't forget to label the new tube! **Put TRANSFER pipette aside, without rinsing.** Keep the tip from touching anything.
6. Repeat steps 3-5 3 more times. Total volume transfered will be 2 ml. Allow the clean $CHCl_3$ to run down the sides of the tube for the 2^{nd}, 3^{rd} and 4^{th} additions.

   **If the sample is cloudy when it is all transferred, then it has water in it. Very bad. Water will attack the double bonds in the fatty acids. Add MeOH with a dropper, until solution clears (approx. 0.5ml).
7. After the final transfer, rinse the TRANSFER pipette as in steps 1-2. TRANSFER pipette is only rinsed in between samples.
8. Dry down all samples. Samples containing MeOH will take longer to dry. Heat from a sand bath helps greatly.

Notes: 1) Avoid transfering particulate matter. 2) This transfer step may not be absolutely necessary, but I think it's worth it because it cleans up the lipids, and allows you to remove water from the sample. Water is worse than any loss of fatty acids during this step.

IF DESIRED, CAN STORE UNDER N_2, COLD, IN THE DARK, (–20°C) AT THIS POINT.

Step 4. Solid Phase Extraction (SPE) disposable cartridge: separate the lipid classes

DAY 4 (about 3/4 day for 40 samples)

1. In fume hood: Set-up SPE cartridges in a rack suspended over glass tubes (15ml) These tubes are for mixed solvent waste. We use a vacuum 'tank' and the tubes are on a rack inside the tank.

2. Add 3 ml $CHCl_3$ to condition the column. (This is approximate volume, use squeeze bottle to 1/2 fill SPE cartridge.)

3. Transfer lipids from the glass tube to the cartridge with 4 (4X) 250 µL transfers of $CHCl_3$. This step is tricky, takes a long time. Follow the transfer protocol from Step 3, using smaller volume of CHCl3.

   May need to cap the tube after adding chloroform, or it evaporates. The first aliquot is directly to the bottom, the next three rinse down the sides prior to transfer. May need to whirl the liquid around the tube to get all the stuff on the sides. 1 ml. total, but depending on the amount of soil, organic matter and/or lipids in the tube, may need to use more $CHCl_3$. If this is the case, use less CHCl3 in the next step.)

4. Add approximately 5 ml $CHCl_3$ to the cartridge unit – (almost fill the cartridge), using $CHCl_3$ squeeze bottle. Allow to drain by gravity.

5. Add approximately 10 ml acetone (2 aliquots of 5 ml each) – COMPLETELY drain column. (It helps to have vacuum capability to drain tubes for this step)

6. Clean-off the bottom of cartridge tips with MeOH (take the rack off, and use a KimWipe with MeOH on it.

7. Switch collection tubes to sample set. Next fraction is what you keep. Discard the waste from the waste tubeset.

8. Add 5 ml MeOH to cartridge. (Use MeOH squeeze bottle, almost fill cartridge). **SAVE THIS ELUTANT (most important one)!!!** Label tube with *sample ID+date*

9. Evaporate the MeOH with N_2. Store at –20°C, in the dark.

NOTE: If desired, you can also collect the other fractions. The Neutral fraction (first one) contains sterols used for traditional fungal biomass estimates. The Glycolipid fraction has PHAs in it, for analyzing microbial carbon storage compounds. See White and Ringelberg, 1998 reference for more detail.

Step 5. Make Methyl-Esters (Transesterification)

DAY 5 (approx. 3/4 day for 40 samples)

1. In hood: Redissolve dried lipid in 1 ml 1:1 MeOH:Toluene and 1 ml *methanolic* KOH. Add reagents to the bottom of the sample tube with glass pipettes, vortex briefly.

(use 1 ml glass pipette; mix MeOH, Toluene ahead of time; shake well to mix the two phases; make methanolic KOH fresh each day)

2. Incubate at 35°C for at least 15 minutes. (use a sand bath, or water bath, or oven)

(If use water bath, make sure there is no toluene or other organic solvents on the outer surface of tubes, to avoid contaminating water bath.)

3. Let samples cool to room temperature!

4. Add 2ml hexane:$CHCl_3$ (4:1), (use glass pipette), mix.

5. Neutralize sample with approx. 1 ml 1M acetic acid. Test pH by putting micro-drop from one sample onto litmus paper.

(1M Acetic Acid=58ml conc. HOAc/1L water) (HOAc = Glacial Acetic Acid)

6. Add 2 ml milliQ ultra-pure water, then cap and shake test tube prior to vortex. Vortex on setting 5 for 30 seconds!

7. Separate phases by centrifuge 5 min at 2000 rpm.

8. Remove upper layer (hexane) to 4 ml screw-cap amber GC vial. Use disposable glass pasteur pipettes, 1 per sample. Label each pipette or keep it next to the tube, so that it can be reused with the appropriate sample.

   DO NOT TRANSFER ANY OF THE LOWER (AQUEOUS) PHASE!!!! EVEN IF THAT MEANS LEAVING BEHIND SOME OF THE UPPER PHASE.

9. Repeat steps 4, 7, 8 once more,(adding 2 ml of the hexane:$CHCl_3$ mix). This is to 'wash' the aqueous phase, and remove any remaining lipids.

10. Add the second hexane fraction to the first. (~4 ml total)

11. Dry with N_2, takes about 3/4 hour. Store at –20°C, dark.

Step 6. Transfer to GC vials with glass inserts

DAY 6 (approx. 3-4 minutes per sample)

Transfer samples to 2ml GC vials (vials have a glass insert). There are two options: 1)Suspend the dried lipids in three aliquots of 100µL each, and transfer all to the insert. Then remove 100µl and put into a replicate GC vial. this is more work, but gives you a back-up sample in case something goes wrong in the analysis. 2) Suspend the sample in 2 aliquots of 100µl, and add to GC vial, no replicates.

Specific procedure for transfer and addition of internal standards:

Have on hand

- Amber vial with dried sample
- Labeled amber GC vials with glass inserts (they are sold as 100µL inserts, with polyethylene 'feet', or springs: we found that they will hold 300µL of liquid)
- Two 40 ml bottles with mininert valves containing
    1. Internal standard in hexane;
    2. hexane rinse for transfer needle.
- 0.1ml glass pipet with 3" needle
- Waste container for hexanes

Transfer Protocol:

- open mininert
- draw < 100µL of Rinse, squirt into waste beaker
- repeat twice more
- draw >100µL Standard (do not allow backwash into bottle!), squirt into waste beaker (i.e. rinse with standard)
- for REAL: draw 100µL Standard, insert needle into 4ml vial with dried sample. With hole of needle against the side of vial, turn the vial while express the standard. (Thus wash down the sides of vial.)
- Vortex 4ml vial, lowest setting – NO SPLASH! 5 seconds.
- Draw sample+standard from vial, put into insert in 2ml vial, cap vial.
- REPEAT 1 or 2 more times (depends on if you want a back up or not.)
- If a replicate is desired, remove 100µL of sample from 2ml vial, and put it into a replicate 2ml vial/insert.
- Purge with N_2 and seal.

Store sealed G.C. vials in freezer prior to analysis.

Materials

Step 1. Prepare soil and glassware:

- Containers for soil samples,
- Freezer,
- freeze-drier,
- mortar and pestle,
- teflon tubes,
- hexane to rinse glassware
- squeeze bottle for hexane
- Vortexer to mix hexane in glassware

Step 2. Extract fatty acids from whole soil

- Shaker (orbital or other)
- Centrifuge
- Repipettors (3), One must be glass and $CHCl_3$ resistant
- Phosphate Buffer
- Chloroform
- Methanol
- Glass tubes for separation (30ml, screw caps with teflon liner) (1 per sample)
- Aspirator set-up (Sidearm flask, tubing, disposable glass pipettes)
- Sand bath or dry bath

Step 3. Transfer to clean tube

- $CHCl_3$
- MeOH
- dropper bottle for MeOH, to add to cloudy samples
- 6-10 ml glass tubes with screw cap (does not need teflon liner) (1 per sample)
- 0.5 ml volume glass pipettes with rubber bulbs (at least 2 pipettes, 2 bulbs)
- N_2 gas source
- Gas manifold to dry-down samples (with as many ports as possible)
- Sand bath or dry bath

Step 4. SPE cartridge: separate lipid classes

- SPE cartridges (1 per sample) (500mg Si gel, 6 ml size)
- Vacuum 'tank' for cartridges
- 15 ml glass tubes to catch waste (needn't be screw cap) (1 per port in the vacuum tank, can be reused over and over again without washing)
- 8-10ml screw cap glass tubes for MeOH fraction (1 per sample)
- $CHCl_3$
- acetone
- MeOH
- Squeze bottles for solvents (1 per solvent = 3)
- 0.5 ml volume glass pipettes with rubber bulbs for transfer (at least 2 pipettes, 2 bulbs)
- N_2 gas source
- Gas manifold to dry-down samples (with as many ports as possible)
- Sand bath or dry bath

Step 5. Make Methyl-Esters

- toluene:MeOH (1:1) mixture
- hexane:CHCl3 (4:1) mix
- 0.2 M KOH, made in methanol instead of water (methanolic KOH)
- Vortexer to mix hexane in glassware
- incubater (oven or water bath set to 35C)
- hexane
- acetic acid
- Deionized water
- pH paper
- Glass bottles for reagents (need 5, 100-150 ml volume)

- Centrifuge
- Transfer upper phase to 4 ml amber vial
- Re-extract lower phase as above 1 more time and save upper phase
- N_2 gas source
- Gas manifold to dry-down samples (with as many ports as possible)
- Sand bath or dry bath

Step 6. Transfer to GC vials (with 100µl liners)
- 2ml amber GC vials with screw tops and red rubber septa
- 150µL glass inserts with polyethylene spring 'feet' for the GC vials
- 100-150µL syringe (glass body with stainless steel needle)
- hexane for rinse
- Hexane with internal standard
- 40 ml flat bottom glass bottles with mini-nert valve on top, teflon lined (need 2)
- N_2 gas source

Reagent Volumes (approximate totals, per 5g soil sample)

CHCl3	20 ml
MeOH	15 ml
Hexane:CHCl3	2 ml
Hexane standard	300 µL
Acetone	10 ml
Acetic Acid	0.3 ml
MeOH:Toluene	1 ml
Me-KOH	1 ml

RECIPES (All glassware should be rinsed with Hexanes prior to use for PLFA)

P-Buffer

Phosphate-Buffer; 0.1M & pH 7.0

39ml 1M K_2HPO_4 stock, sterile
61ml 1M KH_2PO_4 stock, sterile
Fill to **one** liter
Adjust pH to 7.0

(The stock solutions are just bottles of 1M K_2HPO_4 and KH_2PO_4)

1:1 MeOH:Toluene, 4:1 Hexane:$CHCl_3$

Add equal volumes of MeOH and Toluene to a clean (hexane rinsed) bottle. Add 4 parts Hexane to 1 part Chloroform to hexane rinsed bottle. 100 ml bottles will work.

0.2M Methanolic KOH

Make a small quantity of 0.2M KOH in methanol**. This must be made fresh every time you use it, to keep water out of it.** We weigh the KOH first, then quickly calculate how much methanol to add for that mass of KOH.

$(X\ g_{KOH}/0.2*mol.wt._{KOH})*1000=mL_{MeOH}$ to add

Internal Standard for final step

- Purchase the methyl-ester form of 2-3 fatty acids: 10:0, 14:0, and 18:0 are good ones. (Methyl decanoate, Methyl tetradecanoate, and Methyl octanoate)

- Make a stock solution by dissolving known quantities of each acid in hexane, in a 4ml amber vial.
- Add a known amount of stock solution to 30ml hexane

(I do not have the recipe with me at the moment. It needs to be added here)

A note on washing the glassware: I use a scrub brush but no soap. Soap is made of phospholipids. I rinse all glassware afterward with hexane, to remove any contaminant lipids. Other groups will wash with soap, but then will bake out the glassware in an oven at 250C for two days. Then they hexane rinse.

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