

## METHODS FOR STUDYING BIOLOGICAL SOIL CRUSTS

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### SAMPLE COLLECTION

Samples can be collected by:

- (1) pressing a sterile test tube into the crust,
- (2) pressing a petri dish into the crust,
- (3) collecting crust with a spatula and placing in a whirl-pak bag or petri dish.

The depth of sampling can be either a standard depth (such as 1 cm) or determined by initial studies to characterize the depth of crust. The choice of method depends on the measurement to be made. For example, samples are often collected in whirl-pak bags for culturing, in test tubes for chlorophyll *a* analysis, and in petri dishes for nutrient content or nitrogen fixation studies.

### CULTURING

(modified from Hawkes and Flechtner, *In Review*)

For site characterizations by culturing, crust subsamples (1-2 cm deep) are taken from approximately 25 positions in each site of interest. These subsamples are typically distributed along transects of interest or are collected from random positions. The subsamples are combined in a sterile bag and homogenized by crushing and mixing.

From each composite sample, one hundred-fold dilutions are made by adding 1-g of crust to 99 ml of 0.7% saline solution. Aliquots of 0.1 and 0.2 ml are spread in triplicate on two agar-solidified (1.5% agar) media: Z-8 (Carmichael 1986) for quantification of cyanobacteria and Bold's Basal (BBM; Bold and Wynne 1978) for quantification of non-diatom eukaryotic algae. Use agarose with no nutrients added (e.g., Difco bacto-agar). Cultures are allowed to dry overnight before inversion, sealed with parafilm, and incubated in constant light at 20-23 °C until good growth had been obtained (3-6 weeks). This can be done in an incubator, or on a dark countertop under fluorescent light. The number of colony forming units on each plate is counted

For the identification of cyanobacteria, wet mounts prepared directly from individual isolates on Z-8 plates are examined under the microscope (e.g., Olympus BH-2 photomicroscope with Nomarski DIC optics) and photographed using Kodak PKL-135 film. Identifications are made on the basis of cell and colony morphology using standard authoritative references (Desikachary 1959, Geitler 1930-32, Kantz & Bold 1969).

Because many cyanobacteria grow poorly on artificial media, additional identification of cyanobacteria can be made directly from wet mounts of wetted soil samples incubated 48-72 hours in the light (See Moistened Soil Method below).

For identification of non-diatom eukaryotic algae, individual isolates are picked from the plates, placed into 5 ml liquid BBM, and incubated for 2-4 weeks. Identifications can be made on the basis of life history and morphological criteria using standard authoritative references (Ettl & Gärtner 1995, Komárek & Fott 1984).

#### MEDIA PREPARATION

**Bold's Basal Medium** (BBM; Bold and Wynne 1978)

Salt	g salt/500 ml H <sub>2</sub> O for stock	ml stock/L H <sub>2</sub> O for media
NaNO <sub>3</sub>	12.5	10
CaCl <sub>2</sub> · 2 H <sub>2</sub> O	1.25	10
MgSO <sub>4</sub> · 7H <sub>2</sub> O	3.75	10
K <sub>2</sub> HPO <sub>4</sub>	3.75	10
KH <sub>2</sub> PO <sub>4</sub>	8.75	10
NaCl	1.25	10
Trace element solutions (4)	see below	1 of each solution

NOTE: For 1L of media, add stock and trace solutions to 936 ml of distilled water.

#### Trace element solution 1 for BBM

Salt	g/500 ml H <sub>2</sub> O
EDTA	25
KOH	15.5

#### Trace element solution 2 for BBM

Salt	g/500 ml acidified H <sub>2</sub> O (1 ml H <sub>2</sub> SO <sub>4</sub> /1 L H <sub>2</sub> O)
FeSO <sub>4</sub> · 7 H <sub>2</sub> O	2.49

#### Trace element solution 3 for BBM

Salt	g/500 ml H <sub>2</sub> O
H <sub>3</sub> BO <sub>3</sub>	5.71

#### Trace element solution 4 for BBM

Salt	g/500 ml H <sub>2</sub> O
ZnSO <sub>4</sub> · 7 H <sub>2</sub> O	4.41
MnCl <sub>3</sub> · 4H <sub>3</sub> O	0.72
MoO <sub>3</sub>	0.36
CuSO <sub>4</sub> · 5 H <sub>2</sub> O	0.79
Co(NO <sub>3</sub> ) <sub>2</sub>	0.36

**Z-8 MEDIUM** (Charmichael 1986)  
pH 6.5-7.7

Salt	g salt/500 ml H <sub>2</sub> O for stock	ml stock/L H <sub>2</sub> O for media
NaNO <sub>3</sub>	23.35	10
Ca (NO <sub>3</sub> ) <sub>2</sub> · 4H <sub>2</sub> O	2.95	10
MgSO <sub>4</sub> · 7H <sub>2</sub> O	1.25	10
K <sub>2</sub> HPO <sub>4</sub>	1.55	10
Na <sub>2</sub> CO <sub>3</sub>	1.05	10
FeEDTA	see below	10
Trace element solution	see below	1

Fe-EDTA...5.0 ml from a FeCl<sub>3</sub> solution (2.8 g FeCl<sub>3</sub>·6H<sub>2</sub>O dissolved in 100 ml 0.1 N HCl) and 4.75 ml from EDTA solution (3.9 g EDTA-Na<sub>2</sub> dissolved in 100 ml 0.1 N NaOH) are mixed and filled up to 500 ml.

#### Trace element solution for Z-8

Dissolve the following salts in 360 ml distilled H<sub>2</sub>O:

Salt	g/500 ml
Na <sub>2</sub> WO <sub>4</sub> · 2H <sub>2</sub> O	0.165
(NH <sub>4</sub> )Mo <sub>7</sub> O <sub>24</sub> · 2H <sub>2</sub> O	0.440
KBr	0.600
KI	0.415

Then add the following:

Salt	g/L for stock solution	ml/ 500 ml trace element solution
H <sub>3</sub> BO <sub>3</sub> (boric acid)	3.1 g/l	50
MnSO <sub>4</sub> ·4H <sub>2</sub> O	2.23 g/l	50
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	2.87	5
Cd(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O	1.55	5
Co(NO <sub>3</sub> ) <sub>2</sub> ·6H <sub>2</sub> O	1.46	5
CuSO <sub>4</sub> ·5H <sub>2</sub> O	1.25	5
NiSO <sub>4</sub> (NH <sub>4</sub> )SO <sub>4</sub> ·6H <sub>2</sub> O	1.98	5
O		
Cr(NO <sub>3</sub> ) <sub>3</sub> ·9H <sub>2</sub> O	0.41	5
V <sub>2</sub> O <sub>5</sub>	0.089	5
Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> ·K <sub>2</sub> SO <sub>4</sub> ·24H <sub>2</sub> O	4.74	5
H <sub>2</sub> O		

Bring the total volume of the trace element stock to 1 liter.

The ingredients in the medium cannot be stocked or autoclaved together. The components need to be filter sterilized separately, and then added to sterile water.

**REFERENCES:**

- Bold, H.C. and M.J. Wynne. 1978. Introduction to the algae. Prentice-Hall, Inc., Englewood Cliffs, N.J. 706 pp.
- Carmichael, W.W. 1986. Isolation, culture, and toxicity testing of toxic freshwater cyanobacteria (blue-green algae). Pp. 1249-1262 in V. Shilov (Ed.). Fundamental research in homogenous catalysis. Vol. 3. Gordon and Breach, New York, N.Y.
- Desikachary TV (1959) Cyanophyta. Indian Council of Agricultural Research, New Dehli, India, 686 p
- Ettl H, Gärtner G (1995) Syllabus der Boden-, Luft- und Flechtenalgen. Gustav Fischer Verlag, Stuttgart 721 pp
- Geitler L (1932) Cyanophyceae. In: L Rabenherst (ed.^eds.), Kryptogamen Flora von Deutschland, Osterweich und der Schweiz. Reprinted 1985, Koeltz Scientific Books, Königstein, Germany, 1196 pp
- Hawkes, C.V. and V.R. Flechtner. In Review. Biological soil crusts in a xeric Florida shrubland: composition, abundance, and spatial heterogeneity of crusts with different disturbance histories. Microbial Ecology.
- Kantz T, Bold HC (1969) Phycological Studies. IX. Morphological and taxonomic investigations of *Nostoc* and *Anabaena* in culture. University of Texas, Austin, TX, 67 pp
- Komárek J, Fott B (1983) Chlorococcales. In G Huber-Pestalozzi (Ed) Das Phytoplankton des Süßwassers, vol.7, Schweizerbart, Stuttgart, pp 1-1043
- deep. We also use standard 22x22 mm or 18x18 mm No. 1 coverslips, which give better results with fluorescence. An inexpensive but effective counting chamber can be made with a slide, 2 coverslips, and some super glue (Tchan 1952, Johansen and Rushforth 1985). To construct this counting cell, glue a No. 1 thickness 18x18 mm (or 22x22 mm) coverslip near the middle of the slide, with its top and bottom edges centered and parallel to the slide edges. Glue a second coverslip such that a channel exactly 1 cm wide is created between the two coverslips. Be sure the coverslips are pressed down firmly against the slide so that the glue creates negligible height. No. 1 coverslips that we have used have always been 0.1778 mm thick, and this will be the height of the water in the well when you use the counter. You can check this thickness with a caliper.
3. A tissue grinder (15ml) with a plunger rod of steel and plastic head rather than a glass plunger. The glass ones break regularly when sand grains get jammed in the grinder.
  4. Disposable plastic pipets in 1 ml, 5 ml and 10 ml sizes, distilled water, one-sided razor blades. The 1 ml pipets should have their tips cut off so that the sandy aggregates can easily be sucked into the pipet. This is done by scoring the pipet at the 0.9 ml mark by rolling the razor blade over it a few times. The tip can then be broken off easily.
  5. Although not necessary, we find the use of the 15 ml plastic centrifuge tubes to be very helpful. The samples can be measured into these tubes, and when capped, very vigorous shaking can be used to break up the aggregates to some degree.

**ENUMERATION OF SOIL ALGAE**

(from Jeffrey Johansen, John Carroll University, OH)

**I. EPIFLUORESCENCE MICROSCOPY****Recommended equipment/supplies:**

1. Epifluorescence microscope, with light source properly aligned and centered for maximum fluorescence. The blue excitation filter is needed. If using a microscope with Nomarski DIC optics, the DIC prisms in the light path between the objectives and eyepieces must be pulled out of the light path.
2. Hemacytometer. We use an AO Bright Line Hemacytometer, improved Neubauer, 0.1 mm

**Procedure:**

1. Measure 1 gram of dry soil into a centrifuge tube. If growing algae on a wet soil/sand surface, calls/g dry soil is not attainable. In this case I recommend that a known area of soil surface be sampled and cell counts can be reported as concentrations per unit area. Some adjustment of dilutions recommended for 1 gram may need to be made in this case, based on the counts obtained. If cells/g dry soil is needed even when the soils are wet, subsamples from the main sample can be taken, weighed wet, dried in a drying oven, and

- reweighed. Then a conversion factor can be applied to the cells/wet soil to give cells/dry soil.
2. Pipet 5 ml distilled water into the tube, cap, and shake vigorously (by hand or vortexer).
  3. Using the cut-off 1 ml pipet, pipet 1 ml into the tissue grinder by sucking the solution up to the -0.1 mark. Be sure to get as representative sample as possible. This is best achieved by blowing air into the tubes to thoroughly mix the soil solution and then immediately pulling up the 1 ml.
  4. Break up the visible cyanobacterial aggregates in the tissue grinder by using both a plunging and twisting action with the plunger.
  5. Place a coverglass on the Hemacytometer. Thoroughly mix the sample in the tissue grinder by blowing air into it. Pull up some of the liquid and bleed it under the coverglass as quickly as possible so that the sand grains do not all settle in the bottom drop before bleeding that drop on the Hemacytometer. Holding the pipet at a near horizontal angle will help to avoid this problem.
  6. Examine the Hemacytometer by transects across the silvered surface. We do not use the grid on the Hemacytometer, but make full transects across the full length of the silvered surface. Algae are examined at 400X magnification. Count 20 transects per sample if using a Hemacytometer. This will likely take 2-4 Hemacytometer preparations, as they will dry out after about 5-7 transects and need to be remade. When using the Tchan chamber 10 transects is sufficient, as the chamber is both deeper and wider.
  7. Biovolume of cyanobacterial filaments is determined by measuring length of filaments. We categorize filaments by class size, the size classes being 1-3.8  $\mu\text{m}$ , 4-5.8  $\mu\text{m}$ , and 8-12 in diameter. On a sheet of paper we note these size classes and then record the length for each filament. It is important to remember the conversion factors for the ocular micrometer. For most microscopes, you must multiply the micrometer units by 2.5 to get microns at 400X magnification. Calibrating your microscope with a stage micrometer is a good idea. We do not convert the lengths as we go, but record the uncovered lengths and apply the correction later into the computer database. If a whole column is converted at once it is easier and avoids rounding errors. For most microscopes, the unconverted size classes at 400X are 0.4-1.5, 1.6-2.4, 2.5-3.3, and 3.4-5.0 units in diameter. For the Olympus BH-2 in our lab the unconverted size classes at 400X are 0.5-1.9, 2-2.9, 3-3.9, and 4-6 units in diameter. Every filament should have a number on the sheet; do not add numbers in your head and write them down. In this way a frequency or density value can also be determined for the filaments. If you are working with a single species of cyanobacteria, then determine the average diameter of your species and do not worry about size classes.
  8. Diatoms and coccoids are usually not measured. Their frequency is recorded by making a check for every cell counted. Colonial coccoids are recorded as cell counts/estimates within the colony rather than by colonies. If you wished to calculate biovolumes for the coccoids and diatoms, this could be done with some careful measurements.
  9. *Nostoc* species can be counted as frequency of colonies. Biovolume can be estimated by measuring observed length vs. width of the colonies and then calculating the volume of an oval. Very small *Nostoc* colonies can be confused with coccoids, and some expertise is required to differentiate them. Use the length of the transect and width of the microscope to calculate the volume of liquid counted per transect (remember depth is 0.1 mm in a Hemacytometer, 0.1778 mm in the Tchan chamber). The total volume examined is number of transects times volume per transect. You can calculate the total cells/g soil (or biovolume/g soil), by determining the fraction of the 5ml original sample in your 20 transects. Divide your cell count etc. in the 20 transects by this fraction to get cell count etc. per g dry soil.
  10. The formula for frequency (F) is :
 
$$F = \frac{(\# \text{ filaments observed}) (\text{amount of dilutant})}{(\# \text{ transects}) (\text{volume/transect}) (\text{amount of sample})}$$
 where volume/transect is in ml (cubic centimeters).
- For example, the Olympus B-Max at Jon Carroll University this volume is:

(0.1 mm deep) (6 mm long) (0.530 mm high)  
 $(0.001 \text{ ml/mm}^3) = 0.000318 \text{ ml}$

and the Olympus BH-2 at John Carroll University this volume is:

(0.1mm deep) (6 mm long) (0.530 mm high)  
 $(0.001 \text{ ml/mm}^3) = 0.000228 \text{ ml}$

The amount of dilutant is 5 ml, amount of sample is 1 g for these particular instructions. These can be adjusted if area measurements are used.

11. For biovolume of filaments, the midpoint of each diameter range is used as the diameter in calculations. A radius is determined from this diameter. The total length of the filaments in each size class is determined by summing the unconverted lengths and converting the sum to length in microns. Biovolume is equal to (total length)  $(\Pi r^2)$ . This value can then be put into the formula for biovolume.

$$B = \frac{(\text{total biovolume}) (\text{amount of dilutant})}{(\# \text{ transects}) (\text{volume/transect}) (\text{amount of sample})}$$

Biovolume will be in cubic microns, and to keep units appropriate it is best to report this in terms of micrograms of sample (1,000,000 ug/g). The other option is to convert biovolume to cubic mm per gram of soil.

## II. MOISTENED SOIL METHOD

This method is particularly useful in quantifying cyanobacteria and diatoms. Cyanobacteria do not grow well on plates (particularly the cosmopolitan species *Microcoleus vaginatis*). Furthermore, they cannot generally be identified to species as readily on plates, as almost every colony must be checked under a compound microscope. This method does not give the rigorous quantification that the epifluorescence method gives, although more accurate taxonomy is possible. The method is based on frequency of occurrence in microscope fields, and has been used effectively in a number of studies (Johansen and St. Clair 1986, Johansen et al. 1984, 1986, 1993, St. Clair et al. 1986).

Gently crush the soil clods in each soil sample to a maximum diameter of 5 mm. Depending on how you sampled, place either 20 cm<sup>3</sup> or 2.5-3.0 g

soil in a sterile petri dish (100 X 15 mm). Saturate soil with 20 ml of sterile deionized water. Incubate samples under continuous cool-white fluorescent light at room temperature for 8 (Johansen et al. 1993, St. Clair et al. 1986) to 10 (Johansen and St. Clair 1986, Johansen et al. 1984) days. Samples can be incubated in growth chambers at controlled temperatures and with light dark cycles, but it is not necessary. With a light-dark cycle, it may be good to let the samples incubate longer (14 days). Greenhouses are generally too warm and too bright, and they should be used with caution until some experimentation shows that they give satisfactory results. Soils must remain moist, and should be supplemented with more sterile distilled water if they begin to dry out. Check them daily.

Percent visible algal cover in each petri dish can be estimated at the end of the incubation period. Frequency and relative abundance of living algal species (particularly cyanobacteria and diatoms) can be estimated sampling 3-5 points in the plate. Decide on a replicate number and make a template cover plate with 3-5 small circles marked on it. Place the template over the petri dish to be sampled, sight where the sample is to be taken (one of the 3-5 circles) and remove a small amount of the surface soil from that spot. If the method is going to be unbiased, it is important to have an objective sampling methodology so that the temptation to take a sample from a nice blue-green patch can be resisted, even though there are only a few patches on the plate. Make a slide of the sample by putting the sample on the slide, adding water, and teasing the sample apart. Large sand grains should be moved to the edge of the slide. Put on a coverslip. The less thick the water on the slide, the better optics will be when examining the sample.

Read 20 microscope fields on the slide, making sure the fields are chosen while you are not looking through the microscope. Identify all algae in each field and note their presence on a data sheet. At the end of the 20 fields, if an alga appeared in 15 fields, it would have a frequency value of 75%. Make the next slide, and read 20 fields from that as well. The advantage of having a higher number of replicate slides is that you can compensate for the patchiness of the algae in the plate. The disadvantage is the loss of time making the slides. You can read fewer microscope fields if you make more slides. We

have quantified the algae using from 25-100 total microscope fields per sample, and the number needed in a particular study is determined empirically, taking time availability into consideration.

### III. DIATOM SLIDE PREPARATION

The following quantitative method has been used successfully in several studies (Johansen and St. Clair 1986, Johansen et al. 1982, 1984). Unpublished observations indicate that it can be applied to desert, forest, salt marsh, and alpine soils.

Soil samples be of uniform depth; 1 cm is recommended. A composite sample of several samples taken to 1 cm depth yields better results, as algal heterogeneity can be very high when using small samples (Grondin and Johansen 1993). From a well-mixed, screened (1 mm mesh), oven-dried composite sample remove 1 g of soil and place in a beaker (100 ml or larger) with 20 ml distilled water. Add about 10 ml of concentrated nitric acid. Place on a hot plate and bring to a boil. If the soil has a moderate to high amount of organic material, then a second oxidation can be achieved by adding 1/8 tsp. of potassium dichromate to the solution after 10 minutes of boiling. Watch samples carefully while on the hotplate. If they begin to spatter add distilled water or remove from heat immediately. When the sample has evaporated to at or below the 20 ml mark, remove from heat and allow to cool.

The acid must be removed from the solution by repeated rinsing in deionized or distilled water to avoid acid halos that obscure the diatom frustules and make identification difficult. Rinsing is most easily accomplished by centrifuging in 15 ml tubes, decanting the supernatant, refilling with water, and centrifuging again. Six rinses are recommended. All of the sample must be added to the tube; if more than 15 ml are present in the beaker, add the remainder after the first centrifugation. If a centrifuge is not available, rinse by adding the deionized water to the beaker and allow the diatoms to settle 24 hours. Decant or siphon the supernatant and fill with water again. Five rinses are sufficient for method because higher quantities of water can be used.

After the final rinse, bring volume of cleaned soil mixture to 10 ml. Dilute by adding 0.5 ml of this solution and to 9.5 ml deionized water. Place 0.5 ml of the dilution on an 18 mm square coverslip. Carefully use the pipet to spread the solution to the edges of the coverslip to ensure an even mount. Air-dry overnight. Multiple coverslips can be prepared for replicates.

Preheat a hot plate in a safety hood. Place 2-3 drops of diatom mounting medium (Hyrax or Naphrax are recommended) on a glass microslide. Place on the hot plate. Hot plate should be hot enough to boil mountant in 5-20 sec. Boil a few seconds until boiling slows (but does not stop). Remove from hotplate and drop coverslip diatom side down into the mountant. Put back on the hot plate and let come to a boil, tapping lightly with a teasing needle to spread the medium to the edges. Drop slide from 3 cm several times on to the hood edge to aid in ridding the slide of air bubbles. If mountant does not cover the whole coverslip, more mountant should be used. Excess hardened mountant can be scraped from the edge of the coverslip with a razor blade.

Diatoms should be enumerated at 1000X. Determine the width of field of your microscope at 1000X and convert the width to mm. This width times 18 mm is the area of the slide covered by one transect. To obtain quantitative counts, start at the top or bottom of the slide at the nearest even whole number on the vernier scale of the microscope stage. Count all whole diatoms which have their central area in the field of view. Since every diatom has two valves, count the number of valves and divide by two to get the number of frustules represented. Many diatoms will still have both valves together; others will be consistently separate. Count the total diatoms in 9 transects, each 1 mm apart. The edges will have far fewer diatoms than the middle, so it is important to count 9 transects to compensate for these differences. Counts of less than 50 valves are not very reliable, and more transects should be added until at least 50. The number of cells per gram of dry soil can be calculated using the following equation.

$$\frac{FC}{TAM}$$

where F=number of frustules counted; C=area of coverslip (324 mm<sup>2</sup>); T=number of transects scanned; A=area of a transect (18 mm X width of microscope field in mm); M=mass of dry soil on coverslip (0.0025 g). Identification of diatoms is greatly facilitated with either phase contrast or Normarski DIC optics, though brightfield can be used to identify most common soil species. We recommend using some of the illustrated floras of soil diatoms to identify the species present in soils (Anderson and Rushforth 1976, Ashley et al. 1985, Bock 1963, Johansen et al. 1981).

#### REFERENCES:

- Anderson, D.C. and S.R. Rushforth. 1976. The cryptogamic flora of desert soil crusts in Southern Utah, U.S.A. *Nova Hedwigia* 28:691-729
- Ashley, J., S.R. Rushforth and J.R. Johansen. 1985. Soil algae of cryptogamic crusts from the Uintah Basin, Utah, U.S.A. *Great Basin Naturalist* 45:432-442
- Bock, W. 1963. diatomeen extreme trockemer Standorte. *Nova Hedwigia* 5:199-254 plus plates 28-30
- Carmichael, W.W. 1986. Isolation, culture, and toxicity testing of toxic freshwater cyanobacteria (blue-green algae). In Shilov, V. [Ed.] *Fundamental Research in Homogeneous Catalysis*, Vol. 3 Gordon and Breach, New York, NY, pp. 1249-1262
- Grondin, A.E. and J.R. Johansen. 1993. Microbial spatial heterogeneity in microbiotic crusts in Colorado National Monument. I. Algae. *Great Basin Naturalist* 53:24-30
- Johansen, J.R., J. Ashley and W.R. Rayburn. 1983. Effects on rangefire on soil algal crusts in semi-arid shrub-steppe of the Lower Columbia Basin and their subsequent recovery. *Great Basin Naturalist* 53:73-88
- Johansen, J.R. and S.R. Rushforth. 1985. Cryptogamic soil crusts: seasonal variation in algal populations in the Tintic Mountains, Juab Count, Utah. *Great Basin Naturalist* 45:14-21
- Johansen, J.R. and S.R. Rushforth and J.D. Brotherson. 1981. Subaerial algae of Navajo National Monument, Arizona. *Great Basin Naturalist* 41:433-439
- Johansen, J.R. and L.L. St. Clair. 1986. Cryptogamic soil crusts: recovery from grazing near Camp Floyd State Park, Utah, U.S.A. *Great Basin Naturalist* 46:632-640
- Johansen, J.R. and L.L. St. Clair., B.L. Webb and G.T. Nebeker. 1984. Recovery patterns of cryptogamic soils crusts in desert rangelands following fire disturbance. *Bryologist* 87:238-243
- St. Clair, L.L. J.R. Johansen, and B.L. Webb. 1986. Rapid stabilization of fire-disturbed sites using soil crust slurry: inoculation studies. *Reclamation and Revegetation Research* 4:261-269
- Tchan, Y.T. 1952. Study of soil algae. I. Fluorescence microscopy for the study of soil algae. *Proceedings of the Linnean Society London* 77:265-269

#### CHLOROPHYLL *a* EXTRACTION

Chlorophyll *a* is often used as an estimate of the abundance of photosynthetic biomass in crusts, particularly when crust organisms are not visible to the naked eye (e.g., Belnap 1993, Bell and Sommerfeld 1987). To do so, samples are collected in test tubes by pressing the tubes into the crust. These are immediately capped and kept in dark or in dim light conditions. Extractions should be made as soon as possible. To each tube, add 5 ml dimethyl sulfoxide (DMSO) and then incubate in the dark at 65 °C for one hour. Remove the liquid by pipetting, transfer the liquid to a new tube, and centrifuge with an additional 2.5 ml DMSO for 5 min to remove any suspended sediment. Transfer 2.5 ml of supernatant to a cuvette and measure absorbance at  $\lambda = 665$  nm (the peak for chlorophyll *a*) and  $\lambda = 750$  nm (an estimate of sample turbidity) in a spectrophotometer. The extracts are then acidified with 3 drops of 1N HCl, left to sit in the dark for 10 min, and absorbance is measured once more at both 665 and 750 nm.

Chlorophyll *a* content on a per volume basis is then calculated as:

$$\text{Chlor } a \text{ (ug} \cdot \text{cm}^{-3}\text{)} = \frac{26.73 d v}{a L},$$

where

$d = (A_{665} - A_{750})_{\text{before acid}} - (A_{665} - A_{750})_{\text{after acid}}$ ,  $v$  is the extract volume,  $a$  is the volume of the crust

sample,  $L$  is the length of the light path, and 26.73 is a constant accounting for the absorbance coefficient of chlorophyll  $a$  and a correction for acidification. Surface area can also be used in place of volume where more appropriate.

#### REFERENCES:

- American Public Health Association (APHA). 1981. Standard methods for the examination of water and wastewater, 15th edition. American Public Health Association, Washington, D.C. 1134 pp.
- Bell, R.A. and M.R. Sommerfeld. 1987. Algal biomass and primary production within a temperate sandstone. *American Journal of Botany* 74: 294-297.
- Beymer, R.J. and J.M. Klopatek. 1991. Potential contribution of carbon by microphytic crusts in pinyon-juniper woodlands. *Arid Soil Research and Rehabilitation* 5: 187-198.
- Belnap, J., K.T. Harper, and S.D. Warren. 1994. Surface disturbance of cryptobiotic soil crusts: nitrogenase activity, chlorophyll content, and chlorophyll degradation. *Arid Soil Research and Rehabilitation* 8: 1-8.
- Ronen, R. and M. Galun. 1984. Pigment extraction from lichens with dimethyl sulfoxide (DMSO) and estimation of chlorophyll degradation. *Environmental and Experimental Botany* 24: 239-245.

### MEASUREMENT OF NITROGEN FIXATION IN CRUSTS

(from Christine Hawkes and Donald Herman, Univ. of California, Berkeley)

#### I. ACETYLENE REDUCTION METHOD

##### Introduction

The discovery that the nitrogenase enzyme responsible for  $N_2$ -fixation also reduced  $C_2H_2$  to  $C_2H_4$  (Dilworth, 1966) provided a useful assay for the quantification of the  $N_2$ -fixation process. For quantitative determinations of  $N_2$ -fixation,  $^{15}N_2$  techniques should be used, however, the acetylene reduction assay provides a highly sensitive and inexpensive way to quantify relative nitrogenase enzyme activity in  $N_2$  fixing samples.

##### Materials

calcium carbide ( $CaC_2$ )	gas chromatograph configured as follows:
500 mL volumetric flask	injection: splitless
rubber septum	injector temperature: 40° C
2-sided needle	column: GS-Q (J&W Scientific)
gas collection bag	30 m × 530 μm × 20 μm
soil crusts	carrier: ultra high purity $N_2$
225 mL jelly jar with lid fitted with Hungate septum	column flow: 3.9 mL min <sup>-1</sup>
1000 μL pipette	oven temperature: 35° C
distilled water	detector: flame ionization
vacuum grease	detector temperature: 200° C
60 mL gas-tight syringe	$H_2$ : 30 mL min <sup>-1</sup>
100 μL gas-tight syringe	air: 400 mL min <sup>-1</sup>
	makeup ( $N_2$ ): 30 mL min <sup>-1</sup>

##### Procedure

1. Make acetylene with approximately 13 rocks of calcium carbide and 1 cup water in a flask ( $CaC_2 + H_2O \rightarrow C_2H_4$ ). Cover flask opening with a flexible septa. Insert one end of a two-sided needle into the septum and the other end into a collection bag. Fill with acetylene.
2. Prepare samples: Add 10 g of sample (soil crust) into jar and record the exact weight ( $W_{OD}$ ). Add 2 ml ( $V_{water}$ ) distilled water. Grease the lip of the jar and seal.
3. Add acetylene to create a 10% acetylene atmosphere.
4. Take time zero ( $t_0$ ) readings on 100 μL of sample injected into the GC with a gas-tight syringe. Record time. Be sure to run ethylene standards as well.
5. Incubate samples and take additional readings: Put sample jars in low fluorescent light at 25 °C for the time period of interest (usually 24 hrs, but for the lab we will use 2 hrs.). Remove 100 μL from jars and inject into GC. Record the time. (This is the  $t_f$  sample.) Again, be sure to run ethylene standards.

##### Calculations

1. Calculate  $\Delta t = t_f - t_0$
2. Assuming a particle density of 2.6 g mL<sup>-1</sup>, calculate the volume of solids:  $V_{solid} = W_{OD} / 2.6$
3. Calculate the headspace volume:  $V_{headspace} = V_{total} - V_{water} - V_{solid}$
4. Calculate the ethylene concentrations for the  $t_0$  and  $t_f$  measurements from the calibration:  $\mu\text{mol } C_2H_4 \text{ mL}^{-1} = a + bx$  ( $x$  is the peak area from the gas chromatograph. The T.A. will provide the  $a$  and  $b$  coefficients.)

5. Calculate the  $\mu\text{mol}$  of ethylene in the jar at  $t_0$  and  $t_f$ .  $E_{\text{total}} = (\mu\text{mol C}_2\text{H}_4 \text{ mL}^{-1}) \times V_{\text{headspace}}$   
(For simplicity, we are ignoring any  $\text{C}_2\text{H}_4$  dissolved in pore water)
6. Calculate the rate of acetylene reduction to ethylene:  $\text{Rate} = [(E_{\text{total}})_{t_f} - (E_{\text{total}})_{t_0}] / (\Delta t \times W_{\text{OD}})$

## II. MEASUREMENT OF $\text{N}_2$ FIXATION USING $^{15}\text{N}$ LABELING

The N in biological systems is composed predominantly of two stable isotopes,  $^{15}\text{N}$  and  $^{14}\text{N}$ , which constitute about 0.3663 and 99.6337%, respectively, of the N on earth. The nearly constant  $^{15}\text{N}/^{14}\text{N}$  ratio in nature makes it possible to use materials with artificially altered  $^{15}\text{N}/^{14}\text{N}$  ratios to trace biological pathways and quantify rates and products in biological systems. Materials containing  $>0.3663\%$   $^{15}\text{N}$  are referred to as  $^{15}\text{N}$ -enriched or labeled; materials with  $<0.3663$  atom %N are depleted. Here we describe the use of enriched  $^{15}\text{N}_2$  to trace the fixation of N into biological material. To measure uptake of  $^{15}\text{N}_2$  gas, it is necessary to enclose the system in a gas-tight container with a sufficient enrichment of  $^{15}\text{N}_2$  and for long enough to detect enrichment. We then measure the total amount of N in the sample using an automated N-analyzer and the  $^{15}\text{N}$  incorporated using an Isotope Ratio Mass Spectrometer.

### Materials

10 mL serum vials	$\text{O}_2$ in gas bag
serum vial stoppers (Geomicrobial)	$^{15}\text{N}_2$ (99 atom % $^{15}\text{N}$ )
crimp seal	Incubator set at $27^\circ\text{C}$
crimp tool	Isotope ratio mass spectrometer (IRMS)
100 $\mu\text{L}$ pipette	20 mL liquid scintillation vials
10 mL syringe with stopcock and 23 gauge 1" needle	$\frac{1}{4}$ " x 2" stainless steel bars
5 mL syringe with stopcock and 23 gauge 1" needle	Grinding mill
1 mL syringe with stopcock and 23 gauge 1" needle	8 x 5 mm tin capsules
100 $\mu\text{L}$ gas-tight syringe	

### Procedure

1. Measure 1 g of crust material into 10 mL serum vial, and record the exact weight.

2. Pipette 50  $\mu\text{L}$  of distilled-deionized water onto the crust; crimp-seal the vial with a rubber stopper
3. Remove 5 mL of headspace using a 10 mL plastic syringe fitted with a stopcock and needle. Refill with 1 mL of  $\text{O}_2$  and 4 mL of  $^{15}\text{N}_2$ . Record the time of  $^{15}\text{N}_2$  addition. Incubate at  $27^\circ$  for 1 hour.
4. Using a 100  $\mu\text{L}$  gas-tight syringe, inject 10  $\mu\text{L}$  into the IRMS.
5. Open vial, record time. Calculate  $\Delta t$  (difference between the start and end times). Dry at  $70^\circ$  overnight.
6. Transfer as much of the crust material as possible to a glass scintillation vial. Add two  $\frac{1}{4}$ " grinding bars and cap the vial. Mill overnight, or until the material is pulverized to a fine powder.
7. Measure 25 – 30 mg of the pulverized crust material into an 8 x 5 mm tin capsule.
8. Measure the isotope ratio and %N using an automated nitrogen-carbon analyzer coupled to an IRMS.
9. Calculate the fraction of N in the soil derived from fixation:  
 $f = (S - 0.367) / (A - 0.367)$   
where  
S is the atom%  $^{15}\text{N}$  of the headspace air (measured)  
A is the atom%  $^{15}\text{N}$  of the soil crust (measured)
10. Calculate the nmol of N fixed per gram of crust:

$$N_{\text{fixed}} = f \times (\%N_{\text{crust}} / 100) \times (10^9 \text{ ng N nmol}^{-1} \text{ N}) / 14 \text{ ng N nmol}^{-1} \text{ N}$$

11. Calculate the hourly fixation rate:

$$\text{rate} = N_{\text{fixed}} / \Delta t \text{ (units of nmol N g}^{-1} \text{ h}^{-1}\text{)}$$

### REFERENCES:

- Dilworth, M.J. 1966. Acetylene reduction by nitrogen fixing preparations from *Clostridium pasteurianum*. Biochem. Biophys, Acta 127:285-294.
- Weaver, R.W. and S.K.A. Danso., 1994. Dinitrogen fixation. pp 1019--1045. *IN*: Weaver, R.W., J.S. Angle, and P.S. Bottomley (eds). Methods of soil analysis. Part 2. American Society of Agronomy, Madison, WI, USA.

Zuberer, D.A. 1998. Biological dinitrogen fixation: introduction and nonsymbiotic. *In* Principles and Applications of Soil Microbiology. D.M. Sylvia, J.J. Fuhrmann, P.G. Hartel, and D.A.

Zuberer Eds. Prentice Hall, Upper Saddle River, NJ.