

Geochemistry & Ecology of Red Mat Systems (GERMS) Summer Science Educator Program

Red Layer Microbial Observatory (RLMO)
National Science Foundation
Western Oregon University
Yellowstone National Park



Back in the lab, Part One Pigment Analysis

Goals – Big Picture

Characterize photosynthetic pigments in red and green mat layers and compare to available ID tables
The methanol method extracts naked pigment – good for phylum-level characterization
In vivo method extracts membrane/protein-bound pigments – good for genus-level characterization

Common Bacteriochlorophyll Absorption Properties

<u>Bacteriochlorophyll</u>	<u>in vivo abs.(nm)</u>	<u>methanol abs. max (nm)</u>	<u>Typical Phylogenetic Lineage</u>
Bchl a	805, 830-890	771	Purple/Proteobacteria**
Bchl b	835-850, 1020	794	Purple/Proteobacteria
Bchl c	745-755	660-669	Green Sulfur Bacteria
Bchl c-s	740	667	Green Nonsulfur Bacteria
Bchl d	705-740	646	Green Sulfur Bacteria
Bchl e	719-726	646	Green Sulfur Bacteria
Bchl g	670, 788	756	Gram Positive Heliobacteria

Mat Layer Processing – This Will Overlap With Microscopy

Retrieve and thaw 0.1 g samples of all mat layers collected
In general, one member of each team will assess red; the other green
Add 0.1 g sample to disposable mortar and pestle with 550 ul GTE
Grind sample until broken up and evenly-suspended
Remove 50 ul to one microfuge tube and place in freezer for microscopic viewing
Remove 250 ul to a 1.5 ul microfuge tube on ice - for in vivo/bead-beater processing
Pellet remaining 250 at 10,000 rpm for 5 minutes – for methanol extraction

Methanol Extraction

After 5 minute spin (designed to pull down cells) – carefully remove all supernatant
Add 1 ml methanol (do not touch on bare skin!) and resuspend cells (vortex and inversion)
Wrap in foil and place on ice for 10-30 minutes – this is when methanol “extracts” the pigments
Proceed to other procedures while waiting...
After complete, spin 5 minutes, 10,000 rpm (designed to pull down cell debris but not molecules)
Carefully remove supernatant to a clean 1.5 ul tube and wrap in foil
Place on ice until in vivo samples are processed
Then proceed to spectrophotometer and pigment absorption analysis

In vivo Bead-Beating (BB)

Fill special BB tube half full of .1mm zirconium beads, add 800 ul GTE, cap and invert to “wet” beads
Transfer 250 ul sample to tube and top off with GTE until no air space remains
Beat about 2-3 minutes (1 minute bead, 1 min ice, repeat) – use judgment to visually assess lysis
Spin 5 min. 5000 rpm, remove supernatant to a clean 1.5 ml tube and wrap in foil; place on ice

Spectrophotometric Analysis

Review how a spec works – emphasizing the use of blanks and the X/Y axes of output graphs
Prepare a 1 ml methanol only blank and a 1 ml GTE only blank – use for respective samples
Gather blanks and samples (on ice), p1000 and tips, and EXPENSIVE quartz cuvettes
I will walk you through spectrophotometer once; you will complete other runs on your own

- use methanol blank when analyzing methanol extracts; GTE for in vivo preparations
- if in doubt or if you need to start over, hit "main menu"
- use "chlorophyll program"
- rescale, trace, and print data to obtain better resolution and print peaks
- understand what the two axes mean and be prepared to explain them
- clean up everything when through; print-outs MUST appear in your notebooks!

Back in the lab, Part Two Microscopy and Digital Imaging

Goals – Big Picture

Simple microscopic assessment demonstrates color, cell shape, relative abundance
Fluorescence assessment allows some sorting of phototrophs based on pigment content
Compare and document red and green mat suspensions for cell and pigment types
Digitally capture 10X and 40X images of same sample view under normal light and fluorescence

Basic Microscopy

For this program, you will only be using the digital microscope; understand basic features
Always begin with 4X objective and focus sequentially to 40X objective
In general, we view wet mounts and large filaments that cannot be examined past 40X

Principle of Fluorescence Microscopy

Fluorescence microscopy requires an expensive UV light source, mirrors, and filters
High energy UV light is passed through different filters (R or F – see below) and shone upon sample
Certain molecules – like chlorophyll – is excited by light and emits lower energy light (= fluorescence)
A second set of barrier filters (each associated with R or F – see below) screens emitted light
Chlorophylls – associated with oxygenic phototrophs (plants, Cyanobacteria) fluoresce in F and R
Green bacteriochlorophylls – associated with some anoxygenics – fluoresce slightly in R, not in F
Red bacteriochlorophylls – associated with some anoxygenics – do not fluoresce in F or R

	Excitation Filter	Dichroic Mirror	Fluorescence Barrier
F = Ch. 2	Emits 460-490 nm	Passes < 505 nm to sample	Transmits > 510 nm
R = Ch. 3	Emits 530-550 nm	Passes < 570 nm to sample	Transmits > 590 nm

Image – Visible Light

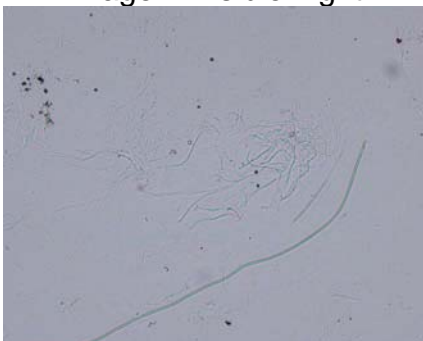


Image – F Fluorescence

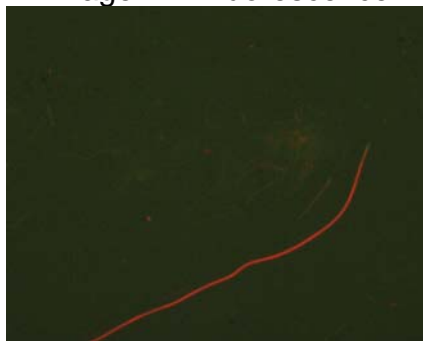
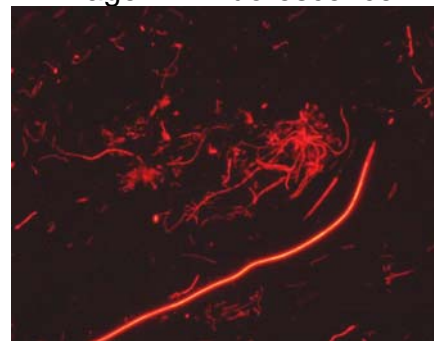


Image – R Fluorescence



Using the Fluorescence Microscope

Only your instructor will turn on the UV lighting system prior to this activity

Note the filter selection wheel: (1) no filter; (2) F filter system; (3) R filter system

When viewing with fluorescence, make sure the visible light source is off and the shutter is open

Image Selection and Capture

Make sure there is a CompactFlash card in the video capture system before you begin

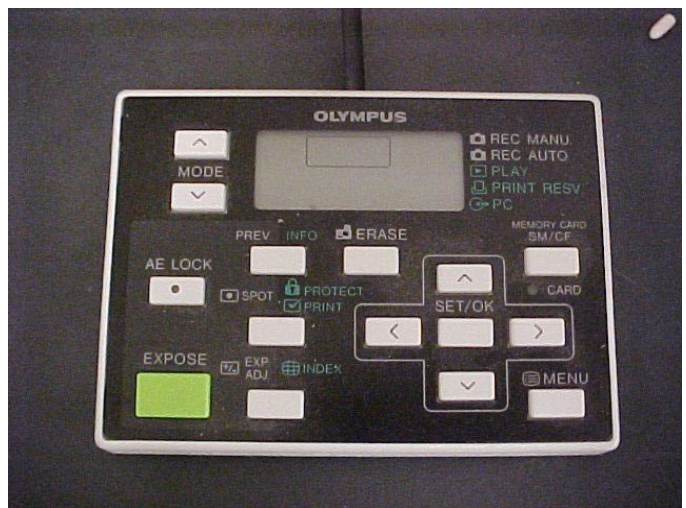
For all samples, search carefully until you find an area that contains well-dispersed filaments

You will be capturing the SAME sample view under 10X and 40X so check fields of view carefully

When you are ready to capture image, firmly press "PREV" to see what the image will look like

To get out of "PREV," firmly press "PREV" again (this will un-still the image back to streaming video)

To capture, press GREEN "Expose" button and make sure there was a big pause/capture moment



Mat Layer Processing - FINALLY

Retrieve and thaw 0.1 g samples of all mat layers collected

In general, one member of each team will assess red; the other green

Add 0.1 g sample to disposable mortar and pestle with 550 ul GTE

Grind sample until broken up and evenly-suspended

Remove 50 ul to one microfuge tube and place on ice for microscopic viewing

Place remaining 500 in another microfuge tube, label, and refrigerate for pigment analysis

Wet Mount Microscopy for Mat Layers

Obtain clean slide and coverslip; add 20 ul of mat suspension to naked slide

Carefully lay edge of coverslip on suspension and "slide" over drop to create thin film

Focus through 10X objective and search slide for appropriate areas for image capturing (see earlier)

Check same view using 40X objective before proceeding to digitally capture images

When completely satisfied with all views:

- (1) Shoot same view with 10X objective with light, R and F channels
- (2) Increase to 40X objective (same view) and shoot with light, R and F

Back in the lab, Part Three

Chemical Analysis of Acid-Digested Samples for Metal Assessment

Acid Digestion of Samples - Metals

Acidify sample at hotel - 5 ml 50% nitric acid per liter sample (SHOULD BE DONE)

At WOU, volumetrically transfer 250 ml acidified sample to flask (DONE FOR YOU THAT MORNING)
 Add 12.5 ml distilled 1:1 HCl (DONE FOR YOU THAT MORNING)
 Using a steam bath or hot plate, reduce volume to 30-35 ml. DO NOT BOIL! (IN PROGRESS)
 CAREFULLY adjust to pH 4 by drop-wise addition of 5.0 N NaOH
 DO NOT EXCEED pH 5 as some metals will precipitate and further tests will not work
 Quantitatively transfer the sample with deionized water to a 250 ml volumetric flask
 Proceed to specific metal protocols below

Note: you will have to adjust your final data because of the combined additions of the acid and then the base. If you do not keep track of how much base you are adding, you will be screwed.

For Example: (1000+5)/1000 [First addition of nitric acid] MULTIPLIED BY
 Final concentration read for each metal
 = Corrected concentration

Specific Post-Acid Digestion Protocol

Test Protocol With Acid Digested (AD) Sample	Special Notes
<p><u>Aluminum</u> Sample should be collected in glass or plastic bottle PROGRAM 1 Fill 50 ml cylinder with AD-sample Add 1 Ascorbic Acid Powder Pillow - invert to dissolve Add 1 AluVer 3 Aluminum Powder Pillow - invert to dissolve Press TIMER ENTER (3 minutes) - keep inverting Pour 25 ml into one sample cell (SAMPLE) Add 1 Bleaching Powder Pillow to cylinder Now TIMER will ask for ENTER (30 seconds) - SHAKE Pour cylinder into second sample cell (BLANK) TIMER ENTER (15 minutes) - wait ZERO BLANK and then READ SAMPLE Red-orange indicates aluminum</p>	<p>Test must be read immediately after timer. Actual detection limit: 0.013 mg/L. Phosphates interfere. No sites should interfere, however. Clean equipment IMMEDIATELY after using - soap and water.</p>
<p><u>Copper</u> Sample should be collected in glass or plastic bottle PROGRAM 20 Fill sample cell with 10 ml AD-sample (BLANK) and ZERO Fill CuVer2 AccuVac with sample (SAMPLE) - invert Press TIMER ENTER (2 minutes) - READ Sample Purple indicates copper</p>	<p>Actual detection limit: 0.02 mg/L. Ferric Iron will interfere; while we don't measure ferric per se, total iron is detectable.</p>
<p><u>Total Iron</u> Sample should be collected in glass or plastic bottle PROGRAM 33 Fill sample cell with 10 ml AD-sample (BLANK) Fill TPTZ AccuVac with sample (SAMPLE) - invert Press TIMER ENTER (3 minutes) ZERO BLANK and then READ SAMPLE Blue indicates ferrous iron</p>	<p>Actual detection limit: 0.04 mg/L. Known interfering compounds from sites: copper, molybdenum, and likely nitrite.</p>
<p><u>Manganese</u> Sample should be collected in a plastic bottle PROGRAM 41 Fill sample cell with 10 ml sample (BLANK) ZERO BLANK and remove BLANK cell To BLANK, add 1 Sodium Periodate Powder Pillow - invert 10 sec Press TIMER ENTER (2 minutes) and READ SAMPLE Violet indicates manganese</p>	<p>Actual detection limit: 0.12 mg/L. Deviations from acid digestion procedures will screw up this test.</p>

<p><u>Zinc</u> - DO NOT PERFORM ON HIGHLY ACIDIC SAMPLES Sample should be collected in a plastic bottle PROGRAM 97 Fill sample cell with 20 ml AD-sample (SAMPLE) Add 1 ZincoVer Powder Pillow* - invert to completely dissolve A positive sample will be orange; if brown - you will have to dilute Remove 10 ml of final cell contents to another cell (BLANK) Add 0.5 ml cyclohexanone to SAMPLE - SHAKE 20 seconds Press TIMER ENTER, add 21 drops PAN Indicator to each - Swirl Press TIMER ENTER (2 minutes) ZERO BLANK and READ SAMPLE</p>	<p>Actual detection limit: 0.02 mg/L.</p> <p>*CONTAINS CYANIDE - handle and dispose of properly (see note below).</p> <p>Two known metals at these sites - aluminum and copper (1-5 mg/L) - can interfere.</p>
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Cyanide Handling and Disposal Notes

NEVER use with a strongly acidic sample

Handle ONLY in a fume hood

After use, dispose of by mixing with strong solution of NaOH (pH = 11)

NEVER mix cyanide waste with other waste batches

Consult with chemistry to facilitate hazardous waste disposal (D003 level)