**Stream Team Analytical Lab**

**Standard Operating Procedure**

**SPECTROPHOTOMETRIC ANALYSIS OF CHLOROPHYLL *a* AFTER ETHANOL EXTRACTION**

ISSUE DATE: 11JUN2019

SUPERCEDES: 08DEC2017

I. Principle/Background

This is one of many similar methods estimating algal biomass by extracting phytopigments in solvent then quantifying them either spectrophotometrically or fluorometrically.

There are ongoing lively debates about best methods. While acetone extracts yield narrower absorbance peaks for the various chlorophylls, ethanol is a more efficient extractant, particularly for recalcitrant organisms, including cyanobacteria and green algae, even when samples are not physically disrupted. And while fluorometry can detect pigments at lower concentrations, it is subject to some uncorrectable interferences if the sample contains much chlorophyll *b* or *c* although methods are developing.

This method uses 96% ethanol at room temperature to extract pigments and is based on Wasmund et al. (2006) and adopts the acidification recommendation of Parker et al (2016). The quantitation used is the monochromatic spectrophotometric method for chlorophyll *a* after correction for the presence of pheopigments (Lorenzen 1967). The extinction coefficient (83.4 l g-1 cm-1) and acid factor (1.72, unitless) used in calculations are from Sartory and Grobbelaar (1984).

Collection and storage of samples will vary based on the study objectives and are only generally described here.

II. Equipment and Supplies

Glass fiber filters with appropriate effective pore size

Vacuum pump

Ethanol

Hydrochloric acid

Magnesium carbonate, basic

Screw-Capped 15-ml Polypropylene Centrifuge Tubes

Diamond-tipped pencil

Graduated cylinders or bottle top dispensers

Test tube/centrifuge tube rack

Forceps

Sharpie

Vortexer

Centrifuge

Spectrophotometer with a band width <=2nm

1-cm or larger cuvettes

III. Safety

The hazards of each reagent used in this method have not been fully established. Each chemical should be regarded as a potential health hazard and exposure should be minimized. A reference file of Safety Data Sheets is available and a Laboratory Chemical Hygiene Plan is in place. Chemicals used in this procedure that have the potential to be highly toxic or hazardous include:

Ethanol

Hydrochloric Acid

IV. Range and Rate of analysis

A. Range--depends on amount of material concentrated on filters and the length of the light path. Previous work suggests the lower detection limit of chl a by spectrophotometry with a 1 cm path length is ~34 µg/L in the extract (see Salinas 1988 and Excel spreadsheet “Chla detection limits”). It follows that if you are routinely concentrating 167x (1000 mls of water on a filter extracted in 6 mls ethanol) the detection limit will be ~0.2 µg/L. The detection limit for pheopigments is similar.

B. Rate—after extraction and centrifugation, the spectrophotometric analysis will take ~5 min per sample.

V. Reagents

1. Saturated water solution of basic magnesium carbonate- Add~200 mg of basic magnesium carbonate (also known as light magnesium carbonate or magnesium carbonate hydroxide or magnesium hydroxide carbonate. It can be confusing so check the CAS # 39409-82-0) to 200 mls reagent grade water. Mix well and let settle for > 48 h. Decant the clear solution into a new container for subsequent use. Use only the clear “powder free” solution in subsequent steps.
2. 96% Ethanol—Dilute100% ethanol with saturated water solution of basic magnesium carbonate, i.e., for 200-mls add 192-mls of buffered 100% ethanol to a graduated cylinder and add saturated water solution of basic magnesium carbonate to bring to 200-mls.
3. 0.1 M Hydrochloric Acid—Work under a fume hood and wear coat, apron, goggles, and gloves. Put ~500 mls of Type I reagent grade water in a 1 L volumetric flask. Add 8.3 ml concentrated HCl. Mix and allow to cool if necessary, then bring to a final volume of 1-L with Type I reagent grade water.

VI. Procedure

1. General information on collecting samples
2. **IMPORTANT NOTE**: Chl *a* will break down into pheopigments with exposure to light, heat, or acidic conditions. It is very important to protect your samples as much as possible during all stages of processing and storage, so work under subdued light as much as possible and keep samples cool.
3. **Err on the side of extracting too much, rather than too little sample** because it is possible to dilute your samples after pigment extraction but it’s impractical to concentrate a sample after extraction. Whenever possible, you want a sample that will have an initial absorbance >0.100. That solution will be visibly green or gold. This has been hard to achieve with our typical samples.
4. Suspended material like plankton or epilithon brushed off rocks is concentrated by filtering material onto a glass fiber filter. Different projects use filters with different effective pore sizes. Make sure you are using the correct filter for your project. Generally, vacuum filtration should not exceed ~20 kPa (15 cm Hg) or take much longer than 10 min. Keep cool until filtering and filter as soon as possible and within 24 h. Typical water volumes to filter for plankton in heterotrophic systems are 1 L or more. Use previous results to guide you as to volume required. You may need to use more than one filter for a single extraction.
5. For extractions from soil or sediment, typically soils are dried and sieved. Then 10 or more g are weighed into a 50-ml centrifuge tube and 30 ml of 96% ethanol added, and mixed.
6. When possible, prepare a duplicate sample for at least one sample in each batch.
7. To preserve samples, generally we have frozen at -20 C.
   1. For filters, fold the filter in half with the material on the inside. Wrap in aluminum foil to exclude light. Collect wrapped filters in a zipper-lock freezer bag. Freeze at -20 C. If colder storage space is available, it is preferred, but be consistent over the length of a project. Note that while we (like many labs) freeze the filters, Wasmund et al (2006) found lesser changes with storage with samples stored as extracts (although it looks like the effects might be dependent on algal composition). There is considerable disagreement over storage lengths. Salinas (1988) found no significant difference in chl a of filters stored frozen at -9 C over 238 days. Both APHA and EPA methods suggest storage times up to a month. But other researchers suggest that storage at -20 C is only adequate for days to weeks, and storage at -80 C is adequate for months.
   2. For sediments or soils, the sample in ethanol is frozen.
8. Extracting samples
   1. Pull the samples from the freezer and keep them in the dark to warm up to room temperature. When possible include a Chlorella reference sample.
   2. Place one or more filters into 6 mls of buffered 96% ethanol in a 15-ml centrifuge tube. Curl the filter to fit in the tube and insert into the tube using a pair of forceps and/or gloved hands– be sure that the filter is not folded too tightly and make sure it is completely submerged (the ethanol has to be in close contact with the material on the filter). Agitate the tube.
   3. Prepare 3 additional tubes with just the ethanol and a clean filter and process them with the samples as blanks.
   4. Leave the samples at room temperature in the dark for 3 to 24 h for extraction to complete. Leaving them overnight is usually convenient. Agitate the tube.
   5. After extraction, agitate the tube, then remove the filter using forceps. The filters are dried, then disposed of in the lab debris.
   6. Centrifuge the tubes for ~5 min at ~1000 x g (USEPA 1997). (This is 2500 RPM on the Centra CL2 using the 6 place swinging bucket-see centrifuge notes and manual for details). If the solution still looks cloudy, centrifuge at up to 3900 RPM for up to 15 min.
9. Analyzing samples - Measure absorbance of each extract on the spectrophotometer at at least 3 wavelengths (750, 664, 665 nm) before and after acidification
10. Set the spectrophotometer up for 1 cm cuvettes. The 1 cm cuvette holder has two upright towers for holding cuvettes, and can only be inserted into the machine one way. Both cuvette holders (1 cm and 10 cm) are kept in the drawer below the instrument.
11. Find a matched pair of quartz cuvettes in the drawer below the instrument. Check that the cuvettes are clean. They can be washed with ethanol. Only if necessary, rinse well then wipe with lens tissue. Do not rub with material that can scratch. Be sure to insert them into the spectrophotometer facing a consistent direction from one sample to the next.
12. Operating spectrophotometer
    1. Make sure there are no cuvettes in the spectrophotometer before you turn it on.
    2. Turn on computer monitor, computer CPU, and spectrophotometer (power switch is on right side towards the front of the instrument).
    3. Start UVProbe software on computer.
    4. The instrument will go through self-check and you can see the progress on the instrument.
    5. When it is finished with the self-check, the instrument will ask for a password. There is none. Just push enter.
    6. The Instrument will now show the mode selection screen. The 4th hot-key (along the bottom right of the screen is PC-CONTROL. Push F4 to allow computer control.
    7. In software, select the mode (i.e. Spectrum, Photometric, Kinetic, Report). You want Photometric Mode. The icon is a red volumetric flask.
    8. Now push “Connect” button. If this is successful, the Photometer status box in lower left corner will no longer say “OFF” and will instead display a wavelength and absorbance. The command buttons along the bottom of the screen (AutoZero, Baseline, Go To WL, Read Unk, Disconnect) will no longer be grayed out.
    9. Let the spectrophotometer warm up for at least 30 min.
    10. Basic Photometric Mode is straightforward. You will open or create a method to define wavelengths. Fill in the “Sample Table” to assign IDs to samples. Use the buttons at the bottom of the screen (Cell Blank, AutoZero, Baseline, Go To WL, Read Unk, Disconnect) to proceed through the analyses.
    11. Open one of 2 methods already set up for chlorophll (e.g., File<Open, UVProbe-Data\Methods\Chl 3 ways.pmd). You will be prompted to immediately save under a new name as a data file (file extension is .unk). Use your initials and the date in the name (i.e., BRNsamples10jun19). Alternately, you can open an old data file using the correct method, then rename it and clear the sample table.
        1. “Chl 3 ways.pmd” is set up to read each sample at 8 wavelengths needed for 3 methods of calculating chlorophyll a; pheophytin corrected method (Lorenzen 1967), trichromatic method (Jeffrey and Humphries 1975), and scytonemin containing method (Garcia-Pichel and Castenholz 1991).
        2. “Chl Pheo.pmd” is set up to read at 3 wavelengths for the Lorenzen calculation and has a 120 sec delay programmed in so you can add the acid, click “Read Unknown” and the instrument will count down 120 sec before initiating the reading. This will slow down the before acidification reading, but it saves having to have a timer.
    12. Click anywhere in the sample table to activate it. “Active” should display in the header bar. Enter identifiers into the Sample ID column. The program eliminates spaces, so put in underscores if you prefer. You will need one line for sample before acidification and another line for the sample after acidification. The program will put an asterisk next to the active line.
    13. Autozero with nothing in the compartment, no liquid, no cuvettes.
    14. Fill both cuvettes with matrix (i.e., 96% ethanol). Autozero twice. Then click “Cell Blank”. (This is most commonly done on empty cuvettes, but we want to correct absorbances for extractant absorbance as well. The corrected absorbances for the reagent blank ideally should be zero. A pop up box titled “Cell Blank Data List” will detail the absorbances that will be subtracted from every reading. From Manual: “When Cell Blank clicked on the Photometer Button bar, the spectrophotometer takes a reading and subtracts this reading from all subsequent readings during data acquisition. The purpose of the cell blank operation is to eliminate the effect of the cuvette from the results. The cell blank reading remains valid until another Cell Blank or an Auto Zero is performed. “ This procedure can be repeated during the run if needed.
    15. Leave the cuvette with 96% ethanol in back position. It is the reference. **On long days, check every couple of hours to make sure evaporation has not reduced the liquid level enough to interfere with absorbance readings.**
    16. Start analyzing samples.
        1. Add 3 ml of sample extract to the cuvette using a pipet. The same tip may be used throughout the procedure if it is rinsed between uses. You may need to blot (NOT rub) droplets off the outside of the cuvette with a kimwipe.
        2. Place the sample in the front holder. Close the lid. Wait ~10 sec for any turbidity to settle out (you can see this happening as the absorbance at 750 nm displayed on the screen stabilizes) then click “Read Unk” on the PC. You will see the sample table populated with the absorbance readings at each wavelength.
        3. Remove the sample cuvette from the spectrophotometer. Add 240 µl of 0.1 N HCl with a pipet and start timing 120 seconds. Then **thoroughly** mix the sample with a Pasteur pipette. **Failure to mix the sample after acidification is a very common problem with this analysis, so take care in this step**. Replace the cuvette in the spectrophotometer.
        4. At 120 sec after having added the acid, click “Read Unk” on the PC.
        5. Collect cuvette contents into a container for later disposal and **rinse the cuvette > 2x with 96% ethanol** so there is no residual acid to affect the next sample. Collect the rinse in the same container.
        6. Repeat.
    17. As you are collecting data, monitor for quality problems and flag problems.
        1. If absorbance at 750 nm is not close to zero (~+ 0.005), there is a problem. You may need to check your sample for turbidity and possibly re-centrifuge or filter the sample. You may need to rerun the “Cell Blank” on the instrument with 96% ethanol in front and rear cuvettes.
        2. **If the reading before acidification at 664 nm is less than ~0.030 absorbance units, the chlorophyll and pheopigment in the sample may be below detection.** Next time, concentrate more water onto the filter or extract 2 filters per vial. **The best determinations come from samples with initial absorbances >0.100.**
        3. The absorbances at 664 nm and 665 nm should be similar.
        4. The absorbances before and after acidification will be roughly similar, but the readings after acidification will likely be a little lower (from 100% to 60% of the before absorbance). If you see another pattern, there is likely to be a problem.
        5. If the reading before acidification at 664 nm is >2.0, dilute with 96% ethanol before proceeding.

# Save your data any of several ways. Save the UVProbe file in the software (file type will .unk and can only be opened in UVProbe). Save as an ASCII file to get a common delimited .txt file you can import into R. Cut and paste from sample table into Excel. Or create a report.

# To turn off the instrument:

# In UVProbe software click “Disconnect”.

# Then Click the X button on upper right of UVProbe window.

# Instrument screen prompts “Press RETURN to switch to mode menu”. Press RETURN.

# Instrument will ask for a password. There is none. Just push enter.

# Turn off power to instrument.

# Fill out appropriate information in the instrument log book.

# Rinse the cuvettes with clean ethanol and leave them to dry on a kimwipe. Replace them in the box and in the drawer as soon as they are dry.

# Wipe down the lab bench with water and paper towels.

1. Cleaning up
   1. Extracts and all rinses should be collected in a well labeled screw cap container and, when full, picked up for disposal as hazardous waste.
   2. Centrifuge tubes should be emptied and dried, then picked up for disposal as lab debris (blue bag).
   3. Filters are also dried, then picked up for disposal and as lab debris (blue bag).

VII. Data analyses, reporting, and management.

A. Calculations (see USEPA 1997 Section 12.2 for a summary, lab Excel workbook titled “Chla calculations using 3004 spec” for an example, and lab document “More than you ever wanted to know about Chl a calculations” for details). An example of typical data to help illustrate Lorenzen calculations is below.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Sample ID | Before 750 | Before 664 | Before 665 | After 750 | After 664 | After 665 |
| Blank 1 | 0.000 | 0.000 | 0.000 | -0.001 | -0.002 | -0.002 |
| Blank 2 | 0.000 | 0.000 | 0.000 | -0.001 | -0.002 | -0.002 |
| Blank 3 | 0.000 | 0.000 | 0.000 | -0.002 | -0.002 | -0.002 |
| Sample 1 | 0.004 | 0.134 | 0.133 | 0.005 | 0.095 | 0.096 |
| Sample 2 | 0.003 | 0.048 | 0.047 | 0.003 | 0.037 | 0.037 |
| Sample 3 | 0.001 | 0.087 | 0.087 | 0.002 | 0.079 | 0.079 |

B. Before acidification, 96% ethanol typically has an initial absorbance of 0.000 for all wavelengths if “Cell Blank” was used to zero out absorbances. This absorbance can drift slightly over the course of the run. Typical absorbances for the blank after acidification are slightly lower, ~-0.002,

C. Correct the absorbances for turbidity

Abs 664b = Before 664 – Before 750

and

Abs 665a = After 665 – After 750

D. NOTE: if you will be calculating pheopigment concentration, any absorbance signal from the blank does not drop out in the calculation. If the ethanol blank has a non-zero absorbance after turbidity correction, you may need to correct for blank absorbance before calculations. This is likely only important at low levels where any signal in the blank will be mistakenly interpreted as pheopigment.

E. Using the corrected absorbances, calculate the concentrations of chlorophyll a and pheopigments in the extract as µg/L (Ce and Pe, respectively). The 28.64 in the equations incorporates the absorption coefficient of chlorophyll a and a factor describing the reduction in absorbance with acidification for pure chl a. This factor is specific to extractant. The 1.72 in the equations is the maximum ratio of absorbances before and after acidification that is typical of chlorophyll a in ethanolic extracts. See Wetzel and Likens (1991), Jeffrey and Welchmeyer 1997, or lab document “More than you ever wanted to know about Chl a calculations” for details.

Ce = 1000 x 28.64 x (Abs 664b – Abs 665a)

and

Pe = 1000 x 28.64 ((1.72 x Abs 665a) – Abs 664b)

Where

C = concentration (µg/L) of chlorophyll *a* in the extract

solution measured,

P = concentration (µg/L) of pheophytin *a* in the

extraction measured.

F. Calculate pigment concentrations (µg/L) in the original sample as

Where DF is dilution factor (if any). If you want to express your pigment concentrations on an areal or mass basis, substitute those units into the denominator of the equation.

For Sample 1 in the example, 500 mls of water was concentrated on the filter, then extracted in 6 ml ethanol:

chlorophyll a in µg/L =1000 \*(28.64 \* ((0.134-0.004) – (0.096-0.005) \* 0.006) / (0.5\* 1) = 13.4

and

pheopigments in µg/L = 1000 \* (28.64 ((1.72 \* (0.096-0.005)) – (0.134-0.004)) \* 0.006) / (0.5\* 1) = 9.1

Note: In many stream samples, pheopigments will constitute a large fraction of the material extracted (e.g. Lorenzen 1967, p. 346). There can be a lot of senescent cells in epilithon. Don't be surprised if you have more pheopigment than chlorophyll.

G. Vet the data.

* 1. If the pigment concentration in the extract is <34 ug/L, report the concentration as below detection
  2. Other data to flag and reevaluate are those for which the absorbance before acidification is <0.030 and any for which the before/after absorbance ratio is not between 1 and 1.72.

VIII. Supporting Information

A. Technical Support

1. Hardware-- Shimadzu UV-1800 spectrophotometer Instruction Manual

2. Software—UVProbe manual

B. Expendable supplies

1. 15-ml polypropylene centrifuge tubes--Fisher 14-959-49B Case of 500 <$166.00.

2. Ethanol from Chemistry Stockroom- Catalog # SOL-140, Ethanol, 100%, 20L ~$80.00. Take a PO number from a completed Interdepartmental Services Request.

3. QC/QA samples--Chlorophyll a (CAS # 479-61-8, FW 893.5) From Sigma- Aldrich C6144. And Chlorella as reference material.

C. Waste Labeling

|  |  |
| --- | --- |
| Constituent | % of waste |
| Ethanol | 96 |
| Water | 4 |
| Magnesium carbonate, basic | trace |
| Hydrochloric Acid | trace |
| Chlorophyll | trace |
| pH of waste | NA |

X. REFERENCES:

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