WOW-5-15-03

CHLOROPHYLL AND PHAEOPHYTIN DETERMINATION
Spectrophotometric Method

General: Estimating the concentration of chlorophyll-*a* remains the most common method for assessing algal biomass. The concentration of chlorophyll-*a* has also been shown to relate to primary productivity (Wetzel 1983) and can be used to assess the physiological health of algae by examining its degradation product, phaeophytin. This degradation product has been shown to contribute 16-60% of the chlorophyll-*a* content in seawater and freshwater. Despite numerous innovations such as HPLC, and dozens of conflicting reports regarding extraction and analytical protocols, most state resource and regulatory agencies still appear to rely on, and in fact require, methods listed in Standard Methods (1992 or more recent) and in some cases EPA (1987). Therefore, spectrophotometry and fluorometry, utilizing 90% acetone extraction, remain the most commonly used methods. Spectrophotometry is most widely used but fluorometry is more sensitive and may be used when low levels of chlorophyll are anticipated or collection and filtration of large volumes of water pose major logistical problems.

This method was adapted from the Lab Manual of the Central Analytical Laboratory at Natural Resources Research Institute, University of Minnesota-Duluth (Ameel, J., E. Ruzycki and R.P. Axler. 1998. Analytical chemistry and quality assurance procedures for natural water samples. 6th edition. Central Analytical Laboratory, NRRI Tech. Rep. NRRI/TR-98/03 revised annually). These methods are based on American Public Health Association (APHA) and US EPA compilations of water quality methods and the Lab and its Methods Manual are certified biannually by the Minnesota Department of Health. For ease of use by teachers using the *Water on the Web* curriculum, some simplifications have been added since class data will likely be used only for instructional purposes. Where appropriate, the *WOW* lecture module slides will include troubleshooting tips and techniques suggestions.

Safety: It is assumed that instructors are proficient in the handling of hazardous chemicals. Dangerous concentrated acids and toxic and carcinogenic chemicals may be used in some methods.

Sample Collection: Lakes- collect whole water samples usually from the surface or via a 2 meter tube sampler for monitoring purposes or from different depths using remote closing samplers (Van Dorn, Kemmer etc). Streams- periphyton can be scraped from a known surface area off rocks, logs or other substrates. Specific details are in the appropriate *WOW* Field Modules (8/9 and 10/11, respectively).

Equipment:
- Extaction:

1. Prewashed 47 mm glass fiber filters (GF/C or AE or equivalent)
2. Gelman polycarbonate filtration tower (or equivalent)
3. Vacuum pump
4. Centrifuge (Clinical is sufficient)
5. DIW / acetone washed 15 mL Corex centrifuge tubes with caps

- Spectrophotometry

1. UV/VIS spectrophotometer (1 nm spectral band width preferred but useable data may be obtained with a bandwidth of ~5-10 nm if necessary)

2. Optically matched 4 cm micro-cuvettes (1 cm will suffice although the detection limit will increase accordingly)

Reagents:

1. 90% acetone **[*T]**: (900 ml acetone + 100 mls MQW (MQW) + 0.1 ml conc. NH_4OH **[*C]**
2. Chlorophyll-*a* stock (10,000 ppb) Chlorophyll-*a* from *Anacystis nidulans* (Sigma chemical C6144) (see Working Standards)
3. 0.12 N HCl: (1.92 mL concentrated HCl **[*C]**+ 200 mL MQW)
4. Saturated $MgCO_3$ solution: (1.0 g $MgCO_3$ + 100 mL MQW)

Hazards:

***T = toxicity hazard.** This is a type of chemical hazard but is flagged separately since the chemical may be extremely toxic to biological systems if ingested or inhaled. Special handling procedures are listed in the Material Safety Data Sheet (MSDS).*

***C = chemical hazard**. The reactivity of the chemical or chemicals with each other or tissue involved through contact may create a reaction that is potentially injurious through the creation of heat, explosion, toxic gases and/or destruction of exposed tissue.*

Filtration Procedure:

1. Concentrate chlorophyll by filtration as soon as possible after collection (< 24 hours and store water in refrigerator or in the dark on ice). Filter an appropriate volume of water (depending on the specified analytical procedure and the estimated chlorophyll concentration) through a pre-washed 47mm glass fiber filter (Whatman GF/C or Gelman AE), using Gelman polycarbonate filtration towers, under low to moderate vacuum (< 8 PSI).

2. Add 0.15 mL of a saturated $MgCO_3$ solution to the sample just prior to the end of filtration. The $MgCO_3$ acts to buffer the sample preventing premature phaeophytinization. If added prematurely, it may greatly increase the filtration time.

3. Fold the moist filter into quarters, wrap in aluminum foil and store frozen at $-10^{o}C$.

4. Record volume of water filtered and sample date and location with a permanent marker pen on a piece of tape wrapped around the foil packet. Packets may then be stored in ziplok baggies or in a jar in the freezer until analyzed. If filtered in the field, be sure to double bag or better yet use a jar with a water-proof lid to store the foil packets – ice melts in field ice coolers and if it soaks the filters it will ruin them.

Extraction Procedure:

<u>Under subdued light</u>.

1. Tear filters into quarters with forceps*.
2. Place filter in a 15 ml centrifuge tube.
3. Add 10 mL 90% acetone.
4. Cap and extract overnight in the dark at 4 ^{o}C (refrigerated).

** Sometimes periphyton samples contain blue-green or green algal filaments. Often the chlorophyll within these filaments will not extract completely without physical disruption. This can be done using a tissue grinder but care must be taken not to overheat the sample (remember that heat degrades chlorophyll). Place the filter in the grinder, then place the grinder into a beaker filled with crushed ice.*

Working Standards:

*Make up stock and working standards under subdued light conditions. Keep stock solution in dark at all times.

Stock chlorophyll-*a* solution (10,000 ppb): Place 1 mg of purified chlorophyll-*a* in 100 mL of 90% acetone (make stock solution at least 24 hours prior to analysis).

500 ppb chlorophyll-*a* solution: Add 2.5 mL of stock solution to 50 mL of 90% acetone.

300 ppb chlorophyll-*a* solution: Add 1.5 mL of stock solution to 50 mL volumetric flask. Dilute to mark with 90% acetone.

150 ppb chlorophyll-*a* solution: Add 0.75 mL of stock solution to 50 mL volumetric flask. Dilute to mark with 90% acetone.

Spectrophotometric Determination of Chlorophyll-*a* and Phaeophytin:

Chlorophyll-*a* and phaeophytin concentrations in the extract are determined spectrophotometrically using the standard method for chlorophyll-*a* in the presence of phaeophytin (as detailed in Standard Methods 1998). A Perkin-Elmer Lambda 3B UV/VIS spectrophotometer with a 1 nm spectral bandwidth and optically matched 4 cm micro-cuvettes are used at NRRI.

1. Measure absorbance of a 90% acetone solution blank at 750 nm and at 664 nm to correct for primary pigment absorbance.

2. To measure phaeophytin, acidify the 4 mL of solution in the cuvette to a final molarity of 3×10^{-3} by adding 100 ul of 0.12N HCl to the 4 mL of extract in the cuvette. Allow acid to react for 60 seconds and record the absorbance at 665 nm and again at 750 nm. This is a correction made for the 665 nm wavelength (for phaeophytin).

3. Run working standard solutions of purified chlorophyll-*a* (Sigma Chemical Co. *Anacystis nidulans* by the procedure used for the blank).

4. Clarify sample filter extracts by centrifuging at 4,000 rpm for 20 minutes. Then read sample and standard absorbances at 750 nm and 664 nm before acidification (750_b and 664_b) and 750 nm and 665 nm after acidification (750_a and 665_a). Chlorophyll-*a* and phaeophytin concentrations are calculated by the following equations from Standard Methods (1998) :

$$\text{Chlorophyll-}a\ (\mu g / L) = \frac{26.7[E_{664b} - E_{665a}] \times V_{ext}}{V_{sample} \times L}$$

and

$$\text{Phaeophytin}\ (\mu g / L) = \frac{26.7[1.7E_{665a} - E_{664b}] \times V_{ext}}{V_{sample} \times L}$$

where:

b = before acidification

a = after acidification

E_{664b} - $[\{Abs_{664b(sample)}-Abs_{664b(blank)}\}-\{Abs_{750b(sample)}-Abs_{750b(blank)}\}]$

E_{665a} - $[\{A_{665a(sample)}-Abs_{665a(blank)}\}-\{Abs_{750a(sample)}-Abs_{750a(blank)}\}]$

V_{ext} = Volume of 90% Acetone used in the extraction (mL)

V_{sample} = Volume of water filtered (L)

L = Cuvette path length (cm)

References

Standard methods for the examination of water and wastewater. 1998. 20th edition. American Public Health Association, Washington, D.C.

Axler, R. C. and C. J. Owen. 1994. Measuring chlorophyll and phaeophytin: whom should you believe? Lake and Reservoir Management 8:143-151.

Axler, R., C. Rose, and C. Tikkanen. 1991. An assessment of phytoplankton nutrient deficiency in northern Minnesota acid sensitive lakes. NRRI Tech. Rep. NRRI/TR-91-18, Natural Resources Research Institute, University of Minnesota, Duluth, 55811. 114 pp.

Baker, K.S., R.C. Smith, and J.R. Nelson. 1983. Chlorophyll determinations with filter fluorometer: Lamp/filter combination can minimize error. Limnol. Oceanogr. 28:1037-1040.

Brown, J.M. and C.D. Goodyear. 1987. Acid Precipitation Mitigation Program: Research

methods and protocols. U.S. Fish and Wildlife Service, Nat. Ecol. Res. Center, Leetown, WV. NERC 87/27.

EPA. 1987. Handbook of methods for acid deposition studies: Laboratory analysis for surface water chemistry. EPA 600/4-87/026, September 1987, U.S. Environ. Protec. Agency, Washington, DC.

Wetzel, R.G. 1983. Limnology. 2nd Edition. Saunders College Publishing, Philadelphia, PA.

Note: A Sequoia-Turner 450-003 "factory equipped" for high chlorophyll sensitivity with NB430 excitation filter, SC665 emission filter and a red-sensitive photomultiplier tube could also be used with the following modifications: The instrument is zeroed with 90% acetone on gain 5 and the fluorescence recorded for all other gains. The factor, D = 50 = gain 5 x 10 mL extract volume in the calculations.