Analysis of Phytoplankton Pigments: Determination of Chlorophyll a

Phytoplankton biomass can be estimated by the photosynthetic pigment, chlorophyll $a$, which is found in all phytoplankton cells. The procedure described here allows one to measure the chlorophyll $a$ from phytoplankton cells using a fluorometer, which detects the fluorescence of the chlorophyll molecule. The procedure consists of isolating the cells, extracting the chlorophyll, and measuring the fluorescence.

The most typical way to separate phytoplankton cells from seawater is to filter the seawater sample to concentrate all the particles onto a filter. The filters are then soaked in a solvent that will extract the pigments from the cells. The solvent most commonly used for this is 90% acetone, which has been shown to give the most efficient extraction without pigment alteration (Mantoura and Llewellyn, 1983).

There are three commonly used methods of extraction: sonication, grinding, and freezing. The idea is to rupture the chloroplasts so that the pigments can elute into the acetone without degradation. A sonicator does this by emitting 3000 watts of acoustic energy to disrupt the cellular membranes, whereas grinders employ mechanical force to shear the cells. Another method is to simply freeze the cells in acetone for 24-48 h, to allow the pigments to diffuse out of the cell. Some researchers have found that the freezing extraction technique can be more variable, so use of a sonicator is recommended. Sonication has been found to be just as efficient as grinding and because no sample handling is involved accuracy is improved.

Once the cells have been disrupted the samples need to sit to ensure a complete extraction; then the extracts are centrifuged to clear away all particulate matter (e.g., filter, cells) so that a pure extract of pigments in 90% acetone remains. This extract can then be measured in a fluorometer (to detect chlorophyll fluorescence) or a spectrophotometer (to detect light absorbance by chlorophyll), or it can be used for chromatography to separate chlorophyll $a$ and all the other phytoplankton pigments. The most common phytoplankton pigment analysis in oceanography is the fluorometric determination of chlorophyll $a$ to estimate phytoplankton biomass.

Fluorescence is the ability of a substance to absorb light at one wavelength and emit light at another longer wavelength. Chlorophyll $a$ in 90% acetone receives maximum excitation at 430 nm and yields maximum emission at 670 nm. A fluorometer is an instrument that excites the solution and can read the subsequent emission.

There are many models of fluorometers and each is a bit different. However, all emit light from a fluorescent lamp that passes through a primary blue filter ($\lambda = 430$ nm) to excite the sample. The emitted light from the sample passes through a secondary red filter ($\lambda = 670$ nm) and on to the red-sensitive photomultiplier tube, which sends numeric output based on the signal strength. The readings are unitless, commonly reported as relative fluorescence units. Different fluorometers have different ways of controlling the sensitivity so that readings are kept on scale; some do it automatically.

The fluorometric method (Lorenzen, 1966) assumes that there are two pigments in the extract that fluoresce: chlorophyll $a$ and phaeopigment. The initial reading of the extract ($F_0$) is the fluorescence of chlorophyll $a$ and any phaeopigment present. Then 10% HCl is added to convert all of the chlorophyll $a$ to phaeopigment, and another reading is taken ($F_a$). Both readings are used in the calculations explained below.
In reality there may be other pigments fluorescing: chlorophyll \( b \) and \( c \) and their respective phaeopigments. Although chl \( c \) has been shown to not significantly affect the calculations (Vernet, 1983), chl \( b \) can cause an overestimation of phaeopigment by up to a third (Vernet and Lorenzen, 1987). Welschmeyer (1994) developed a fluorometric method to avoid chl \( b \) interference. Because chl \( b \) is typically rare in temperate marine waters, we will use the original method. If working in lakes or in the deep chlorophyll maximum of subtropical waters, other methods should be considered.

**Fluorometric determination of Chlorophyll a**

*Concentration of Cells and Extraction via Sonication:*

1. In all steps, remember to avoid degradation of pigments. Minimize exposure of pigments to light and heat.
2. Set up filtering apparatus with glass fiber filters (Whatman GF/F, nominal pore size = 0.7 \( \mu \)m). Cover filter with a small squirt of saturated MgCO\(_3\) solution (1 g in 100 mL distilled water).
3. Filter water of suitable volume (in coastal waters, this can be as little as 60 mL; in oceanic, try 250 mL) using a pressure less than 13 cm Hg.
4. Fold filter, place into centrifuge tube making sure the middle of the filter is facing down, cover with 10 mL of 90% acetone, and cap tightly. (90% acetone is 100 mL of distilled water brought to 1.0 L in a volumetric flask with reagent grade acetone. Be sure to shake mixture and then re-adjust volume to 1.0 L.)
5. Place centrifuge tubes into a cold ice-water bath with the partially submerged sonicator probe. Turn on sonicator probe power for 7 minutes.
6. Let samples sit in the cold and dark 10 minutes or more. At this point, the samples can be stored in freezer or refrigerator for several days, if necessary.
7. Shake the samples well—this is very important (and easily forgotten), otherwise the extract will remain concentrated around the filter and your reading will be erroneous.
8. Place samples in centrifuge for 5 minutes at about 5000 rpm (high speed). Ensure that the centrifuge tubes are the correct type for the rotor head you are using.
9. Keep extracts in the cold and dark until reading.

*Fluorometric Measurement of Chlorophyll a and Phaeopigments:*

1. Turn the fluorometer on approximately 30 min before use.
2. Fill a cuvette with 90% acetone and zero the fluorometer. This "blank" should be repeated every 10-15 samples or whenever the scale is changed, unless your fluorometer auto-scales.
3. Pour sample extract into cuvette, being careful to not disturb the filtrant at the bottom. Take a reading. If your fluorometer does not auto-scale, be sure the reading is between 25-90% of full scale. The readings are non-linear outside of this range. If the reading is too high, use a less sensitive scale or dilute; if it is too low, use a more sensitive scale. **This is the \( F_0 \) reading.**
4. Add two drops of 10% HCl to the cuvette and take another reading after the fluorometer has stabilized. **This is the \( F_a \) reading.** \( F_a \) will be lower than \( F_0 \), that is normal. Read both \( F_0 \) and \( F_a \) on the same scale.
5. IMPORTANT—be sure to rinse cuvette with acetone very well (3 times) before the next sample to remove all traces of the acid.

**Calculation of Chlorophyll a and Phaeopigment Concentrations:**

Equations (Lorenzen, 1966):

\[
\text{Chl}_a \left( \mu g/L \text{ or mg/m}^3 \right) = \frac{F_0/F_a \max}{F_0/F_a \max - 1} \cdot K_x \left( F_0 - F_a \right) \cdot \frac{\text{volume of water filtered (in liters)}}{}
\]

\[
\text{Phaeo} \left( \mu g/L \text{ or mg/m}^3 \right) = \frac{F_0/F_a \max}{F_0/F_a \max - 1} \cdot K_x \left( F_0/F_a \max \cdot F_a - (F_0) \right) \cdot \frac{\text{volume of water filtered (in liters)}}{}
\]

where:

- \( F_0/F_a \max = \) the ratio of \( F_0 \) to \( F_a \) for a sample which contains only chlorophyll and no phaeopigments, around 1.8 to 2.2 (variability is due to different instruments)
- \( K_x = \) calibration factor, to be determined for each fluorometer.
  - Usually \( K_{1x} \) is determined, then
  - \( K_x = \frac{K_{1x}}{\text{factor for scale used}} \)

These equations assume you extracted in 10 mL acetone volume. If you used a different extract volume, you will have to adjust the chl and phaeo concentrations by a factor of (volume used (mL)/10 mL).

If the sample extract was diluted, you will have to adjust by the dilution factor. For instance, if you used 1 mL of extract to 4 mL of acetone for a total volume of 5 mL, this is called a dilution of 1 in 5. Chl and phaeo concentrations are calculated as above and then the results are multiplied by the inverse of the dilution ratio, or, in this case, 5.

**Calibration of the Fluorometer:**

Often the fluorometer has already been calibrated for you and the coefficients will be provided along with the instrument. The calibration technique is described here:

1. Use pure \( \text{chl}_a \) (available from Sigma) or an exponentially growing culture of an alga without \( \text{chl}_b \) (diatoms are good candidates). Filter a large quantity, enough so that there is good color on the filter. Extract in 10 mL 90% acetone, as usual.
2. Determine \( \text{chl}_a \) concentration with a spectrophotometer, see equations below.
3. Make a precise dilution so that you get a good reading on the 1x scale of the fluorometer. Be sure you record this dilution, and make your dilutions in small
volumes or you'll waste a lot of acetone. Wet the pipette with acetone first; this improves pipetting accuracy.

4. Determine K1x as:

\[ K_{1x} = \frac{\text{Chl} \ a \text{ (from spectrophotometer)(dilution factor)}}{1 \times \text{reading (from fluorometer)}} \]

5. F_0/F_a max should be determined from the above extract by acidifying.

6. For non-autoscaling fluorometers, the proportionality between scales (doors) is not exactly the nominal values, e.g., 1:3:10:30. The true values (door factors) need to be determined by taking a high reading on the 3x scale, re-zeroing, and taking it again on the 1x scale. Then, K_x for a given door is calculated as: \( \frac{K_{1x}}{\text{door factor}} \).

**Determination of Chlorophyll a via Spectrophotometry:**

A spectrophotometer measures the absorption of light at a particular wavelength; diode array specs can scan the entire visible wavelength range. Chl a has two absorption maxima, one at 432 nm and another at 664 nm. Since many accessory pigments absorb light in the 400's and only chls and phaeos absorb in the 600's, we use the red maximum for optical density readings (o.d.).

There are many published equations for determining the concentration of chl (trichromatic equations). In all, an extinction coefficient is multiplied by the o.d. at 664 nm (for chl a), then to correct for interference from other pigments, the o.d. at 647 nm (for chl b) and at 630 nm (for chl c) are subtracted. If one is using a culture with no chl b, use an equation that doesn't correct for it. All o.d.s should have a reading at 750 nm subtracted from them to correct for solvent absorption.

**Common Spectrophotometric Equations for Chlorophyll Determinations:**

(Strickland and Parsons, 1972)

\[
\text{Chl} \ a \left( \frac{\mu g}{10 \ \text{ml}} \right) = (11.6 \ \text{o.d.}_{664} - 1.31 \ \text{o.d.}_{645} - 0.14 \ \text{o.d.}_{630}) \left( \frac{\text{extract vol. (ml)}}{\text{cuvette width (cm)}} \right)
\]

(Jefferey and Humphrey, 1975)

\[
\text{Chl} \ a \left( \frac{\mu g}{10 \ \text{ml}} \right) = (11.47 \ \text{o.d.}_{664} - 0.40 \ \text{o.d.}_{630}) \left( \frac{\text{extract vol. (ml)}}{\text{cuvette width (cm)}} \right)
\]

(Mitchell and Kiefer, 1984)

1. Diatoms, chrysomonads and brown algae containing chlorophylls a, and c_1 and c_2 in equal proportions (solvent 90% acetone)
   - Chlorophyll a \( = 11.47 \ E_{664} - 0.40 \ E_{630} \)
   - Chlorophylls c_1 + c_2 \( = 24.36 \ E_{630} - 3.73 \ E_{664} \)

2. Dinoflagellates and cryptomonads containing chlorophylls a and c_2 (solvent 100% acetone)
   - Chlorophyll a \( = 11.43 \ E_{663} - 0.64 \ E_{630} \)
3. Mixed phytoplankton populations containing chlorophylls $a$ and $b$ and equal amounts of chlorophylls $c_1$ and $c_2$ (solvent 90% acetone)

\[
\text{Chlorophyll } a = 11.85 \ E_{664} - 1.54 \ E_{647} - 0.08 \ E_{630} \\
\text{Chlorophyll } b = -5.43 \ E_{664} + 21.03 \ E_{647} - 2.66 \ E_{630} \\
\text{Chlorophylls } c_1 + c_2 = -1.67 \ E_{664} - 7.60 \ E_{617} - 24.52 \ E_{630}
\]

4. Higher plants and green algae containing chlorophylls $a$ and $b$ (solvent 90% acetone)

\[
\text{Chlorophyll } a = 11.93 \ E_{664} - 1.93 \ E_{647} \\
\text{Chlorophyll } b = 20.36 \ E_{647} - 5.50 \ E_{664}
\]

References:


