Extractive Fluorometric Chlorophyll analysis.

1. Preparation Steps.
    a. Make up sufficient volume of 90% acetone solution for sample extraction. Each centrifuge tube requires 10 ml; we generally analyze triplicates for each sample.
        i. To make up 500 ml of 90% acetone, pour 450 ml of acetone in a graduated cylinder. This is not a volumetric reaction (i.e. adding 50 ml distilled water will not result in 500 ml of 90% solution). Bring up to 500 ml volume with deionized or distilled water ensuring that the solution is well mixed.
        ii. Work quickly and ensure all containers with acetone are capped, as it is very volatile.
        iii. Work with acetone in the hood only and use gloves.
    b. Make up sufficient volume of 10% HCl solution for the fluorometric readings. Each sample reading requires 3 drops. The dropper bottle holds 30 ml but it is important that the acid is freshly made (<1 month old) to ensure complete reaction.
    c. Label sample centrifuge tube (3 per sample).
    d. Using the bottle-top pipettor, pipette once or twice into *Acetone* squirt bottle to clear line. Dispense 10 ml (sometimes we will use 5 ml) of 90% acetone into each tube. Be very accurate in this step, this is the largest source of error in the analysis. Cap tube tightly. Cap the pipettor after usage.
2. Water sample processing.
    a. Place a glass fiber filter (size GF/F is nominal pore size of 0.7 µm) in each filter holder. Attach the filter cups ensuring that they are correctly seated. Don't over tighten.
    b. Check the level on the filter trap; it should be empty before you begin.
    c. Check the pump pressure, the intake should not exceed 5 mm Hg when blocked, the outtake should not exceed 20 when blocked.
    d. Gently swirl your sample three times in each direction, three times. Allowing sample to settle is one of the other largest sources of random error in the analysis. Shaking or vigorous swirling can break particle aggregates.
    e. Filter a pre-determined volume of sample through each filter. The filtered volume should be sufficient to just see faint color on the filter. The fluorometric method is very sensitive so only a small amount of material is necessary. If too much material is captured on the filter, the extraction efficiency will be reduced, and large errors can result.
    f. Collect triplicates for each sample until you are confident that you can obtain <5% coefficient of variation in replicates, then you may do duplicates.
    g. Turn the vacuum dial off for each sample as it finishes, preventing air from being drawn through the filter.
    h. Fold filter and place each filter in its respective centrifuge tube; ensuring the filter is well below the acetone level. Take care not to scrape material off filter with forceps.
    i. Shake the sample vigorously a few times, then, Vortex the sample for about 15 seconds. Again, ensure filter is below acetone level.

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j. Store centrifuge tubes in the freezer for 24 to 48 hours. Keep them covered in foil to prevent light damage.

3. Readings with a calibrated Turner Fluorometer (see protocol for fluorometer calibration).
   a. Turn on fluorometer; warm up at least 15 minutes.
   b. Remove sample tubes from the freezer, keeping them protected from light. Process in batches of 6 as possible
   c. Shake sample vigorously and vortex 5s.
   d. Centrifuge the tubes for five minutes on setting 5. Keep samples vertical to prevent filter material from resuspending. Centrifuge tubes have to arranged in a balance distribution. If all six slots are not filled, place 4 such that the six slots are Y Y N N Y Y. 3 would be arranged as Y N Y N Y N; and 2 would be Y N N Y N N.
   e. Run 90% acetone blanks on each scale of the fluorometer. Read only the 0-10 dial scale, record the sensitivity.
   f. Pour a sample from the centrifuge tube into the glass fluorometer vials, minimizing the amount of filter material in the tube. While sample is stabilizing in fluorometers, dump remaining sample in waste container, remove filter and wipe sample name from tube.
   g. Take a reading on the fluorometer, setting the sensitivity so that the reading is between 2 and 8 if possible. Record both the scale (and the X1 or X100 setting) and the dial reading to the nearest 0.1.
   h. Add 3 drops of 10% HCL, invert with parafilm, set aside.
   i. Repeat f-h for the remaining 5 samples.
   j. Take a reading on the first acidified sample. Record both the scale (and the X1 or X100 setting) and the dial reading to the nearest 0.1, the value will be lower than the original reading and likely on a more sensitive scale. Pour sample into waste container when done.
   k. Process the remaining samples from c to j.
   l. Wash tubes and vials with micro cleaner and rinse well with RO water. Dry upside down in rack or on towels. Regarding tubes on the fluorometers, the assumption is that if they are upside down they are clean, right side up are dirty
4. Calculations.
   a. Record the calibration coefficients written on the Turner Fluorometer (M = ______ and A= ________) and note the date of those coefficients.
   b. $$\text{Chl (mg m}^3\text{)} = M * (Fo - Fa) * \frac{V_{acOH}(ml)}{V_{filt}(ml)} \qquad (1)$$

   $$\text{Phaeo (mg m}^3\text{)} = M * ((A*Fa) - Fo) * \frac{V_{acOH}(ml)}{V_{filt}(ml)} \qquad (2)$$

   Where M and A are the calibration coefficients noted in step 4b. V_{acOH} is the volume of acetone in the centrifuge tube (10 ml) and V_{filt} is the volume of sample filtered in ml. Fo and Fa are calculated as follows:

   $$Fo = \frac{Ro - R_{blank}}{scale} \qquad (3)$$

   $$Fa = \frac{Ra - R_{blank}}{scale} \qquad (4)$$

Where Ro and Ra are the dial reading values for the sample before and after acidification, respectively, scale is the scale that the reading was made at and R_{blank} is the dial reading for pure 90% acetone on the scale that Ro or Ra was made at. For example, if Ro was made on the 100 scale, R_{blank} in equation (3) is the dial reading for acetone on the 100 scale; if Ra was made on the 316 scale, R_{blank} in equation (4) is the dial reading for acetone on the 316 scale.

References for original extractive technique and applications:

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Lorenzen, C.J. 1966. A method for the continuous measurement of the in vivo chlorophyll concentration. Deep-Sea Res. 13: 223-227.

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Reference for original observational paper:

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