WET Labs ECO Chlorophyll Fluoreometer Calibration Protocol

Overview

The principle of calibration of the ECO Chlorophyll Fluorometer is to relate the fluorescence response of the instrument (digital counts) to absolute chlorophyll concentration (mg/m^3) in order to determine the slope factor for the sensor. The approach is to:

(1) select a monospecific phytoplankton culture of known extracted chlorophyll concentration,

(2) measure the fluorescence response to a dilution series of this culture,

(3) derive calibration coefficients of the digital count response to chlorophyll using a type II regression (where uncertainties in the estimation of extracted chlorophyll concentration from triplicates and the uncertainties associated with a 30 second burst sampling of the sensor are both considered in the weighting of the regression), and

(4) use these calibration coefficients to estimate in situ chlorophyll concentration from measured fluorescence digital counts.

This calibration approach for the DFLS differs from the calibration of a bench-top fluorometer because the DFLS cannot be calibrated with purified chlorophyll in a solvent (i.e. acetone). The reasons are threefold. First, acetone can damage the optical face of to the instrument. In addition the fluorescence of chlorophyll in extract is very different that the fluorescence by chlorophyll in vivo (e.g. wavelength shift in solvent, cellular packaging of the pigment). Thirdly, most single channel fluorometers excite with an LED with nominal 470 nm peak wavelength. This wavelength does not excite chlorophyll a in vitro, but does excite the accessory pigments in vivo that subsequently lead to chlorophyll a fluorescence in photosystem II.

While there are a number of approaches that can be taken to calibrate the DFLS the one employed here is based upon the following. We are interested in an estimate of phytoplankton biomass in situ. We estimate the biomass by the concentration of chlorophyll contained in that biomass using in vivo fluorescence. The in vivo fluorescence is not equivalent to the chlorophyll that we would derive if we took a water sample and extracted it as there are variations in chlorophyll-specific in vivo fluorescence due to cell size and photoacclimation (i.e. pigment packaging). However, we can relate the in vivo fluorescence of a healthy algal culture to its extracted chlorophyll concentration, thereby accounting for the packaging effect. However, the derived calibration coefficients are specific to that culture and thus it should be recognized that variations between the culture optical properties and the in situ algal optical properties could lead to variations of order factor of 10 in the extreme (Roesler et al. 2017). These are minimized to some extent by selecting calibration cultures that are representative of the in situ community or representative of some median response.

In order to minimize variable calibrations over time and to track instrument responses over time, we use a single species, *Thalassiosira pseudonana,* a centric diatom ubiquitous in the ocean that yields repeated optical properties over time (years) in response to constant growth conditions. Then we can use matchup in situ HPLC TChla values to quantify the fluorescence efficiency of the in situ community relative to an invariant and unbiased calibrated fluorometer.

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Relating the ECO fluorescence signal to the in vivo fluorescence of the culture, we are actually measuring the effective chlorophyll fluorescence, which contains the packaging signal. Taking that sample and extracting it provides the total chlorophyll concentration, removing the packaging. Thus, a calibration using this method provides the in vivo equivalent total chlorophyll biomass and should be interpreted as such. We note that these same issues are inherent to a greater extent in the in vitro approach as well. The only way to sort out the packaging effect is to quantify the pigment per cell ratio either by direct measurement of absolute cell and pigment concentrations, or qualitatively by measuring the pigment-specific phytoplankton absorption coefficient.

Approach

Culture Preparation – A single monospecific culture of *Thalassiosira pseudonana* is maintained in exponential growth and harvested at moderate concentrations (~30 mg chl m^{-3}). The culture is grown in f/2 (Guillard) media with added silica, and maintained at standard temperature (18°C), salinity (33°/$_{oo}$) and light (150 µmol photons m^{-2} s^{-1}) conditions under 24 hour light to avoid synchronize diel cycles. The culture is stepped up to a large volume by inoculating ~2 ml of culture into 2 L of media. It takes about 3 to 5 days for the culture in exponential growth to reach desired concentrations. The approximate chlorophyll concentration in the culture is monitored using in vivo fluorescence measured in the Turner fluorometer to determine when the culture has achieved an optimal biomass concentration. The relationship between in vivo fluorescence and in vivo chlorophyll is such that a reading of 5 on each scale, and corrected for a blank, is approximated by the following:

Scale	Chl
1	7700
3.16	2400
10	770
31.6	240
100	75
316	22
1000	5

The absolute values are determined by annual Turner calibration.

Dilution Series – Serial dilutions of the harvested culture to span the range that is expected in the Gulf of Maine (0 to 20 mg m^{-3}). We perform serial dilutions by using 0.2 µm-filtered seawater (the same used for growth medium of the culture and the blank reading for the ECO) as the diluent. Approximately 10 dilutions are made, resulting in approximate chlorophyll concentrations of 25, 10, 5, 2.5, 1, 0.5, 0.25, 0.1, 0.05 and 0.025 mg m^{-3}. As we need approximately 1 L for each fluorometer reading, we perform the dilutions as we go, to minimize the amount of culture material and filtered seawater that we need. Because serial dilution is prone to error propagation, we collect triplicate subsamples from each dilution for fluorometric chlorophyll analysis. We also collect a particulate absorption subsample (Roesler 1998; Roesler et al. 2018), from the undiluted culture to maintain a database on the chlorophyll-specific

phytoplankton absorption efficiency of the culture and HPLC pigment profile (measure by NASA OBPG).

Measurements

1. Using the Hyperterminal communication protocol to communicate with the ECO via the test cable with 9V battery box. Set the communication protocols to Comm 1 with 9600 baud, 8 bits, 1 stop bit, no parity and no flow control. Switch the box to On and Terminal. The instrument should turn on, cycle the shutter and take ~30 seconds worth of readings. Turn switch off.
2. Use the Transfer>Capture text command to open a file. The naming procedure is ECO_IT_SN_yyyymmdd_medium.dat where IT is the instrument type (e.g., 3x1m, bbfl2, f2wb), SN is the serial number, yyyymmdd is the numeric calibration date (20190701 for 1 July 2019) and the medium code is air, MQ, fsw, D1, D2…Dn.
3. Collect an air reading by holding the sensor such that the excitation beam is not reflecting off any surface (it also helps generally to work in a low light environment). Switch On to collect the data. Switch off when shutter has returned to initial position.
4. Use the Transfer>Capture>stop to end file. Collect separate files for each instrument and each measurement.
5. Collect data for each instrument for each reading, thus on the first round all instruments will be used to collect air reading, then MilliQ, then filtered seawater (the same that is used in the dilution). For the second and subsequent rounds, each instrument will be used in dilution 1 (the pure culture), then in dilution 2 (the 1:2 dilution) and so on.
6. Collect reading on liquid media, fill casket chamber (Dall'olmo et al. 2009) with the well mixed medium (MQ, fsw or culture dilution), ensuring that it was well mixed by swirling three times in each direction, do not stir or shake as this will not mix adequately or damage the cells, respectively. In the case of MQ, ensure that it has been degassed to minimize bubble formation on the glass and instrument surfaces. For cultures make sure the culture remains well mixed.
7. Debubble the chamber, use inport and outport tubing to mix the medium during measurement. This ensures that the phytoplankton fluorescence is not quenched during measurement. Rinse casket between each dilutions as you are working from most concentrated to least and the potential for contamination is greatest.
8. For each dilution, collect sufficient volume for filtering triplicate extracted chlorophyll samples and one particulate absorption sample. Place each chlorophyll filter into a centrifuge tube with 5 or 10 ml of 90% acetone. Cap tightly and shake vigorously to displace the particles on the filter, vortex for about 10 seconds. Ensure the filter is below the level of acetone and freeze for 24 to 48 hours before reading on the Turner fluorometer following standard protocols. Place the absorption filter in a TissueTek®, freeze immediately in the liquid nitrogen dewar.
9. Repeat steps 6-8 on each dilution until complete.
10. Make one more reading in MQ water or in air to assess stability.
11. Use the template/code to calculate the median and standard deviation ECO counts for each dilution, MQ, FSW, air readings.

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Computations

1. Regression. Assuming that the filtered seawater has zero chlorophyll, plot the median counts versus derived chlorophyll for each dilution for each instrument, putting error bars for both the counts and the triplicate chlorophyll estimates on the graph. Obvious methodological outliers should be removed from the calibration coefficient estimates. Determine the slope and intercept which are the calibration coefficients using type II regression (Figure 1). In the example of Figure 1 the calibration coefficients are given by the **slope** and intercept of the linear regression (34.5 counts (mg m^{-3})$^{-1}$ and 202 counts, respectively). The regression is performed to minimize the errors in y (counts) and the calibration coefficients represent the sensitivity of the fluorometer to chlorophyll and the instrument signal (at zero chlorophyll), respectively. Note that the intercept is the culture medium blank and not the instrumental **dark** reading, which is the value observed in MQ which should be within instrumental uncertainty with the air reading. In this example, the dark reading was 192 counts, with standard deviation, σ_{dark}. The regression yields uncertainty values for slope and intercepts, σ_{slope} and $\sigma_{intercept}$.

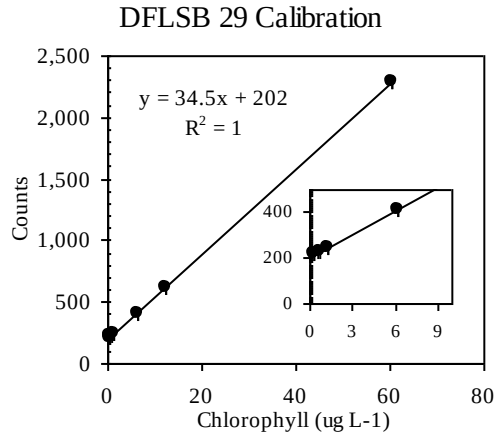

Figure 1. Example of an ECO-DFLS calibration curve, where the digital counts are on the dependent axis and the known chlorophyll concentrations are on the independent axis. Inset shows curve at low values. X and Y error bars represent standard deviation of triplicate chlorophyll estimates and standard deviation of digital

2. Calibrated in vivo chlorophyll equation and calibration uncertainty. In vivo chlorophyll is estimated from digital counts (DC) using the following equation:

$$Chl\left(\frac{mg}{m^3}\right) = \frac{(DC - dark)}{slope}$$

For the case shown in Figure 1:

$$Chl\left(\frac{mg}{m^3}\right) = \frac{(DC - 192)(counts)}{34.5\left(\frac{mg}{m^3}\right)}$$

The uncertainty in the calibration is determined from

$$\sigma_{Chl}\left(\frac{mg}{m^3}\right) = Chl\sqrt{\left(\frac{\sigma_{DC}}{DC}\right)^2 + \left(\frac{\sigma_{dark}}{dark}\right)^2 + \left(\frac{\sigma_{slope}}{slope}\right)^2}$$

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References

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