Frequently Asked Questions About Fluorometric Chlorophyll Analysis

Q: **Why measure chlorophyll?**

A: All plant life contains the primary photosynthetic pigment chlorophyll *a*. The quantitation or estimation of chlorophyll *a* supplies information on this abundance of microscopic primary producers growing in all types of water bodies. Since chlorophyll-containing organisms are the first step in the food chain, the health and /or abundance of these primary producers will have cascading effects to all organisms. The determination of chlorophyll concentration is one of the key indices of monitoring the health of a natural system. The estimation of algal biomass supplies information on the general health of a natural systems which is used researchers and technicians in all aquatic environments.

Chlorophyll measurements are also used to directly monitor phytoplankton populations. Examples include, but are not limited to, the monitoring of chlorophyll in natural marine and freshwater environments, reservoirs, water and sewage treatment plants, and aquacultural systems.

Microscopic plants occupy the lit zone of all water bodies, covering over 70% of the surface area of the planet. These primary producers consist mainly of planktonic algae and photosynthetic bacteria and are responsible for the great majority of O2 evolved, as well as act as the main CO2 sink on earth. For these reasons alone, it is obvious that there needs to be an effective means of quantitating the concentrations of microscopic photoautotrophs through measuring chlorophyll *a* concentrations in water.

Q: **How do fluorometers detect and quantitate chlorophyll in water?**

A: Fluorescence is the phenomena of some compounds to absorb specific wavelengths of light and almost instantaneously emit longer wavelengths of light. Chlorophyll *a* naturally absorbs blue light and emits red light. Fluorometers detect chlorophyll by transmitting an excitation beam of light in the 440nm (blue) range and by detecting the light emitted by the sample in the 680nm (red) range. Generally, this emitted light is directly proportional to the concentration of the material in question.

Q: **What is the difference between *in vivo*, *in vitro*, and extracted chlorophyll analysis?**

A: *In vitro*, meaning "within", chlorophyll analysis is the same as extracted analysis. It entails concentrating chlorophyll-containing cells onto a filter followed by extracting the chlorophyll *a* from the concentrated cells. *in vivo* chlorophyll analysis simply refers to the analysis of chlorophyll in the natural environment or in our case, in the living algal cells.

Q: **What is *in vivo* chlorophyll analysis?**

A: *In vivo* chlorophyll analysis is the fluorescent detection of chlorophyll in living algal and cyanobacterial cells in water. In this technique, the excitation light from the fluorometer passes through the untreated sample water and excites chlorophyll within the living cells of the algae present. Due to the nature of light, the excitation light will be affected by cells and other dissolved and particulate materials in the water before it reaches the chlorophyll molecules. Examples of interfering materials include other plant pigments and degradation products, dissolved organic matter, turbidity, and cell morphologies. Therefore, *in vivo* analysis is a semi-quantitative tool. *in vivo* numbers should correlate well with each other but rarely can they be used as actual chlorophyll *a* concentration measurements until correlated with extracted chlorophyll *a* data.

In vivo detection has several very useful applications. An example is the monitoring of general trends in chlorophyll concentrations in real time. It is very easy to obtain large amounts of data using *in vivo* instrumentation and is an excellent means of following trends and estimating chlorophyll concentration. This type of *in vivo* measure can be conducted continually along a ship's track, in vertical profiling, using discrete samples, or in long term deployments of in situ instruments on moorings. If water samples are taken, the *in vivo* data can be correlated to extracted chlorophyll *a* data to estimate actual concentrations. Otherwise, the *in vivo* data can be used as a relative measurement to identify trends and patterns. Another application is the direct monitoring of natural or laboratory phytoplankton populations and growth. Examples of direct monitoring applications include aquaculture and hatchery systems, water treatment facilities, reservoir, and aquatic research.

Q: How do you calibrate a fluorometer for *in vivo* chlorophyll analysis?

A: Due to the fact that *in vivo* chlorophyll analysis is a semi-quantitative measurement, calibration with extracted chlorophyll *a* data must occur in order to estimate actual concentration from the data. *in vivo* data is normally "calibrated" after the field measurements have been taken by correlating the *in vivo* data with extracted chlorophyll *a* data obtained from water samples collected during *in vivo* sampling (see Figure 1).

In laboratory cultures in which the phytoplankton assemblage is relatively constant and the environmental condition is controlled, actual chlorophyll *a* concentration can be estimated from *in vivo* data with a small degree of error (less than 10%) after initial correlation between extracted and *in vivo* data from different stages in the growth cycle.

Calibration of a field instrument can occur immediately preceeding field sampling by taking grab samples of the natural water body and extracting the chlorophyll to correlate the *in vivo* signal with the actual concentration. The fluorometer can then be set to read actual chlorophyll *a* but it must be understood that changing environmental conditions can introduce significant changes in readings even within one day.

Q: How do you calibrate a fluorometer for extracted chlorophyll *a* analysis?

A: Allow the fluorometer to warm-up for the time specified in the User's Manual. Measure the fluorescence of each standard at sensitivity settings that provide mid-scale readings (refer to your User's Manual for proper calibration procedures). Follow directions under section 10.0 from E.P.A. Method 445.0 (Revision 1.2) for the calibration and standardization procedure using the traditional acidification technique or the non-acidification method. E.P.A. Method 445.0 calls for filtering onto glass fiber filters (GFF) filters and grinding of the filters. This step may not be necessary in some systems and tests should be run to

compare extractions with and without grinding. Non-grinding techniques can use either GFF filters, which can be torn and allowed to steep in the solvent for 12-24 hours, or membrane filters can be used that will dissolve in the solvent.

The most commonly used extraction solvent is a 90% acetone 10% DI water solution. Other solvents, such as methanol and acetone/DMSO solutions are also commonly used and can improve extraction efficiency with specific phytoplankton or may be found useful for the extraction of sediment samples.

Q: **What environmental factors cause error in *in vivo* chlorophyll analysis?**

A: Light, temperature, water quality, and dissolved components can have significant effects on fluorescent readings independent of the chlorophyll concentration. However, all of these factors can be controlled and/or corrected to a degree if the user is aware of their effects.

Temperature has an inverse relationship with fluorescence. For example, in a vertical profile, as the temperature decreases, the fluorescence will increase independent of chlorophyll concentration. The *in vivo* chlorophyll fluorescence response changes at a rate of 1.4% per °C. A temperature drop of 10 °C in a vertical profile would result in a 14% overestimation of chlorophyll due to temperature changes alone.

Light history can have significant affects on the fluorescence in algal cells. For example, at low light levels, algal cells can optimize the light uptake by pushing chloroplasts to the outer edge of the cell or by producing more chlorophyll per cell. Both of these responses can result in data that falsely represents the algal biomass. To avoid this, opaque hose should always be used when sampling natural waters. The transport time of the water in the hose will dark-adapt cells to an extent, significantly reducing fluorescence error caused by variations in the light history of the cells.

Dissolved organic matter (DOM), chlorophyll degradation products, and turbidity can also affect fluorescence response. If these factors are suspected to be significant it is worth conducting a quick study to look at the effects by comparing the fluorescence from filtered and non-filtered water samples from below the photic zone where chlorophyll concentrations would be at a minimum.

Q: **What is the effect of varying species on fluorometric chlorophyll analysis?**

A: Different species of phytoplankton have varied morphologies (cell packaging), physiological rates, size, and chlorophyll *a* : carbon ratios. All of these factors can affect the fluorescence emitted from a cell under a specific excitation light intensity. Luckily, in natural environments, the phytoplankton assemblage is diverse enough that much of the variation in fluorescence resulting from the factors above balance eachother out so the net effect on the fluorescence reading is reduced. Nevertheless, the researcher needs to be aware of the potential for error in readings caused by these factors to more accurately assess the *in vivo* data.

Q: **How do other chlorophylls and degradation products affect chlorophyll analysis?**

A: All chlorophyll pigments and their degradation products (pheophytins) have their own unique excitation and emission spectra. Unfortunately, these spectra can overlap significantly with

the spectra of chlorophyll *a* due to the similar chemical structure of the various pigment molecules (see Figure 2). Due to the overlap in spectra, the presence of one can result in an interference in the measurement of another, resulting in an under or overestimation of the pigment in question.

In the case of chlorophyll *a*, interfering pigments to be aware of are chlorophyll b, chlorophyll c, and pheophytin a (see figure 3). Chlorophyll b causes the most interference in freshwater systems with high concentrations of chlorophytes and/or prasinophytes and in marine systems with high concentrations of prochlorophytes. The interference results during the acidification step of the traditional extraction technique. Chlorophyll b undergoes a wavelength shift when acidified, resulting in an underestimation of chlorophyll *a* and an overestimation of pheophytin. In environments with high chlorophyll b concentrations, we strongly recommend using the Welschmeyer (non-acidification) method.

High concentrations of chlorophyll c can result in a slight overestimation of chlorophyll *a* and an underestimation of pheophytin a sometimes even resulting in negative pheophytin readings. It has been reported that a chl a : chl b ratio of 1 : 1 , which is the highest ratio which could occur in nature, would result in a chl a overestimation of 10%.

Q: **What is a secondary standard?**

A: A secondary standard is used as an alternative to a primary calibration standard. It is often used when primary standards are expensive, difficult to obtain, or unstable. In the case of chlorophyll *a*, a secondary standard is used the majority of time for calibration because liquid chlorophyll *a* standards are expensive, time-consuming, and photosensitive. To properly use a secondary standard, you must first calibrate with a primary liquid standard of the analyte of interest. You can then obtain the equivalent value of the secondary standard. Henceforth, you may calibrate using the secondary standard using the value you obtained for it initially. Occasional calibrations using a primary standard to recheck the stability is recommended.

In the past, secondary standards have been more stable than the primary standards they mimic, but have still required special storage and handling conditions with relatively short lifetimes. Examples of these include coproporphrin and fluorescent dyes such as Rhodamine WT.

Turner Designs has developed a new solid secondary standard that is stable under ambient light and temperatures with no special treatment or storage required. This new secondary standard will greatly reduce time, cost, and trouble in fluorescent chlorophyll analysis procedure.

Q: What is the best solvent and procedure for the extraction of chlorophyll *a*?

A: There is no ‘best’ solvent or procedure for chlorophyll extraction. Several work well and have their own pros and cons. However, steps need to be taken by the scientific community to move toward a generally-accepted extraction technique for specific situations. The large number of extraction and analysis techniques make data comparisons more difficult and can create opposing factions between research groups. E.P.A. Method 445.0 describes the recommended step-by-step process for analysis using 90% acetone and acidification for the pheophytin correction. There are many factors in the extraction process which can lead to different results. Several examples of these factors will be discussed below in hopes of

generating discussions within the chlorophyll community to more toward widely accepted analysis procedures.

Water collection, storage, and filtration: Because pigment is being extracted from living cells, it is critical to use consistent techniques of obtaining water, filtering and storing filters. The living cell is sensitive to changes in the environment such as temperature and light. Conditions leading to cell death, lysis, or leakage will affect chlorophyll concentrations.

In the collection of water samples, it is important to make certain that the collection containers are clean of all chemicals. They should be rinsed several times in the sample water. Once collected, if samples cannot be filtered immediately, they should be stored quickly on ice in the dark. The time between collection and filtration should be as brief as possible and should not exceed 4 hrs.

Specifics on the recommended filtration and storage procedure can be found in E.P.A. Method 445.0.

Q: **How do I take and store discrete water samples in the field?**

A: For discrete *in vivo* analysis, water samples should be measured as soon as possible after collection. The same time constraints should be placed on the filtering of water samples that are to be used for extracted analysis. From the time of collection to measurement, the samples should be stored in the dark on ice. Remember that the cells are living and significant time in a container will alter the physiological state of the sample, resulting in misrepresented chlorophyll data compared to the natural situation.

Discrete samples need to be kept at the same temperature. This is most easily accomplished through the use of a water bath. The bath should be covered from direct light. When the samples are being measured in the fluorometer, a 'time in the instrument' must be established. Use the discrete sample averaging function on the 10-AU Fluorometer or wait for the reading to stabilize (~10 seconds) and record the fluorescence. If this time is not monitored, the heat in the instrument will cause the sample temperature to increase thus decreasing the fluorescence.

Run your own experiment to test change in fluorescence over a given transport time by analyzing samples from a given sample at hour or half-hour intervals.

Q: **How do I calculate actual chlorophyll *a* concentrations from my fluorometric data?**

A: For Acidification Method:

Prior to running sample on the fluorometer, the instrument must be calibrated with a pure chlorophyll *a* standard and the maximum acid ratio must be determined by measuring the fluorescence of the standard before and after acidification. If a fluorometer other than the 10-AU is being used, the fluorometer sensitivity coefficient must also be determined. For further information please refer to EPA Method 445.0.

chl a $= K (F_m / F_m - 1) \times (F_b - F_a) \times (v/V)$

pheo a $= K (F_m / F_m - 1) \times [(F_m \times F_a - F_b)] \times (v/V)$

If necessary, the result can be multiplied by a dilution factor.

A: Non-Acidification Method (Chlorophyll *a* concentration only)

1) Collect fluorescence data (one number/sample). ** **DO NOT ACIDIFY****

2) Plug data into following equation:

$$\text{Chl a} = \frac{KF_oV}{V}$$

where:

K= sensitivity coefficient, equal to 1 on 10-AU or TD-700

F_m **=** max acid ratio F_b/F_a of pure chlorophyll *a* standard

F_b **=** fluorescence before acidification

F_a = fluorescence after acidification

F_o **=** fluorescence signal of sample

v = extract volume (L)

V= volume filtered (L)

Q: **Does the E.P.A. approve fluorometric chlorophyll analysis?**

A: Yes, the E.P.A. has published Method 445.0 which covers the *in vitro*(extraction) fluorometric analysis of chlorophyll *a*. In the most recent revision (Rev 1.2 Sept., 1997), the E.P.A. also approves the use of the Welschmeyer (non-acidification) optical kit, which is recommended for use with high concentrations of chlorophyll b. This filter kit supplies only chlorophyll *a* concentrations with no information on pheophytin concentration.

Q: **How can I compare chlorophyll data obtained through different measurement techniques?**

A: All detection instrumentation used in chlorophyll analysis will result in chlorophyll concentrations which are directly comparable. A side-by-side comparison between a fluorometer and a spectrophotometer is easily done but would require dilution of the chlorophyll sample to put it within the linear range of the fluorometer. A sample that is in range on a spectrophotometer will be over-range on a fluorometer.

Q: **Why use a fluorometer over a spectrophotometer for extracted chlorophyll analysis?**

A: Benefits of fluorescence over spectrophotometry include the capability of *in vivo* detection, durability, versatility (accepts a wide range of discrete sample cells and flow cells, accepts AC or DC power, and the user can choose and quickly change several optical kits), easy to use making it an excellent teaching tool, stability, easily transported and used in the field, and a small footprint.

For oceanographic research, the greater sensitivity of fluorescence results in less time and work in the analysis because much less water must be filtered for extracted analysis. The superior sensitivity also enables *in vivo* detection of chlorophyll concentration of Freshwater researchers now have an extremely accurate and easy way to measure chlorophyll *a* even with high chlorophyll b concentrations using the non-acidification optical kit. Fluorometers also allow for in-line monitoring to collect data in real time.

Q: **What are the chlorophyll detection limits of Turner Designs Fluorometers?**

A: The TD-700 Laboratory Fluorometer and the 10-AU Field Fluorometer with red sensitive PMT, have extracted chlorophyll detection limits of approximately 20ppt using a 13mm diameter test tube and 5ppt using a 25mm test tube.

Q: **Do I need to verify the concentrations of Turner Designs liquid chlorophyll standards (P/N 10-850) on a spectrophotometer?**

A: No. The stock standard from which the liquid chlorophyll *a* standards were diluted from was already verified on a spectrophotometer. In addition, the concentrations of the liquid chlorophyll *a* standards are too low to be accurately measured on a spectrophotometer.

Calibration of the Fluorometer
(milligrams per cubic meter)

Calculated Chlorophyll

Measured Chlorophyll

FIGURE 1 - USGS, 1998

Fluorescence Response (relative)

Wavelength (nm)

Pheo a, Chl a, Chl b, Chl b, Chl a

FIGURE 2

chlorophylls and derivatives

chlorophyll *a*

chlorophyll *b*

phaeophytin *a*

chlorophyllide *a*

FIGURE 3

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