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White Paper

In Vivo Measurement of Chlorophyll and the YSI 6025 Wiped Chlorophyll Sensor

Introduction

Chlorophyll, in various forms, is bound within the living cells of algae, phytoplankton, and other plant matter found in environmental water. Chlorophyll is a key biochemical component in the molecular apparatus that is responsible for photosynthesis, the critical process in which the energy from sunlight is used to produce life-sustaining oxygen. In general, the amount of chlorophyll in a collected water sample is used as a measure of the concentration of suspended phytoplankton, the magnitude of which can significantly affect the overall quality of the water. The use of the measurement of phytoplankton as an indicator of water quality is described in Section 10200 A of *Standard Methods for the Examination of Water and Wastewater*.

The classical method of determining the quantity of chlorophyll at a particular site is to collect a fairly large water sample and analyze it in the laboratory. The procedure involves filtration of the sample to concentrate the chlorophyll containing organisms, mechanical rupturing of the collected cells, and extraction of the chlorophyll from the disrupted cells into the organic solvent, acetone. The extract is then analyzed by either a spectrophotometric method using the known optical properties of chlorophyll or by high performance liquid chromatography (HPLC). This general method is detailed in Section 10200 H of *Standard Methods* and has been shown to be accurate in multiple tests and applications as long as a competent laboratory analyst carries out the protocol. The procedure is generally accepted for reporting in scientific literature. This method is time-consuming, however, and usually requires an experienced, efficient analyst to generate consistently accurate and reproducible results. It also does not lend itself readily to continuous monitoring of chlorophyll, and thus phytoplankton, since the collection of samples at reasonable time intervals, e.g., every hour, would be extremely tedious.

YSI has developed the 6025 chlorophyll sensor for the determination of chlorophyll in spot sampling and continuous monitoring applications. It is based on an alternative method for the measurement of chlorophyll which overcomes these disadvantages, albeit with the potential loss of accuracy. In this procedure, chlorophyll is determined *in vivo*, i.e., without disrupting the cells as in the extractive analysis. The YSI 6025 chlorophyll sensor is designed for these *in vivo* applications and its use allows the facile collection of large quantities of chlorophyll data in either spot sampling or continuous monitoring applications. It is important to remember, however, that the results of *in vivo* analysis will not be as accurate as those from the certified extractive analysis procedure.

The limitations of the *in vivo* method are outlined below and should be carefully considered before making chlorophyll determinations with your YSI sonde and sensor. Some of the sources of inaccuracy can be minimized by combining the data from the YSI 6025 with data from extractive analysis of a few samples acquired during a sampling or monitoring study. However, the *in vivo* studies will never replace the standard procedure. Rather, the estimates of chlorophyll concentration from the easy-to-use YSI chlorophyll system are designed to complement the more accurate (but more difficult to obtain) results from more traditional methods of chlorophyll determination.

Measurement of Chlorophyll *In Vivo*

One key characteristic of chlorophyll is that it fluoresces; that is, when irradiated with light of a particular wavelength it emits light of a higher wavelength (or lower energy). The ability of chlorophyll to fluoresce is the basis for all commercial fluorometers capable of measuring the analyte *in vivo*. Fluorometers of this type have been in use for some time. These instruments induce chlorophyll to fluoresce by shining a beam of light of the proper wavelength into the sample, and then measuring the higher wavelength light which is emitted as a result of the fluorescence process.

Most chlorophyll systems use a light emitting diode (LED) as the source of the irradiating light that has a peak wavelength of approximately 470 nm. LEDs with this specification produce radiation in the visible region of the spectrum with the light appearing blue to the eye. On irradiation with this blue light, chlorophyll resident in whole cells emits light in the 650-700 nm region of the spectrum. To quantify the fluorescence, the system detector is usually a photodiode of high sensitivity that is screened by an optical filter that restricts the detected light. The filter prevents the 470 nm exciting light from being detected when it is backscattered off of particles in the water. Without the filter, turbid water would appear to contain fluorescent phytoplankton, even though none were present. Figure 1 depicts the general principal of operation of YSI's Model 6025 chlorophyll sensor.

Most commercial fluorometers fit into two categories. The first category is benchtop instruments that generally have superior optical flexibility and capability but are relatively expensive and are often difficult to use in the field. The second category is sonde-type fluorometers that have a fixed optical configuration but are less expensive, can be more easily used in the field, and are usually compatible with data collection platforms. The use of a pump is recommended for many sonde

fluorometers and this can result in the need for large capacity batteries for field use.

The unique YSI chlorophyll system available as an option for use with YSI sondes consists of a sensor which is similar in concept to the sonde-type fluorometers, but is much smaller, making it compatible with the sensor ports of the YSI 6820, 6920, and 6600 sondes. The output of the sensor is automatically processed via the sonde software to provide readings in either generic fluorescence units (percent full scale; % FS) or µg/L of chlorophyll. No pump is required for the YSI system, allowing the sensor to operate off of either the sonde internal batteries or the batteries in the YSI 650 display/logger. Like the widely-used YSI 6136 turbidity sensor, the YSI 6025 chlorophyll sensor is equipped with a mechanical wiper to periodically clean the optical face either by manual or automatic activation (Figure 2). With these features, the YSI chlorophyll sensor provides the same level of performance as the sonde fluorometers, but is much easier to use and can be deployed in environmental water for several weeks without the need for service. In addition, the sensor will be a component in sondes that can acquire up to ten other parameters simultaneously with chlorophyll, rather than just providing the single parameter.

Effect of Temperature on Readings

While the effect of temperature on the chlorophyll sensor itself is very small, YSI experiments have indicated that the fluorescence of phytoplankton suspensions can show significant temperature dependence. For example, the apparent chlorophyll content of our laboratory test samples of algae increased from 185 to 226 µg/L when the temperature was dropped from 21°C to 1°C even though no change in phytoplankton content took place. In the absence of compensation, this effect would obviously result in errors in field chlorophyll readings if the site temperature were significantly different from the calibration temperature. This temperature error can be reduced by employing a chlorophyll temperature compensation routine ("Chl tempco")resident in the sonde software under the Advanced-Sensor menu.

From our studies, it appears that entry of a value of 1 to 2 % per degree C for "Chl tempco" is appropriate to partially account for changes in the fluorescence of environmental phytoplankton with temperature. This value can be estimated in the above example as follows:

- Change in Temperature = 21–1 = 20°C
- Change in Fluorescence = 226-185 = 41 µg/L
- % Change in Fluorescence = (41/185) x 100 = 22.1
- Chl Tempco Factor = 22.1/20 = 1.11% per degree °C

Note that the use of this empirically derived compensation does not guarantee accurate field readings since each species of phytoplankton is likely to be unique with regard to the temperature dependence of its fluorescence. Changes in fluorescence with temperature are a key limitation of the in vivo fluorometric method (see below) which can only be reduced, not eliminated, by this compensation. In general, the best way to minimize errors is to calibrate with phytoplankton standards of known chlorophyll content that are as close as possible in temperature to that of the environmental water under investigation.

Effect of Fouling on Optical Measurements

Field optical measurements are particularly susceptible to fouling, not only from long-term buildup of biological and chemical debris, but also to shorter-term formation of bubbles from out-gassing of the environmental water. These bubbles can sometimes be removed in short-term sampling applications by simply agitating the sonde manually. For studies longer than a few hours where the user is not present at the site, the quality of the chlorophyll data obtained with a fluorescence sensor that has no capability of mechanical cleaning is likely to be compromised.

The YSI 6025 probe is equipped with a mechanical wiper that makes it ideal for unattended applications. The wiper can be activated in real-time during discrete sampling operations or will function automatically just before each sample is taken during long-term unattended monitoring studies. The number of wiper movements and the frequency of the cleaning cycle for the unattended mode can be set in the sonde software. Generally, one wiper movement is sufficient for most environmental applications, but in media with particularly heavy fouling additional cleaning cycles may be necessary.

Effect of Turbidity on Chlorophyll Readings

As described above, the filters in front of the photodiode in the YSI 6025 chlorophyll probe prevent most of the 470 nm light which is used to excite the chlorophyll molecules from reaching the detector after being backscattered off non-fluorescent particles (turbidity) in environmental water. However, a minor interference on chlorophyll readings from suspended solids may result. Laboratory experiments indicate that a suspension of typical soil measured with a YSI 6026 sensor will have a turbidity interference characterized by a factor of about 0.03 µg/L per NTU. For example, the turbidity of the water must be above 100 NTU to produce an apparent chlorophyll reading equal to 3 µg/L. In very cloudy water, the user may wish to utilize the independently-determined turbidity value and the above compensation factor to correct measured chlorophyll values.

Limitations of *In Situ* Chlorophyll Measurements

As noted above, the measurement of chlorophyll from *in situ* fluorescence measurements will always be less reliable than determinations made on molecular chlorophyll that has been extracted from the cells using the procedures described in *Standard Methods*. This section describes some of the known problems with *in situ* chlorophyll measurement.

Interferences from Other Fluorescent Species

The analytical methods described in *Standard Methods* for chlorophyll involve disruption of the living organisms present in suspension, followed by extraction of molecular chlorophyll into a homogeneous solution in an organic solvent. Acidification of the extract helps to minimize the interferences caused by a number of other, non-chlorophyll species. In addition, readings can be taken at various wavelengths on a spectrophotometer or a laboratory fluorometer to differentiate between the various forms of chlorophyll (a, b, c) and pheophytin a.

In contrast to this fairly controlled situation, all *in vivo* sensors operate under whole-cell, heterogeneous conditions where the sensor will measure, at least to some degree, everything which fluoresces in the region of the spectrum above 630 nm when irradiated with 470 nm light. Therefore, the sensor is actually quantifying overall fluorescence under these optical conditions, rather than chlorophyll specifically. While it is probable that most of the fluorescence is due to suspended plant and algal matter and that much of the fluorescence from this biomass is due to chlorophyll, it is impossible to exclude interferences from other fluorescent species using the approach described above. Note that *in vivo* fluorometers usually cannot differentiate between the different forms of chlorophyll.

Lack of Calibration Reagents

The usual reagents which are used for the calibration of fluorometric measurements for chlorophyll after extraction into organic solvents are purchased as "purified chlorophyll a" from chemical supply vendors such as Sigma. These standards are not soluble in aqueous media and, even if they were, their fluorescence is unlikely to be the same as when the chlorophyll is present in the whole living cell. Therefore, for even a semi-quantitative calibration, the user needs a "substitute" standard such as Acridine Orange or Rhodamine WT (see YSI 6-Series manual) to provide a method for estimating the sensitivity of the sensor. However, field readings based on this type of calibration will provide only an estimate of chlorophyll in environmental water where the measurement is taken on whole cell suspensions *in vivo*. The calibration standard that provides the best measure of accuracy for *in vivo* chlorophyll sensors is a portion of a phytoplankton suspension that has been analyzed for chlorophyll by the extractive procedure.

We recommend the use of this procedure and further recommend that the phytoplankton suspension is taken from the monitored site so that the species producing the fluorescence in the standard are as close as possible to the field organisms. To truly assess data reliability in a long-term monitoring study, grab samples should be taken periodically, e.g., weekly, and analyzed in the laboratory as the study progresses. These data can then be used to "post-calibrate" the readings logged to the instrument during the study, perhaps using a spreadsheet for the simple mathematical treatment. In any case, obtaining quantitative chlorophyll data from any *in vivo* fluorometric sensor is more difficult than with most other environmental sensors. For this reason, it is difficult to provide an accuracy specification for chlorophyll measurement made with *in vivo* fluorometers and therefore no accuracy specification is quoted for the YSI 6025.

Effect of Cell Structure, Particle Size, and Organism Type on *In Vivo* Measurement

If the only fluorescent species present for *in vivo* measurements is chlorophyll, and reliable calibration standards are available, its absolute quantification would still be difficult because samples are not homogeneous. Differing species of algae with differing shape and size will likely fluoresce differently even if the type and concentration of chlorophyll are identical and this significantly limits the accuracy of *in vivo* measurements.

Effect of Temperature on Phytoplankton Fluorescence

YSI experiments indicate that phytoplankton fluorescence increases as temperature decreases. Thus, readings taken on a phytoplankton suspension at cold temperature would erroneously indicate the presence of more phytoplankton than when the suspension is read at room temperature. Unless this effect is taken into account, most field readings will be somewhat in error, since the field temperature will differ from the temperature of calibration. The use of the "Chl Tempco" factor found in the Advanced-Sensor menu will help to reduce this error, but must be used with caution since each species of phytoplankton is likely to have a slightly different temperature dependence.

Effect of Photosynthetic Activity on Phytoplankton Fluorescence

Chlorophyll is a key factor in the photosynthetic apparatus of phytoplankton, participating in the production of oxygen during the day and "resting" at night during the respiration cycle of the cells. It seems likely that the fluorescence of the phytoplankton will vary in intensity depending on the state of the chlorophyll. Empirical data indicate that, at constant phytoplankton level, the fluorescent signal can change significantly

on a diurnal schedule, showing less fluorescence when oxygen is being produced and more fluorescence during the "resting" phase of the organisms. If further data support this hypothesis, it is clear that this effect will produce errors in the absolute values of chlorophyll nless it is accounted for by the user.

Photoinhibition of Phytoplankton Fluorescence

It is well known that *in vivo* fluorescence of the chlorophyll in phytoplankton varies in intensity depending on the light conditions at the site. This factor is known as "photoinhibition" and results in lower fluorescence readings when the phytoplankton is exposed to light. From a practical point of view, this effect results in lower fluorescence during daylight hours and higher fluorescence at night even though the actual phytoplankton content of the water is invariant. Empirical data from the YSI 6025 (and competitive fluorometers such as WetLabs and Turner) indicate that, at constant phytoplankton level, the fluorescent signal can change significantly on a diurnal schedule. Unless this effect is accounted for by the user, it is clear that errors (the extent depending on the type of algae and the depth of deployment) will result. In shallow deployments during bright sunlight, this photoinhibition effect can, in fact, be the most significant error in the correlation of *in vivo* measurements with chlorophyll determinations by extractive analysis.

The chlorophyll section of *Standard Methods* and application notes that are offered by other fluorometer manufacturers substantiate these limitations. The limitations result in the realization that any *in vivo* "chlorophyll" sensor will be much less quantitative than any of the other sensors offered for use with YSI sondes.

References

Standard Methods for the Examination of Water and Wastewater, 20th Edition, APHA-AWWA-WPCF. 1999. American Public Health Association.

YSI 6-Series Environmental Monitoring Systems Manual. Revision A. May, 1999. YSI Inc. 264 p.

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