AN ABSTRACT OF THE THESIS OF

John T. Salinas for the degree of Master of Science

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TITLE: A CRITICAL COMPARISON OF METHODS FOR THE DETERMINATION OF PHYTOPLANKTON CHLOROPHYLL

Abstract approved: James D Ingle, Jr.

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The concentration of chlorophyll in natural bodies of water is commonly determined as a means to rapidly estimate the phytoplankton biomass. The literature gives numerous warnings, however, as to the problems involved with accurately determining chlorophyll concentrations. The author's work at Crater Lake, Oregon enticed him to explore critically the spectrometric methods for determining chlorophyll.

Four spectrometric methods for the determination of chlorophyll have been investigated. These are the spectrophotometric method, the 'in vitro' fluorometric method, the 'in vivo' fluorometric method and the 'in situ' fluorometric method using fiber optic cables (remote fiber fluorometry).

The spectrophotometric trichromatic and monochromatic methods depend on absorption measurements made with a spectrophotometer. The spectral bandpass of the spectrophotometer is a critical variable in the determination of chlorophyll. A spectral bandpass of 2.0 nm has been suggested and shown to be adequate to measure the concentrations of chlorophyll-a. The chlorophyll concentration determined is 15%

and 36% too low with spectral bandpasses of 10 and 20 nm, respectively. Increasing the spectrophotometric cell pathlength from 1.0 to 5.0 cm improves the detection limit of the method by a factor of 5. With a 1-cm pathlength cell, the detection limit for chlorophyll-a is 34 µg/L in an extract or 0.34 µg/L in lake water with a concentration factor of 100.

Of the fluorometric methods studied, the 'in vitro' uncorrected fluorometric method was shown to be the most precise and to provide the lowest detection limit (4 ng/L in an extract and 0.04 ng/L chlorophyll-a in lake water with a concentration factor of 100). The detection limits for the 'in vivo' and the enhanced 'in vivo' method (using DCMU) fluorometric methods are 5 and 3 ng/L, respectively.

The effect of several variables in the sample preparation method for the spectrophotometric and 'in vitro' fluorometric methods were studied with samples of Cronemiller Lake water. No difference in filter retention efficiency at the 95% confidence level was observed when the Millipore HA membrane, S & S glass and Whatman Glass GF/F filters were compared with a solution of titanium dioxide or a natural phytoplankton sample.

Following 65 days of storage at 0^{o} C or 238 days of storage at -9^{o} C, the chlorophyll concentration determined did not significantly change from that determined at the beginning of the study. The use of $MgCO_3$ did not change this condition.

The 'in vivo' fluorometric technique, applied to water samples from Crater Lake, Oregon, was shown to be influenced by sample temperature and irradiance history. The addition of the herbicide DCMU to a sample has been reported to decrease the dependency of the

fluorescence signal on temperature and irradiance history of the sample. This was shown not to be the case. A 10^{o} C decrease of sample temperature resulted in an average 1.8% increase in sample fluorescence. Exposure of a set of samples to solar radiation decreased the fluorescence signal for chlorophyll in the samples. A period of great change in fluorescence signal was followed by an extended period of slower change. After 50 minutes of sample irradiation, the average fluorescence signal decreased over 50% relative to the original signal.

A remote fiber fluorometer was constructed to investigate its use for the 'in situ' fluorometric determination of chlorophyll. Transmission characteristics of the fiber showed that light attenuation increased as the wavelength decreased. With a jig that held the excitation and emission fibers at varying distances and angles, it was found that maximum fluorescence signals were recorded as the fiber ends were moved as close as possible to each other and at an angle of about 10^{o}. The 'in situ' detection limit for chlorophyll-a was determined to be 0.64 μg/L using 1-m excitation and emission fibers.

A CRITICAL COMPARISON OF METHODS FOR THE DETERMINATION OF PHYTOPLANKTON CHLOROPHYLL

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I hope researchers determining chlorophyll in lakes, streams and oceans find answers to problems they encounter here in these pages. I also hope that the use of fiber optic remote probes help future researchers to detect materials and deepen our understanding of our many diverse ecosystems.

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INTRODUCTION

To understand the biological patterns of a lake or ocean, aquatic biologists must characterize the physical and biological composition of the aquatic system. Important characteristics include physical features, nutrient balance, primary producers, herbivores and fish activity. Nutrients, such as nitrate, sulfate, and phosphorous, are routinely determined through spectrophotometric or colorimetric methods utilizing autoanalyzers (Coffey, 1985). The herbivore population is determined using various collection techniques followed by microscopic examination. The fish population is estimated directly through the use of sonar type fish finders or indirectly by examining their effect on other populations such as invertebrates or zooplankton.

Primary producers or phytoplankton are quantitated either by using microscopic cell counting, cell volume, and identification procedures or by measuring chlorophyll concentrations. Chlorophyll is determined spectrophotometrically, fluorometrically, or more recently using high performance liquid chromatographic (HPLC) techniques. Phytoplankton populations can be estimated with instrumental chlorophyll measurements more rapidly than with cell counting procedures. The determination of chlorophyll yields an

estimate of the phytoplankton population or autotrophic biomass because the amount of chlorophyll-a in the many types of phytoplankton varies from 0.5 to 3.0% (w/w) of the dry weight (APHA, 1985; Loeb, 1985). Even if the concentration of chlorophyll in a sample is known to high accuracy, it still only represents an estimation of the phytoplankton population present. Zooplankton populations might alternatively be estimated by spectrometrically determining phaeophytin, a degradation product of chlorophyll, which is found in zooplankton and their excretion products.

This research is concerned with the critical comparison of different spectrometric methods for the determination of chlorophyll. The limitations of different techniques are delineated. Finally, the results of preliminary experiments to access the possibility of using fiber optics for remote sensing of chlorophyll in a water body are evaluated.

HISTORICAL

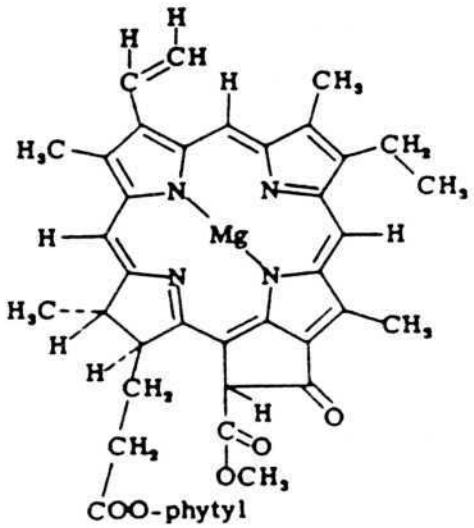
General Characteristics of Chlorophyll and Its Determination

Researchers have used various methods to quantitate the phytoplankton population in a specific aquatic system. Since 1952, a relatively rapid spectrophotometric method for the determination of chlorophyll has been used as an indirect means to estimate the phytoplankton population in a lake. It involves the collection of a water sample, filtration for phytoplankton, grinding of phytoplankton coated filters, extraction of chlorophyll into an organic solvent, centrifugation to remove the cell and filter parts, and the spectrophotometric determination of the chlorophyll concentration.

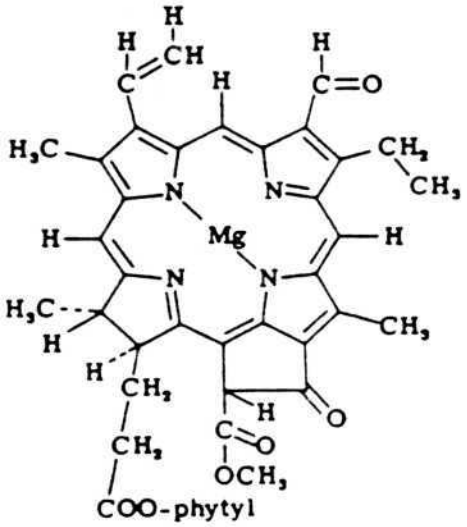
For purposes of comparison, standard extraction volumes are used throughout this paper. It will be assumed that in sampling a water body, five liters of water are collected, filtered and extracted with ten milliliters of an organic solvent. This results in an overall concentration factor of 500.

The chlorophylls are large pyrole complexes as shown in Figure 1. The structures of chlorophyll-a and b were determined by Goedheer (Goedheer, 1966), and the structure of chlorophyll-c was determined by Dougherty (Dougherty, 1966).

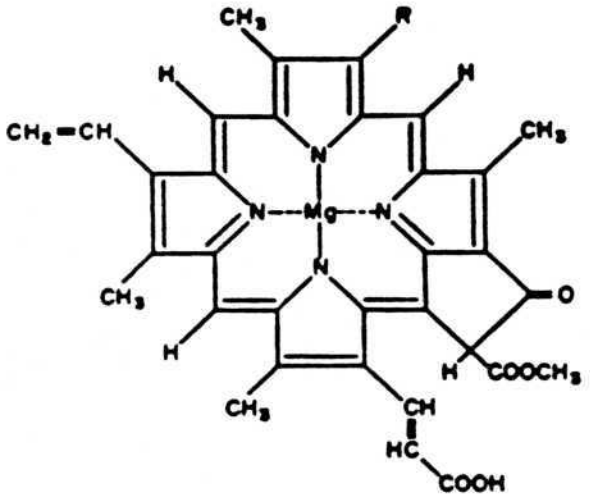
The chlorophylls absorb light in two major portions of the visible spectrum, the blue region between 400 and 460 nm and the red region between 640 and 670 nm, and fluoresce in the 630 to 670 nm region as shown in Figures 2, 3 and 4. Spectrophotometric determination of chlorophyll is normally conducted in the red region of the spectrum as many other organic species interfere with its



Chlorophyll-a



Chlorophyll-b



Chlorophyll-c

(a) R = -CH = CH_2
($C_{35}H_{28}N_4O_5Mg$)
(b) R = $-CH_2-CH_3$
($C_{35}H_{30}N_4O_5Mg$)

Figure 1. Structures of chlorophyll-a and chlorophyll-b and chlorophyll-c. The phytyl group is 3,7,11,15-tetramethyl-2-hexadecene. Bonding to chlorophyll occurs at the number 1 carbon. (Aronoff, 1966 and chlorophyll-c from Dougherty, 1966)

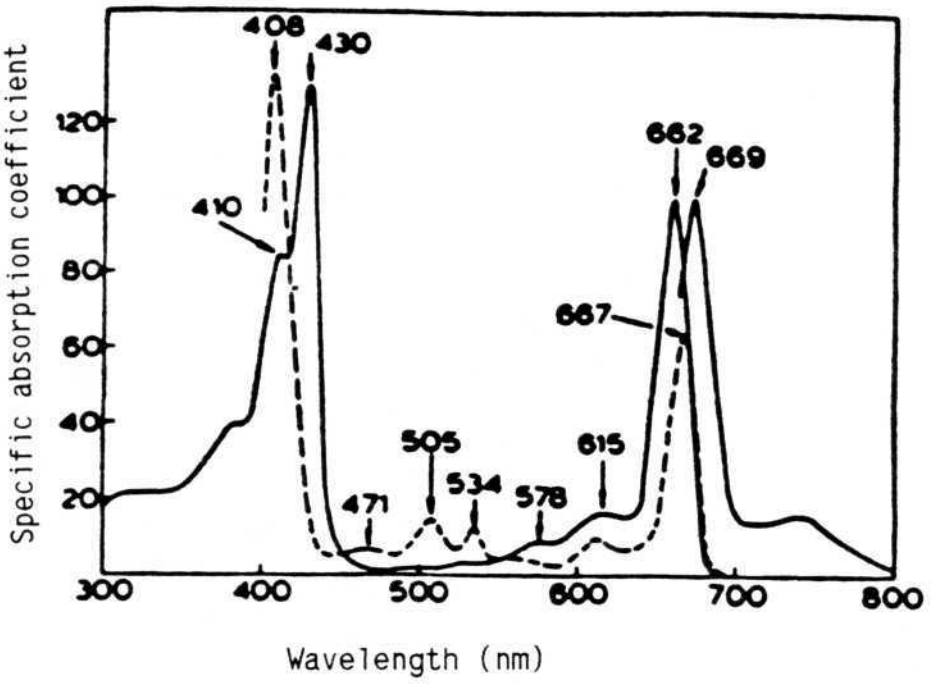


Figure 2. Absorption spectrum (peak maxima at 430 and 662 nm) and fluorescence emission spectrum (peak maximum at 669 nm) of chlorophyll-a (————) and absorption spectrum of phaeophytin-a (- - - -). All species dissolved in ether. The specific absorption coefficient has units of L/g-cm (Goedheer, 1966). The fluorescence spectrum is plotted as relative fluorescence signal.

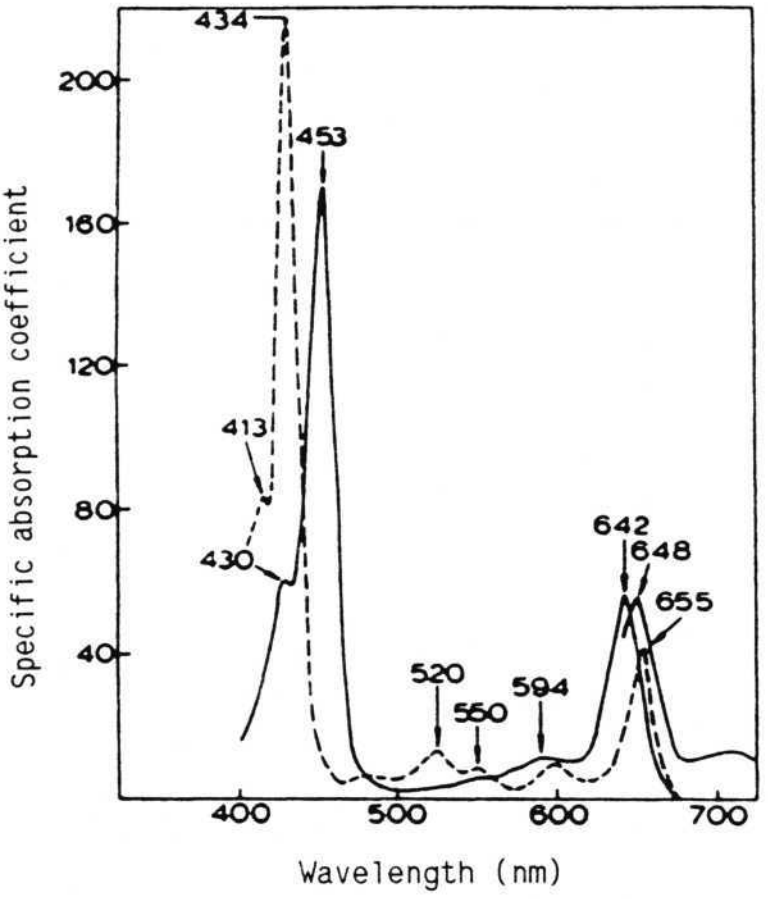


Figure 3. Absorption spectrum (peak maxima at 453 and 642 nm) and fluorescence emission spectrum (peak maximum at 648 nm) of chlorophyll-b (———) and absorption spectrum of phaeophytin-b (- - -) (Goedheer, 1966). All species dissolved in ether. The fluorescence spectrum is plotted as relative fluorescence signal.

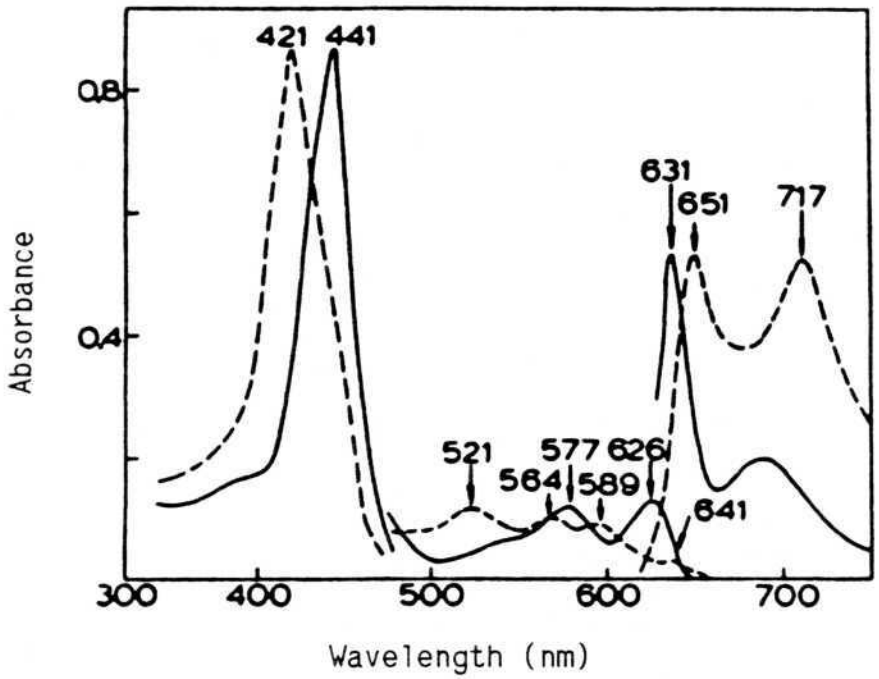


Figure 4. Absorption spectrum (peak maxima at 441 and 626 nm) and fluorescence emission spectrum (peak maximum at 631 nm) of chlorophyll-c (———) and absorption spectrum of phaeophytin-c (- - - -) (Goedheer, 1966). All species dissolved in ether. The fluorescence spectrum is plotted as relative fluorescence signal.

determination in the blue region (Richards and Thompson, 1952).

Chlorophyll, as found in nature, exists in many forms and its degradation products are also present. The pathways to some degradation products are shown in Figure 5. More recently HPLC has been used to separate as many as fourteen chlorophylls, their associated breakdown products and seventeen other pigments collectively called carotenoids (Mantoura, 1983).

In an extract, the chlorophylls easily lose their central magnesium atom upon acidification. This irreversible process produces a series of degradation products called phaeo-pigments as is shown in Figure 5. Chlorophyll is also degraded by an enzyme present in living phytoplankton called chlorophyllase. It catalyzes the removal of a phytyl group from a chlorophyll molecule which results in the production of chlorophyllide. Both the phaeo-pigments and chlorophyllides absorb light in the region of the visible spectrum used to determine chlorophyll spectrophotometrically and so lead to errors in the estimation of chlorophyll-a concentration.

Fluorometric methods allow chlorophyll to be determined more selectively and at lower concentrations. Three fluorometric methods are currently used to determine chlorophyll in a water body which depend upon the native fluorescence of chlorophyll and its associated pigments. The extraction of chlorophyll from phytoplankton, as in the spectrophotometric technique, followed by the fluorometric analysis of the extract is denoted the 'in vitro' or the extractive fluorometric technique. Because of the better detectability and selectivity provided by the fluorometric technique, chlorophyll in either extracts or phytoplankton can be determined. The collection

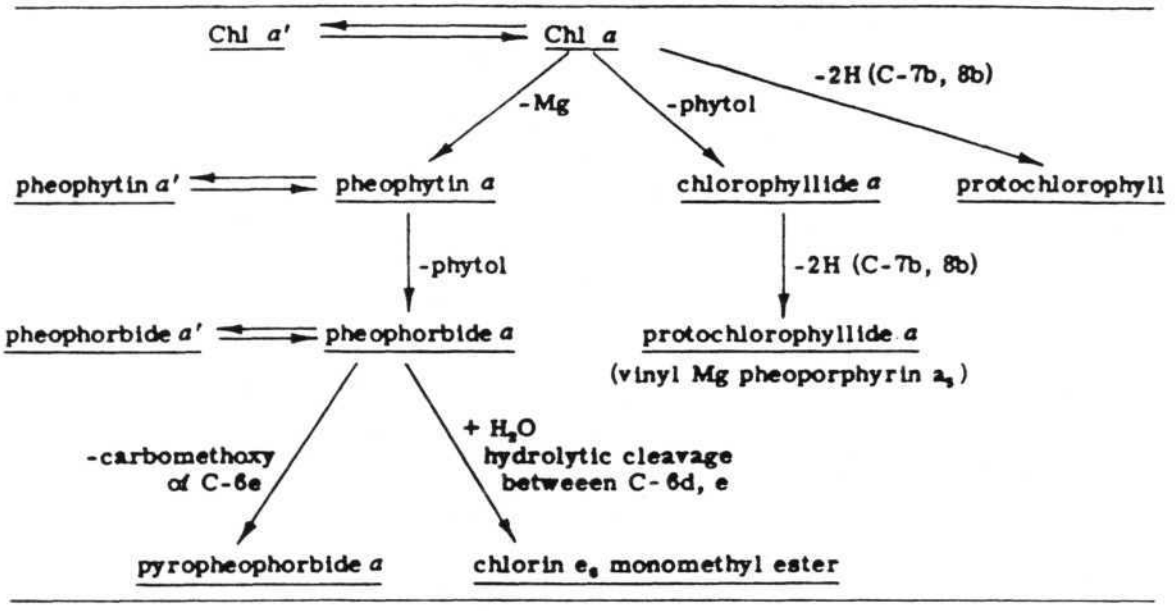


Figure 5. Degradation of the chlorophylls (Aronoff, 1966).

and direct measurement of chlorophyll fluorescence in the living cells with little sample pretreatment is an 'in vivo' technique.

Lastly, the measurement of the fluorescence of phytoplankton directly in a water body is called the 'in situ' technique. If one were to launch a fluorometer into a water body, an 'in situ' fluorometric signal would be recorded. This, indeed, has been demonstrated (Abbott, 1984). More recently it has been suggested that researchers might use fiber optics to direct excitation light into a water body and to collect and detect a fluorometric signal proportional to the chlorophyll concentration (Lund, 1983). This is attractive because most of the expensive instrumentation remains protected on shipboard with only the fiber optic material actually in the water body.

Spectrophotometric Methods

Trichromatic Method

Richards and Thompson in 1952 developed the first practical method to determine the concentration of chlorophyll in water (Richards and Thompson, 1952). This spectrophotometric technique was used on shipboard to estimate and characterize phytoplankton populations.

Richards and Thompson's original method involved the collection of a water sample, separation of the phytoplankton using a Foerst plankton centrifuge, extraction of the chlorophylls into a solvent of ten percent aqueous acetone (90:10, acetone:water), and the determination of the peak absorbances of chlorophyll-a, b, and c of

the extract at 665, 645, and 630 nm, respectively, in a 1-cm pathlength cell. From the known specific absorption coefficients for each chlorophyll at the selected wavelengths, Appendix 1, the concentration of the chlorophylls-a, b, and c are estimated from the empirical equations below.

$$\text{Chl-a (mg/L)} = 15.6\ A_{665} - 2.0\ A_{645} - 0.8\ A_{630} \quad (1)$$

$$\text{Chl-b (mg/L)} = 25.4\ A_{645} - 4.4\ A_{665} - 10.3\ A_{630} \quad (2)$$

$$\text{Chl-c (MSPU/L)} = 109\ A_{630} - 12.5\ A_{665} - 28.7\ A_{645} \quad (3)$$

Here A symbolizes the absorbance of the extract at a wavelength in nanometers indicated by the subscript. In the literature the antiquated symbol OD (optical density) is often used to represent the absorbance of a sample. The unit MSPU stands for a millispecific plant unit that was first defined when the structure and molecular weight of chlorophyll-c were unknown. The term SPU was defined as an amount of pigment approximately equal to one gram of chlorophyll-c. The absorbances at wavelengths 510 and 480 nm were also recorded to quantitate the concentrations of astacine and nonastacine carotenoids. These are groups of accessory pigments related to the chlorophylls which have since been individually identified. Equations 1 through 3 have become known as the trichromatic equations.

Assuming one were working with a sample containing only chlorophyll-a and defining the detection limit as the chlorophyll concentration that yields an absorbance of 0.010, a detection limit of chlorophyll-a in an extract is 160 µg/L. If the standard extraction volumes are assumed, this corresponds to a chlorophyll concentration of 0.3 µg/L in water. Richards and Thompson stated

that a linear relationship existed between the absorbance and the concentration of chlorophyll up to an absorbance of 0.8. This yields a range of linearity from 0.3 to 25 µg/L chlorophyll in water, for the standard extraction volumes.

It was quickly discovered that other species in the extract also absorb at these wavelengths making the calculated concentrations of chlorophyll inaccurate. Therefore the method was modified to include an absorption measurement at a fourth wavelength outside the absorbance range of the chlorophylls (Strickland and Parsons, 1960). This method will be called the Strickland and Parsons modification. The absorbance at this fourth wavelength, 750 nm, is then subtracted from each of the first three absorbances to give absorbance values corrected for turbidity. Strickland and Parsons also suggested that absorption measurements be done with a 10-cm pathlength cell.

The degradation of chlorophyll decreases the accuracy of a chlorophyll determination. For this reason, it was originally suggested by Richards and Thompson that a small amount of a *suspension of magnesium carbonate be added to the sample to buffer* it. This halts the degradation of chlorophyll to phaeophytin. It was also thought that coating a filter with this solid might improve the filtering efficiency of the filter (Richards and Thompson, 1952).

As the Strickland and Parsons method of the chlorophyll determination was popularized, researchers soon had to justify conflicting data. In the Strickland and Parson's method, water was filtered through either a membrane or glass filter. Questions arose as to the effect of the type and treatment of filters, the storage of filters, the type of extraction technique, and the extraction

solvent. These questions, born in the field, lead to a series of experiments beginning in 1971, by Long and Cooke. The type and treatment of filter to separate phytoplankton was shown to affect critically the amount of chlorophyll later determined. Membrane filters have the advantage of a published and controlled pore size and of being soluble in acetone. However, glass filters help in the grinding process as they form an abrasive slurry increasing the efficiency of the chlorophyll extraction. Glass filters were shown to produce higher pigment yields and to require shorter extraction times. Glass filters are also less expensive than membrane filters (Long and Cooke, 1971).

In filtering phytoplankton, one has to be concerned with the condition of the filtering apparatus. Any trace of acid, even from ones fingers, causes the degradation of chlorophyll to phaeophytin. Most field researchers either coat the filter to be used with a small amount of magnesium carbonate or add the same amount to the last 100 mL of water to be filtered so as not to retard the filtration process initially. It has also been reported, however, that chlorophyll in cells collected on filters without magnesium carbonate does not suffer the degradation effects alluded to by Strickland and Parsons (Holm-Hansen, 1978). Holm-Hanson believes that the phytoplankton form a complex with the magnesium carbonate on the filter which then makes the complete extraction of chlorophyll more difficult. This results in less chlorophyll being extracted from magnesium carbonate coated filters. It has been stated that the usefulness of magnesium carbonate both as a buffer and as a filtering aid is superfluous (Holm-Hansen, 1980).

After filtration, filters are often stored because field researchers might choose to complete the analysis at a later time. It has been documented that the chlorophyll concentration determined from wet filters stored in the dark and cold does not noticeably decrease for up to twenty-four hours (Marker, 1980). It has been further shown that there is no appreciable loss of chlorophyll determined when filters are stored frozen at -20^{o} C for up to two months (Marker, 1980). Freeze-drying filters has, however, resulted in a 30-40% decrease in the chlorophyll concentration determined (Lenz, 1980).

An organic solvent is used to remove the chlorophyll from the phytoplankton retained on a filter after grinding. Richards and Thompson originally suggested that ten percent aqueous acetone be used for this purpose. Ten percent aqueous absolute methanol and ethanol have been suggested as better solvents since they provide a larger chlorophyll extraction efficiency than acetone (Holm-Hansen, 1980). However, the stability of chlorophyll in methanol is uncertain and may result in breakdown and transformation products (Mantoura, 1983). Methanol is also a potential health hazard to researchers. Absorption coefficients for the chlorophylls and phaeophytins in the acetone are known and widely used. However, the absorbance coefficients in methanol and ethanol have not been thoroughly characterized.

The actual extraction of chlorophyll from phytoplankton retained on the glass filter with acetone generally involves a tissue grinding technique, a sonification technique, or a combination of both techniques. In a tissue grinder, a Teflon pestle is spun inside a

glass test tube like mortar as shown in Figure 6. Within a minute or two the filter is completely macerated. This slurry is then transferred into a capped, calibrated centrifuge tube and brought up to a specific volume using 90% (v/v) aqueous acetone. The filter parts, along with the disrupted cell parts, are generally stored for 3 to 12 hr in a cold, dark refrigerator to allow the chlorophyll to enter the acetone solvent. Up to a certain limit, longer storage times were shown to increase the amount of chlorophyll extracted (Marker, 1980). Once the steeping is complete, the tubes are centrifuged at high speed until the supernatant is clarified. The supernatant is then poured into a cuvette and the absorbances at the suggested wavelengths are recorded.

To determine the concentration of the chlorophylls, the Environmental Protection Agency suggests the use of the Richards and Thompson equations as modified by Jeffrey and Humphrey in 1975 (Collins, 1985). These are shown below using the absorbances corrected for turbidity by subtracting the extract's absorbance at 750 nm from the absorbances at 664, 647 and 630 nm.

$$\text{Chl-a (mg/L)} = 11.85\ A_{664} - 1.54\ A_{647} - 0.08\ A_{630} \quad (4)$$

$$\text{Chl-b (mg/L)} = 21.03\ A_{647} - 5.43\ A_{664} - 2.66\ A_{630} \quad (5)$$

$$\text{Chl-c (mg/L)} = 24.52\ A_{630} - 7.6\ A_{647} - 1.67\ A_{665} \quad (6)$$

With very low concentrations of all pigments in mixtures, less than 0.2 mg/L in the extract, the recovery of chlorophyll may be in error by up to 60% (Jeffrey and Humphrey, 1975). This corresponds to a chlorophyll concentration in water of 0.4 µg/L using the standard extraction volumes.

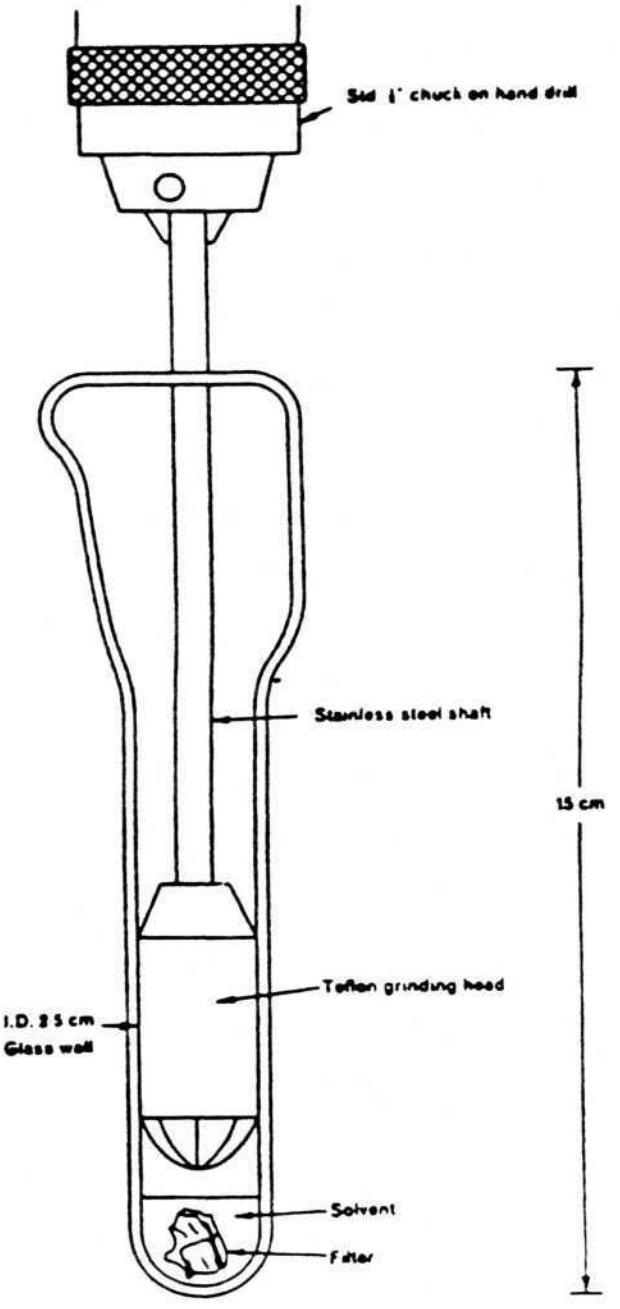


Figure 6. Grinding apparatus for chlorophyll extraction. Conventional tissue grinder (IBP Handbook, 1969).

The absorption bands of the different chlorophylls overlap and the absorption bands are relatively narrow. To measure the absorbance at a given wavelength accurately and to distinguish best among each specific chlorophyll, the spectrophotometer's spectral bandpass should be adjusted to a small enough value. A spectrophotometer having a spectral bandpass of 2 nm is recommended. With a spectral bandpass of 20 nm, the error in the estimate of chlorophyll-a concentration has been stated as being as large as 40% (ASTM, 1984).

A computer simulation was conducted to demonstrate the effect of spectral bandpass on the absorption spectrum of a chlorophyll-a solution (Weber, 1976). The absorption spectrum was initially measured using a Beckman ACTA V spectrophotometer with a small spectral bandpass (0.5 nm). The peak absorbances and absorption spectra for larger spectral bandpasses were computed and are shown in Figure 7. For this simulation the SCOR-UNESCO 1966 trichromatic equations were used. These equations are shown below.

$$\text{Chl-a (mg/L)} = 11.64\ A_{663} - 2.16\ A_{645} - 0.10\ A_{630} \quad (7)$$

$$\text{Chl-b (mg/L)} = 20.97\ A_{645} - 3.94\ A_{663} - 3.66\ A_{630} \quad (8)$$

$$\text{Chl-c (mg/L)} = 54.22\ A_{630} - 14.81\ A_{645} - 5.53\ A_{663} \quad (9)$$

The percent recoveries using the SCOR-UNESCO 1966 trichromatic equations are shown in Table I. Clearly, the spectral bandpass should be 2 nm or less to obtain results accurate to 1.2%. Today the accepted trichromatic equations are the modified Jeffrey and Humphrey equations (APHA, 1985).

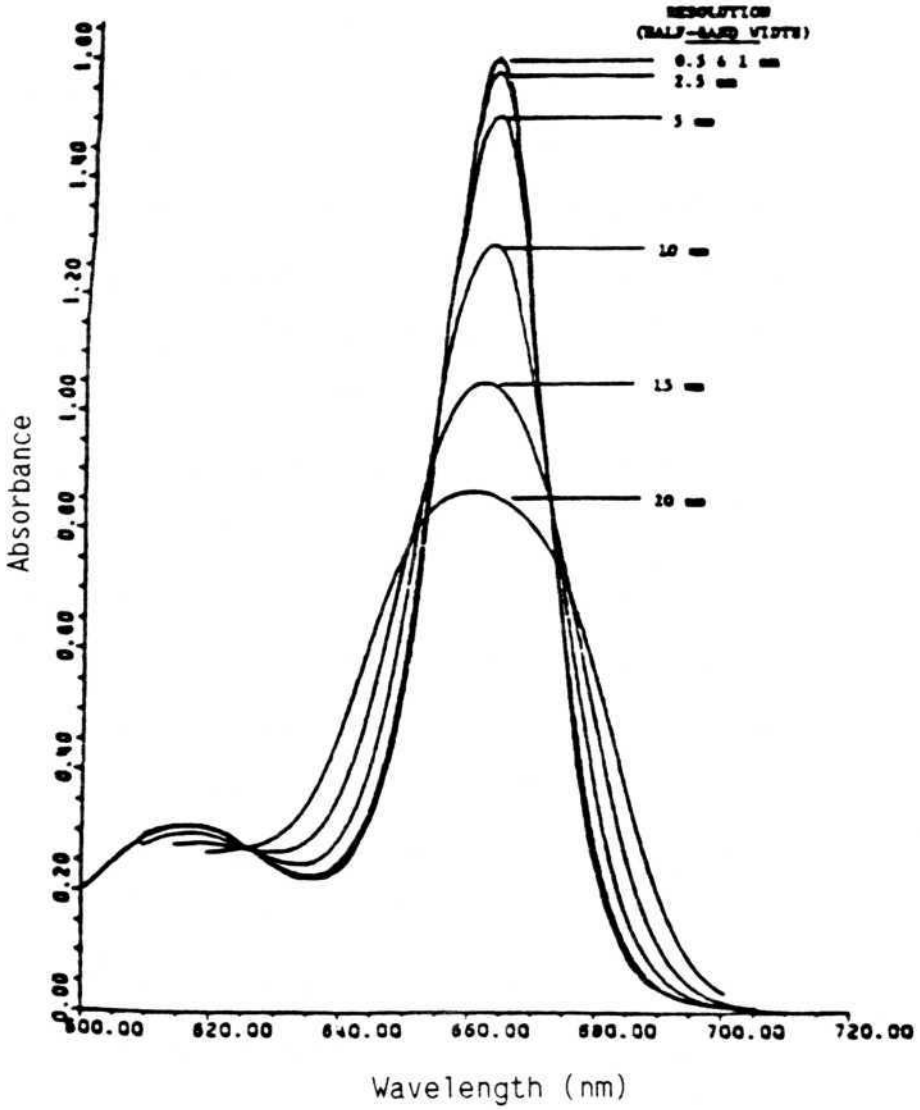


Figure 7. Effect of spectrophotometer resolution on the chlorophyll absorption spectrum (Weber, 1976).

Table I. The Apparent Concentration of Chlorophyll-a as a Function of Simulated Spectral Bandpass[a]

Spectral Bandpass (nm)	Recovery of Chl-a (%)
0.1	100
1.0	99.6
2.0	98.8
10	78.6
15	62.0
20	48.5

[a] (Weber, 1976)

Monochromatic Method

In 1967, Lorenzen developed a method for the correction of the interference due to the degradation product, phaeophytin, and for the determination of phaeophytin. Upon acidification, the absorbance of a sample of pure chlorophyll-a at a wavelength of 665 nm is reduced by a factor of 1.7 and is unchanged for pure phaeophytin-a. The decrease in absorbance at 665 nm is due to the extraction of a magnesium atom from the chlorophyll molecule and the conversion of chlorophyll to phaeophytin. Lorenzen also suggested that the usefulness and accuracy of a new set of hexachromatic equations to quantitate chlorophylls-a, b, and c and phaeophytins-a, b, and c, was not justified. Therefore he developed the empirical equations for the determination of chlorophyll-a and the group of phaeo-pigments based on the absorbances measured at one wavelength as shown below.

$$\text{Chl-a (mg/L)} = 26.7\ (A_{665b} - A_{665a}) \qquad (10)$$

$$\text{Phaeo (mg/L)} = 26.7\ (1.7\ (A_{665a} - A_{665b})) \qquad (11)$$

The subscripts a and b symbolize the absorbances after and before the addition of acid to the chlorophyll extract, respectively. Lorenzen used a 1-cm pathlength cell for all measurements. These equations are now known as the monochromatic equations and the method is also referred to as the acidification method. Absorbances at 750 nm are also measured and subtracted from the absorbances used in these equations to correct each for turbidity (Parsons and Strickland, 1963). Recently, it was shown that the use of this method is actually preferred to the trichromatic equations (Nusch, 1980) because the trichromatic equations poorly quantitate the concentrations of chlorophylls b and c and sometimes leading to

"negative" results for chlorophyll-a. The monochromatic equations estimate the chlorophyll-a concentrations as accurately as the trichromatic equations with only one additional absorbance measurement needed for correction. Because it has been shown that chlorophyll-a is usually the largest component of the chlorophylls in phytoplankton, the monochromatic method is the method which is used to estimate most accurately phytoplankton biomass.

The EPA endorsed the use of the monochromatic equations in 1983. The range of linearity extends from 0.27 to 21 mg/L for a pure chlorophyll-a extract. This range is based on the assumption that one can determine chlorophyll absorbances from 0.01 to 0.80 for an extract of pure chlorophyll-a in a spectrophotometer with a 1-cm sample cell. A detection limit of 0.27 mg/L corresponds to a concentration of 0.54 µg/L of chlorophyll in water assuming the standard extraction volumes.

Fluorometric Methods

Fluorometric methods may be broken into three distinct categories, the 'in vitro' or extractive method, the 'in vivo' method, and the 'in situ' methods.

The 'in vitro' Method

An 'in vitro' fluorometric method for the quantitation of chlorophyll has been discussed in detail (Yentsch and Menzel, 1963). After a similar extraction technique as used in the spectrophotometric technique, samples are excited with a band of

radiation centered at 435 nm using Corning CS 5-60 or 47B excitation filters. The fluorescence (band maximum at 663 nm) is measured using Corning CS 2-60 or CS 2-64 emission cutoff filters which transmit radiation of wavelengths longer than 660 or 640 nm, respectively. Transmission spectra for two of the filters are shown in Figure 8. The fluorescence emission spectra of the chlorophylls are shown in Figures 2-4.

Yentsch and Menzel recommended calibrating the fluorometer with chlorophyll extract solutions whose concentrations have been determined with the spectrophotometric technique. This leads to a correlation diagram similar to that shown in Figure 9. The total chlorophyll concentration in a water sample is then calculated from equation 12

$$Chl_t\ (\mu g/L) = D/10 \cdot k/1000 \cdot V_{ext}/V_{sam} \qquad (12)$$

where D is the equivalent absorbance measured in a 10-cm cell and k is an average of the specific absorptivities for chlorophyll-a at 666 and 655 nm in μg/L per A.U. and recommended to be 56.6 (Vernon, 1960). The symbol V_{ext} is the milliliter volume of the extract used to calibrate the fluorometer and V_{sam} is the liter volume of seawater filtered. Here D is calculated from equation 13

$$D = F \cdot m \qquad (13)$$

where F is the fluorescence signal of the sample and m is the slope of a correlation curve (A.U./fluorescence readout units) similar to Figure 9.

The EPA recommends calibrating the fluorometer directly with a chlorophyll standard in 90% (v/v) aqueous acetone (Collins, 1984). In this case the chlorophyll concentration is calculated from

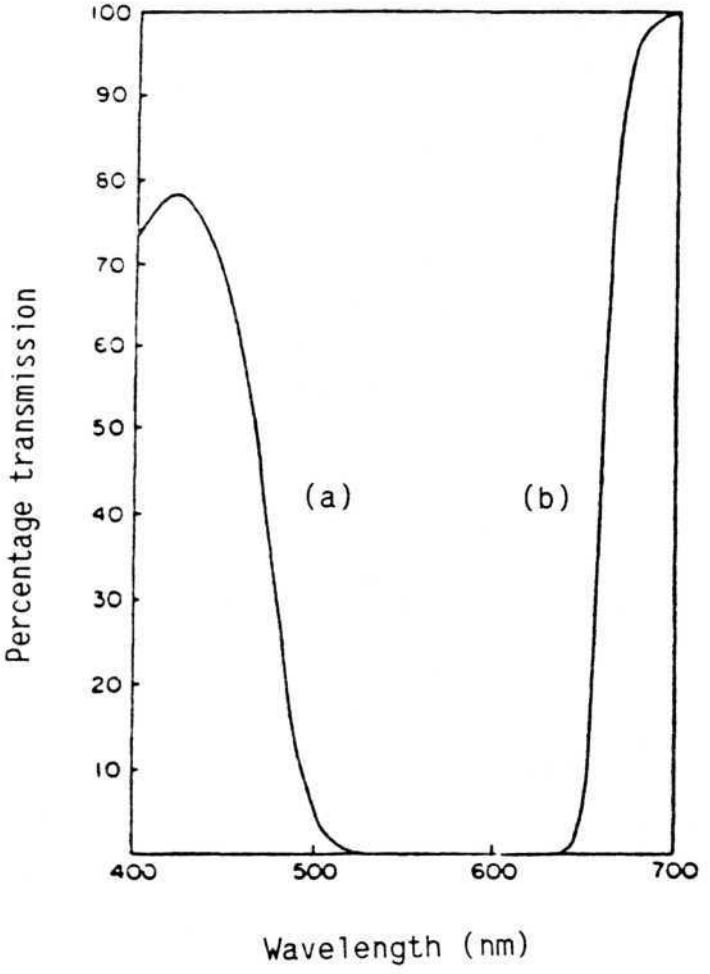


Figure 8. Transmission properties of the CS 2-64 filter (curve a) and the CS 5-60 filter (curve b). These filters are used in the Turner Designs fluorometer.

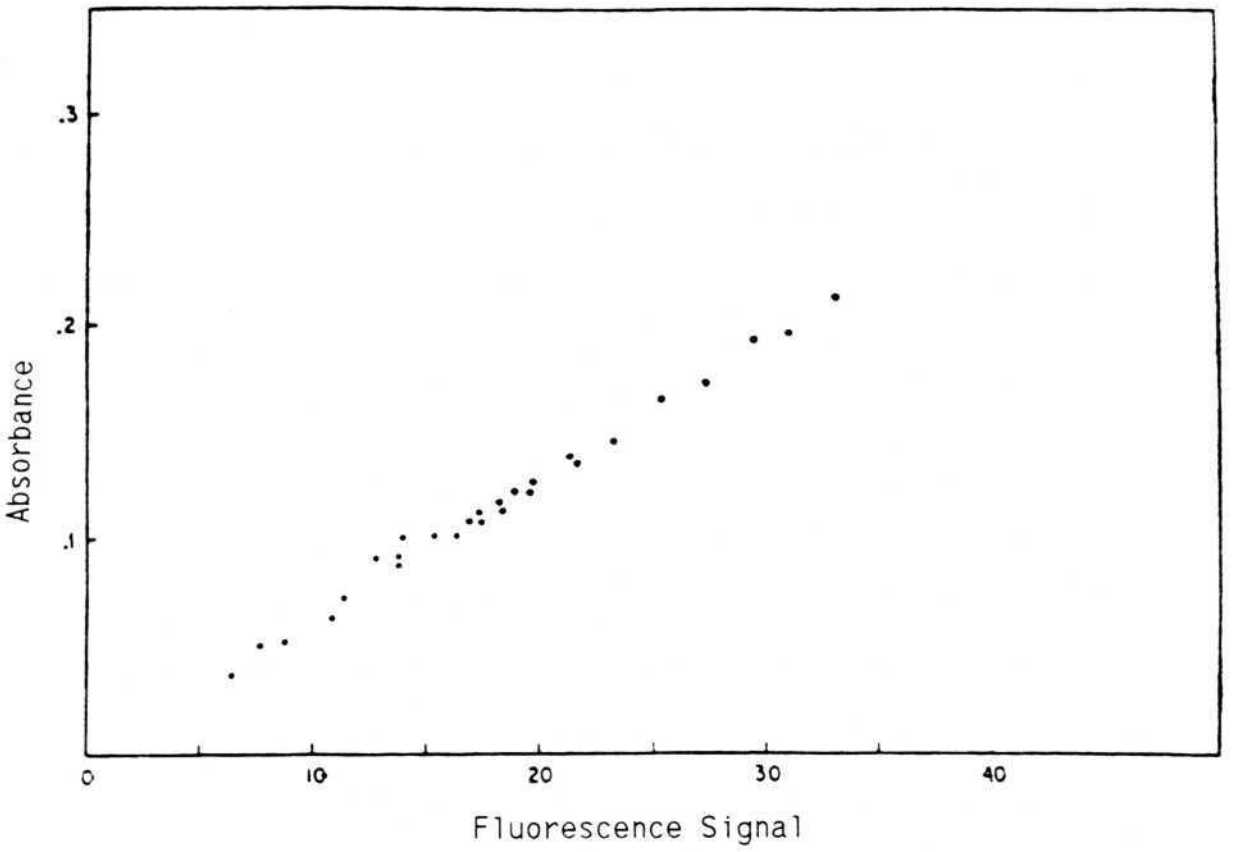


Figure 9. Correlation diagram between 'in vitro' fluorescence signal and absorbance. Absorbances measured using a 10 cm pathlength cell at λ = 665 nm of 85% (v/v) aqueous acetone extracts of natural phytoplankton populations in seawater between the coast of Montauk Pt., Long Island, and the north central Sargasso Sea (Yentsch and Menzel, 1963).

equation 14.

$$Chl_t(\mu g/mL) = F \cdot M_f \qquad (14)$$

where M_f is the conversion factor for standards in µg/L per fluorescence readout unit. It is the inverse of a normal calibration curve slope.

A secondary standard, coproporphytin, is sometimes used. Coproporphytin is much more stable than chlorophyll and so serves well as a long term standard. It may be kept in a diluted state in a dark refrigerator at 0^o C for several months (Turner, 1981). The fluorescence signals of coproporphytin and a primary standard of chlorophyll-a are measured with a fluorometer with the same excitation and emission conditions. Thereafter, the coproporphytin standard is used to calibrate the instrument. The chlorophyll-a concentration in a sample is determined from its fluorescence signal, the calibration curve of fluorescence signal versus coproporphytin concentration, and the ratio of the fluorescence signals for equivalent concentrations of chlorophyll and coproporphytin.

The 'in vitro' fluorometric technique provides a lower detection limit than the spectrophotometric technique (Yentsch and Menzel, 1963, ASTM, 1984). Therefore it is the recommended technique when working with small populations of phytoplankton and therefore low concentrations of chlorophyll. One might simply filter more water, to increase the concentration of chlorophyll in the extract; however, an upper limit of about 10 L of water has been suggested because of the resulting increase in the time of filtration. The fluorometer manufacturer reports that the fluorometric detection limit for

chlorophyll is over twenty times better than that obtained by spectrophotometry (Turner, 1981). The American Society for Testing and Materials (ASTM, 1984) states that the fluorometric determination of chlorophyll has a 10 to 1000 times better detection limit than the spectrophotometric determination. This assumption leads to a detection limit in the range of 0.27 to 27 μg/L in the extract and would correlate with a chlorophyll concentration in a water sample of 0.54 to 54 ng/L assuming standard extraction volumes.

As discussed previously, researchers have been interested in the phaeophytin concentration and so have developed a fluorometric acidification technique (Yentsch and Menzel, 1963). The fluorescence signal of chlorophyll-a (F_{Chl-a}) is estimated from the following equation

$$F_{chl-a} = F_b\,[(F_b/F_a) - 1.0]/0.7 \qquad (15)$$

where F_a and F_b symbolize the fluorescence signal after and before acidification, respectively, of the chlorophyll sample tested. Here F_a is the fluorescence signal due to the phaeophytins and it is assumed that for equivalent amounts of chlorophyll and phaeophytin, the fluorescence signal for chlorophyll is a factor of 1.7 greater. To determine the concentration of pure chlorophyll-a, this corrected fluorescence signal (F_{Chl-a}) is substituted for F in equation 13 to calculate D, which is then substituted into equation 12. The difference between the total chlorophyll (Chl_t), obtained from equations 12 and 13 with $F = F_b$, and the corrected chlorophyll-a concentration, obtained using equation 15, is the concentration of phaeophytin-a.

In 1984, the EPA recommended the following procedure to differentiate the concentrations of chlorophyll-a and phaeophytin-a (Collins, 1984). The fluorescence signal of a pure sample of chlorophyll-a is determined before and after acidification. The before:after acid ratio, $r = F_b/F_a$, depends on the individual fluorometer where F_a and F_b have the same meanings as before. The ratio, r, is then used in the following equations

$$\text{Chl-a } (\mu g/L) = M_f \, (r/r-1) \, (F_b - F_a) \qquad (16)$$

$$\text{Phaeo-a } (\mu g/L) = M_f \, (r/r-1) \, (r \cdot F_a - F_b) \qquad (17)$$

where M_f in the conversion factor between fluorometric readout units and chlorophyll concentration. This is calculated using samples of pure chlorophyll-a supplied by the EPA. The range of linearity was not reported. The EPA provides quality control standards to evaluate the instrument calibration in a specific laboratory.

The 'in vivo' Method

Because of the increased sensitivity afforded by the fluorometric method, a technique to quantitate the phytoplankton in a water body with very little sample preparation has been developed. In this method, the fluorescence signal of chlorophyll in living phytoplankton is measured directly. The water sample is manually placed in the fluorometer cuvette or is pumped to a fluorescence flow cell. As shown in Figure 10, the 'in vivo' signal correlates with the amount of chlorophyll-a in the sample, (Lorenzen, 1966). The 'in vivo' fluorescence method provides a detection limit of 0.04 µg/L and linearity up to 15 µg/L (Lorenzen, 1966). Lorenzen suggested that

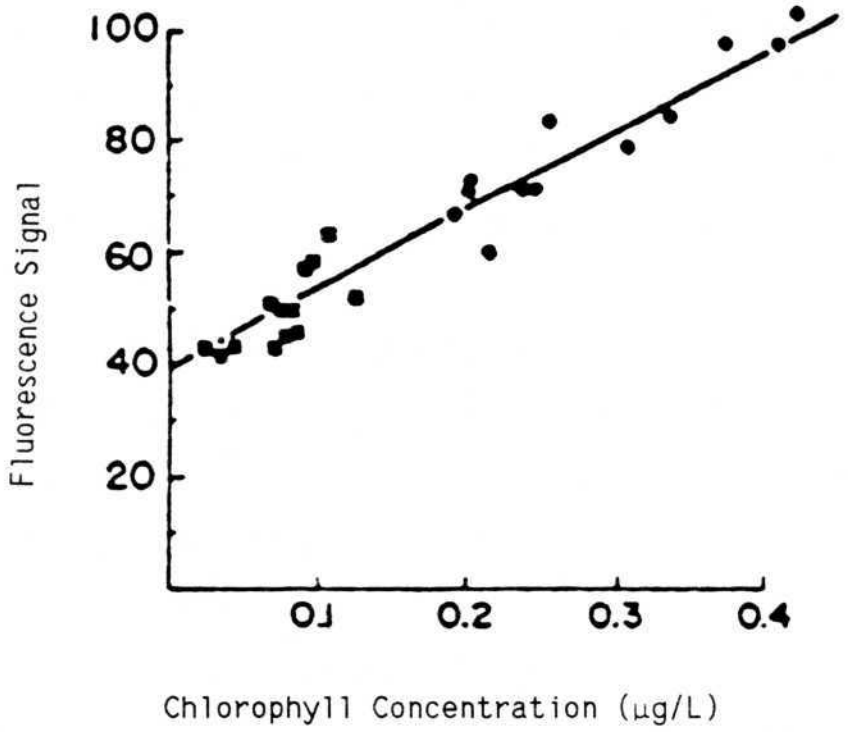


Figure 10. Calibration curve for 'in vivo' fluorescence measurements of chlorophyll-a. The chlorophyll-a values were determined fluorometrically from extracts using the 'in vitro' method. Regression: Y = 38.6 + 146 X. Squares denote samples from offshore and circles from near shore (Lorenzen, 1966).

the non-zero intercept observed was probably the result of light scattering and light leakage through the emission filters.

Others have realized that the 'in vivo' fluorescence is a remarkably complex phenomenon (Prezelin, 1980). Since the phytoplankton are not only intact but photosynthesizing, the fluorescence signal depends not only on their number and chlorophyll content, but also on their irradiance history, nutrient balance, age, and species. The fluorescence signal for a given sample also depends upon the type of sample handling, the type of detection procedure, and time of day (Prezelin, 1980). The intensity of fluorescence when using a flow cell has been shown to vary as a function of flow rate. Above a flow rate of 500 cm^3-min^{-1}, the 'in vivo' fluorescence increases with flow rate. This effect suggests that the phytoplankton fluorescence is a function of illumination time or time of exposure to excitation light (Sweet and Guinasso, 1984).

When living phytoplankton are illuminated with light of a wavelength absorbed by chlorophyll, the absorbed energy can be used for photosynthesis or fluorescence. Thus the fluorescence yield of chlorophyll in a living cell depends upon the cell's ability to photosynthesize (Samuelsson, 1977). Because of this, a variable has been described which relates the 'in vivo' fluorescence signal to the sample's 'in vitro' fluorescence signal. The 'in vitro' fluorescence signal is normalized to a concentration factor of unity. This ratio, $F_{'in\ vivo'}/F_{'in\ vitro'}$, has been called "R" or the fluorescent number (Kiefer, 1973). Patterns have been recognized in data which suggest that the phytoplankton's health, age, nutrient availability, and light history might indeed be related to a quantity similar to

this ratio. As shown in Figure 11, the fluorescent number varies typically from 0.25 to 0.30 for measurements taken at night and from 0.10 to 0.15 for measurements taken at midday.

A method to halt the passage of energy onto the photosynthetic pathway has been demonstrated. The herbicide 3(3,4)-dichlorophenyl-1,1-dimethyl urea, DCMU, was shown to inhibit photosynthetic electron transport (Papageorgiou, 1971). It was further shown that the use of DCMU removed the dependency of the 'in vivo' fluorescent signal on the phytoplankton's light and nutrient history, but not differences specific to individual species. If a cell can not pass the absorbed excitation light energy onto its related photosynthetic mechanism, it is more likely to fluoresce. Indeed, this new signal has been referred to as the enhanced 'in vivo' fluorescence signal. This signal allows comparison of cells with differences in physiological state (Samuelson, 1978; Bjaronborn, 1980). If the phytoplankton are capable of a high rate of photosynthesis, the enhancement factor (E) in the fluorometric signal,

$$E = F_{DCMU}/F_o$$

is the greatest where F_{DCMU} is the enhanced 'in vivo' fluorescence signal using DCMU and F_o is the untreated 'in vivo' fluorescence signal. However, if the algae are old or somehow reduced in their capacity to photosynthesize, the increase in fluorometric signal is minimal (Samuelson, 1977). Correlation between the productivity determined by the standard C-14 method and the fluorescence enhancement factor has been reported to be very high using a growing laboratory culture of Chlorella pyrenoidosa over a period of nineteen

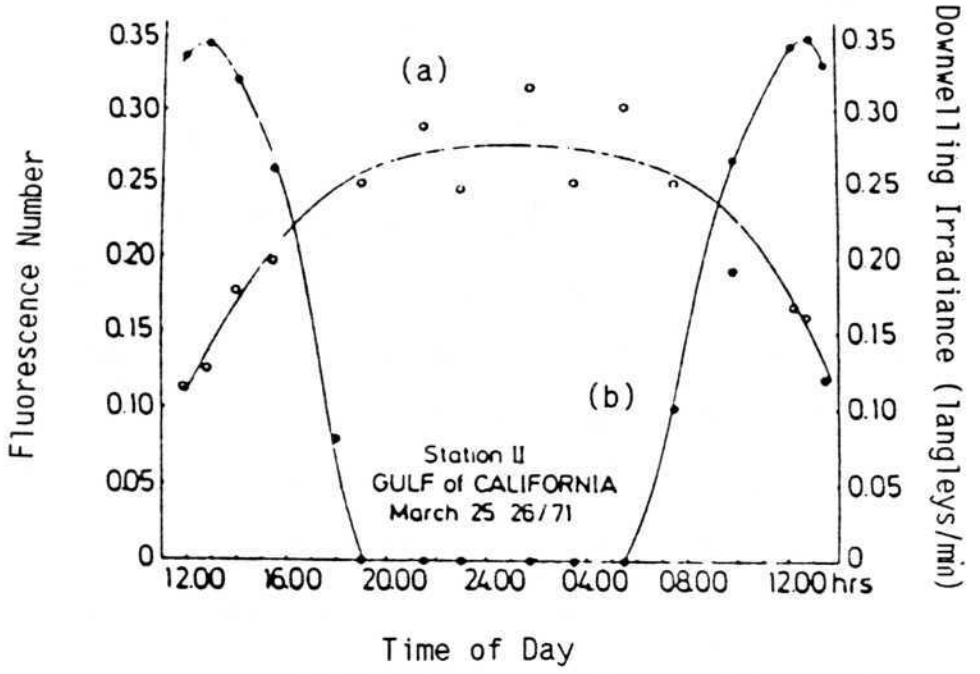


Figure 11. Diel changes in fluorescence number (a) and estimated downwelling irradiance (b). Down-welling irradiance was measured with on-deck irradiance meter, and phytoplankton were sampled at depth of 1 m in the Gulf of California (Kiefer, 1973). The concentration factor for the 'in vitro' measurements was 4.

days (Samuelson, 1977).

In searching for meaningful relationships between the 'in vivo' and the enhanced 'in vivo' fluorescence signals, the fluorescent response index, FRI, has been defined as

$$FRI = (F_{DCMU} - F_o) / (F_{DCMU}) \quad (18)$$

where F_{DCMU} and F_o have the same meanings as above. Its value usually lies in the range 0.0 to 1.0 because of the way it is defined. A very low value of FRI suggests a low photosynthetic ability in a phytoplankton sample (Cullen, 1979).

The 'in situ' Technique

The 'in situ' fluorometric method of determination of chlorophyll is an extension of the 'in vivo' technique and involves the direct measurement of fluorometric signals without bringing the water sample to the surface. Researchers have employed pumps, flow cells, and tubing to follow the 'in vivo' fluorometric patterns of a phytoplankton population in a body of water. But this is not a true 'in situ' fluorometric determination.

Three true 'in situ' methods deserve description. In one case, the fluorometer, encased in a submersible container along with a battery, flow cell, pump, depth sensor, and data recorder have been attached to a hydrographic cable and lowered into the sea. Depth data taken concurrently provided information on the fluorescence signal and depth in that profile (Abbott, 1984). This type of fluorometer, at greater cost, might also be in continuous communication with the ship above. This would have allowed real time data to be collected. This fluorometric device is limited to a depth

of about 100 m determined by the strength of the case and, more importantly, the strength of the flow cell.

A second type of fluorometer has been used and consists of two watertight containers. One houses a strobe light source which is oriented at right angles to a second container housing a detector, battery, and recorder. This type of fluorometer was used to obtain a deeper profile but was still limited to a depth of about 500 m (Mendes, 1985).

Yet a third type of 'in situ' fluorometric probe has been suggested. This method involves the use of fiber optics to channel excitation light into a water body and to return the fluorescence signal to a detector (Lund, 1983). This is a type of remote fiber fluorometry (RFF). The detection limit depends upon the type of algae was shown to be as low as 0.02 μg/L for chlorophyll-a.

Chromatographic Methods

Historically the separation of photosynthetic pigments was accomplished using thin layer chromatography (Holden, 1976). This technique allows isolation of many specific chlorophyll pigments and degradation products, but is considered too time consuming and labor intensive for routine determinations (Mantoura, 1983).

The preparative separation of chlorophyll-a and b follows a procedure designed by Strain (Strain, 1963). The pigments of green leaf extracts are separated on a powdered sugar column. The column is then disassembled and the individual bands of chlorophyll are extracted with petroleum ether.

To increase the speed of separation for routine applications, several HPLC techniques have been developed. Abayshi and Riley in 1979 used normal-phase HPLC after evaporation of the acetone in the extract. Detection was carried out spectrophotometrically using a wavelength of 440 nm. Brown used reverse-phase HPLC but the resolution was very poor with many of the polar compounds not being separated (Brown, 1981). A spectrofluorometric detector was incorporated using an excitation wavelength of 412 nm and emission wavelengths of above 550 nm.

Mantoura and Llewellyn in 1983 described an HPLC technique which was able to separate many of the major photosynthetic pigments and degradation products. They used a reverse phase HPLC technique in conjunction with an ion-pairing agent to separate and quantitate fourteen chlorophylls and their associated breakdown products and seventeen carotenoids from acetone extracts of phytoplankton (Mantoura, 1983). The ion-pairing agent, P, was prepared by mixing 1.5 g of tetrabutylammonium acetate with 7.7 g of ammonium acetate and diluting to 100 mL with water. Mobile phases used in the gradient elution consisted of primary eluant, A, made up of 10:10:80 mixture, by volume, of solution P:water:methanol, and a secondary eluant, B, made up of 20:80 acetone:methanol, by volume. They used a linear gradient elution from 100% solution A to 100% solution B in 10 minutes followed by a 12 minute isocratic hold at 100% B. A 25 X 0.5 cm column was packed with octadecyl-silane bonded 5-μm ODS-Hypersil. A fluorescence detector was employed with an excitation wavelength range of 430 $\pm$ 40 nm. The fluorescence emission at wavelengths greater than 600 nm was monitored. This detector was used in

conjunction with an absorption detector to obtain chromatograms monitored at 440 nm and absorption spectra of the chromatographic peaks from 380 to 600 nm. They report a detection limit of 0.1 ng/L of chlorophyll in water with 100 µL injections from a 10 mL extract of 1 L of seawater. Using the standard extract volumes, this would *correspond to a detection limit of 20 pg/L. This represents a improvement in the detection limit of more than four orders of* magnitude over the spectrophotometric monochromatic method and about one order of magnitude over the uncorrected 'in vitro' fluorometric method. They also state that for comparison purposes, that the spectrophotometric method has a limit of detection of 0.1 µg/L for a 1-L extraction or 0.02 µg/L for a 5-L extraction. This is four times better than that predicted using the Jeffrey and Humphrey equations.

The HPLC method has been used to prepare the chlorophylls-a and b, their epimers-a' and b', and their phaeophytins at the 20-50 mg level (Watanabe, 1984). Silica gel was used as a reverse-phase stationary phase even though it was previously thought to be overly reactive leading to erroneous results (Braumann, 1981). The resulting chlorophyll-a was actually of much higher purity than the best samples commercially available as shown by elemental analysis, analytical HPLC, and spectrometric measurements (Watanabe, 1984).

Remote Sensing With Fiber Optics

General Principles

In 1970 Corning glass works developed a fiber optic material capable of transmitting one percent of the incident light a distance

of one kilometer (Gunderson, 1983). Since that time, fiber optics have been extensively developed and improved because of their application in the communication industry. Communication of digital data at one billion baud for 50 to 100 km using radiation in the 600 to 1600 nm wavelength range is possible today. The properties of the glass used to produce fiber optics has been improved allowing transmission of data over longer distances before a repeater is needed. Vapor deposition is used to produce high purity silicon and germanium oxides. This high purity material is made into fused silica fiber optics and can transmit radiation at wavelengths as low as 220 nm (Seitz, 1984).

An early spinoff of fiber optic technology is the use of fiber optics in remote sensing applications. In the last decade researchers involved with the detection and determination of analytes at remote sites became aware of the possibility of using fiber optics in spectroscopic applications. The advantages of using fiber optics for remote sensing have been cited in many recent articles and include:

use to quantify fluorophores, quenchers and analytes that may be made to fluoresce (Klainer, 1983)

reasonable cost and availability of interfacing materials

small size and potential for miniaturization and clinical applications

light weight and environmental ruggedness and the potential to probe explosive, radioactive, physically or chemically harsh environments

ability to couple many fibers into a central monitoring system allowing investigators to interrogate several remote sites with fiber optics sensors, the inexpensive part is duplicated (Hirschfeld, 1983b)

'in situ' use decreases the possibility of sample alteration in collection, transport and storage until analyses are initiated

near real time data acquisition eliminates logistical and record keeping problems

immunity to large magnetic and electric fields

independence from any reference electrode, ability to select wavelengths and detection times

use at times and locations where no other instruments are available.

The limitations of fiber optics in remote sensing applications include the attenuation of light in the fiber optics, the complexity of focusing light into the fiber optic, interference from ambient radiation, and the difficulty of making the distal end of the fiber optic selective for one species or property. Physical damage to the fiber optic end may result as source power is increased. However, more distant sites may be probed using increased source intensities. This may be accomplished without affecting sample response (Chuduk, 1985).

The sensing end of the fiber optic probe is often called an optrode in the same sense that the end of an electrical sensor is called an electrode. Optrodes can be classified any number of ways. Milanovich and Hirschfeld have divided optrodes into two broad groups, physical and chemical (Hirschfeld, 1983 b). Physical optrodes respond to mechanical or physical properties directly. These include pressure, temperature, position, acceleration, electric and magnetic fields, or acoustic waves. Chemical optrodes are sensitive to selected chemical species. This usually requires immobilizing reagents at the distal sensing end of the fiber. The reagent can be chemically bound to or in a porous material at the

fiber end or confined in a reservoir in contact with a sample through a semipermeable membrane. If the reaction between the reagent and the analyte is reversible, the optrode signal increases or decreases as the analyte concentration goes up and down. Thus, the response of the sensor is similar to that observed in potentiometric analyses. However, if the reaction is irreversible, the reagent is consumed in the process and the optrode signal changes only in one direction. The rate of change is related to the analyte concentration. This response is similar to that observed with amperometric analyses.

Chemical fiber optic probes can be based on absorption, fluorescence or chemiluminescence. This thesis is concerned primarily with fluorescence-based optrodes.

Remote Fiber Fluorometry

The use of fiber optics to sense the fluorescence of a chemical species at a distance is called remote fiber fluorometry (RFF). Fiber optics probes for remote fluorescence sensing are useful for several reasons. Fluorometry offers selectivity through choice of both the excitation and emission wavelength. Fluorescence or phosphorescence lifetimes may be used to increase selectivity. With modern detectors and signal processing techniques, very low levels of sample fluorescence can be measured and a large linear dynamic range response is achieved. Excellent detectability, good selectivity and well characterized response combine to make fluorometry and attractive method for remote sensing (Seitz, 1984).

RFF seems to have begun with the use of fiber bundles to channel

light for short distances into test tubes or cuvettes (Mitchell, 1976). A bifurcated fiber optic bundle was used to channel excitation radiation from a quartz halogen lamp through an excitation filter and into a blackened test tube containing the sample. The emission radiation was directed from the test tube by a second branch of the same fiber optic bundle through an emission filter and on to a photomultiplier tube (PMT). Early studies focused on correcting fluorescence measurements for the absorption of light by interferences in the sample matrix. The use of a fiber optic probe eliminates pre-filter effects and minimizes effects due to cell wall inconsistencies and surface contamination.

The use of a fiber optic probe for the determination of phytoplankton in water was investigated (Lund, 1983). This was accomplished using a xenon flashlamp, a broad band 420 nm excitation filter (100 nm bandpass), and an emission filter selected to transmit light at 690 nm but absorb light used to excite the sample. An RCA 31034A PMT was used to sense the emission signal. A 5 mm bifurcated fiber optic bundle was used to channel radiation to and from the sample. This probe was lowered to a depth of 0.3 m and towed behind a boat. Detection limits for a lab grown sample of the phytoplankton Selenastrum capricornutum were found to be as low as 0.02 µg/L.

The use of bifurcated fiber bundles over long distance is cost prohibitive. Because of this, individual fiber optics with core diameters of 200 to 600 µm diameter were used to increase the distance between the sensor and the instrument at a reasonable cost (Hirschfeld, 1983 a). In this case the excitation radiation is channeled to the sensing end of the fiber optic probe and the

emission radiation is directed back to the detector (Sepaniak, 1983; Hirschfeld, 1983a). This approach is claimed to increase the coupling of excitation radiation and collection of emission radiation.

For the single-fiber approach, a coupling device is needed to separate the excitation and emission beams. The three methods used by Hirschfeld's group are illustrated in Figure 12. These are the perforated mirror (hole-in-the-mirror) technique, the small prism method and the dichroic filter technique. The first two methods are used with laser excitation. The small diameter laser beam can be focused on the fiber optic. The size of the "hole" or prism is small compared to the emission beam diameter such that most of the emission beam is collected. In the dichroic filter method, the filter transmits the excitation radiations but reflects the emission radiation which has a longer wavelength.

With single-fiber technology, fibers can be used to channel light up to 1 km at certain wavelengths. It has been suggested that Raman spectrometers be used as detector systems for long fiber optics probes because of their increased sensitivity (Hirschfeld, 1983a). The overall calibration sensitivity of the optrode depends on the diameter of the fiber core. The effective pathlength (L_e) or depth of penetration of the fluorometric measurement into the sample by an optrode with a plane perpendicular termination is given by the equation

$$L_e = 1.303\ r \cot a \qquad (16)$$

where r is the fiber radius and a is the acceptance angle of the fiber optic material (Deaton, 1983). The effective pathlength is the

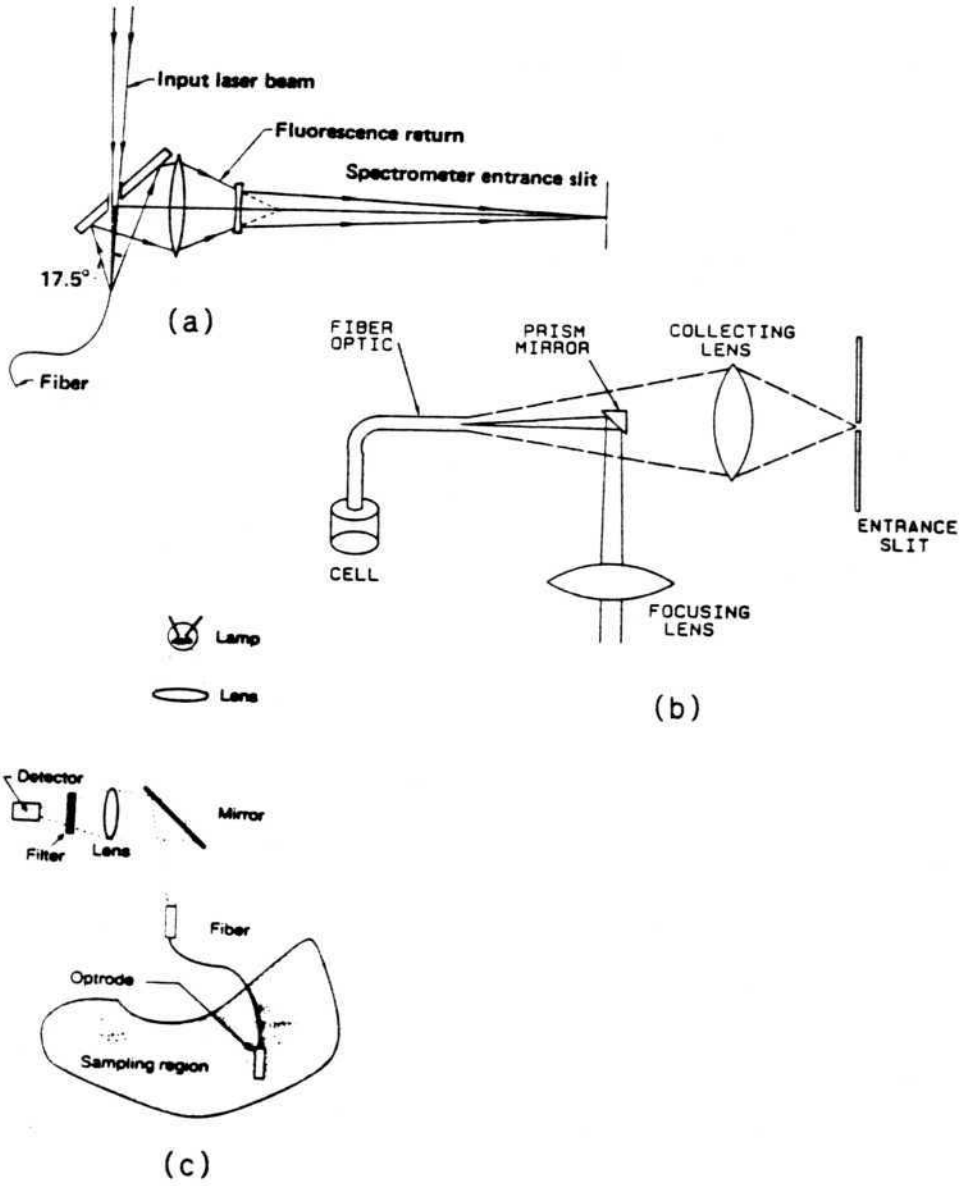


Figure 12. Three arrangements to separate the excitation and emission radiation for single-fiber remote sensing fluorometers (Hirschfeld, 1983a). (a) Hole in mirror, (b) small prism, (c) dichroic filter (Angle, 1987)

length of an idealized cylinder of solution with a radius of the fiber optic which yields the same fluorescence signal as observed with the optrode.

In air the numerical aperture (NA) is equal to the sine of one half the acceptance angle. Consider a 600-μm diameter single fiber optic cable with a perpendicular face and a numerical aperture of 0.22. In this case a is 25.4^{o} and the effective pathlength of a single fiber optic optrode would be 0.825 mm. If more sensitivity is required, the diameter of fiber probe is increased. Fiber diameters up to 1000 μm are available.

INSTRUMENTATION

Spectrophotometric Measurements

Both a Cary 118C scanning spectrophotometer and an HP 8451A diode array spectrophotometer were used for spectrophotometric measurements of chlorophyll standards and the determination of chlorophyll in extracts from Crater Lake and Cronemiller Lake. Both 1-cm and 5-cm pathlength cells were used for spectrophotometric measurements.

Typical operating parameters for the Cary 118C spectrophotometer are listed in Table II. For the monochromatic and trichromatic methods, the absorbance readings from the digital readout were taken at selected fixed wavelengths.

Table II. Instrumental Parameters For Measurements with the Cary 118C Spectrophotometer

Scan Rate	1 nm/s
Period	1 s
Chart Speed	20 nm/in
Spectral Bandpass	2 nm
Geometric Slitwidth	0.044 mm
Absorbance	1.0, full scale

The Cary 118C spectrophotometer was also used to acquire absorption spectra and to determine the influence of the spectrophotometer bandpass on the width and maximum absorbance of chlorophyll absorption bands. The monochromator slitwidth was set to give the desired spectral bandpass using equation 19.

$$s = R_d\ (W + 0.005) + s_m + s_d \qquad (19)$$

where s is the spectral bandpass in nm, W is the geometric slitwidth in mm, R_d is the reciprocal linear dispersion of the monochromator in nm/mm, s_m is the slit mismatch in nm, and s_d is the diffraction limited spectral bandpass measured in nm. Because the Cary 118C is based on a prism monochromator s, R_d, s_m, and s_d vary with wavelength. The values of these variables to use in equation 19 to calculate W for the desired spectral bandpass were determined using data from the instrument manual (see appendix 2) at a wavelength of 650 nm.

The HP 8154A diode array spectrophotometer has a spectral bandpass of 2 nm and was programmed to use a 1-s integration time. It was configured to report the the absorbances at wavelengths of 630, 646, 664 and 750 nm.

Fluorescence Measurements

The Turner Designs fluorometer, model 10, was used for the fluorometric determination of chlorophyll and a Varian spectrofluorometer, model SF-330, was used to acquire emission spectra. The Turner fluorometer was equipped with a Corning CS 2-64 emission filter and a Corning CS 5-60 excitation filter. The transmittance spectra of these two filters are shown in Figure 8. This instrument was also equipped with a red sensitive R-446 photomultiplier tube and a coated Hg blue light source, F4T.5.

Attenuation plates of different diameters can be selected to adjust the excitation radiant power striking the sample. If a sample contains little chlorophyll, the attenuation plate with the largest opening is used to increase the emission radiant power

detected. The largest diameter attenuation plate (47-mm diameter) was used for all 'in vivo' measurements. The 7-mm diameter attenuation plate was used for all 'in vitro' measurements.

The above instrumental conditions (i.e., filters, detector, source) are recommended made by both the EPA and Turner Designs for the 'in vivo' detection of chlorophyll. Turner further recommends the use of Corning Wratten CS 70 and Corning Wratten CS 16 emission filters in place of the CS 2-64 filter for determining chlorophyll-a by the 'in vitro' method. These filters are recommended where instrument temperature variations are a problem. A color filter's cutoff wavelength varies with temperature. The Wratten filters are stable over a wider temperature ranges. Since the EPA does not recommend this substitution, the CS 2-64 emission filter was also used for 'in vitro' measurements.

Fiber Optic Measurements

Three experimental configurations were employed to study the focusing characteristics of the source, transmission of the fiber optic cables, and the use of fiber optic cables in a remote-sensing fiber fluorometer (Figure 13). In all cases, a Photon Technology International (PTI) model LPS 200 power supply and a model LP-100 lamp housing were used. Both a 100-W Hg and a 75-W Xe arc lamp were also used to provide high intensity white light focused to a small image. The lamp housing was used with both f/4.5 and f/2.5 elliptical reflectors. The f/2.5 elliptical reflector produces a focal spot that more closely matched the acceptance angle of the fiber optic and was used in all fiber optic studies. The

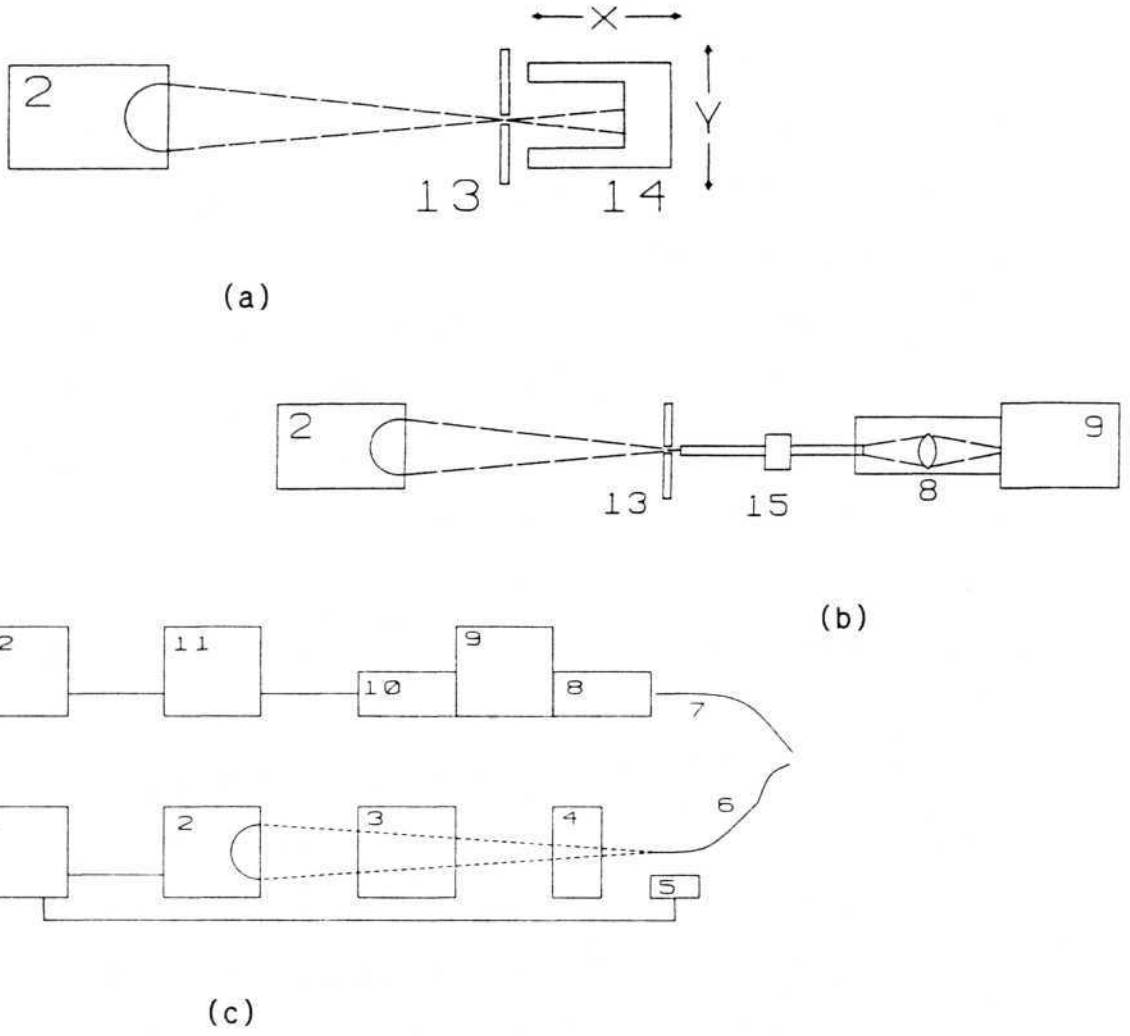


Figure 13. Fiber Optic Instrumentation. Configuration for focusing studies (a), transmission studies (b) and fluorescence studies (c). 1, Lamp Power Supply; 2, Lamp Housing; 3, IR Filter; 4, Excitation Filter; 5, Feedback Sensor; 6, Excitation Fiber Optic Cable; 7, Emission Fiber Optic Cable; 8, FO Focusing Adapter; 9, Emission Monochromator; 10, PMT; 11, Current-to-Voltage Converter; 12, Digital Voltmeter; 13, Aperture; 14, Radiometer; 15, Coupler.

xenon lamp was powered at 13.5 V with a current of 4.7 A. Under these conditions the lamp is operated at about 63 W which is below its 75-W rating.

To study the focusing characteristics of the source and its housing, the configuration shown in Figure 13a was used. The source, the aperture and radiometer were mounted on separate carriers on an optical rail. Two perpendicular translation stages allow positioning of the aperture in the plane perpendicular to the optical axis. Focusing the Xe lamp involved adjusting the lamp-to-reflector distance and the lamp-housing-to-aperture distance (x). Three adjusting screws on the lamp housing were used to change and center the position of the image of the Xe arc. The image was centered on a 1-mm aperture behind which a radiometer converted transmitted radiant power to a voltage (Scientech Pyroelectric Radiometer, model 36-0203). The aperture was moved along the optic axis toward and away from the lamp housing. The radiant power at different distances from the lamp housing window to the aperture (x) were recorded. Once the x-distance that yielded the greatest transmitted power was determined, the radiant power transmitted as a function of position of the aperture as it was moved across the optical axis (y) was determined. The dependence of the transmitted radiant power on aperture size was also investigated.

For transmission measurements, the configuration shown in Figure 13b was used. A 1-m length of fiber optic cable was mounted directly behind the aperture at the x-distance yielding the greatest power transmission. A second fiber optic cable was coupled to the first to study transmission characteristics of this cable. The second

cable was either a 1- or 45-m cable and was coupled to a monochromator-based detection system as discussed below. The fibers used were 600-μm diameter fused silica. Three pieces (1, 1 and 45 m in length) were cut and polished as later described.

A block diagram of the configuration used for remote sensing fluorometry is shown in Figure 13c. This system was used to study the remote detection of quinine sulfate and chlorophyll fluorescence and the scattering effects in solutions. The optical feedback is designed to maintain the intensity of the lamp constant. A portion of the unfocused lamp radiation is impingent on the photodiode off the beam axis and close to the beginning of the fiber optic cable.

A water filter was used to absorb a portion of the infrared radiation from the source in transmission, scattering, and fluorescence studies. The heat filter is required since the fiber optics and any colored excitation filters could be damaged if exposed to unfiltered IR radiation. The IR filter is a 7.0-cm long cylinder with quartz windows that is filled with deionized water. The IR filter degrades the quality of the focused image and reduces the amount of radiation reaching the fiber optic cable. It was placed in the optical path at a position to optimize the amount of light input into the fiber optic.

Because the fiber optic was attached to an x-y translator mounted to an optical rail, it could be aligned with respect to the focused source image. The fiber optic was properly aligned when the amount of light transmitted through it was a maximum as determined by the detection system.

For transmission, scattering and fluorescence measurements, a Heath model EU-700 monochromator was used as a wavelength selector. The RCA 1P28 PMT in the PMT housing attached to the monochromator was replaced by a RCA 4840 PMT which has higher responsivity in the red region of the spectrum where chlorophyll fluoresces. The monochromator slitwidth and the PMT voltage were set to different values depending on the study as tabulated in Table III. The reciprocal linear dispersion of the monochromator is 2 nm/mm.

An operational amplifier was used in the current-to-voltage configuration to convert the photoanodic current from the PMT into a proportional voltage monitored by a Fluke digital multimeter. The operational amplifier was used with different feedback resistors and feedback capacitors (see Table III). For spectral scans during the transmission study, the voltage signal was also monitored with a strip chart recorder.

The second fiber optic used in the transmission, scattering and fluorescence studies was coupled to the monochromator using the adjustable adapter shown in Figure 14. The adapter used was designed by Jeff Louch, Department of Chemistry, Oregon State University. It holds an asymmetrical aspheric 18-mm glass lens with an f-number of 0.75 and a diameter of 25.4 mm. The coupler allows adjustment of the distance both between the fiber end and the focusing lens and between the monochromator and the lens. The object and image distances are adjusted so that the lens collects the f/2 radiation leaving the fiber optic and focuses it on the entrance slit of the monochromator with an f-number that matches that of the monochromator (f/7).

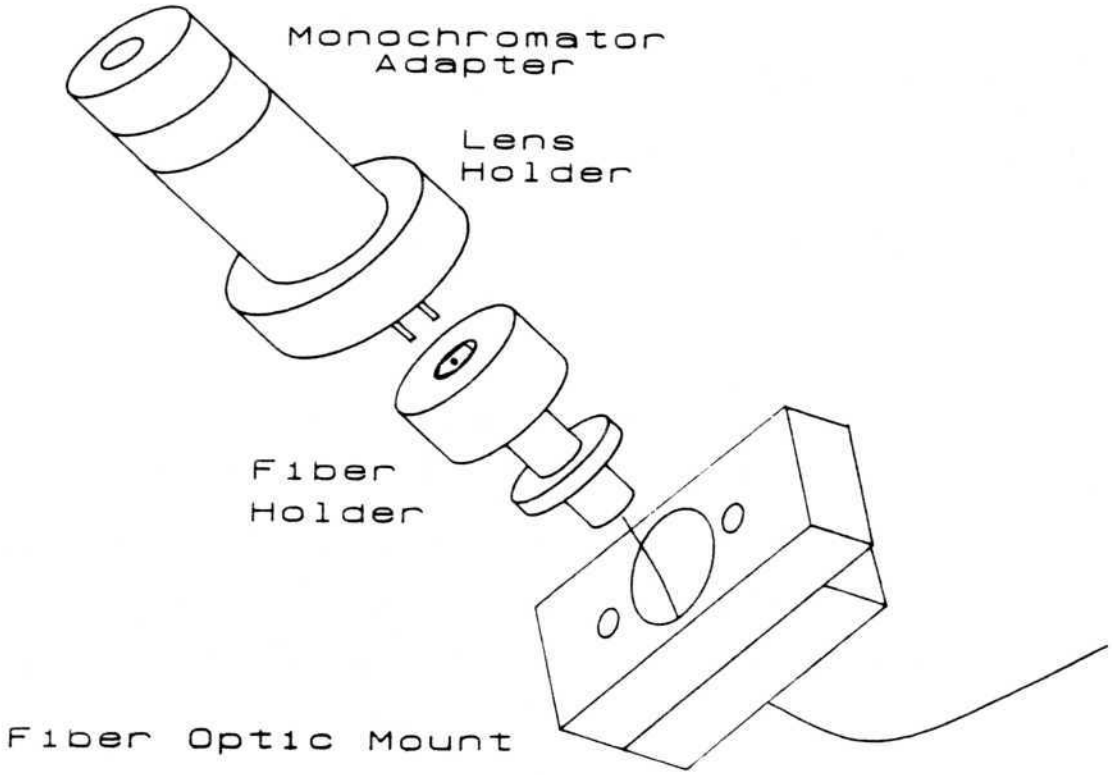


Figure 14. The fiber optic/monochromator adapter. The fiber optic enters through the mount and is fixed to the fiber optic adjust. The fiber optic adjust positions the fiber end with respect to the lens. The monochromator adapter adjusts the lens position with respect to the monochromator entrance slit.

Table III. Fiber Optic Instrumental Parameters

	Transmission Study	Scattering Study	Fluorescence Studies	
			Quinine Sulfate	Chlorophyll
Monochromator Slitwidth (um)	300	100	2000	2000
PMT Voltage (V)	320	700	1120	1220
R_f (MΩ)	0.992	100	9.95	5.0
C_f (μF)	0.01	0.10	0.10	0.10
Excitation Conditions filter	Unfiltered	Unfiltered	754 filter	5-60
Emission Wavelength (nm)	300-700	450	450	683

A jig was constructed to hold the excitation and emission fiber optic cables in a plane but at variable angles and distances with respect to one another as shown in Figure 15. Typically, the excitation light fiber holder was fixed and the emission fiber holder was adjusted to positions around a semicircle with respect to the excitation fiber.

A fiber optic coupler was used to connect two sections of fiber optic cable. This coupler oriented the two fiber's distal ends along an axis and could hold their ends within a millimeter of each other (see Figure 16). For maximum coupling efficiency, the fibers should be as close as possible. This should be done with care since if the fibers make contact, they can be chipped, in which case, they both must be recut and repolished.

The excitation filters used to select the excitation wavelength

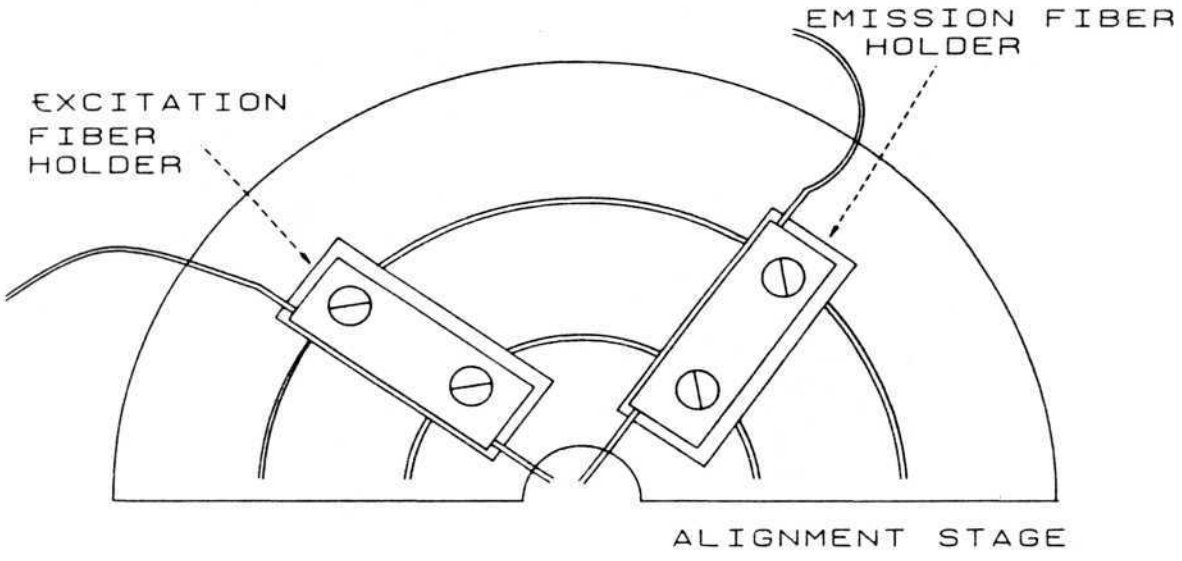


Figure 15. A fiber optic orientation jig. The jig holds fiber optic cables at varying distances and angles.

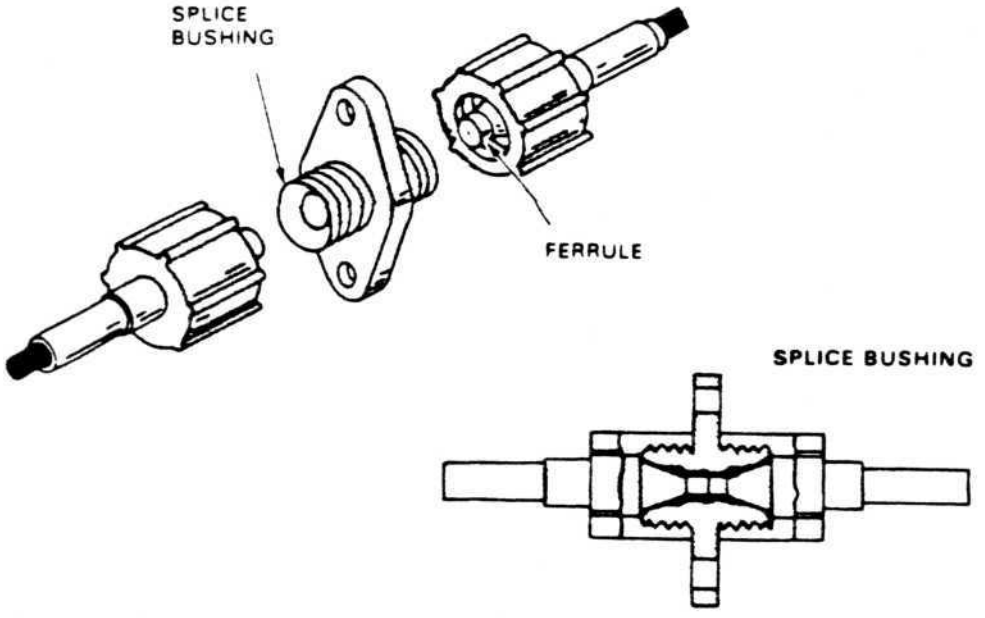


Figure 16. Fiber optic coupler (Boyd, 1982).

range in the two fluorescence experiments are shown in Table III. Emission wavelengths were selected after scanning with the monochromator to determine the wavelength yielding the greatest emission signal (see Table III).

EXPERIMENTAL

Sampling Techniques

Samples were collected from the locations and at the dates indicated in Table IV. The specific details are given below.

Table IV. Sampling Locations and Dates

Location	Date	Study
Crater Lake	31 July 1984	Fluorescence Profile
	31 July 1985	'in vivo' Temperature
	12 August 1985	'in vivo' Sunlight
	12 September 1985	Precision
	26 June 1986	Fluorescence Profile
Cronmiller Lake	20 May 1985	Filter Storage
		Filter Efficiency
		Detection Limit
		Comparison of Methods
Redwood Pond	8 February 1986	Fiber Optic 'in situ'

Crater Lake, Oregon

Crater Lake is located in Crater Lake National Park in southwest Oregon. It has many unique qualities which make it interesting and yet difficult to study. The fact that it is an oligotropic lake requires researchers to collect large quantities of water for preconcentration to measure accurately low concentrations of many species. Its location in a caldera and limited trail access challenge all who study it in transporting instruments and bottles to and from the lake. Winter study has just begun with the completion of a shelter and boathouse on Wizard Island.

Samples were taken of Crater Lake using a boat supplied by the National Park Service and using Van Dorn 4-L sampling bottles. As many as 22 bottles could be placed on a cable at one time. Brass messengers closed the bottles when dropped from the surface. Each bottle released its own messenger to continue the collection sequence down the cable. A power winch brought the cable up and the bottles were mounted on a rack. Individual samples were then drawn from the Van Dorn sampling bottles and stored overnight in different sized bottles as described later.

Cronemiller Lake

A 40-L sample of water was collected from Cronemiller Lake. This limited access lake is in the McDonald Forest and is just west of Peavy Arboretum north of Corvallis, Oregon. Water samples were collected just off shore with plastic bucket and dumped into a 50-L water cooler until 40 L were collected. This sample was not stored in separate sampling bottles. Portions of the sample were removed from the cooler when needed.

Redwood Pond

A small sample of pond water was taken from Redwood Pond in Grants Pass, Oregon. This sample was collected by bucket and stored in a 4-L polystyrene bottle.

Sample Preparation Techniques

Chlorophyll was extracted from lake samples for the spectrophotometric and 'in vitro' fluorometric methods with the

following procedures.

Filtration

Filters were used to separate phytoplankton from lake water samples. The 47- to 50-mm diameter glass and membrane filters were held between a fritted glass platform and a funnel assembly (Millipore, model numbers XX1004704 and XX1004703). The sample was poured into the funnel and drawn through the filter with a vacuum of 25 in of Hg. The funnel was rinsed between samples with deionized water. Unless noted otherwise, all water used was deionized water from a Millipore Milli-Q water system fed by house deionized water. This water will be denoted as Millipore water.

For most studies, S&S filters were used and the filters were extracted immediately. Studies of the extraction efficiency of different types of filters, the use of $MgCO_3$ on filters, and of the storage time and storage temperature for the filters on the amount of chlorophyll determined are discussed later. Routinely, 0.5 mL of an aqueous suspension of $MgCO_3$ was added to samples from Crater Lake as they were filtered. This solution was made by mixing 1.0 g of $MgCO_3$ with 100 mL of deionized water.

Extraction

The extraction of chlorophyll from the phytoplankton separated on the filter involves three processes: grinding of the filter in 90% (v/v) aqueous acetone, steeping the ground filter in the solvent for some time, and separating the filter parts from the chlorophyll containing solvent using centrifugation. A test-tube-like mortar was

used with a Teflon pestle (see Figure 6). The pestle was spun with a variable speed electric motor. The filter was macerated with 5 to 6 mL of 90% (v/v) aqueous acetone. This mixture was quantitatively transferred into a capped centrifuge tube and made up to a volume of 10 to 15 mL.

The capped tubes were stored in a dark cool refrigerator at a temperature of 1^{o} C for 4 to 12 hr. During this time the chlorophyll leaves the cell and filter parts and dissolves in the solvent.

Centrifugation

Each capped centrifuge tube was spun in an Clay Adams analytical centrifuge (model CT-3200) for 10 to 15 min before spectrophotometric or fluorescence measurements were taken. The sample was used to rinse and fill a cuvette without disturbing the solid at the bottom of the tube.

Preparation of Chlorophyll Standards and Quality Control Samples

To prepare the chlorophyll standards, the following procedure was used. A sealed glass ampule was opened to weigh its contents. Chlorophyll in the solid form appears the same as a piece of ground black pepper. In this researcher's first attempts to open the ampule, glass pieces became mixed with the chlorophyll and had to be manually separated before the chlorophyll could be weighed. A balance with a resolution of 0.001 mg was needed to weigh the chlorophyll since the ampule contained only a 1-mg sample. The weighing procedure had to be carried out quickly as the sample could

be seen to increase in weight as it adsorbed moisture.

Reagent grade acetone was mixed with Millipore water to make a 90% (v/v) aqueous acetone solution, 90:10, acetone:water. This solution was used to dissolve 1.00 mg of pure chlorophyll-a crystals (Sigma Chemical Company, Lot No. 114F-9650) to a final volume of 50 mL to give a concentration of 20.0 mg/L of chlorophyll-a. This solution was then diluted with 90% (v/v) aqueous acetone solution to make standard solution concentrations of 10.0, 5.00, 2.50, 1.00, 0.50, 0.25, 0.10, 0.010 mg/L chlorophyll. A second chlorophyll-a standard was prepared by the dissolution of 0.611 mg of chlorophyll-a in 100 mL of 90% (v/v) aqueous acetone solution. Further dilutions produced a series of solutions containing 191, 95, 47, 24, 12, 6.0 and 3.0 µg/L chlorophyll. The blue-green color of chlorophyll solution is easily visible in solutions with concentrations of 1 mg/L or greater.

An EPA supplied ampule containing a standard chlorophyll-a concentration of 80 µg/L was diluted with 90% (v/v) aqueous acetone to concentrations of 4.0 and 16 µg/L. Two other ampules supplied by the EPA as quality control samples were certified to contain 4.4 and 16.7 µg/L chlorophyll-a concentrations when analyzed using the uncorrected fluorometric method. The same ampules were specified to contain 3.2 and 16.7 µg/L chlorophyll-a and 1.4 and -0.1 µg/L phaeophytin-a when analyzed using the corrected fluorometric method. The quality control samples were used to evaluate the calibration of the fluorometer.

Spectrometric Measurements

In this section, the general procedures used for spectrometric measurements are outlined.

Spectrophotometric Measurements

Extracted samples and standards containing chlorophyll in 90% (v/v) aqueous acetone were used to rinse and fill cuvettes of 1- or 5-cm pathlengths. Crater Lake samples were routinely measured in the 5-cm pathlength cuvette. For the trichromatic method the absorbance was recorded for each sample, standard, or blank at individual wavelengths of 630, 646, 664 and 750 nm. The concentration of chlorophyll-a was calculated using the Jeffrey and Humphrey trichromatic equations (4, 5 and 6).

The Cary 118C spectrophotometer was blank adjusted using two glass equivalent pathlength cells containing the 90% (v/v) aqueous acetone. The absorbance was set to zero using the set of potentiometers in the wavelength range to be recorded. Five consecutive readings were averaged. The HP 8451A diode array spectrophotometer was blank adjusted by filling a cell with 90% (v/v) aqueous acetone and storing the reference spectrum.

For the monochromatic method, sample extract absorbances were measured with the HP 8451A spectrophotometer at wavelengths of 750 and 664 nm. A second set of absorbance measurements were recorded 90 s after addition and mixing of 0.1 mL of 0.1 M HCl with 5 mL of extract in a second cell. Lorenzen's equations (10 and 11) were used to calculate the concentrations of chlorophyll-a and phaeophytin in

lake water samples.

'In vivo' Fluorescence Measurements

A sample's 'in vivo' fluorescence was measured after storing samples in 250-mL brown polystyrene bottles for one hour at a uniform temperature. A 25 by 150-mm cuvette was rinsed several times with the sample and filled with 20 mL of sample. The cuvette was then placed into the Turner model 10 fluorometer with its scan control set at one revolution CCW from its most sensitive CW position. It was blank adjusted using deionized water.

The Turner fluorometer has two marked scales (0 to 10 and 0 to 31). The scales are marked with sub-divisions of 0.2 and 1 FU, respectively. Electronic scale multipliers can be used to increase each scale 10 times. An optical scale multiplier can be used to increase the range of the readout 100 times. This provides scales of 0 to 10, 31, 100, 310, 1000, 3100, 10000 and 31000. All fluorescence signals are reported in fluorescence readout units (FU) where full scale on the analog readout is 31000 FU.

A separate cuvette was used for the enhanced 'in vivo' measurement incorporating DCMU. Two drops of a saturated aqueous solution of DCMU were dispensed into a previously rinsed cuvette. After the 'in vivo' fluorescence of a sample was measured, the sample was poured into the cuvette containing the DCMU. After mixing, this cuvette was introduced into the fluorometer. A sample of deionized water was treated with DCMU to record an enhanced 'in vivo' blank signal which was used to subtract from the sample's signal. Because the blank standard deviation is small, a digital voltmeter was

connected to the recorder output of the fluorometer to increase the readout resolution. The conversion factor to FU is 2 FU/V.

'In vitro' Fluorescence Measurements

'In vitro' sample fluorescence signals were measured with the same procedure used for 'in vivo' fluorescence measurements except that a smaller sample cuvette (10 X 100 mm) was used in conjunction with a smaller (7 mm) attenuation plate for the excitation radiation. Also, the fluorometer was blank adjusted using a sample of 90% (v/v) aqueous acetone solution. The 80 µg/L EPA standard of chlorophyll-a and the 16 and 4 µg/L dilutions previously described were used to calibrate the fluorometer.

The fluorescence of a chlorophyll extracts were measured after rinsing and filling the cuvette with about 9 mL of sample. The uncorrected chlorophyll-a concentration was determined using the sample's fluorescence signal (F_b) and a conversion factor (M_f). This factor was determined by dividing the known chlorophyll-a concentration in a standard by its fluorescence signal.

The corrected chlorophyll-a concentration was determined by adding 0.1 mL of 2.0 M HCl to a second 10 X 100-mm cuvette, pouring in the 90% (v/v) acetone extract of samples, standards or blanks, mixing, and after 90 s, recording the fluorescence signal, F_a. These additional data for the standards and blanks were used to calculate the factor r ($r = F_b/F_a$). From M_f and r, the corrected chlorophyll-a and the phaeophytin concentrations in the lake water extracts were calculated with equations 16 and 17.

In some studies, the factor R ('in vivo'/'in vitro' fluorescence

signal) was also determined. In this case, the 'in vitro fluorescence signal was normalized by dividing by the concentration factor of the extraction procedure.

Study of Spectrophotometric Parameters

Spectral Bandpass

The spectrophotometric determination of chlorophyll depends on the absorbance measured with a spectrophotometer. The effect of slitwidth on the absorbance measured and the concentration of chlorophyll-a calculated was determined with three series of standard solutions of known chlorophyll concentration. Both the trichromatic equations (i.e., the Jeffrey and Humphrey method) and the monochromatic equations (Lorenzen's method) were used to calculate the chlorophyll-a concentration in the standards.

The operating parameters for the Cary 118C spectrophotometer are listed in Table II. It was used with 1-cm pathlength sample cells to make measurements at 630, 647, 664, 665 and 750 nm and for a spectral scan from 600 to 700 nm at slitwidths of 0.044, 0.272 and 0.558 mm which correspond to spectral bandpasses of 2, 10 and 20 nm, respectively.

Cell Pathlength

Routinely, a cuvette of 1-cm pathlength is used in the 'in vitro' spectrophotometric determination of chlorophyll. To determine the effect of cell pathlength, absorption measurements were made on a series of aqueous acetone chlorophyll standard solutions using both

1-cm and 5-cm pathlength cells in the Cary 118C spectrophotometer. The monochromatic equations were used to calculate the chlorophyll concentrations throughout this study (equations 10 and 11).

Comparison of Spectrometric Methods

Two studies were conducted to compare the different spectrometric techniques for determining chlorophyll. The first study was designed to evaluate the linearity and detection limits of the different techniques. In this study, a portion of the Cronemiller Lake sample was diluted four times by 50% (v/v) by mixing with deionized water. The 50, 25, 12.5 and 6.2% lake water samples were made by mixing 500, 250, 125 and 62 mL of lake water with enough deionized water to produce a final volume of 1.00 L. Three 20-mL portions of each test solution were analyzed by the 'in vivo' and enhanced 'in vivo' fluorometric techniques. Three 300-mL portions of each test solution were filtered, extracted, and concentrated to 12.5 mL in 90% (v/v) aqueous acetone. Each extract was analyzed by the monochromatic spectrophotometric technique and the uncorrected and corrected 'in vitro' fluorometric techniques.

For each technique, the mean and standard deviation of the signal for each test solution were calculated and appropriate equations and calibration factors were used to determine the chlorophyll concentration in each test solution. For each method, plots of signal versus % lake concentration (LC) were made to obtain the slope (signal units/% LC), intercept, and linear correlation coefficient. The standard deviation of 20 blank measurements was calculated to determine the detection limit.

A second set of experiments were designed to determine the precision of each of the five methods used in the first study and additionally of the trichromatic spectrophotometric technique. Two different 20-L samples of lake water from Crater Lake, Oregon were collected (one from the known chlorophyll minimum, about 20 m below the surface, and the other from the chlorophyll maximum, at a depth of about 120 m). Each sample was separately mixed on board the boat in a plastic-lined wooden box and then siphoned into twenty 2-L brown polystyrene bottles and treated as individual samples. The samples were transported to a field lab to be run as 20 separate samples.

Twenty 2-L portions of each of the Crater Lake water samples were filtered and extracted with 90% (v/v) aqueous acetone to produce 15 mL extract solutions. The same extracts were used for the 'in vitro' uncorrected and corrected fluorometric technique and the monochromatic and trichromatic spectrophotometric techniques.

Filter Efficiency Studies

To study the efficiency of the filters used routinely in the filtration of water for phytoplankton, two experiments were conducted. In the first study, a portion of the 40-L Cronemiller Lake sample was divided into 300-mL subsamples which were filtered through three types of filters. In the second study, titanium dioxide of uniform size distribution was filtered through the same three types of filters. The retentive efficiency was determined as mass of TiO_2 retained divided by mass of TiO_2 originally suspended in the solution.

Lake Studies

The ability of Whatman GF/F glass filters, Millipore Type HA membrane filters, and Scheicher and Schuell #30 glass filters, lot #ZE31, to retain chlorophyll from natural lake water was tested. These filters have published pore sizes of 0.7 μm, 0.45 μm, and <0.30 μm, respectively. All tests were run in triplicate with a sample sizes of 300 mL. The blank signal contribution from each filter was determined by filtering 300-mL samples of deionized water.

After grinding, the two glass type filters produced a milky slurry which settled quickly in a centrifuge tube. The membrane filter dissolved in the 90% (v/v) aqueous acetone solution to produce a clear solution which was much more viscous than the original acetone solution. The volume of this slurry, in all extracts, was adjusted to 12.5 mL in capped centrifuge tubes and stored for 12 hr at 1^{o} C.

Spectrophotometric measurements were made in 1-cm pathlength sample cells with the Cary 118C spectrophotometer. Fluorometric measurements were conducted as previously indicated. The EPA endorsed monochromatic equations and the EPA endorsed 'in vitro' fluorometric technique were used to calculate the concentrations of chlorophyll-a as well as of the phaeophytin pigments.

Filter Efficiency Using TiO_2

To define further the ability of the filters to separate phytoplankton from lake water, titanium dioxide was used. This dry

powder is made by Deguass AG of Frankfurt, Germany. The specifications indicate that the TiO_2 consists of particles between 15 and 40 nm in diameter as shown in Figure 17. A mass of 1.0008 grams of TiO_2 was ultrasonically mixed to a volume of 500.00 mL using Millipore water. All filters were weighed before filtering this solution. For each of the three types of filters, a volume of 50.0 mL of this suspension was mixed in the filtration funnel with 100 mL of Millipore water and filtered in triplicate. Note that after filtering, each filter retains at most 0.10008 g of TiO_2.

The filters were allowed to dry in stacked watch glasses of different sizes. These were placed in an oven under a vacuum of 20 in of Hg at 40^{o} C for 18 hr and then the filters were weighed. This process was repeated until the filters achieved a stable weight.

Filter Storage Study

Often researchers find it necessary to collect and filter lake samples and then store the filters until such time as the extraction and determination of chlorophyll can be accomplished. This researcher has seen thousands of dollars ill spent on samples which have been stored and analyzed at a later date, only to find the results unusable. For this reason, a filter storage study was designed and carried out.

A portion of the Cronemiller Lake sample was divided into 63 subsamples of 300 mL, filtered using S & S #30 glass fiber filters, and extracted with 12.5 mL of 90% (v/v) aqueous acetone. For half of the subsamples, 0.5 mL of a saturated solution of $MgCO_3$ was added to the lake water in the filtration funnel as it passed through the

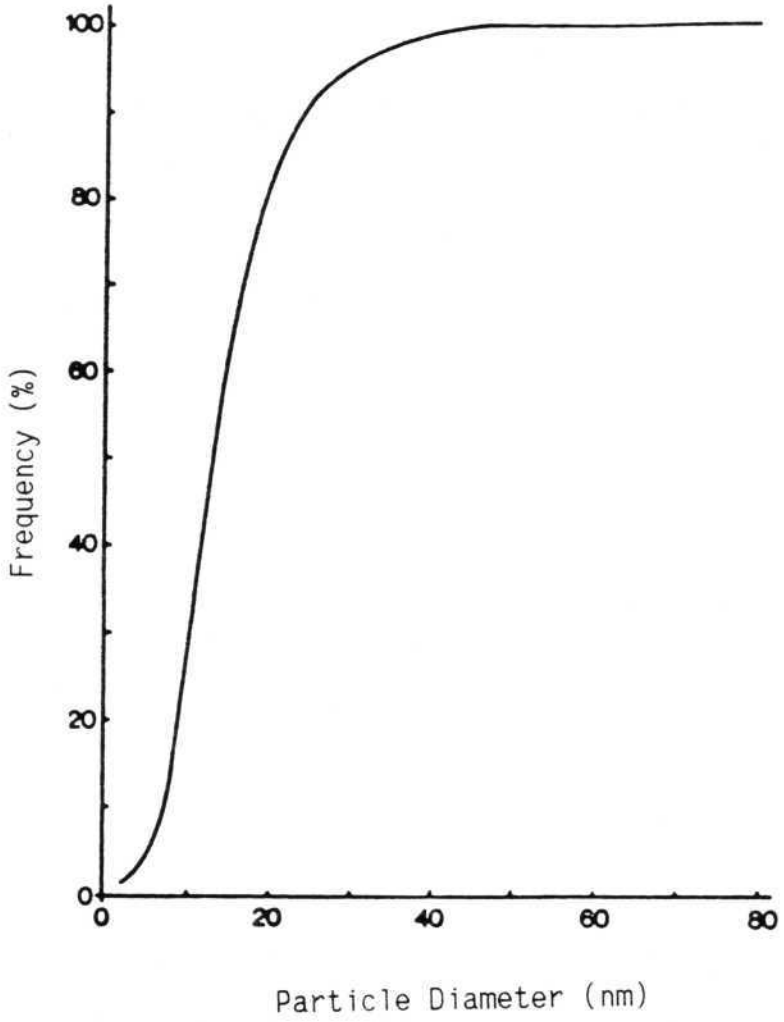


Figure 17. Particle size summation curve of titanium dioxide P25. From Deguass AG Technical Notes.

filter. The $MgCO_3$ solution is added as the last of the sample passes through the filter so as to not increase the time of filtration. Immediately a set of three filters not containing $MgCO_3$ was extracted and the absorbances of the extracts were measured. From spectrophotometric measurements with the Cary 118C or the HP 8451A spectrophotometer and 1-cm pathlength cells, the chlorophyll-a and phaeophytin-a concentrations were calculated using Lorenzen's monochromatic equations. Half of the remaining filters (30) with and without $MgCO_3$ were stored in a refrigerator at a temperature of 1^oC. The other half (30) with and without $MgCO_3$ were stored in a freezer at -9^o C. In this manner twenty sets of six filters were stored for later analysis. The filters were extracted and tested for chlorophyll using the monochromatic method at 0, 1, 12, 65, 194, 212 and 236 days after being placed in storage.

Study of Factors Affecting the 'in vivo' Fluorescence Signal

A chlorophyll depth profile of Crater Lake was obtained. Samples were collected at Crater Lake on July 31, 1984 from the surface to 200 m at intervals of 20 m and at 10 m and the 'in vivo' and 'in vitro' fluorescence signals were measured. The 'in vivo' fluorescence signal for an 20-mL portion of each sample was recorded as the samples arrived on board the boat. Next 100 mL of each sample was filtered on board the boat with an in-line filter (S & S #30) attached to a syringe. The filters were returned to the lab, extracted with 10 mL of 90% (v/v) aqueous acetone and the 'in vitro' fluorescence signals recorded. The 'in vitro' fluorescence signal was normalized to the original volume

filtered.

The fluorescence number (R) or the ratio of the 'in vivo' fluorescence signal and the 'in vitro' fluorescence signal was calculated and was noted to vary with depth. Two affects are thought to be responsible for the change in the fluorescence number. The first is a temperature effect and the second a sunlight or irradiance effect. After investigating these effects as discussed below, the profile study was repeated on June 26, 1986. The samples collected throughout the water column were stored for six hours in opaque plastic containers in a cooler containing water of 0^{o} C (stored with ice). After dark adaptation and cooling to a common temperature, the 'in vivo' and the 'in vitro' fluorescence signals were measured with the same procedure used in the July 31, 1984 study.

Temperature Effect

Twelve lake water samples were collected from Crater Lake on July 31, 1985 using Van Dorn sampling bottles placed every 20 m to a depth of 200 m and at 10 m. These samples were then transferred and stored for 3 hr in 125-mL brown polystyrene bottles. The samples' 'in vivo' fluorescence signals were recorded. Next the samples were cooled in a refrigerator from 20 to 10^{o} C, and their fluorescence signals were recorded a second time. The samples were kept in a controlled environment until the fluorescence signal was measured.

Sunlight Effect

Samples were collected at 10-m intervals from Crater Lake on August 12, 1985. Individual samples were stored in 250-mL opaque polyethylene bottles for 36 hr. The samples were mixed into four working mixtures based on observed peaks in the 'in vivo' fluorescence signal profile. These mixtures contained samples collected from 30 to 60 m, 70 to 110 m, 120 to 160 m, and 170 to 200 m. Each mixture's 'in vivo' and enhanced 'in vivo' fluorometric signals were recorded. The mixtures were then placed into separate 1-L beakers and exposed to direct sunlight of 1.02 $cal \cdot cm^{-2} \cdot min^{-1}$ as recorded using a pyranograph (Weather Tronics, model ####). The beakers were exposed to 9:00 AM sunlight from a cloudless sky filtering through a window with a screen at Crater Lake National Park. The lab was located at the 6200 foot elevation in the headquarters area. The 'in vivo' and enhanced 'in vivo' fluorescence signals were recorded approximately every 5 min for 70 min.

Fiber Optics

People are generally surprised to learn that pure quartz or glass can bend. A sheet of glass without flaws or scratches can be made to bend without breaking. Glass fiber optic cables can also be made to bend. A fiber optic is sheathed with a plastic jacket in an effort to protect the glass from abrasion and to restrict the amount of bending. This jacket is not to be confused with the fiber cladding. This outer material is responsible for the total internal reflection which results in the fiber's ability to transmit light. The fiber

optic used in this research is termed plastic clad silica or PCS (Maxilight, NA ~ 0.33). A 600-μm diameter fiber optic can be made to coil onto a 30-cm diameter spool without danger of breakage. This is not to say the fiber cannot be broken. Breakage may occur if undue pressure is put on a section of fiber as might occur in the fitting of a ferrule over the end of the fiber.

The construction of a fiber optic cable is shown in Figure 18. Termination and polishing of a fiber optic begins with cutting a piece of fiber optic from a large spool with a wire cutter. The cabling jacket is cut and stripped back about 10 cm. Under the protective jacket one will typically find a Kevlar strength member which consists of braided multifilament polymer. This is cut and removed from the section of interest. Beneath this Kevlar jacket lies the plastic which protects the actual silica fiber optic. This plastic sleeve is removed using precision wire insulation strippers. After cutting the fiber optic with a wire cutter, the ends of the fiber optic are chipped and ragged and appear like broken glass.

Once about 2 cm of plastic jacket has been removed from a fiber optic end, the actual fiber optic is cleaved about 1 cm from the end of the jacket as a piece of glass tubing might be cut using a triangular file and thumb pressure. In this case however, the file is replaced with a sapphire crystal mounted on a metal adapter, and extremely light pressure is exerted by holding the fiber within about 1 cm of the intended cleavage point. The pressure bows the fiber optic away from the sapphire crystal. A sapphire crystal and its holder were mounted on a section of heavy wood to prevent it from moving during the cleavage process. Unlike glass tubing the fiber

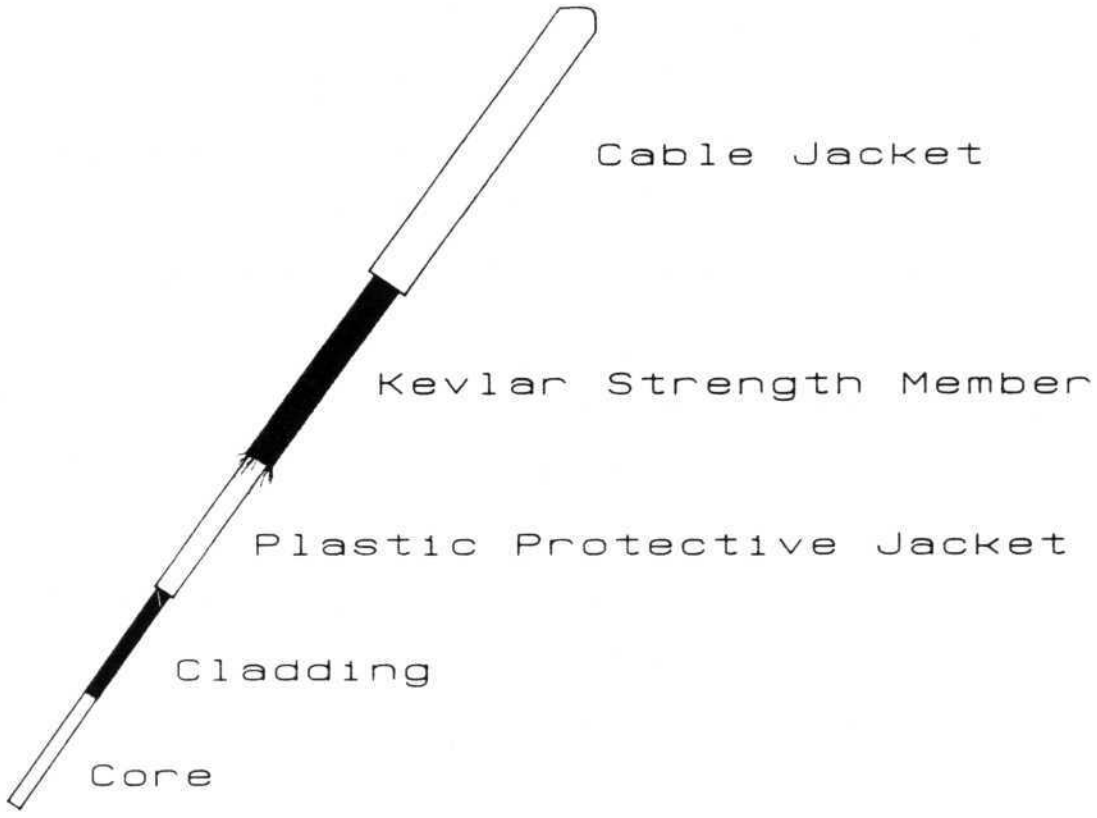


Figure 18. Construction of a fiber optic cable. The different layers must be cut in the cleavage and polishing process.

optic separates cleanly as it is scored by the crystal. If too much pressure is applied, the result is the removal of glass from a section of the newly exposed face of the fiber optic. This is later ground off in the polishing process. However, the lighter the pressure exerted in the cleavage process, the less is the grinding required in the next step. A fiber optic might not have to be polished at all if the cleavage is done so lightly as to cause a negligible score on the fiber optic face.

The fiber is fitted into a ferrule which is used to mount the fiber optic to various connectors as shown in Figure 19. To grind and polish the fiber optic end, the ferrule along with its mated fiber optic is then pressure fit into a metal grinding tool. This tool is described by workers in the industry as a hockey puck because of its appearance. Its main purpose is to hold the end of the fiber optic perpendicular to the grinding surface.

Initially, a rather course grinding medium such as 30-μm grit aluminum oxide paper is used to make the fiber optic face relatively flat across its full cross section. This process takes a minute or two. The result of this first grinding leaves the fiber optic face looking like ground glass. A second grinding medium with a grit size of 3.0 μm is used for about 4 to 6 min to remove all of the ground glass features. The fiber optic face appears darker and darker as the grinding proceeds when viewed with a microscope using a magnification of 100X and incident reflected light. This occurs because of the diffuse reflectance from the irregularities on the surface are reduced by polishing. Care must be taken to keep the grinding surface, as well as the fiber optic face, clean to prevent

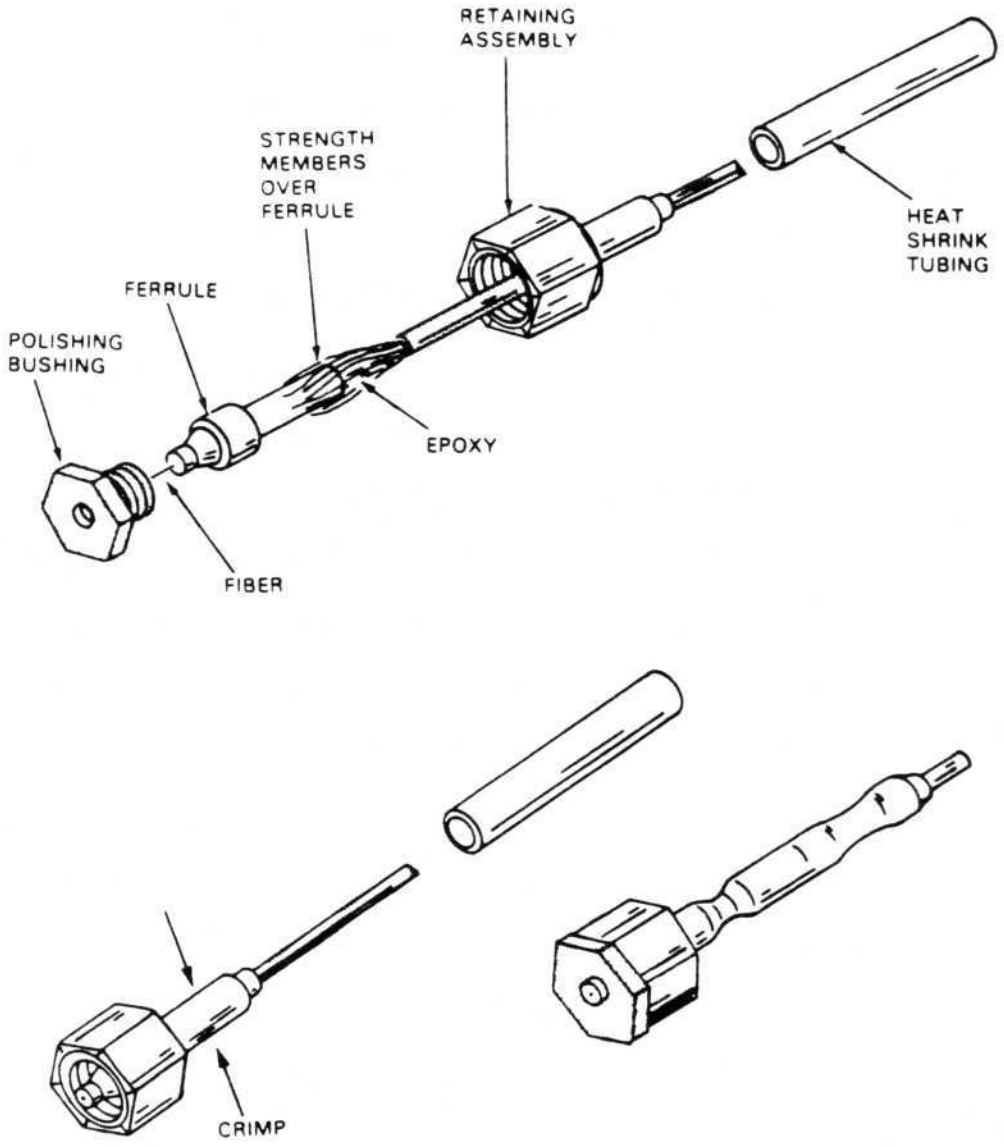


Figure 19. Termination of the end of a fiber optic cable, (Boyd, 1982).

contamination and scratches on the fiber optic face. A camel hair brush is used to remove dust and general contamination from both the fiber and polishing surfaces.

The final grinding is accomplished with 0.3-μm silicon oxide paper which removes the last of the pits and grooves left in the fiber optic face. The fiber optic face appears absolutely black without a blemish if this polishing is done well. At this point light can be transmitted efficiently through the fiber optic. The light transmitted should be of uniform intensity across the fiber optic face as viewed under magnification.

The ferrule used to polish the fiber optic can now be permanently attached to the fiber optic end. Amphenol connectors (retaining assembly in Figure 19) are for both grinding and for permanent attachment. Eastman 910 adhesive is used to adhere the fiber optic to the connecting female ferrule. The epoxy is smeared on the section of outside cabling jacket as well as the remaining Kevlar ends which will contact the ferrule. The ferrule is slipped over the end of the fiber optic to make contact with the fresh epoxy. One must remember to slip the screw collar of the Amphenol connector over the fiber optic end before epoxying the ferrule to the end. This collar fastens the ferrule along with its attached fiber optic to a male adapter on an instrument or connecting device. An hour or two later, the epoxy sets enough to allow the fiber optic to be used.

If any part of the fiber optic face appears rough, pieces of the patch of unpolished surface may chip and flake off. This happens once the fiber optic is placed in service, especially when conducting light of high intensity. The chipping continues until the fiber

optic face returns to the ground glass appearance it had during the initial grinding process. This deep fracturing is caused by the initial grinding process must be completely removed in the second grinding step before the polishing may be started. One could polish a fiber optic face smooth and yet conceal fractures below the surface. Small pieces of fractured glass from the surface of the fiber optic could then chip off due to thermal expansion. Under the microscope, dark areas of the surface glass pop off with heat and become white again as in a ground glass surface.

Fiber optic cables were used to determine transmission characteristics of the cable and scattering characteristics of a dilute solution of milk of magnesia and to transmit excitation and emission radiation to and from a sample of quinine sulfate and pond water.

The milk of magnesia solution was prepared by mixing 2 mL of Fred Meyer brand antacid suspension (Lot #21323) with deionized water to make a 2-L solution. The 10 µg/L quinine sulfate solution was a stock solution available in the laboratory.

A natural water sample from Redwood pond was used to determine the applicability of a remote fiber optic fluorescence probe for the detection of 'in vivo' chlorophyll fluorescence. All solutions used for the fiber optic measurements were contained in a glass container. The fibers were submerged in the sample and the entire sample was covered with a light-tight box. All experiments were run at night to further eliminate stray light.

The chlorophyll concentration in the Redwood Pond sample was determined by filtering 100-mL portions of pond water through S & S

#30 glass filters in triplicate. Each filter was ground and extracted into 12.5 mL of 90% (v/v) aqueous acetone solution. The monochromatic equations were used to determine the chlorophyll concentrations from corrected absorbances measured with the HP 8451A spectrophotometer and a 1-cm pathlength sample cell.

RESULTS AND DISCUSSION

Spectrophotometric Parameters

Spectral Bandpass

The effect of the value of the spectrophotometer spectral bandpass on the absorption band at 665 nm and the absorbance at 665 nm is illustrated in Figure 20 and Table V, respectively. The data in Table V show that the absorbance increases for a given chlorophyll concentration as the spectral bandpass decreases from 20 to 2.0 nm. Calibration curves are shown in Figure 21. With a spectral bandpass of 20 nm, significant negative deviation in the calibration curve is observed. The absorbance for 20 mg/L chlorophyll is about 0.15 A.U. below that expected from extrapolation of the linear response at lower concentrations for the 20-nm bandpass and about 0.55 AU below that obtained with a 2-nm spectral bandpass. The spectra in Figure 20 demonstrate the decrease in absorbance across the entire absorption band that occurs with larger spectral bandpasses. Since the natural width of the chlorophyll band at 665 nm is about 20 nm at half height, using a spectral bandpass larger than one tenth of the half-width would be expected to decrease the measured absorbance and result in negative deviations in the calibration curve due to polychromatic radiation.

The absorbances for the chlorophyll standards measured with the different spectral bandpasses were used to calculate the chlorophyll concentrations based on the Jeffery and Humphrey trichromatic equations (equations 4, 5 and 6). The results are reported in Table

Table V. Absorbance Calibration Data for Chlorophyll-a[a]

Chl-a Conc. (mg/L)	Spectral Bandpass 2 nm	10 nm	20 nm
0.010	0.0010	0.0015	0.0008
0.100	0.0067	0.0074	0.0048
0.250	0.0186	0.0178	0.0139
0.500	0.0348	0.0331	0.0251
1.00	0.0709	0.0657	0.0507
2.50	0.1792	0.1611	0.1263
5.00	0.3555	0.3203	0.2440
10.0	0.7157	0.6361	0.4776
20.0	1.402	1.2302	0.8492

[a] measured at 665 nm, average of 5 readings

VI and plotted as a correlation diagram in Figure 22. In all cases, the calculated chlorophyll concentrations are lower than expected. This may be due in part to inaccuracy in the original stock solution because of the difficulty in weighing sub-milligram quantities, glass particles (see the experimental section) or adsorption of water by the chlorophyll. Also possible is the degradation of chlorophyll in the solution. Chlorophyll solutions are very sensitive to light, acid and heat.

The determined concentration of chlorophyll-a is within 20% of the expected chlorophyll concentrations for concentrations from 0.1 to 20.0 mg/L using a spectral bandpass of 2.0 nm. The fact that the error is relatively constant supports the suggestion that the chlorophyll concentration in the stock solution was lower than calculated. The error in determining the chlorophyll concentration is worse with a larger spectral bandpass because the observed

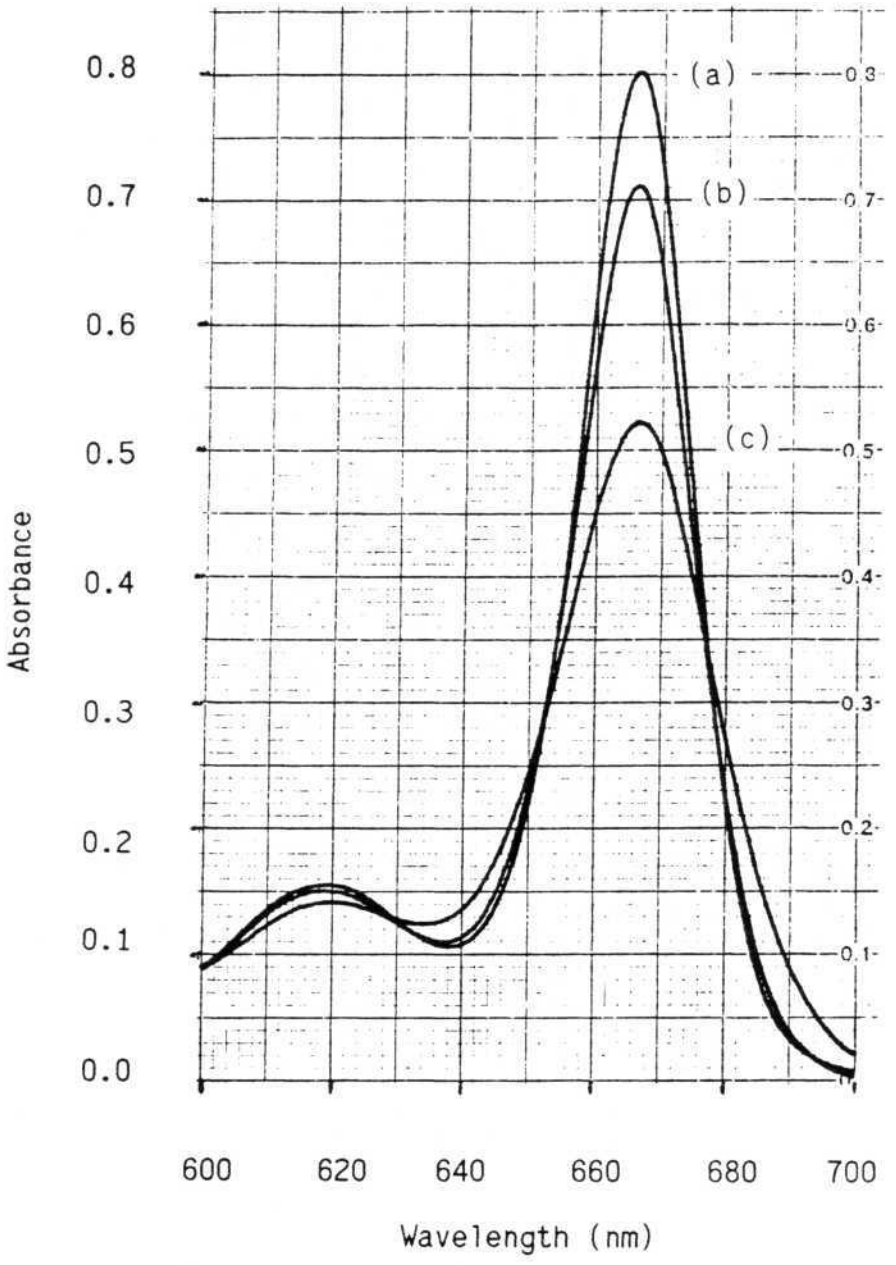


Figure 20. Effect of spectral bandpass on the 665 nm absorption band of chlorophyll-a. The spectral bandpasses are 2.0 nm (a), 10 nm (b), and 20 nm (c). Chlorophyll-a concentration was 1.0 mg/L.

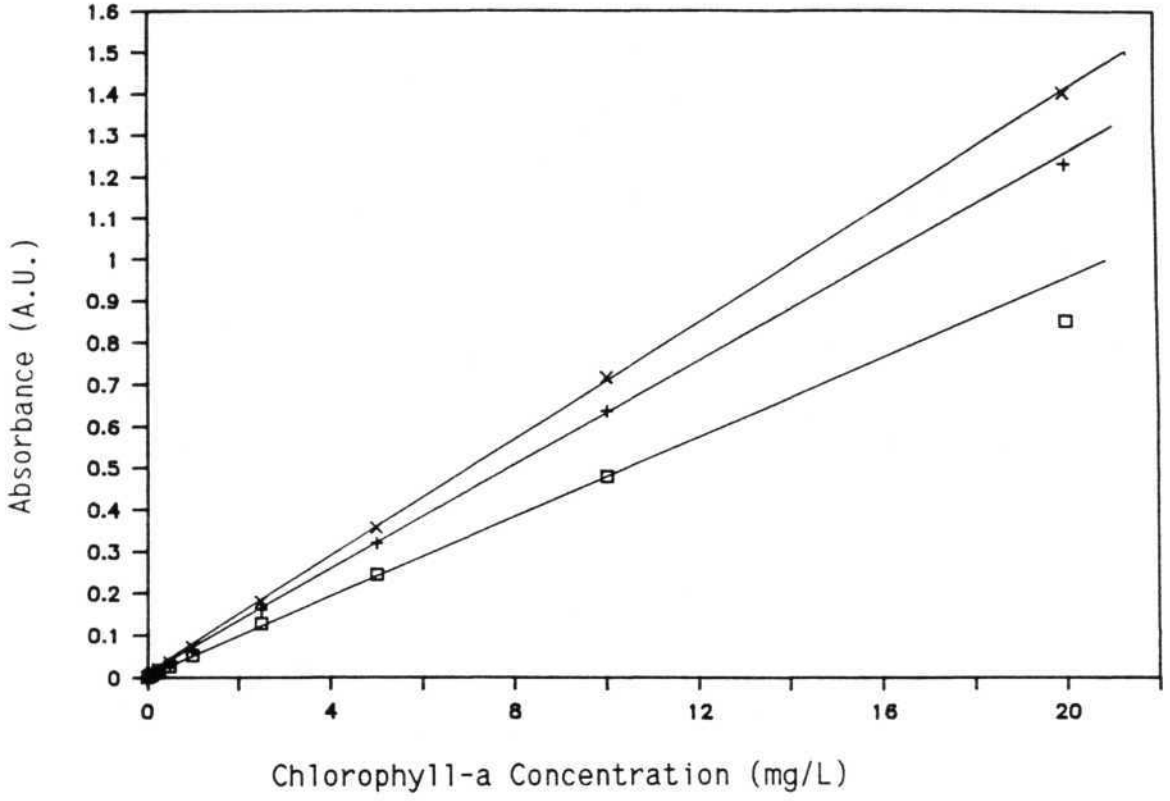


Figure 21. Effect of spectral bandpass on the spectrophotometric calibration curves for chlorophyll-a. Absorbance measurements were made at 665 nm without correction for blank. Spectral bandpasses are 2.0 (X), 10 (+) and 20 nm (▫). Note the deviation from linearity between concentrations of 10 and 20 mg/L with the 20-nm spectral bandpass.

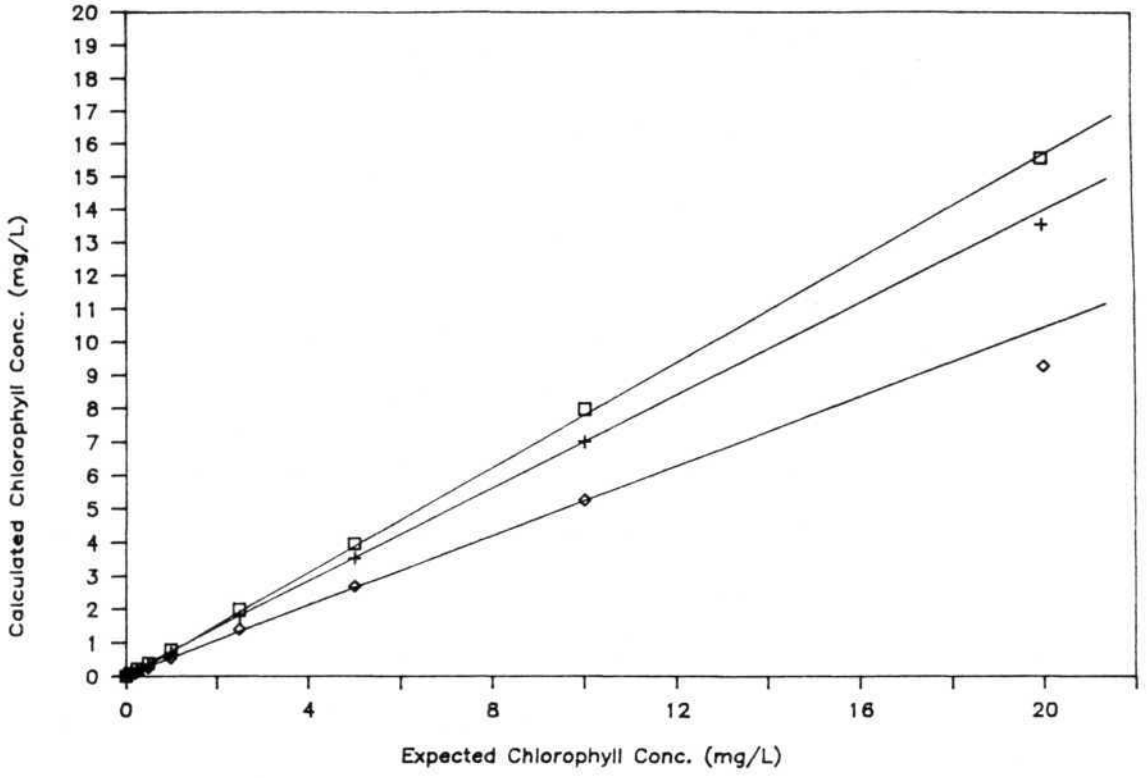


Figure 22. Correlation between calculated and expected chlorophyll concentrations. The spectral bandpasses are 2.0 nm (□), 10 nm (+), and 20 nm (◇).

Table VI. Calculated Concentrations of Chlorophyll-a Using the Trichromatic Equations

Expected Chl-a Conc. (mg/L)	Calculated Chl-a Conc. (mg/L) - - - Spectral Bandpass - - -		
	2 nm	10 nm	20 nm
0.010	0.016 (60%)[a]	0.001 (90%)	0.005 (50%)
0.100	0.085 (15%)	0.063 (37%)	0.061 (39%)
0.250	0.21 (16%)	0.17 (32%)	0.13 (48%)
0.500	0.40 (20%)	0.35 (30%)	0.28 (44%)
1.000	0.79 (21%)	0.70 (30%)	0.55 (45%)
2.500	2.0 (20%)	1.8 (29%)	1.4 (44%)
5.000	4.0 (20%)	3.5 (30%)	2.6 (48%)
10.00	8.0 (20%)	7.0 (30%)	5.3 (47%)
20.00	16 (20%)	14 (30%)	9.3 (54%)

[a] % error in ( )

absorbances are lower due to polychromatic radiation. For 0.01 mg/L chlorophyll the errors are larger due to the low absorbance. For this solution the standard deviation in the absorbance is 0.0002 AU and the average absorbance is 0.0010 AU. This absorbance is similar to the absorbance measured for the turbidity blank correction (A_{750}). The turbidity absorbance was subtracted from the absorbances measured at 630, 646, and 664 nm before substitution into the Jeffrey and Humphrey equations.

A similar spectral bandpass study was conducted with two more sets of chlorophyll standards prepared from different concentration stock solutions. Lorenzen's monochromatic equations were used to calculate the chlorophyll-a concentrations and the results are shown in Table VII. For 0.764 and 0.382 mg/L chlorophyll-a standards, an average decrease of 14% in the calculated chlorophyll concentration occurred when the spectral bandpass was changed from 2.0 to 10 nm. With a spectral bandpass of 20 nm, an average 36%

Table VII. Influence of Spectral Bandpass on the Recovery of Chlorophyll-a Using Lorenzen's Monochromatic Equations

Spectral Bandpass (nm)	Expected Chl-a Conc. (mg/L)	Calculated Chl-a Conc. (mg/L)	Error (%)
2[a]	0.764	0.807	+6
	0.382	0.427	+12
			+9[c]
10[a]	0.764	0.715	-6
	0.382	0.363	-5
			-5[c]
2[b]	5.70	5.88	+3
	1.14	1.18	+4
	0.570	0.616	+8
	0.285	0.312	+9
			+6[c]
20[b]	5.70	3.66	-36
	1.140	0.833	-27
	0.570	0.442	-22
	0.285	0.185	-35
			-30[c]

[a] May 29, 1985

[b] May 27, 1985

[c] Average error

decrease in the calculated chlorophyll-a concentration was found relative to that calculated with a 2.0-nm spectral bandpass. Weber in his simulated spectral bandpass study (Weber, 1976) observed 20.2 and 50.3% decreases in the chlorophyll-a concentration determined with 10- and 20-nm spectral bandpasses when compared to the concentration determined using a bandpass of 2 nm.

The data presented clearly show that the chlorophyll concentrations calculated from the trichromatic or monochromatic equations as recommended presently by the EPA are too low if the spectral bandpass for absorption measurements is greater than 2 nm. Use of a spectral bandpass significantly less than one tenth of the width of the chlorophyll band at half height would increase the baseline noise and degrade the detection limit.

Cell Pathlength

The basis of spectrophotometric analyses is the Beer-Lambert equation,

$$A = abc \tag{18}$$

where A is the absorbance of the sample, a is the absorptivity of the sample at a specific wavelength, b is the pathlength of the spectrophotometric cell, and c is the concentration of the analyte. When working with chlorophyll samples from oligotropic lakes (lakes low in nutrient input and low organic production), the amount of chlorophyll in a sample is very low. In this case one must take special precautions to collect meaningful data. Absorbances below 0.10 are not recommended as cell positioning imprecision and noise cause more imprecision in the absorbance

measured than at higher absorbances (APHA, 1985). To achieve this minimal absorbance value when dealing with low concentrations of chlorophyll, one must either increase the concentration of chlorophyll in the extract or increase the cell pathlength.

The absorbances measured with the Cary 118C spectrophotometer for a series of chlorophyll standards in 90% (v/v) aqueous acetone solution and the resulting calibration curves with 1- and 5-cm pathlength cells are shown in Table VIII and Figure 23. The chlorophyll concentrations calculated from the monochromatic equations are also given in Table VIII.

Table VIII. Effect of Cell Pathlength[a]

	ONE-CENTIMETER CELL		FIVE-CENTIMETER CELL	
Chl-a Conc. (mg/L)	Absorbance	Calculated Chl-a Conc. (mg/L)	Absorbance	Calculated Chl-a Conc. (mg/L)
6.11	0.5829	6.42	2.6084	4.69
3.06	0.2929	3.23	1.4272	3.08
1.53	0.1463	1.60	0.7220	1.59
0.764	0.0766	0.807	0.3663	0.818
0.382	0.0389	0.427	0.1790	0.395
0.191	0.0188	0.220	0.0884	0.196
0.0955	0.0085	0.104	0.0449	0.104
0.0477	0.0048	0.060	0.0195	0.048
0.0239	0.0024	0.017	0.0110	0.028
0.0119	-0.0005	-0.044	0.0026	0.012
0.00597	-0.0002	0.021	0.0009	0.006
0.00298	not read	-	0.0038	0.009

[a] wavelength, 665 nm; spectral bandpass, 2 nm; no acid added.

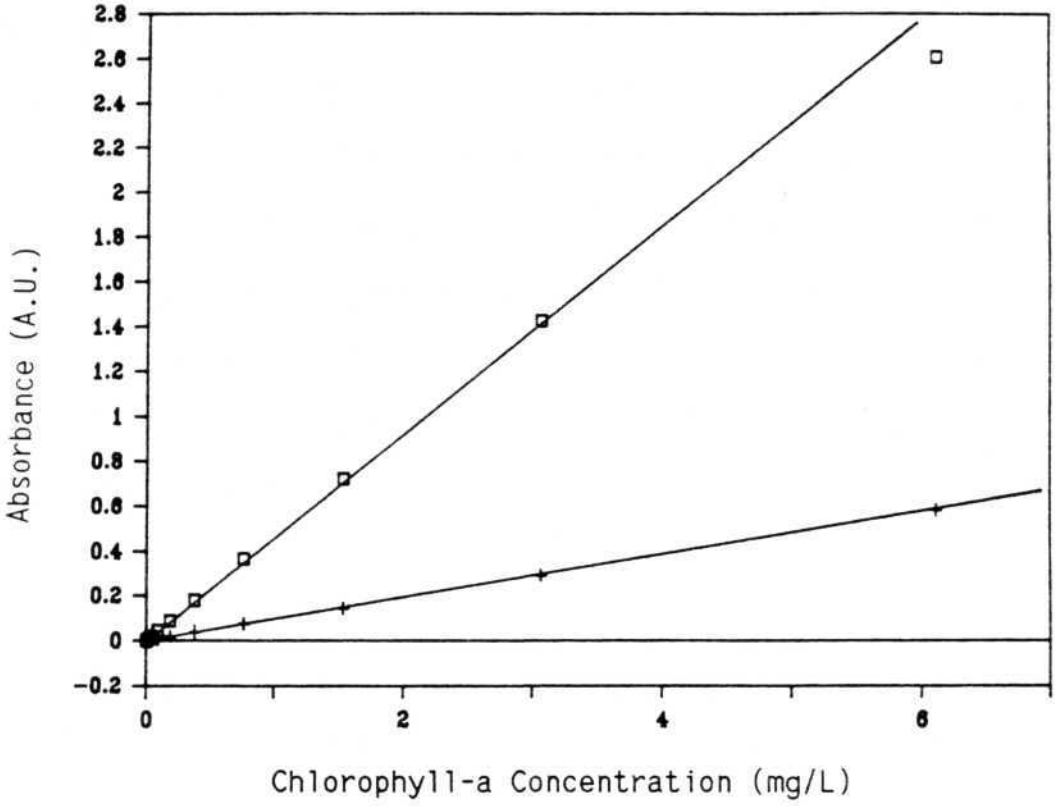


Figure 23. Calibration curves for the spectrophotometric technique. For data from 0.191 to 6.11 mg/L for the 1-cm cell (+), A = 0.0907c + 0.0007 with r = 1.0000. For the 5-cm cell (□) using 0.0239 to 1.53 mg/L, A = 0.454c - 0.0020 with r = 1.0000. (A = absorbance, c = Chl-a concentration and r = correlation coefficient)

Detection limits and calibration sensitivities (slopes) are summarized in Table IX. The blank standard deviation was calculated from twenty blank absorbance measurements with a single sample (stationary cell). The detection limit is calculated as two times the blank standard deviation divided by the calibration sensitivity. This value defines the lower limit of the dynamic range of the technique. The dynamic range of the spectrophotometric monochromatic technique (assuming 2 L of lake water is concentrated into 10 mL of extract) is 35 ng/L to 55 µg/L for the 1-cm pathlength cell and 4.4 ng/L to 11 µg/L for the 5-cm pathlength cell. The upper end is based on the EPA suggested maximum absorbance of 1.0. With the 5-cm pathlength cell linearity extended to chlorophyll-a concentrations of 3 mg/L (15 µg/L in a lake). Linearity extended beyond the most concentrated chlorophyll-a standard, 6 mg/L (30 µg/mL in a lake), for the 1-cm pathlength cell.

Table IX. Performance Characteristics of the Spectrophotometric Technique

Cell Path-Length (cm)	Blank Std. Dev. (A.U.)	Calibration Sensitivity (A.U./(mg/L))	Detection Limit Extract (µg/L)	Detection Limit Lake[a] (ng/L)
1	0.00032	0.0907	7.1	35
5	0.00020	0.454	0.88	4.4

[a] Using the dilution volume of 2 L of lake water phytoplankton filtered and extracted into 10 mL of 90% (v/v) aqueous acetone solution.

As discussed in the historical section, the detection limit is often specified as the chlorophyll concentrations yielding an absorbance of 0.01 A.U.. With this definition and the data for the 1-cm pathlength cell in Table IX, the detection limit is calculated to be 0.11 mg/L in the extract or 0.55 μg/L in lake water. This detection limit is almost 16 times worse than that reported in Table IX. The lower detection limit is overly optimistic for several reasons. Because four absorbances are used to calculate the chlorophyll concentration in the monochromatic method, the blank standard deviation is estimated to be $4^{1/2}$ or 2 times greater. Moreover, the difference in absorbance at 665 nm before and after acidification is calculated. This difference yields an effective calibration slope of about 40% of that reported above. When these two factors are considered, a better estimate of the detection limit would be a factor of 5 times worse or about 35 μg/L in the extract.

Other factors could degrade the detection limit further. These include a greater blank standard deviation due to cell positioning imprecision, variation in absorbance and scattering in real blanks (e.g., distilled water taken through the extraction procedure), or variations in uncompensated absorption and scattering between samples.

Evaluation of Fluorometric Measurements

Table X presents the fluorescence calibration data obtained for EPA chlorophyll standards and quality control samples with and without acidification. In Table XI the chlorophyll and phaeophytin concentrations calculated using equations 16 and 17 are compared to

Table X. Fluorescence Calibration Data

Chl-a Conc. (µg/L)	F_b (FU)	F_a (FU)	M_f[b] (µg/L)/FU	r[b]
80.0	4250	1950	0.0188	2.18
16.0	852	418	0.0188	2.04
4.00	213	98	0.0188	2.17
3.20[a]	241	136		
16.7[a]	895	418		

[a] Quality control samples supplied by the EPA. The concentrations are the reference values specified by the EPA for the corrected measurement procedure. For the 3.2 µg/L sample, the reference value is 4.4 µg/L with the uncorrected measurement procedure.

[b] M_f = Chl-a Conc./F_b and r = F_b/F_a

Table XI. Results for Fluorometric Quality Control Samples

Uncorrected Method:

Reference Chl-a Conc. (µg/L)	Calculated Chl-a Conc. (µg/L)	Confidence Range (µg/L)
16.7	16.9	15.0 to 18.4
4.4	4.53	3.9 to 4.8

Corrected Method:

Ref. Chl-a Conc. (µg/L)	Calc. Chl-a Conc. (µg/L)	Conf. Range Chl-a Conc. (µg/L)	Ref. Phaeo Conc. (µg/L)	Calc. Phaeo Conc. (µg/L)	Conf. Range Phaeo Conc. (µg/L)
16.7	17.4	14.2 to 19.2	-0.1	-1.56	-2.4 to 2.1
3.20	3.65	2.4 to 4.0	1.40	1.90	0.80 to 2.0

the certified values. The concentrations for both quality control samples fall within the confidence interval stated by the EPA. The meaning of the confidence interval (e.g., the confidence level) is not indicated.

Comparison of Spectrometric Methods

Cronemiller Lake Study

Cronemiller lake samples diluted to different degrees were analyzed by five different spectrometric techniques to evaluate and compare each technique's performance characteristics. The data are summarized in Table XII.

The absorbances of the Cronemiller lake test samples and the calculated concentrations of chlorophyll and phaeophytin using the monochromatic method are given in Tables XIII and XIV, respectively. The dependence of the determined chlorophyll concentration on the percent lake concentration (% LC) is shown in Figure 24.

The calibration factor of 26.7 (mg/L)/A.U. from Lorenzen's monochromatic equation is inverted to yield a calibration slope of 3.74×10^{-5} A.U./(μg/L). Here and for the slope calculation the absorbance represents $A_b - A_a$ after the turbidity correction. To calculate the detection limit, the blank standard deviation (s_{bk}) is assumed to be twice that obtained for measurements at a single wavelength to account for the four absorbances used to calculate the chlorophyll concentration.

For the 'in vivo' fluorometric determination, it is recommended that the fluorometer have its blank adjustment set using filtered

Table XII. Comparison of Performance Characteristics of Five Methods Used to Quantitate Chlorophyll in Natural Waters

Method	Slope[a]	Calibration[b] Sensitivity	- - - Blank - - - Signal[e]	Std. Dev.[e]	Detection Limit[c] Extract	Lake[d]
Fluorometric						
'In vivo'	3.69	36	0.090	0.088	-	4.9×10^{-3}
Enhanced 'in vivo'	6.79	67	0.090	0.088	-	2.6×10^{-3}
'In vitro'						
Uncorr.	63.4	24	0.79	0.046	3.9×10^{-3}	1.6×10^{-4}
Corrected	31.9	13	0.48	0.049	11×10^{-3}	4.6×10^{-4}
Spectrophotometric						
Monochromatic	8.75×10^{-5}	3.74×10^{-5}	0.0020	3.2×10^{-4}	34	1.4

[a] The units are FU/%LC for the fluorometric techniques and A.U./%LC for the monochromatic method. The fluorometric corrected slope is $F_b - F_a$ vs. %LC.

[b] The units are FU/(μg/L) for the fluorometric techniques and A.U./(μg/L) for the monochromatic method.

[c] The detection limit is calculated as twice the blank standard deviation divided by the calibration sensitivity, in units of μg/L.

[d] The concentration factor is 24.

[e] From 20 blank measurements on a stationary cell. For the fluorometric methods the units are FU and for the monochromatic method, the units are A.U. at 665 nm.

Table XIII. Absorbances of Cronemiller Lake Samples[a].

Lake Conc. (Percent)	Absorbance 665_b (nm)	Absorbance 750_b (nm)	Absorbance 750_a (nm)	Absorbance 665_a (nm)
100	0.0315 (0.0071)	0.0092 (0.0067)	0.0100 (0.0066)	0.0232 (0.0071)
50	0.0130 (0.0008)	0.0037 (0.0018)	0.0044 (0.0015)	0.0098 (0.0003)
25	0.0080 (0.0026)	0.0031 (0.0019)	0.0042 (0.0018)	0.0067 (0.0021)
12.5	0.0055 (0.0010)	0.0029 (0.0006)	0.0041 (0.0006)	0.0047 (0.0008)
6.25	0.0026 (0.0015)	0.0023 (0.0011)	0.0048 (0.0014)	0.0047 (0.0017)

[a] Average of 3 samples; standard deviation in ( ); 300-mL sample volume, concentration factor of 24.

Table XIV. Calculated Chlorophyll and Phaeophytin Concentrations Using the Monochromatic Spectrophotometric Method for Dilutions of Cronemiller Lake[a]

Lake Conc. (%)	Chl-a Conc. (µg/L)	Phaeo. Conc. (µg/L)
100	10.1 (0.1)	0.2 (0.4)
50	4.3 (1.0)	-0.1 (0.4)
25	2.7 (0.4)	-0.8 (0.3)
12.5	2.3 (0.4)	-1.8 (0.2)
6.25	0.4 (0.2)	-0.5 (0.4)

[a] Standard deviation in ( )

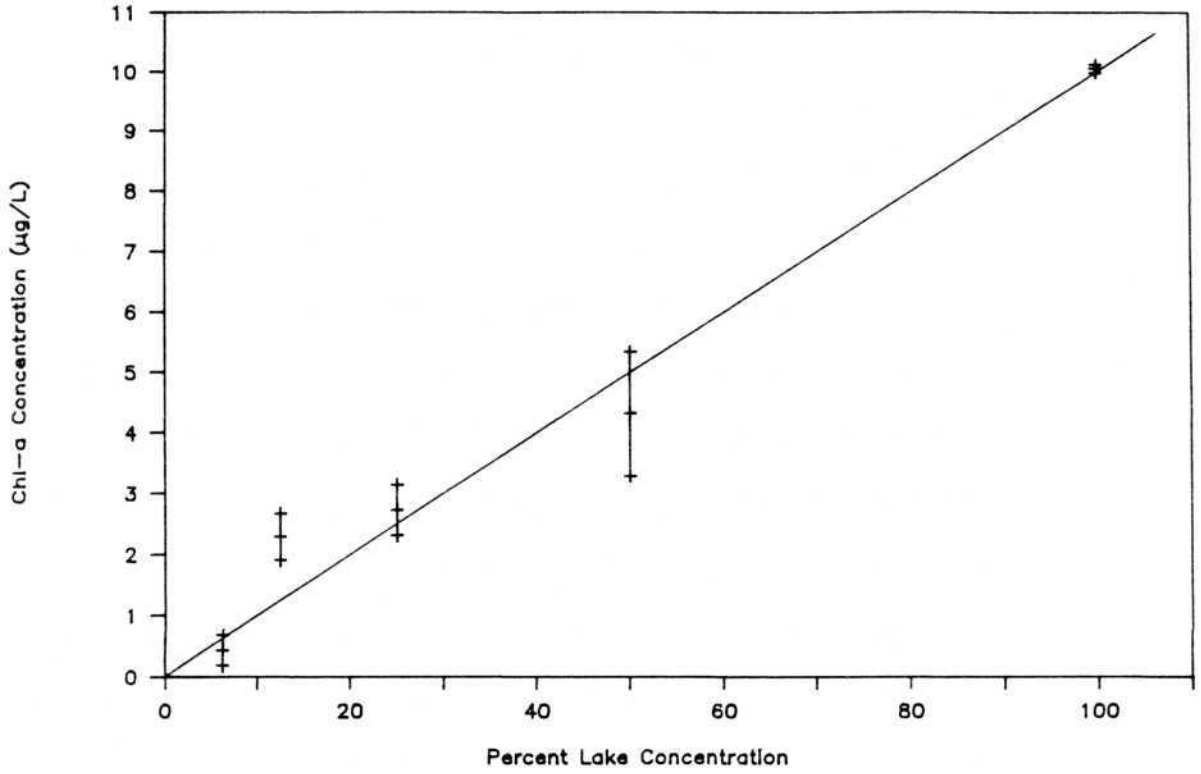


Figure 24. Dependence of chlorophyll concentrations determined using Lorenzen's monochromatic method on lake concentration (May 20, 1985). Error bars indicate ± one standard deviation from the mean. A linear regression fit, excluding the 12.5% data point, yields a slope of 0.101 µg/(L-%LC), an intercept of -0.176 µg/L Chl-a concentration and a correlation coefficient of 0.991.

lake water (Turner, 1981). Lake water filtered through a S & S #30 glass fiber filter resulted in a fluorescence signal of 34.0 FU and a standard deviation of 0.14 FU. The zero of the readout scale was adjusted using deionized water.

After the addition of DCMU, the deionized water blank signal increased from 0.08 to 3.2 FS and the filtered lake water blank signal increased from 34.0 to 39.0 FS with a standard deviation of 0.22 FU. The increase in the blank signal may be due to filter inefficiency or the increased scattering of excitation light from the undissolved DCMU crystals (Turner, 1981).

The fluorometric data and calibration curves for the 'in vivo' fluorescence and the enhanced 'in vivo' fluorescence methods are found in Table XV and Figure 25. The 'in vivo' signals are related to the chlorophyll concentration by using the absolute concentrations determined with the spectrophotometric method. The blank corrected signal was obtained by subtracting the lake blank signal times the percent lake dilution from the corresponding signal for the lake test solution with the same dilution factor.

Table XV. 'In vivo' Fluorometric Data for Dilutions of Cronemiller Lake[a]

Lake Conc.(%)	'In Vivo' (FU)	Enhanced (FU)
100	466 (1.0)	684 (3.9)
50	253 (1.0)	293 (0.5)
25	97 (0.9)	180 (0.8)
12.5	40 (0.3)	86 (0.5)
6.3	19 (0.1)	43 (1.0)

[a] Standard deviation in ( ).

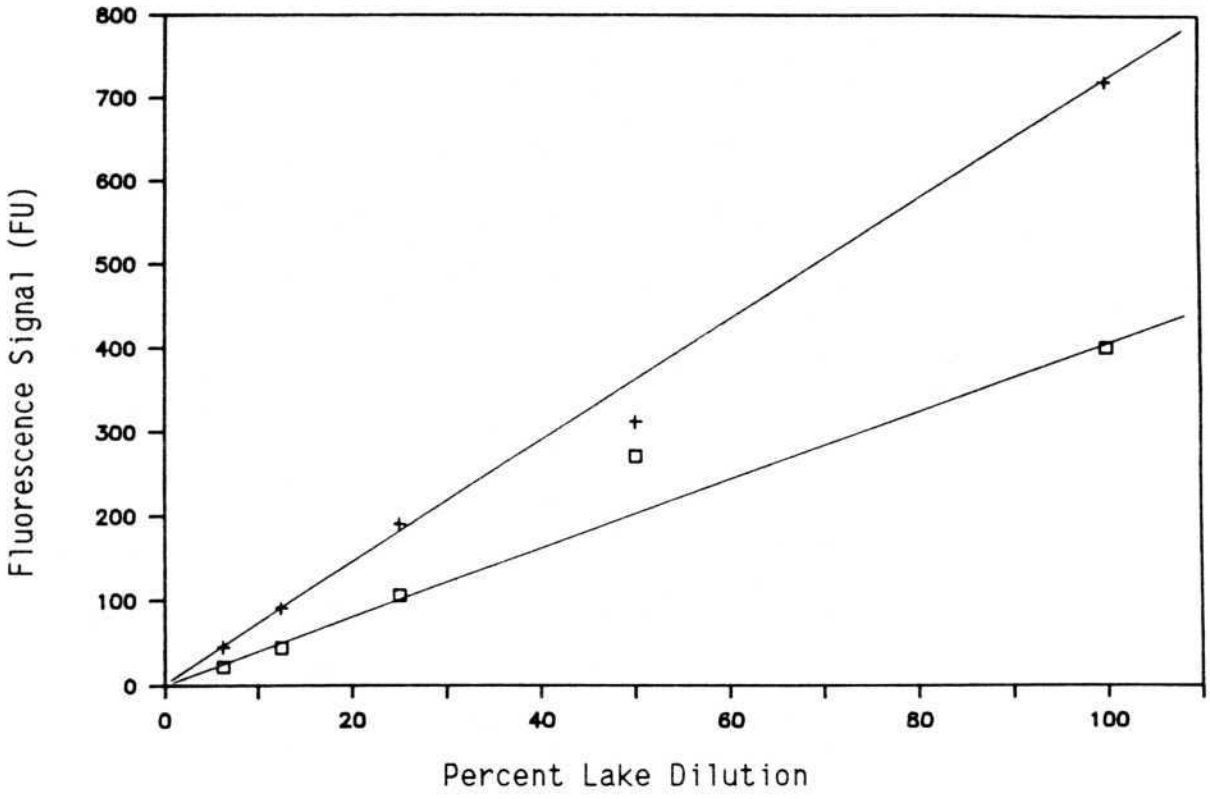


Figure 25. Dependence of 'in vivo' fluorescence signal (□) and enhanced 'in vivo' fluorescence signal (+) on lake concentration (May 20, 1985). A linear regression fit yields slopes of 3.69 and 6.79 for the normal and enhanced 'in vivo' methods with intercepts of -2.33 and 3.59, respectively, and similar correlation coefficients of 1.00 (the 50% LC data point was not used).

The fluorescence calibration sensitivity for the 'in vivo' method of m = 36 FU/(μg/L) is calculated by dividing the slope of the 'in vivo' fluorescence signal versus % LC (3.69 FU/% LC) by the slope of the absorbance-based chlorophyll concentration vs. lake concentration plot (0.101 μg/(L-% LC). The corresponding calibration sensitivity for the enhanced 'in vivo' method is 67 FU/(μg/L). For detection limit calculations, it is assumed the blank deviation for a deionized water blank is the same for the normal and enhanced methods.

The same Cronemiller Lake sample extracts used for the spectrophotometric measurements were also analyzed by the 'in vitro' fluorescence technique and the data are shown in Table XVI and Figure 26. From chlorophyll standards the calibration factor (M_f) is 0.042 μg/L-FU, m is 23.8 FU/(μg/L) and r is 2.18. The calibration slope is the inverse of M_f.

Because the corrected 'in vitro' technique involves two fluorescence measurements, the blank standard deviation used to calculate the detection limit is taken as $2^{1/2}$ X 0.049 = 0.069. The calibration sensitivity is taken as 23.8 FU/(μg/L) X ((r-1)/r) = 12.9 FU/(μg/L).

In general, the data show good linearity over the range of lake dilutions tested (10 to 0.6 μg/L of chlorophyll). The 'in vivo' and 'in vitro' fluorometric methods provide detection limits approximately a factor of 10^3 and 10^4 better, respectively, than the monochromatic method. For higher dilution factors, the precision of the monochromatic method is poorer because the chlorophyll concentrations are at or near the detection limit. The detection limits for the fluorometric methods could be degraded in samples with

a high background fluorescence or scattering signal if the noise in the background signal was greater than the noise measured for the deionized water blank.

Table XVI. 'In vitro' Fluorometric Data for Dilutions of Cronemiller Lake[a]

Lake Conc. Percent	Fluorometric Sig.(FU) F_b	F_a	Concentrations (µg/L) Chl-a uncorr.	Chl-a corr.	Phaeo.
100	6250 (350)	3105 (55)	10.9 (0.61)	10.2 (0.96)	1.7 (0.74)
50	3333 (189)	1713 (90)	5.8 (0.33)	5.2 (0.32)	1.3 (0.02)
25	1510 (94)	778 (51)	2.6 (0.16)	2.4 (0.14)	0.60 (0.06)
12.5	750 (42)	383 (24)	1.3 (0.07)	1.2 (0.06)	0.28 (0.03)
6.3	355 (10)	185 (5)	0.62 (0.017)	0.55 (0.016)	0.16 (0.003)

[a] Standard deviation in ( )

Precision Study

All six spectrometric methods were used to analyze 20 samples from Crater Lake. The mean, standard deviation, and relative standard deviation in the chlorophyll concentration determined by spectrophotometric and 'in vivo' fluorometric methods are reported in Table XVII. The mean, standard deviations and relative standard deviation for the 'in vivo' fluorescence signals are also given.

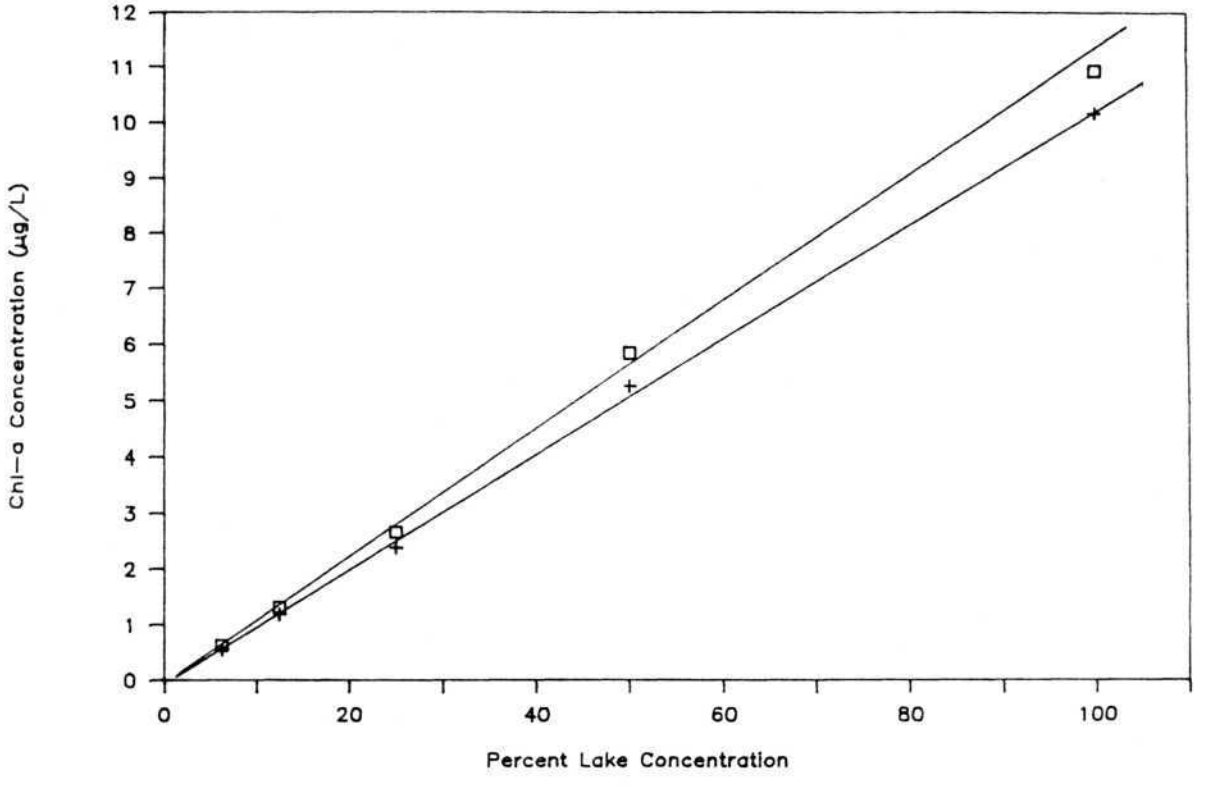


Figure 26. Dependence of the uncorrected (□) and corrected (+) 'in vitro' fluorescence signal on lake concentration (May 20, 1985). A linear regression fit yields a slope of 0.111 µg/L-%LC and an intercept of -0.028 µg/L with a correlation coefficient of 0.998 for the uncorrected method and a slope of 0.103 µg/L-%LC and an intercept of -0.100 µg/L with a correlation coefficient of 0.999 for the corrected method.

Table XVII. Crater Lake Precision Study

Method	Chl-a Conc. (µg/L)	Std. Dev. (µg/L)	Relative Std. Dev. (%)
FLUOROMETRIC			
'In Vivo'[a]			
20 m	12	1.9	16
120 m	38	2.7	7.2
Enhanced 'In Vivo'[a]			
20 m	15	3.0	20
120 m	56	3.9	6.9
Uncorrected 'In Vitro'			
20 m	0.30	0.013	4.3
120 m	0.84	0.043	5.1
'Corrected 'In Vitro'			
20 m	0.31	0.013	4.2
120 m	0.71	0.037	5.2
SPECTROPHOTOMETRIC			
Monochromatic			
20 m	0.36	0.041	11
120 m	0.63	0.058	9.3
Trichromatic			
20 m	0.84	0.21	25
120 m	1.31	0.34	26

[a] All 'in vivo' data represent fluorescence signals, FU.

The chlorophyll concentrations determined from the 'in vitro' fluorescence methods and the monochromatic spectrophotometric method are in reasonable agreement. The trichromatic method yields significantly higher chlorophyll concentrations. The precision of the 'in vitro' methods is about twice as good as the monochromatic method and a factor of 5 better than the trichromatic method.

Filter Efficiency Studies

Lake Studies

Samples of Cronemiller Lake water were filtered in triplicate with different types of filters. After extraction of the filters with 90% (v/v) aqueous acetone, the chlorophyll-a and phaeophytin pigments were determined by the EPA endorsed spectrophotometric monochromatic technique and both the EPA endorsed 'in vitro' uncorrected and corrected fluorometric techniques. The absorbance data and calculated concentrations are shown in Tables XVIII and XIX and the fluorescence data and resultant concentrations are given in Table XX.

The spectrophotometric data in Table XIX show that the S & S glass filter yielded a chlorophyll concentration equal to 91% of that obtained with the Millipore membrane filter and the Whatman GF/F filter resulted in a concentration equal to 79% of that obtained with the Millipore membrane filter. Based on a student-t difference test, these differences are insignificant at the 95% confidence level. The difference in the chlorophyll concentrations determined with the Millipore and Whatman filters is significant at

Table XVIII. Absorbance Data for Filter Efficiency Study[a].

Filter	ABSORBANCES 665_b(nm)	750_b(nm)	750_a(nm)	665_a(nm)
S + S Glass #30	0.0315 (0.0071)	0.0092 (0.0067)	0.0100 (0.0066)	0.0232 (0.0071)
Blank	0.0055 (0.0011)	0.0055 (0.0011)	0.0061 (0.0013)	0.0059 (0.0013)
Millipore HA Membrane	0.0310 (0.0022)	0.0059 (0.0010)	0.0060 (0.0011)	0.0211 (0.0013)
Blank	0.0047 (0.0016)	0.0046 (0.0018)	0.0054 (0.0018)	0.0055 (0.0018)
Whatman Glass GF/F	0.0311 (0.0091)	0.0093 (0.0085)	0.0101 (0.0083)	0.0241 (0.0098)
Blank	0.0101 (0.0034)	0.0082 (0.0027)	0.0085 (0.0019)	0.0103 (0.0027)
Acetone Solution Blank[b]	0.0021 (0.0001)	0.0021 (0.0003)	0.0023 (0.0003)	0.0015 (0.0001)

[a] Absorbance data shown are uncorrected for acetone blank and taken with Cary 118C spectrophotometer using a 1-cm pathlength sample cell. Six absorbance reading were recorded for each of three filters. The average absorbance of 3 samples is reported with the standard deviation in ( ).

[b] Twenty solution blanks read at the end of the analysis

Table XIX. Chlorophyll and Phaeophytin Concentrations Calculated for the Filter Efficiency Study[a]

Filter	Chl-a Conc. (µg/L)	Phaeo. Conc. (µg/L)
S + S Glass #30	10.1 (0.07)	0.23 (0.40)
Blank	0.13 (0.65)	-0.26 (0.83)
Millipore HA Membrane	11.1 (1.3)	0.69 (0.71)
Blank	0.051 (0.39)	-0.01 (0.51)
Whatman Glass GF/F	8.7 (1.1)	2.1 (2.2)
Blank	0.14 (0.09)	1.3 (0.69)

[a] Average for three samples with monochromatic method, standard deviation in ( ), blank corrected using a solution blank.

the 90% confidence level. In all cases, the mean chlorophyll-a concentrations determined for the blanks were not statistically different from zero and were less than 2% of the chlorophyll concentrations determined in the lake samples.

With the fluorometric technique all filters yielded effectively the same chlorophyll-a concentration based on the student-t difference test at the 95% confidence level. All blank signals were less than 0.3% that of the lake sample. The fluorescence methods are less sensitive to the interference caused by filter parts left in suspension after centrifugation than the spectrophotometric method.

Table XX. Fluorometric Data and Chlorophyll and Phaeophytin Concentrations Calculated for the Filter Efficiency Study[a]

Filter	Fluorometric Signal F_b	F_a	Chl-a Conc. Uncorr. (µg/L)	Chl-a Conc. Corr. (µg/L)	Phaeo. Conc. (µg/L)
S & S Glass #30	6250 (350)	3100 (55)	10.7 (0.6)	10.2 (1.0)	1.7 (0.7)
Blank	1.65 (0.05)	1.25 (0.25)	0.0028 (0.0001)	0.0013 (0.0006)	0.0035 (0.0016)
Millipore HA Membrane	5970 (470)	3100 (40)	10.2 (0.8)	9.3 (0.7)	2.6 (0.2)
Blank	16.0 (8.6)	9.1 (4.6)	0.027 (0.015)	0.022 (0.013)	0.012 (0.005)
Whatman Glass GF/F	6330 (170)	3230 (94)	10.8 (0.3)	10.0 (0.3)	2.3 (0.2)
Blank	8.2 (1.1)	9.6 (3.9)	0.014 (0.002)	-0.005 (0.009)	0.041 (0.024)

[a] Average for 3 samples; reported with standard deviations in ( ).

Two additional factors to consider when choosing an efficient filter for the collection of phytoplankton from a lake are the time of filtration and the filter cost. The Millipore membrane filter quickly dissolved in acetone and thus did not aid in the grinding process as when using glass filters. The absence of grinding material did not decrease the amount of chlorophyll in the extract in the spectrophotometric study. The average filtering time is longer with Millipore filters (≈15 min) compared to that of either glass filter (≈10 min). This might seem a small inconvenience, but when one is filtering many samples each taking 15 minutes, a decrease in the time of filtration of 30% is a significant time savings. The

cost of packages of 100, 24 mm diameter, filters are $44.00, $24.65 and $10.40, respectively, for the Millipore, Whatman and S & S type filters.

In conclusion, the three types of filters tested yielded equivalent retentive efficiencies. For the spectrophotometric or fluorometric determination of chlorophyll in lake water, either glass filter is more efficient to use in terms of time and money. For a specific phytoplankton population it is recommended that filter efficiency studies as described above be implemented.

Filter Efficiency Using TiO_2

Filters papers were weighed before and after filtering an aqueous suspension containing 0.10008 g of titanium dioxide. Table XXI summarizes the weighing data.

Table XXI. Data for Titanium Dioxide Suspension Filter Study[a]

Filter Type	Weight Filter + TiO_2	Filter Only	TiO_2 Only
S & S Glass	0.2361 (0.0007)	0.1373 (0.0008)	0.0988[a] (0.0002)
Millipore HA Membrane	0.1861 (0.0006)	0.0874 (0.0006)	0.0987 (0.0000)
Whatman Glass GF/F	0.1992 (0.0007)	0.1005 (0.0007)	0.0988 (0.0000)

[a] All weights in grams, average of 3 filters; standard deviation in ( ).

All values for the mass of TiO_2 retained by each of the filters tested were within 0.1 mg. There is no significant difference among the three filters in their ability to retain TiO_2 particles. For all the filters 1.18 mg or 1.18% of the TiO_2 suspended in 50 mL of Millipore water is not accounted for. The filtrate produced using all filters was visually clear. As the suspension was allowed to flow through each filter, a light TiO_2 film was noticed on the inside walls of the filtration funnels. This film did not rinse off using a light stream of Millipore water and may account for the recovery being about 1% low.

It is believed that the first TiO_2 crystals adsorb to the filter and therefore coat the filter. The TiO_2 acts in a similar fashion on each filter to standardize the size of particles which might subsequently be retained. Biologists often use Millipore Type HA membrane filters for the filtration of lake water. The published pore size of this filter is 450 nm. The titanium dioxide used in this study has a published particle diameter of 20 nm and larger. The smallest phytoplankton have diameters in the range of 3 to 4 μm, (Yentch, 1963).

Filter Storage Study

During the seven months following the storage of several sets of chlorophyll-containing filters, sets of three filters were taken out of storage and analyzed for chlorophyll using Lorenzen's monochromatic spectrophotometric method. The results of calculations are summarized in Table XXII and Figure 27.

Table XXII. Concentrations Determined After Storage of Filters[a]

	Refrigerator (1° C)	+ $MgCO_3$	Freezer (-9° C)	+ $MgCO_3$
Day 0				
Chl-a	10.1 (0.07)			
Phaeo	0.23 (0.40)			
Day 1				
Chl-a	9.23 (0.71)	7.57 (0.54)	8.12 (0.56)	10.5 (0.21)
Phaeo	7.39 (0.71)	2.76 (0.46)	3.24 (0.04)	3.74 (1.1)
Day 12				
Chl-a	11.3 (1.17)	12.3 (0.48)	8.22 (0.86)	10.1 (0.38)
Phaeo	0.61 (0.80)	0.73 (0.46)	2.36 (0.01)	0.62 (0.49)
Day 65				
Chl-a	11.2 (0.85)	11.8 (0.81)	11.6 (0.11)	11.8 (2.3)
Phaeo	-1.07 (0.55)	1.20 (4.2)	-1.77 (0.14)	-0.18 (1.8)
Day 194				
Chl-a			8.24 (0.79)	10.8 (0.40)
Phaeo			5.16 (0.91)	8.22 (2.85)
Day 212				
Chl-a			9.2 (1.4)	11.0 (0.50)
Phaeo			6.1 (2.3)	3.3 (2.8)
Day 236				
Chl-a			8.56 (0.1)	11.5 (0.46)
Phaeo			6.94 (3.2)	4.51 (1.41)

[a] All concentrations reported in the original sample in µg/L; average of 3 sample filters; standard deviation in ( ).

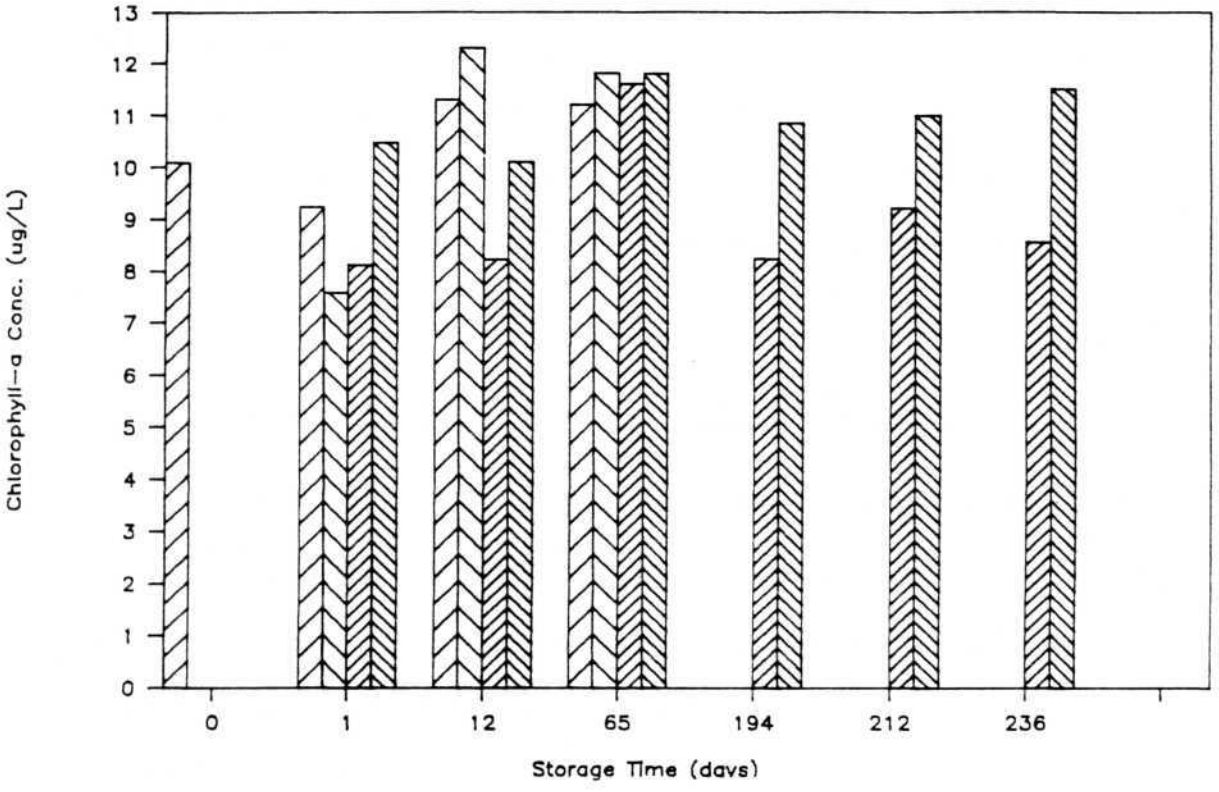


Figure 27. Chlorophyll concentration calculated as a function of storage time. ▨ 1°C, ▨ 1°C with $MgCO_3$, ▨ -9°C and ▨ -9°C with $MgCO_3$.

The data do not indicate any significant difference in the chlorophyll concentration determined when filters were stored with or without $MgCO_3$ or just above freezing or frozen. The average relative standard deviation in the chlorophyll concentration determined is 8.7% which makes it difficult to distinguish differences of 10 to 20%.

Study of the Effect of Temperature and Sunlight on the 'in vivo' Fluorescence Signal

The dependence of the 'in vivo' and the 'in vitro' fluorometric signals on sampling depth is shown in Figure 28. The variations of the fluorescence number (ratio of the 'in vivo' and the 'in vitro' fluorescence signals) with depth is clear in Figure 29. Because of this observation, the following temperature and sunlight studies were conducted.

Temperature Effect on 'in vivo' Fluorescence

The fluorescence of most species depends on the temperature (Wehry, 1973). The fluorescence signal usually increases as the sample's temperature decreases because dynamic quenching due to collisions is reduced at lower temperatures. The 'in vivo' fluorescence signal also shows this dependence on the sample's temperature.

The effect of sample temperature on the 'in vivo ' fluorescence signal is shown in Figure 30. As the temperature decreases from 20 to 10^{o} C, the resulting 'in vivo' fluorescence signal increases at all depths sampled, except at 160 m. Temperatures cooler than 10^{o} C can lead to fogging of the sample cell and erratic results

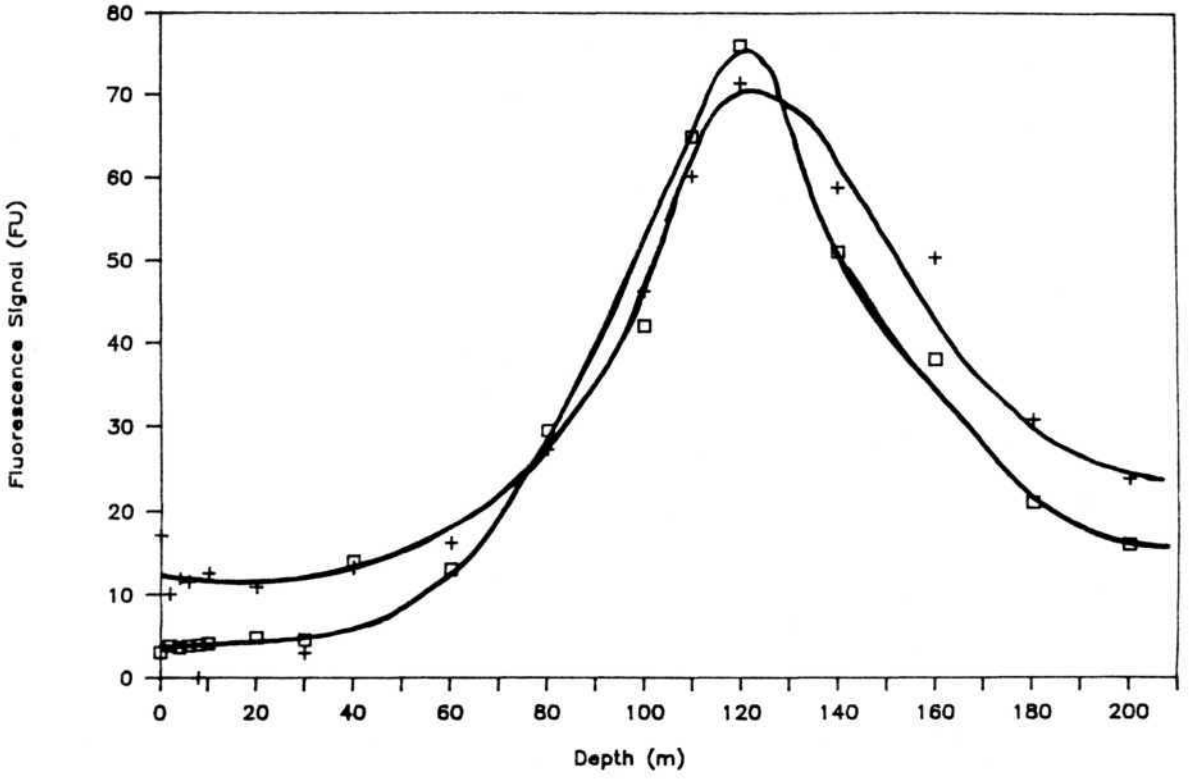


Figure 28. Profiles of the 'in vivo' (□) and 'in vitro' (+) fluorescence signals from Crater Lake (July 31, 1984). The 'in vitro' fluorescence signals are normalized to lake water by dividing the fluorescence signals by the concentration factor of 10.

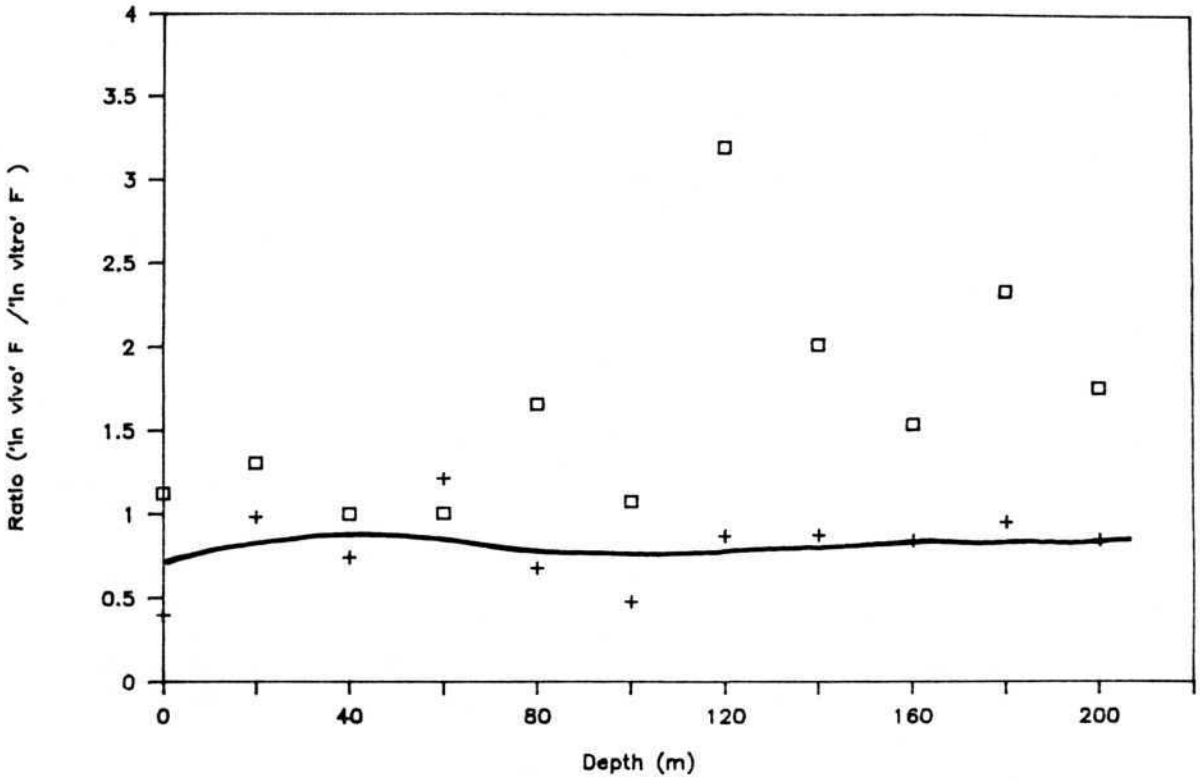


Figure 29. The effect of depth on the fluorescence number. From Crater Lake, Oregon, July 31, 1984 (□) and June 26, 1986 (X).

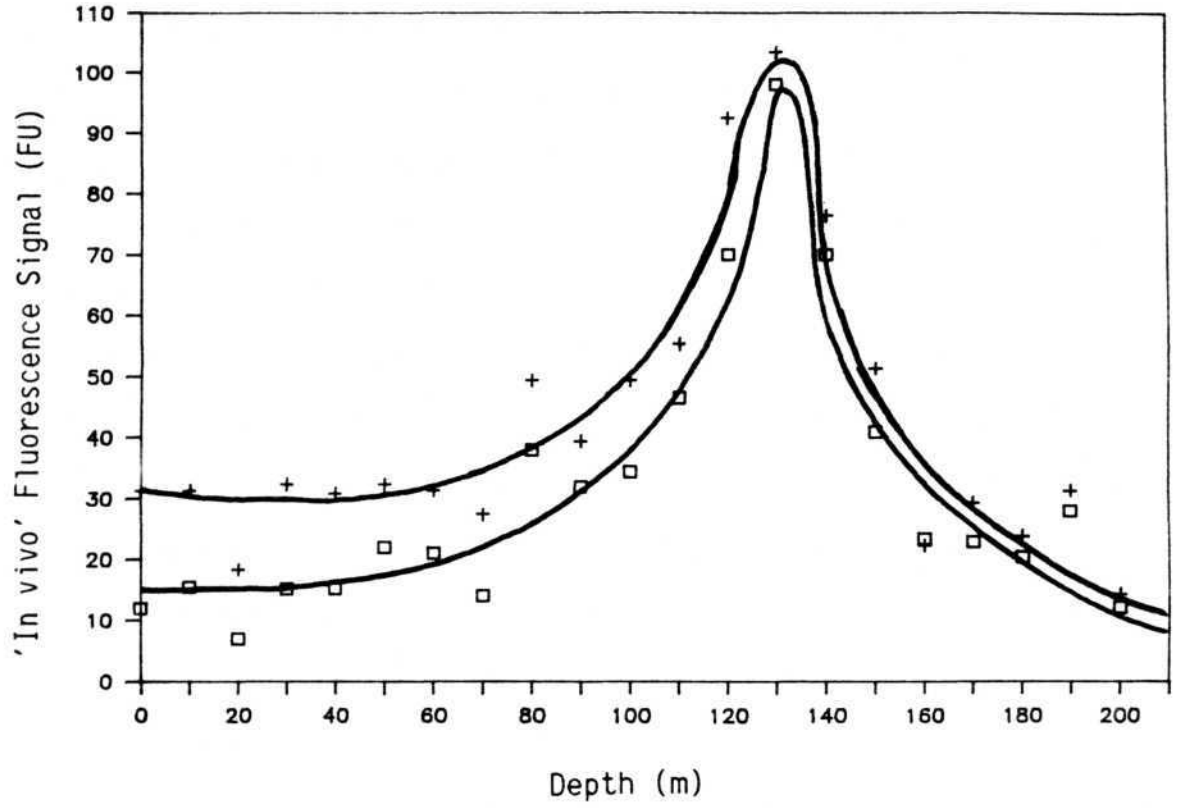


Figure 30. Effect of temperature on the 'in vivo' fluorescence signal. From Crater Lake, Oregon, July 31, 1985; (+) 10°C, (▫) 20°C .

on humid days.

The factor by which the fluorescence signal increases depends on the depth as shown in Figure 31. This factor ranges from 2.63 for samples near the surface to 1.05 for the 130-m sample without DCMU. With DCMU the trend is similar and the enhancement factor varies from 2.34 for samples near the surface to 1.03 at a depth of 120 m.

For every degree decrease in temperature, the 'in vivo' fluorescence signal increases on an average of the entire profile by 0.18%. This study was repeated three times using Crater Lake water. Each time a decrease in temperature resulted in a similar increase in 'in vivo' fluorescence signal.

Because the chlorophyll concentration determined using the 'in vivo' fluorescence method varies not only with chlorophyll concentration but also with sample temperature, it is recommended that the samples be brought to a uniform temperature before the 'in vivo' fluorescence signal is measured. In this way, the 'in vivo' fluorescence signal will be more closely correlated to the concentration of chlorophyll.

Effect of Sunlight on 'in vivo' Fluorescence

Researchers have shown that sunlight incident on phytoplankton causes the cell's chloroplasts to contract. This effect occurs within two to five minutes of exposure (Kiefer, 1973). A longer term decrease in 'in vivo' fluorescence also results and is associated with the migration of chloroplasts to opposite ends of

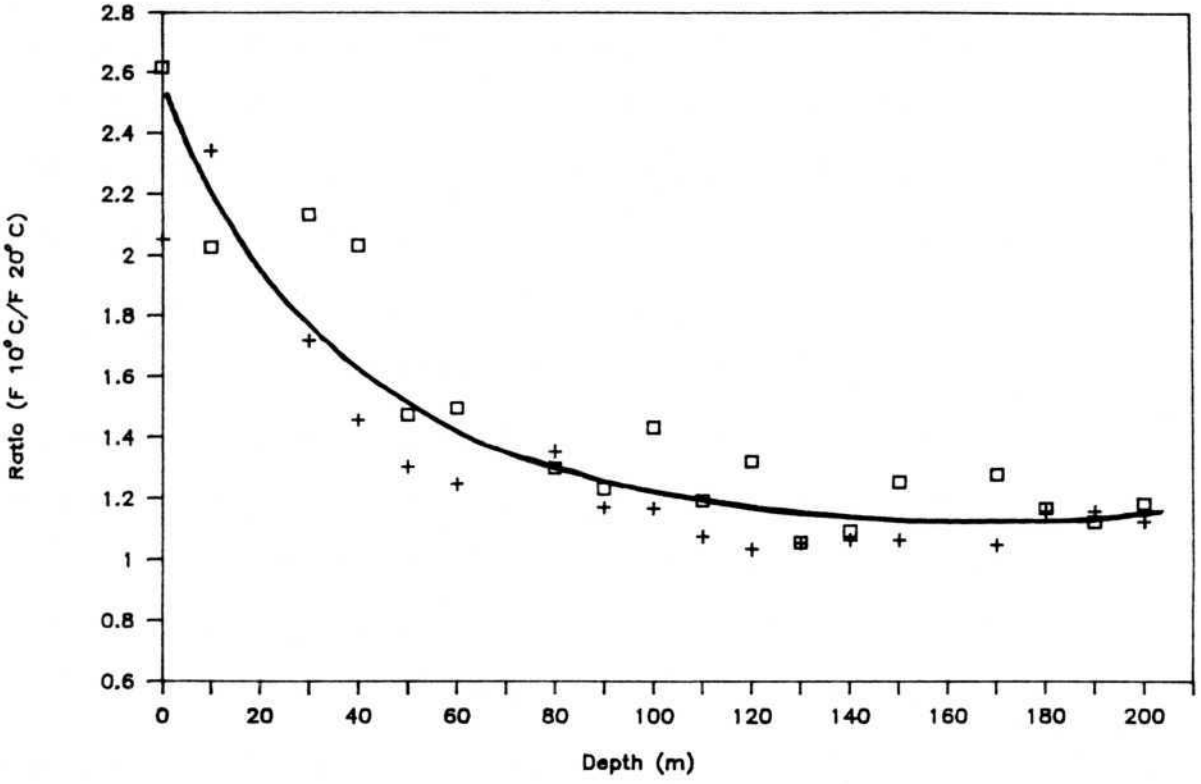


Figure 31. The temperature dependence of the enhancement factor of the 'in vivo' (□) and enhanced 'in vivo' (+) fluorometric signals on depth (July 31, 1985).

the cell in a further effort to protect the cell's chlorophyll. The latter effect begins to become important about five minutes after the initial exposure and continues to affect the 'in vivo' fluorescence for up to about 50 min. Because both mechanisms effect the amount of excitation light absorbed by chlorophyll in the cell, the fluorescence signal is also affected.

The 'in vivo' fluorescence signal of Crater Lake samples exposed to sunlight was observed to decrease markedly in the first 5 min (an average of 3.4%/min) and then more slowly for 40 min as shown in Figure 32. After 40 to 50 min, the 'in vivo' fluorescence signal became constant and the lowest recorded (an average signal 47% that of the original). These data indicate that this response of phytoplankton to sunlight could lead to a decrease in the 'in vivo' fluorescence signal in surface samples taken in a lake. The enhanced 'in vivo' fluorometric signals were recorded under similar experimental parameters. Trends in the enhanced 'in vivo' fluorometric signal were similar to those already discussed for the 'in vivo' fluorometric signal as shown in Figure 33.

The higher temperature and sunlight exposure of surface lake samples decrease the 'in vivo' fluorescence signal recorded relative to that of deeper samples for a given concentration of chlorophyll. Figure 34 depicts typical temperature and light profiles for Crater Lake during the summer. The effect of each variable on the 'in vivo' fluorescence of samples taken directly from the lake might account for the decrease observed in the ratio of the 'in vivo' fluorescence to 'in vitro' fluorescence signals for shallow samples. If the samples are stored for at least 60 min

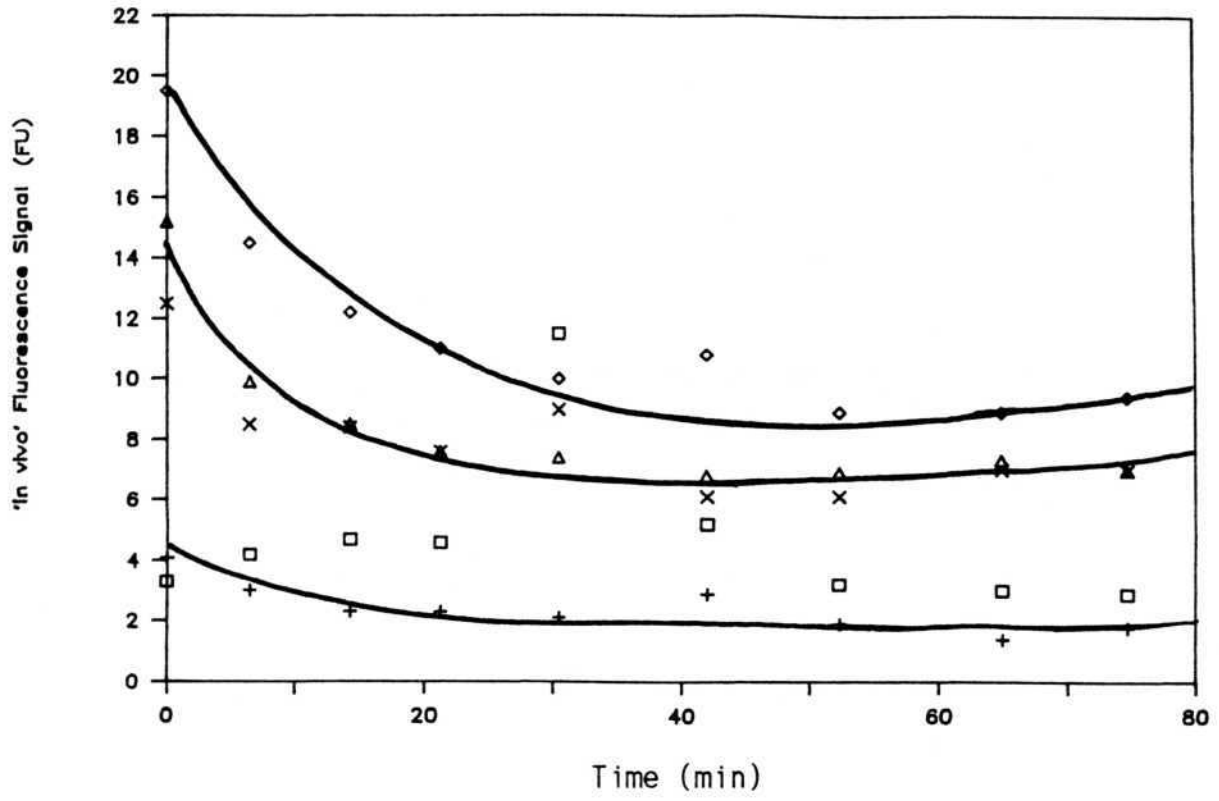


Figure 32. Dependence of the 'in vivo' fluorescence signal on exposure time to sunlight (1.02 cal/(cm^2 · min), August 12, 1985). Mixtures included: (□) 0 to 20 m, (+) 30 to 60 m, (◇) 70 to 110 m, (△) 120 to 160 m, (X) 170 to 200 m.

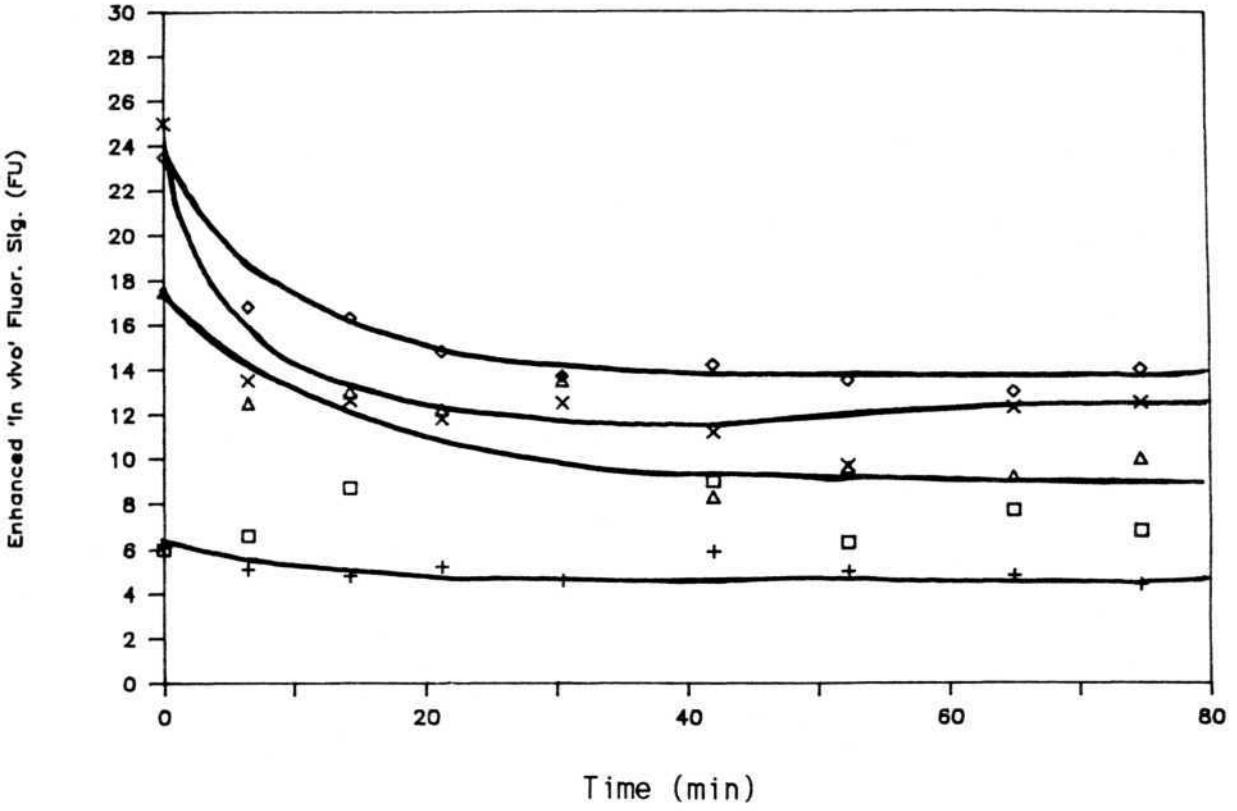


Figure 33. Dependence of the enhanced 'in vivo' fluorescence signal on exposure time to sunlight (1.02 cal/(cm^2 · min), August 12, 1985). Mixtures included: (□) 0 to 20 m, (+) 30 to 60 m, (◇) 70 to 110 m, (△) 120 to 160 m, (X) 170 to 200 m.

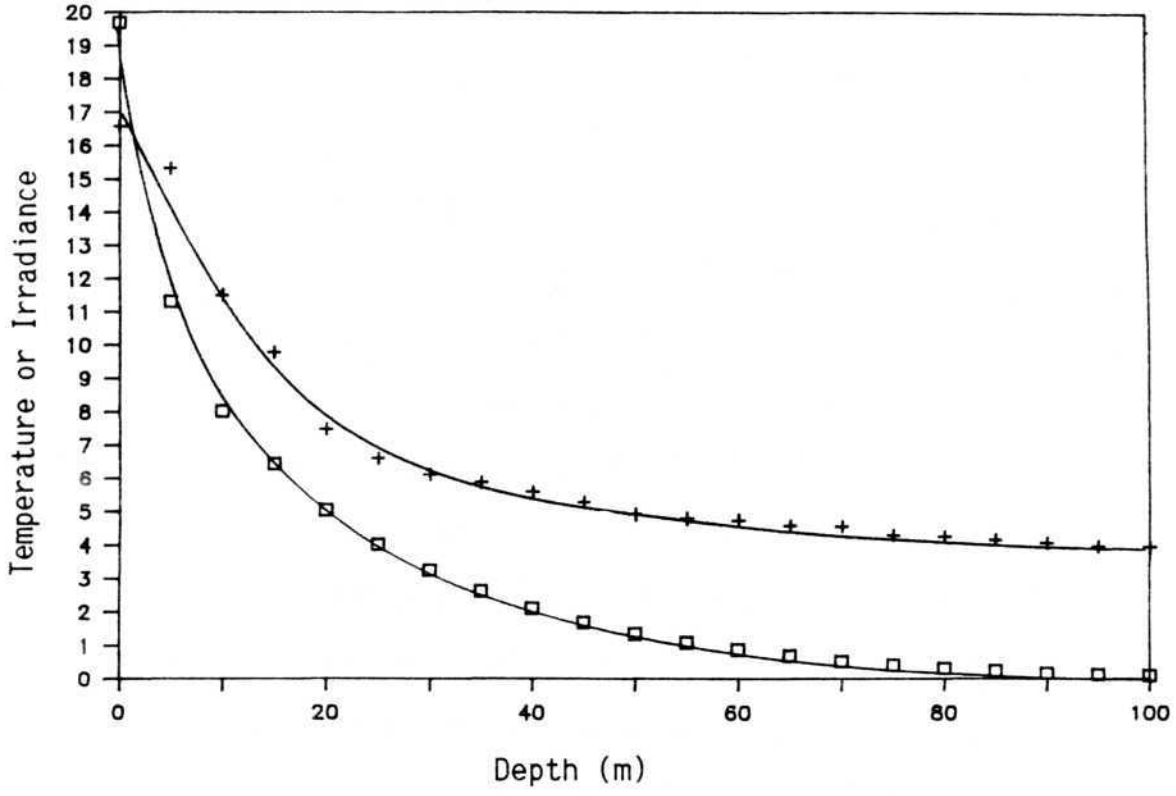


Figure 34. Temperature (July 23, 1985) and irradiance (August 6, 1985) depth profiles for Crater Lake. (□) Temperature ^{o}C, (+) Irradiance (white light, cal/(cm^2·min)/3.1)

in a light tight container and allowed to reach uniform temperature, the 'in vivo' fluorescence signal might more accurately reflect the concentration of chlorophyll in the water sample. Therefore it is recommended that samples collected for an 'in vivo' fluorescence signal measurements be stored in brown plastic bottles for 60 min in a Coleman-type cooler filled with water of a known temperature. This procedure would allow the cells to experience a similar light history, adapt to the low light conditions of deeper waters, and come to uniform temperature before the 'in vivo' fluorescence measurements are made. Under these conditions the 'in vivo' fluorescence should more accurately reflect the samples' chlorophyll-a concentration.

The Crater Lake fluorescence profile experiment leading to Figure 28 was repeated on June 26, 1986. All samples were stored for 60 min in the dark and kept between 0.5 and 2.0^{o} C until the 'in vivo' fluorescence signal was recorded. The data in Figures 29 and 35 show that the fluorescence number remains more constant with depth even for samples collected near the surface.

Fiber Optic Fluorometry

Focusing the Source

To evaluate the possibility of remote sensing of chlorophyll fluorescence, the focusing characteristics of the lamp were first studied. With the f/4.5 reflector and the Hg lamp installed, the lamp to the reflector distance along the major axis of the elliptical reflector was adjusted to focus the lamp arc image about

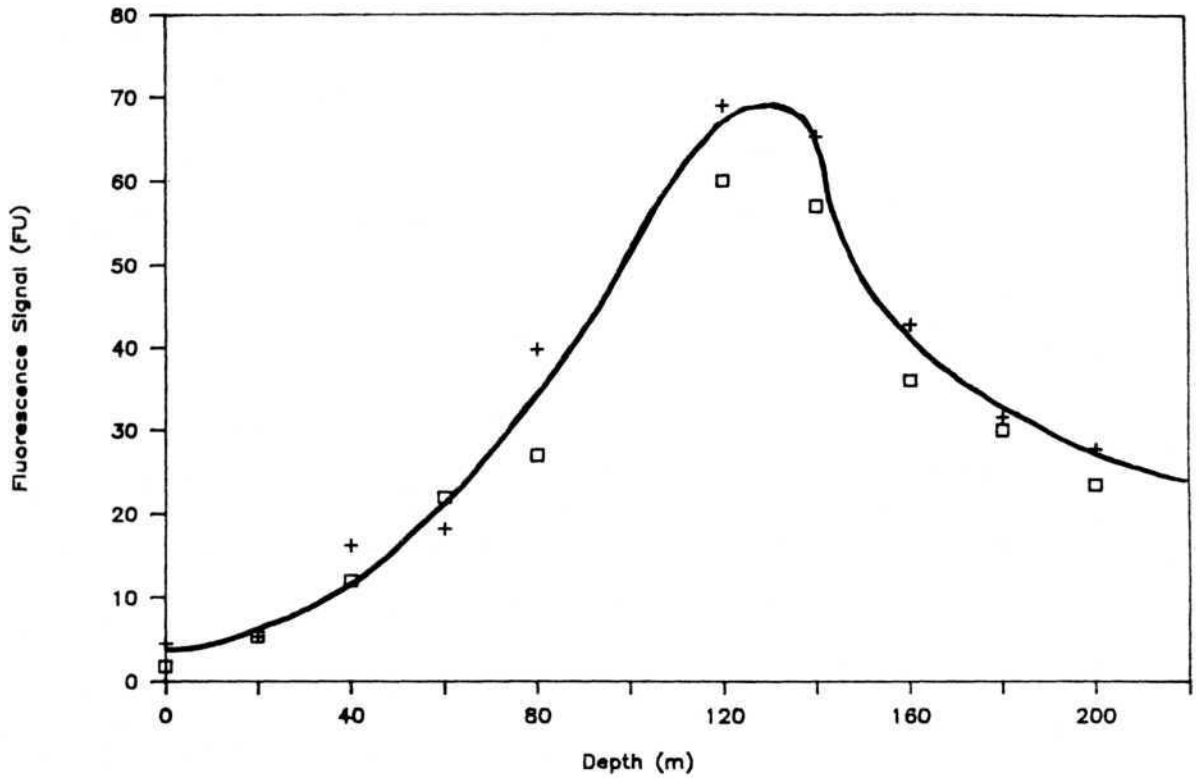


Figure 35. Profiles of 'in vivo' (□) and 'in vitro' (+) fluorescence signals from Crater Lake (June 26, 1986). The 'in vitro' fluorescence signal is normalized to lake water.

11.0 in in from of the lamp housing (x-distance). The proper reflector distance is achieved when the sharpest image is produced with a "hole" in its center.

The radiometer indicated a total power output by the lamp (no aperture) of 8.24 W. With no aperture the detector has an entrance diameter of 3.0 cm. The distance from the lamp to the detector is adjusted so that the entire image entered the detector and essentially none of the beam is blocked by the detector aperture. A 0.5 turn clockwise or counterclockwise on the lamp positioning screws changed the focal point distance in front of the lamp housing by about 1 in. A clockwise turn brings the lamp closer to the reflector and moves the focal point farther away from the housing.

A 1.0-mm aperture was placed in front of the lamp housing with the detector placed directly behind the aperture. For three different positions of the lamp reflector adjustment, the aperture and detector were moved along and perpendicular to the optical rail, x- and y-axes, to maximize the radiant power. This lead to the data shown in Table XXIII.

The dependences of the radiant power passed by a 1-mm aperture on the x and y positions of the aperture are shown in Figures 36 and 37. Here the lamp-reflector distance was optimized to produce the most intense lamp image. At this distance the lamp intensity observed through the 1-mm aperture located at the focal point changes the greatest before and beyond the focal distance as well as for movement of the aperture in the focal plane when compared to other lamp-reflector distances.

Table XXIII. Maximum Radiant Power Observed with a 100-W Hg Lamp and a 1.0-mm Aperture.

x-Distance (in)	Radiant Power (mW)	Peak Width[a] (mm)	Comment
11.5	673	3.7	initial setting
11.0	1047	2.8	0.25 turn ccw of lamp positioning screws
11.5	792	3.4	0.25 turn cw of lamp positioning screws

[a] Peak width at half height measured by sweeping across the optical axis in the y direction.

The peak width at half height (w_h) of the y-axis intensity profile was determined. If the focal spot was 1.0 mm in diameter, a scan with a 1-mm aperture across the spot would have a w_h of about 1.0 mm. The data gathered at the optimum lamp-reflector distance and x-distance (11.0 in) (see Table XXIII) indicate a focal spot half-width of about 2 mm.

With a new Supracil 75-W Xe arc lamp installed with the f/4.5 reflector, a total radiant power of 4.74 W was measured with no aperture. Changes in the radiant power transmitted through a 1-mm aperture resulted as the lamp-to-reflector distance and x-distance were changed as shown in Table XXIV. The optimum x-distance was again 11.0 in.

The dependence of the radiant power passed on the size of the aperture is shown in Table XXV. The radiant power passing through a 1.0-mm aperture is only about 2.5% of the total radiant power

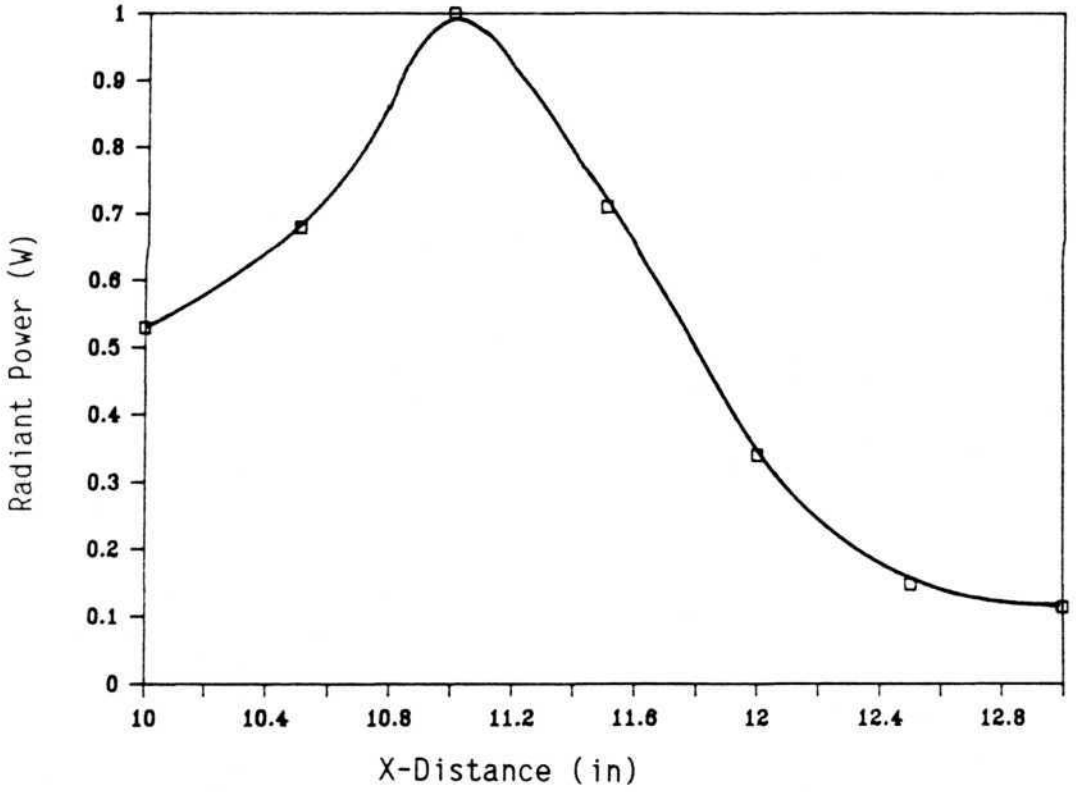


Figure 36. Profiles of lamp intensity observed when the 1-mm aperture and detector were moved along the optical rail in the x-direction (Hg lamp).

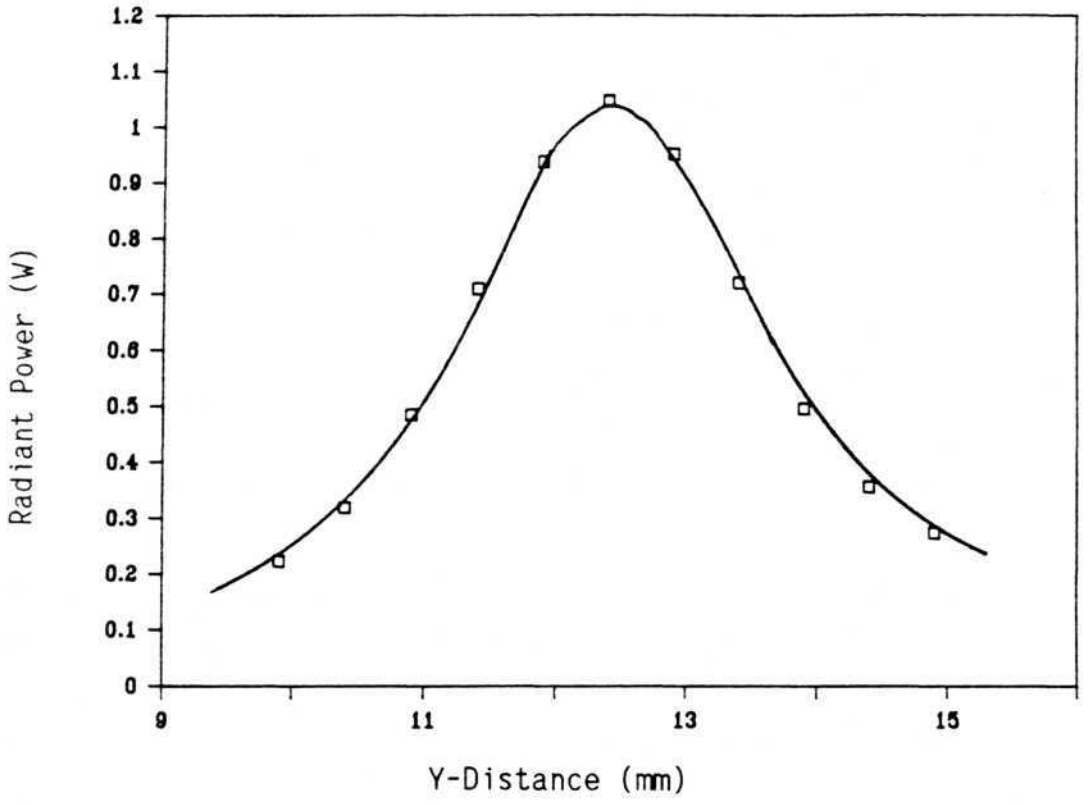


Figure 37. Profile of lamp intensity observed when the 1-mm aperture and detector were moved across the optical rail (y). The x-distance was that yielding maximum radiant power for the data in Figure 36 (Hg lamp).

Table XXIV. Maximum Power Observed with a 75-W Xe Lamp and 1.0-mm Aperture

x-Distance (in)	Power (mW)	Peak Width[a] (mm)	Comment
10.0	57	N/A	initial setting
10.4	104	N/A	0.25 cw turn of lamp positioning screws
11.06	116	2.3	position optimized
11.14	111	N/A	cw 0.25 turn of positioning screws from 10.4 setting
11.97	60	N/A	cw 0.25 turn of positioning screws

[a] Peak width at half height measured by sweeping across the optical axis in the y direction.

Table XXV. Dependence of Radiant Power on Aperture Size

Diameter (mm)	Area (mm^2)	Radiant Power (mW)	Power Density (mW/mm^2)	Percent Radiant Power (percent)
30.0	707	4200	5.94	100
7.0	38.5	2100	54.5	50
2.0	3.14	430	137	10
1.0	0.785	110	86.4	2.6

output by the lamp through its maximum 30 mm aperture.

The lamp and associated elliptical reflector seem to provide a focal spot of light about 2.0 mm in diameter. The lamp focus is between 11.0 and 11.5 in. in front of the lamp housing window using the f/4.5 elliptical reflector. Similar results were obtained using the f/2.5 elliptical reflector but in this case the focus position is 5.4 in in front of the lamp housing window.

Fiber Transmission Characteristics

To further investigate the feasibility of using fiber optics for the 'in situ' detection of a fluorescent materials, the attenuation of the fiber was studied next. The transmission spectra for the 1-m and 45-m fibers are shown in Figure 38. The transmission characteristics at various wavelengths of the fiber optic cables are tabulated in Table XXVI. The attenuation of the 45-m fiber in decibels (dB) was calculated using the equation

$$\text{Attenuation (dB)} = -10 \cdot \log (I_{45}/I_1) \qquad (20)$$

where I_{45} is the intensity of radiation transmitted through the 45-m fiber optic cable and I_1 the intensity transmitted through the 1-m fiber optic cable.

The attenuation increases as the wavelength decreases as shown in Figure 39. This is attributed to absorption and scattering of the light in the cable itself. Because the excitation wavelength is always shorter than the emission wavelength in fluorescence measurements, the excitation radiation would be the radiation most

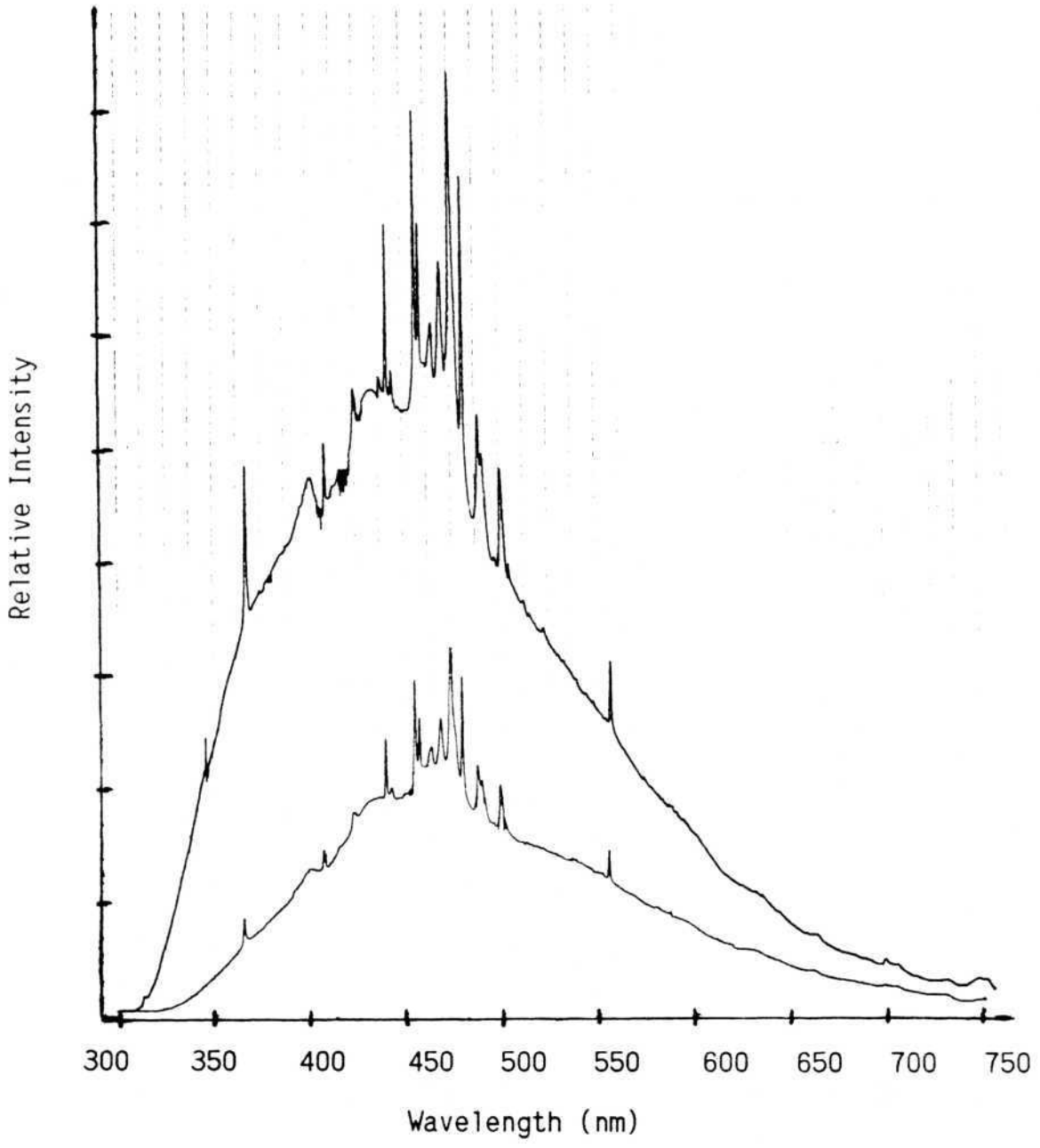


Figure 38. Spectral transmission characteristics of a 600-μm diameter fiber optic cable (upper 1-m fiber, lower 45-m fiber optic cable).

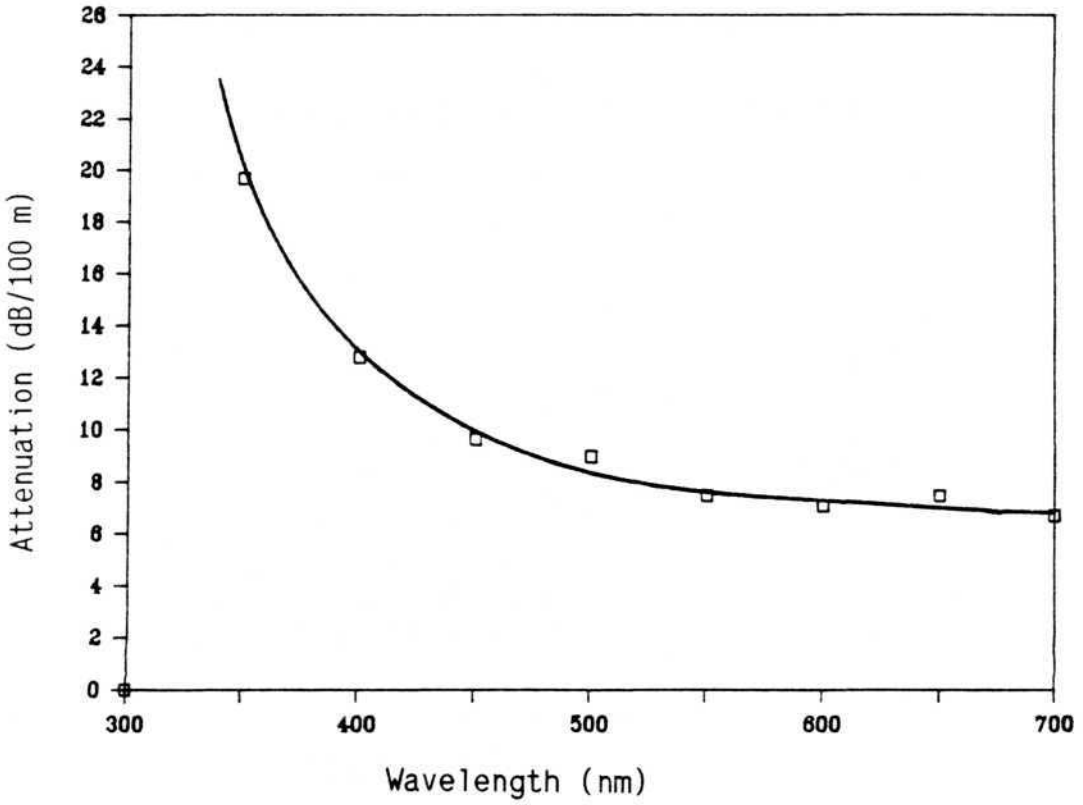


Figure 39. Dependence of attenuation of radiation on wavelength.

attenuated by transmission over long distances in a fiber optic fluorescence probe. Table XXVI also indicates the calculated attenuation for 100- and 1000-m fiber optic cables.

Table XXVI. Transmission Characteristics of 1- and 45-m Fiber Optic Cables

Wavelength (nm)	Chart Values 1-m FO	Chart Values 45-m FO	Attenuation (dB/45 m)[a]	Attenuation (dB/100 m)[b]	Attenuation (dB/1 km)[c]
300	0.0	0.0			
350	23.0	3.0	8.85	19.7	197
400	47.4	12.6	5.75	12.8	128
450	80.0	29.5	4.33	9.63	96.3
500	38.0	15.0	4.04	8.97	89.7
550	31.0	14.3	3.36	7.47	74.7
600	12.9	6.2	3.18	7.07	70.7
650	7.8	3.6	3.36	7.46	74.6
700	3.0	1.5	3.01	6.69	66.9

[a] -10(log(Signal of 1-m FO/Signal of 45-m FO))
[b] -10(log(Signal of 45-m FO/Signal of 1-m FO)/45)100
[c] -10(log(Signal of 45-m FO/Signal of 1-m FO)/45)1000

Remote Fiber Fluorometry

A series of experiments were completed to optimize the angle and distance between the fibers. Test solutions included 10 mg/L quinine sulfate and its blank (0.2 M H_2SO_4), milk of magnesia and its blank (deionized water), and a sample of phytoplankton from pond water with deionized water as the blank.

The dependence of the fluorescence signal of a 10 mg/L solution of quinine sulfate on the fiber angle and separation distance is shown in Figure 40. The fluorescence signal was greatest at an angle of 10^{o}. Beyond an angle of 170^{o}, the excitation

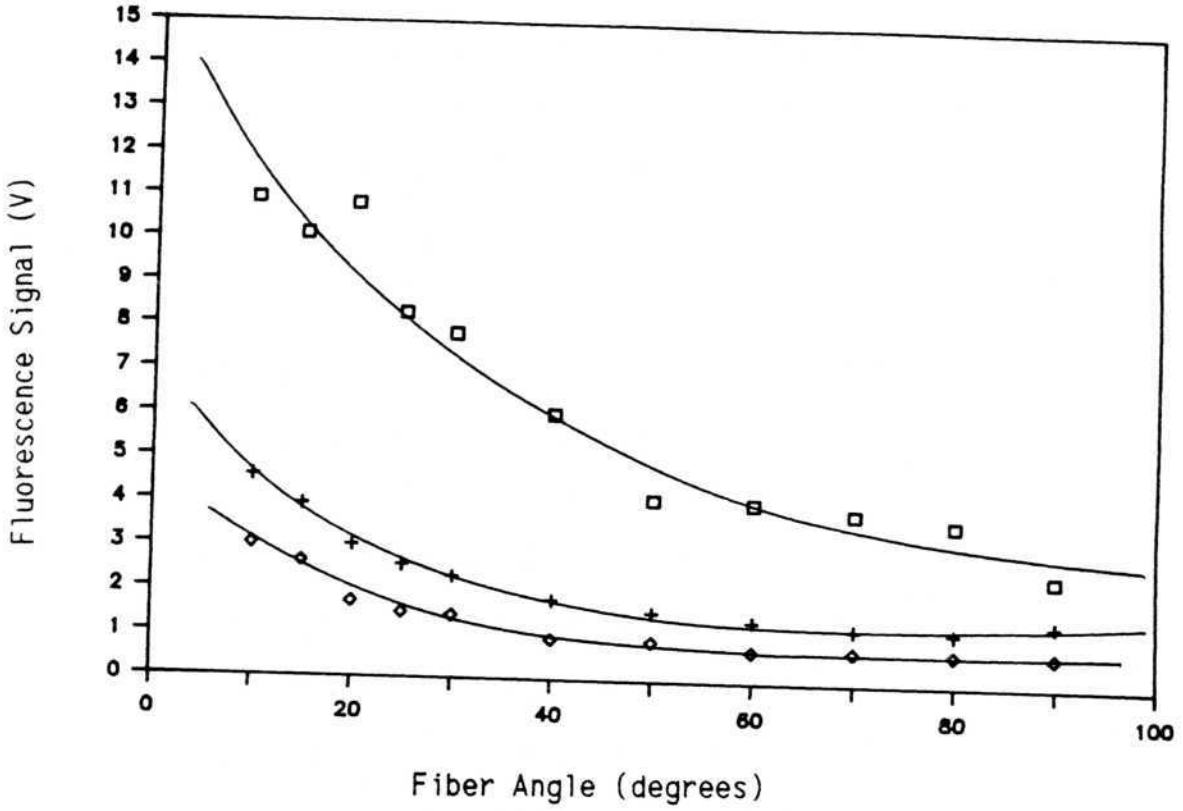


Figure 40. Dependence of the fluorescence signal on the separation distance of the distal ends of the fibers and the fiber angle. For a 10 mg/L quinine sulfate solution. (□) FO very close, (+) FO 0.5 cm apart, (◇) FO 1.0 cm apart.

radiation is directly transmitted into the emission fiber. The fluorescence signal decreases as the fibers are moved farther apart along circles of larger radii. The blank signals are less than 2% of the quinine fluorescence signal at all angles.

With the minimum fiber separation and a fiber angle of 10^{o}, twenty repetitive blank measurements yielded a blank standard deviation of 83.1 μV. The calibration slope is estimated to be 1.09 V/(mg/L) from 10.92 V signal for 10.0 mg/L quinine sulfate solution. These data yield an estimated detection limit for quinine sulfate of 152 ng/L.

The dependence of the scattering signal from a milk-of-magnesia solution on the angle between the fibers is shown in Figure 41. The light scattering signal is minimal at 60^{o}.

A portion of the Redwood pond water was analyzed in triplicate by the spectrophotometric monochromatic method to determine the concentration of chlorophyll. The average chlorophyll-a concentration was 187 μg/L with a standard deviation of 0.047 μg/L.

With another portion of the Redwood pond water and the remote fiber fluorometric jig, an angle of 10^{o} and the shortest separation distance possible provided the maximum fluorescence signal. With these conditions, a 10 MΩ feedback resistor, and 1-m excitation and emission fiber optic cables, the 'in situ' fluorescence signal was 4.97 V with a standard deviation of 0.89 V.

Blank signals were recorded 24 times for deionized water with R_f = 100 MΩ. The mean blank signal was 4.35 V and the standard deviation was 78.1 mV.

When all the above signals are normalized to an R_f = 10 MΩ,

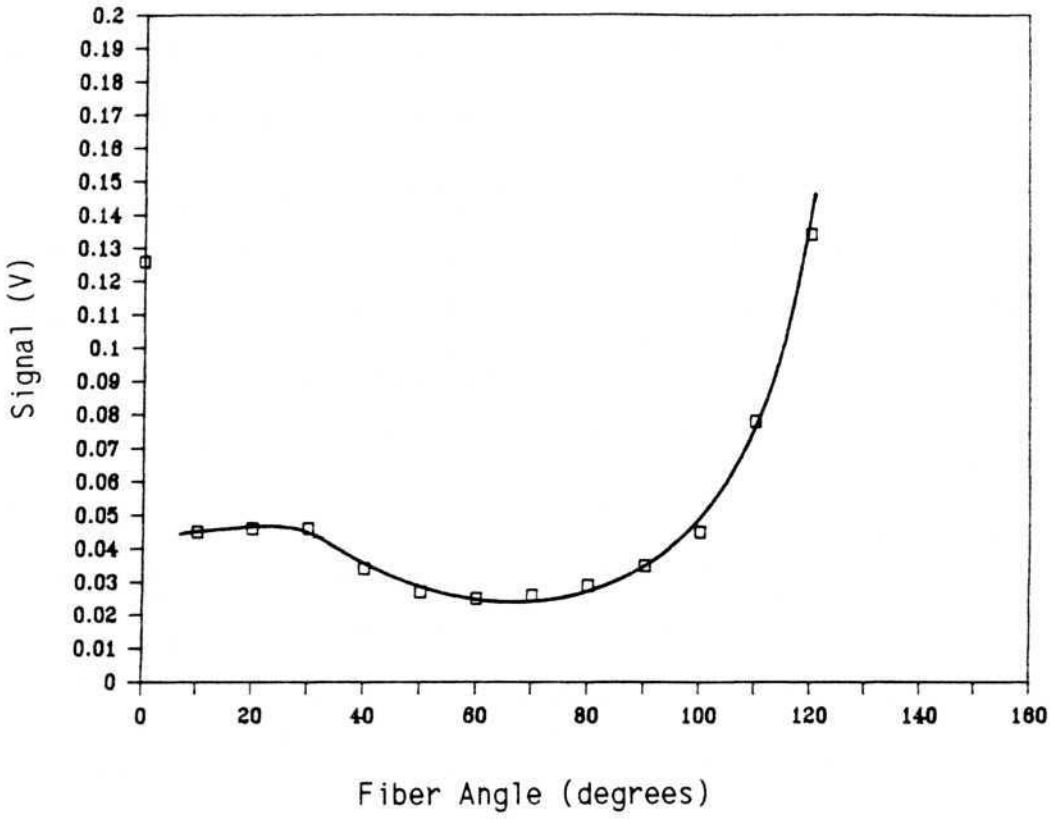


Figure 41. Dependence of light intensity on the fiber angle for a dilute solution of milk of magnesia.

the normalized blank signal and standard deviation are 0.435 V and 7.8 mV, respectively, and the calibration sensitivity is (4.97 - 0.43)/(187 μg/L) = 2.4 X 10^{-2} V/(μg/L). These data yield an 'in situ' detection limit for chlorophyll-a of 0.64 μg/L.

A fiber probe for the 'in situ' detection of phytoplankton chlorophyll fluorescence has been demonstrated. From the attenuation data in Table XXVI, the detection limit with the experimental system using a 100-m fiber optic cable for excitation and emission is estimated to be 32 μg/L chlorophyll. If the source was located at the probe end to provide excitation directly to the sample, the detection limit is estimated to be 3.6 μg/L chlorophyll.

The dependence of detection limit on cable length was investigated using combinations of the 1- and 45-m fiber optic cables. As expected, given the increased attenuation for shorter wavelengths, the detection limit for the 45-m excitation and 1-m emission fiber optic configuration was 2.0 times worse than that obtained with the 45-m emission fiber optic and 1-m excitation fiber optic configuration and 2.8 times worse as that obtained with the 1-m excitation and 1-m emission fiber optic configuration.

CONCLUSIONS

The determination of chlorophyll in natural water bodies has long been known to be a complex analysis. The major spectrometric methods have been critically compared. The techniques studied included the trichromatic and monochromatic spectrophotometric methods, the 'in vivo' and 'in vitro' fluorometric methods, and a new 'in situ' method, remote fiber fluorometry. The parameters studied included the accuracy and precision of analysis, the calibration sensitivity and detection limit.

Rarely does an investigator study such waters as clear as those found in Crater Lake, Oregon. This study originated with this lake and was constantly concerned with the ability of the various schemes of analyses to quantify extremely low levels of chlorophyll.

The collection of a representative chlorophyll sample must begin with a deliberate repeated rinse of the sample bottles with the sample to remove contaminates. Acid causes the irreversibly conversion of chlorophyll to phaeophytin and reduces the concentration of chlorophyll later determined. Samples must be kept on ice ($\approx 0^{o}$ C) and in a dark environment to slow sample degradation.

Filters used for the collection of phytoplankton cannot be recommended without testing the filter efficiency for the phytoplankton community present in the lake under study. When filtering water containing uniform size TiO_2, S & S and Whatman glass filters and Millipore membrane filters were similarly efficient using a student-t comparison test at the 95% confidence level. For

the lake samples studied, the collection efficiency of filters was not statistically different at the 90% confidence level. Filtering time rose 50% when using the Millipore membrane filter as compared to a glass filters.

The concentration of chlorophyll determined after the storage of phytoplankton containing filters at reduced temperatures resulted in a variety of chlorophyll concentrations being determined. Within the uncertainty of the analytical methods used, no significant degradation of chlorophyll on the filters stored at either 1 or -9° C and with or without $MgCO_3$ was noted.

A spectral bandpass of 2 nm or less is required for the accurate measurement of the absorbance and concentration of chlorophyll by the spectrophotometric method. For a 1.00 mg/L solution of chlorophyll-a the absorbance and resulting concentration of chlorophyll-a determined was 35% less using a bandpass of 20 nm as compared to 2 nm.

The use of a 5-cm pathlength cell in place of a 1-cm pathlength cell increases the calibration slope and improves the detection limit by a factor of 5. The detection limit of the spectrophotometric method is lowered to 4.4 ng/L chlorophyll in lake water concentrated *from 2 L to 10 mL in the extract using a 5-cm pathlength cell.* The typical chlorophyll concentrations found in Crater Lake are two orders of magnitude greater than this detection limit. This spectrophotometric method is therefore recommended for use at Crater Lake. The detection limit is 0.035 µg/L when using a 1-cm pathlength cell with a similar concentration factor. This is still an order of magnitude lower than the typical chlorophyll concentrations

determined at Crater Lake and can be recommended for use with caution. Investigators must be alert to the potential problems associated with working close to a method's detection limit. Remember, this detection limit is stated when using a concentration factor of 200. With this concentration factor, a detection limit of 0.17 µg/L is more realistic considering all factors that affect the blank deviation.

The chlorophyll in a sample of lake water can be rapidly estimated using the 'in vivo' fluorescence technique. However, because this technique is influenced by many environmental parameters, it is recommended that samples to be analyzed be stored in an environment which is both isothermal and dark for at least one hour before analysis. This allows chlorophyll containing cells in the various samples to be of common temperature and similar light history. The belief that the herbicide, DCMU, counters these effects was not justified in this study. The exposure of Crater Lake water samples to sunlight lead to an average 47% decrease in a samples' fluorescence over a period of 50 minutes.

When sample temperature was varied from 20 to 10^{o} C, the 'in vivo' fluorescence signal varied by a factor of up to a maximum of 2.63 for surface samples and a minimum of 1.03 for samples collected at 130 m. Enhanced 'in vivo' sample measurements, using DCMU, exhibited a similar trend.

The 'in vivo' fluorometric method has a detection limit for chlorophyll of 4.9 ng/L. Using the herbicide, DCMU, the enhanced 'in vivo' fluorometric method has a detection limit of 2.6 ng/L. Both methods offer researchers at Crater Lake the ability to determine

chlorophyll directly from water samples with minimal sample pretreatment since the chlorophyll concentration is known to be about two orders of magnitude greater than the stated detection limit.

The 'in vitro' fluorometric analyses were shown to be the most precise techniques for the determination of chlorophyll-a and to provide the lowest detection limit. It provides detection limits of 3.9 and 11 ng/L in the extract (before considering the concentration factor) for the uncorrected and corrected methods, respectively. The average percent relative deviation of chlorophyll concentration for 20 similar samples from Crater Lake was about 5% and was half that of the spectrophotometric monochromatic method.

It is recommended that when working with oligotropic lakes, including Crater Lake, which are known to contain unusually low levels of chlorophyll that investigators interested in chlorophyll-a store filters at 1^{o} C without $MgCO_3$ and determine chlorophyll concentrations using the uncorrected 'in vitro' fluorometric method of analysis. Because the detection limit is about 4 orders of magnitude better than the spectrophotometric method, much smaller concentration factors can be used. This saves filtering time.

The remote detection of chlorophyll in lakes could involve the use of a fiber optic cable. The 'in situ' detection of chlorophyll was investigated. It was shown that fiber optic material was easily polished and configured to collect 'in situ' fluorescence signals. Methods to focus a lamp onto a fiber optic face were developed. These methods involved the motion of an elliptical reflector with respect to a lamp while investigating the focal spot with a 1-mm aperture.

The transmission of light through a fiber optic was investigated. It was shown that as the wavelength becomes shorter the attenuation increased for a 45-m length 600-μm diameter fiber optic cable. The attenuation was 12.8 dB at 400 nm and 7.46 dB at 650 nm per 100 m of cable.

The intensity of a light scattered from a dilute milk of magnesia solution was studied. With a 1-m fiber optic source and emission cable and detector, the light scattering was smallest when the fibers were separated by an angle of 60^{o}.

The fluorescence of a solution of quinine sulfate was shown to depend of fiber angle and distance. A maximum fluorescence signal was obtained with the 1-m excitation and 1-m emission fibers were at an angle of 10^{o} and very close together. A detection limit for the quinine sulfate solution was 152 ng/L.

A fiber probe for the detection of phytoplankton chlorophyll was made for its detection in natural bodies of water. With 1-m fiber optics for transmission of excitation and emission radiation, a detection limit for chlorophyll of 0.64 μg/L was estimated. A source might be included at the probe end of the fiber cable to provide excitation directly to the sample and to improve the fiber optic probe detection limit.

The dependence of detection limit on cable length was investigated using combinations of the 1- and 45-m fiber optic cables. As expected, increasing the transmission length for the excitation radiation, the detection limit for the 45-m excitation and 1-m emission configuration was the highest and about 2 times as high as with the 45-m emission and 1-m excitation setup and 2.8 times as

high as with the 1-m excitation and emission configuration.

The instrument used has been improved since this research was completed and detection limits of a factor of 10 or better should be possible. Thus it should be possible to use the fiber optic fluorescence probe in lakes in which the chlorophyll concentration is about 0.1 μg/L. Moreover, Lund has shown that detection limits as low as 0.02 μg/L are possible (Lund, 1983). A fiber probe can be made to detect phytoplankton in lakes as clear as Crater Lake where concentrations of chlorophyll are typically below 1.0 μg/L.

BIBLIOGRAPHY

Abayshi, J.K.; Riley, J.P., Anal. Chim. Acta. (1979), 107, 1-11.

Abbott, M.R.; Denman, K.L.; Powell, T.M.; Richerson, F.J.; Richards, R.C.; Goldman, C.R., Limnol. Oceanogr. (1984), 4(29), 862-878.

Angle, S.M., Spectroscopy, (1987), (2)4, 38-48.

APHA, Standard Methods for the Examination of Water and Wastewater, (1985), 1067-1072.

Aronoff, S., in The Chlorophylls by Vernon and Seely, (1966), 3-6.

ASTM, Annual Book of ASTM Standards, Water and Environmental Technology, (1984), 11.02, Water II, 580-584.

Bjaronborn, B., Ergebnisse Der Limnologie, Heft 16, (1980), 107-116.

Borman, S.A., Anal. Chem., 53(14), (1981), 1616-1618A.

Boyd, W.T., Fiber Optics, (1982), 189-198.

Braumann, T.; Grimme, L.H., Biochim. Biophys. Acta, (1981), 637, 8-17.

Brown, L.M.; Hargrave, B.T.; MacKinnon, M.D., Can. J. Fish Aquat. Sci., (1981), 38, 205.

Chudyk, W.A., Carrabba, M.M., Kinny J.E., Anal. Chem., (1985), 57(7), 1237-1242.

Coffey, D., Analytical Chemist, Forestry Laboratory, OSU, Personal Communication, April, 1985.

Collins, G., Quality Assurance Branch, Environmental Monitoring and Support Laboratory, USEPA, Cincinnati, OH 45268, Personal Communication, 1983.

Collins, G., Quality Control Samples, Instructions for Fluorometric Analyses of Chlorophyll, Quality Assurance Branch, Environmental Monitoring and Support Laboratory, USEPA, Cincinnati, OH 45268, 1984.

Cullen, J.; Renger, E., Marine Biology, (1979), 53, 13-20.

Deaton, T., Ph.D. thesis, University of California, Davis, 1983.

Dougherty, R.C.; Strain, H.H.; Svec, W.A.; Uphaus, R.A; Katz, J.J., Journal of the American Chemical Society, (1966), 88(21), 5037-5038.

Goedheer, J.C., in The Chlorophylls by Vernon and Seely, (1966), 147-185.

Golterman, H.L.; Clymo, R.S., IBP Handbook No 8, (1969), 114-119.

Gunderson, L.C.; Keck, D.B., Technology Review, (1983), 86(4), 1-14.

Hirschfeld, T.; Deaton, T.; Milanovich, F.; Klainer, S., Optical Eng., (1983a), 22(5), 527-531.

Hirschfeld, T.; Milanovich, F., UCRL-89026 preprint, (1983b).

Holden,M., in Chemistry and Biochemistry of Pigments, Godwin, T.W. editor, Vol. 2, (1976), 2.

Holm-Hansen, O.; Reimann, B, Oikos, (1978), 30, 438-447.

Jeffery, S.W.; Humphrey, G.F., Biochem. Physiol. Pflazen (BPP), (1975), BD.167, S.191-194.

Kiefer, D.A., Marine Biology, (1973), 22, 263-269.

Klainer, S.M., Optical Eng., (1983), 22(5), 507-526.

Lenz, J.; Fritsch, P., Ergebnisse Der Limnologie, (1980), 14, 46-51.

Loeb, S., Tahoe Research Group, Department of Environmental Studies, University of California, Davis, Personal Communication, April, 1985.

Long, E.B.; Cooke, G.D., Limnology and Oceanography, (1971), 16, 990-992.

Lorenzen, C.J., Deep-Sea Research, (1966), 151, 223-227.

Lorenzen, C.J., Limnology and Oceanography, (1967), 12, 343-346.

Lund, T., I.E.E. Conference Publication, (1983), 221, 190-194.

Mantoura, R.F.C.; Llewellyn, C.A., Analytica Chem. Acta, (1983), 151, 297-314.

Marker, A.R.H.; Crowther, C.A.; Gunn, R.J.M., Ergebnisse Der Limnologie, (1980), 14, 52-69.

Mendes, D., Oregon State University, Dept of Oceanography, Corvallis, Personal Communication, (1985).

Mitchell, D.G., Garden, J.S., Aldous, K.M., Anal. Chem., (1976), 48(14), 2275-2277.

Mitsuyoshi, M., Calif. Eng., (1984), Dec., 17-19.

Nusch, E.A., Ergebnisse Der Limnologie, (1980), 14, 15-36.

Papageorgiou, B.; Govindgee, Biochim. Biophys. Acta., (1971), 234, 428-432.

Prezelin, B.B.; Ley, A.C., Marine Biology, (1980), 55, 295-307.

Richards, F.A.; Thompson, T.G., Journal or Marine Research, (1952), XI, 2, 156-172.

Saari, L.; Seitz, W.R., Anal. Chem., (1982), 54(4), 821-823.

Samuelsson, G.; Oquist, G., Physiol. Plant., (1977), 40, 315-319.

Samuelsson, G.: Oquist, G.; Halldal, P., Mitt. Internat. Verein. Limnol, (1978), 21, 207-215.

Seitz, R.W., Anal Chem, (1984), 56(1), 16-26A.

Sepaniak, M.J.; Tromberg, B.J.; Eastham, J.F., Clinical Chem., (1983), 29(9), 1678-1682.

Strain, H.H.; Thomas, M.R.; Katz, J.J., Biocimica et Biophysica Acta, (1963), 75, 306-311.

Strickland, J.D.H.; Parsons, T.R., A Manual of Sea Water Analysis, (1960), 185p.

Sweet, S.T.; Guinasso Jr, N.L., Limnol. Oceanogr., (1984), 29(2), 397-401.

Turner, G., Turner Designs Publication, Bulletin 101, (1981).

Vernon, L.P., Anal Chem, (1960), 32(9), 1144-1150.

Vincent, W.R., Br. Phycol. J., (1983), 18, 5-21.

Watanabe, T.; Hongu, A.; Honda, K.; Nakazato, M.; Donno, M.; Saitoh, S., Anal Chem, (1984), 56(2), 251-256.

Weber, C.I., USEPA Analytical Quality Control Newsletter #31, October, 1976.

Wehry, E., in Practical Fluorescence by G. Guilbault, (1973), 79-136.

Yentsch, C.; Menzel, D., Deep Sea Research, (1963), 10, 221-231.

Appendix 1. Specific Absorption Coefficients Of Some Plankton Pigments In 90% Acetone Solution, (Richards, 1952).

Wave Length $M\mu$	Chlorophyll a $\frac{1gm}{E\ 1cm}$	Chlorophyll b $\frac{1gm}{E\ 1cm}$	Chlorophyll c $\frac{SPU}{E\ 1cm}$
665	66.7	6.5	1.1
645	16.4	45.6	4.4
630	11.9	12.7	10.4
510	2.6	3.5†	2.1
480	1.9	13.6	5.4
450	8.9	54.0	78.5
420	70.7	26.8	37.3

APPENDICES

Appendix 2. Reciprocal Dispersion (nm/mm), Slit Mismatch (nm), And Diffraction Limit (nm) From The Cary 118C Operation Manual.