A method for the continuous measurement of *in vivo* chlorophyll concentration*

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Abstract—*In vivo* chlorophyll, like many other organic molecules, possesses the ability to fluoresce. This fluorescence was measured continuously with a modified model III Turner fluorometer at sea. Reliable readings were obtained over the range of 0·04–2·0 mg chlorophyll *a* m^{-3} while on a 21-day cruise off the coast of Baja California. Since the relationship between fluorescence and chlorophyll was linear on all scales, it should be possible to continuously monitor chlorophyll from 0·04 to between 10 and 15 mg m^{-3}, a range adequate for all open ocean studies.

INTRODUCTION

TECHNIQUES currently used for the measurement of chlorophyll usually involve the filtration of discrete samples, solution of the pigment with 90% acetone followed by either measuring the absorption of light by the familiar trichometric method of RICHARDS with THOMPSON (1952), or more recently, by measuring the fluoresence of the sample (YENTSCH and MENZEL, 1963; HOLM-HANSEN *et al.*, 1965). Other though more indirect methods may be used under some circumstances to measure *in situ* or *in vivo* chlorophyll, namely : transparency measurements either with a Secchi disc (SAIJO and ICHIMURA, 1960) or a submarine photometer, or the measurement of light scattering which in oceanic regions bears some relation to the phytoplankton standing crop (BERGE, 1963; TYLER, 1964). A more direct approach would be to measure a property closely associated with the chlorophyll molecule such as fluorescence.

Chlorophyll, like many other organic molecules, possesses the ability to fluoresce. Simply stated, fluorescence is the ability of a substance to absorb light at one wavelength, and emit light at a longer wavelength (see WILLIAMS and BRIDGES, 1964 for a detailed review). For example, chlorophyll in an acetone solution receives maximum excitation at 430 mμ and yields maximum emission at 670 mμ. Green leaves and chloroplast suspensions also fluoresce, yielding maximum emission at 685 mμ at room temperatures when excited by the 436 mμ line from a mercury lamp (GOEDHEER, 1964).

The fluorescence of *in vivo* chlorophyll is considerably less efficient than dissolved chlorophyll, yielding only about 1/10 as much fluorescence per unit weight as the same amount in solution (THOMAS and FLIGHT, 1964). The development of extremely sensitive fluorometers make the routine measurement of low chlorophyll concentrations practicable, and a method for continuous monitoring of *in vivo* chlorophyll is now possible. The purpose of this paper is to describe such a method, which is suitable for shipboard operation.

MATERIALS AND METHODS

A Turner model III fluorometer was modified for *in vivo* chlorophyll measurements by replacing the standard S-4 photomultiplier (RCA 931A) with a red sensitive one (R 136) thus extending the response of the instrument to 750 mμ. The standard photomultiplier normally will not respond to light much beyond 650 mμ, and since *in vivo* chlorophyll has maximum fluorescence at 685 mμ, a photomultiplier sensitive to longer wavelengths was necessary. This modification increased the sensitivity of the instrument about 10 times.

A blue fluorescent lamp (F4T5) was used to excite the sample. The primary filter was blue (Corning, CS 5–60) with maximum transmission at 430 mμ. The red secondary filter (Corning, CS 2–64) was of the sharp cut type being opaque to light at wavelengths shorter than 645 mμ and reaching its maximum transmission at 650 mμ.

At first, surface seawater was pumped into the laboratory with a small submersible pump, but later water was taken from a through-hull fitting (depth about 2 m) in the engine room. The water was passed through a bubble trap and then gravity fed into a 20 ml flow-through cuvette (Turner $\#$ 110–871). A hydrostatic head of 1 m produced a flow of about 100 ml min^{-1}. Water passed through the instrument in less than 1 minute's time after it was picked up. The 0–10 mV output from the fluorometer was put into an appropriate paper recorder which gave a continuous record. Periodically, discrete samples were collected for filtration and acetone extraction and the *in vivo* fluorescence noted.

The discrete samples (including 2 to 5 replicates) were handled in the following manner : a thin layer of Mg CO$_3$ was laid down on a Whatman GC/F glassfiber filter, and the sample was filtered. The wet filter was immediately ground up. After 1 hr of eluting in 90% acetone the sample was centrifuged to remove all solids from suspension, and was then placed in the fluorometer (with the discrete sample door in place, Turner $\#$ 110–005) and the emission noted before and after acidification with 2 drops of 1 N HCl. The concentration of chlorophyll in the sample was calculated from the equations :

$$\text{chlorophyll } a \text{ (mg m}^{-3}) = \frac{\dfrac{F_0/F_{a_{\max}}}{(F_0/F_{a_{\max}}) - 1} (k_x)(F_0 - F_a)}{\text{liters filtered}}$$

$$\text{phaeophytin } a \text{ (mg m}^{-3}) = \frac{\dfrac{F_0/F_{a_{\max}}}{(F_0/F_{a_{\max}}) - 1} (k_x) \left[F_0/F_{a_{\max}} (F_a) - F_0 \right]}{\text{liters filtered}}$$

where : F_0 = fluorescence before acidification
                F_a = fluorescence after acidification
$F_0/F_{a_{\max}}$ = maximum acid factor which can be expected in the absence of phaeophytin
        k_x = calibration constant for a specific sensitivity scale.

It should be noted that k_x, $F_0/F_{a_{\max}}$, and acid ratios are functions of the combination of photomultiplier and color filters.

<div align="center">RESULTS</div>

The present instrument has four sensitivity scales labeled 1X, 3X, 10X and 30X. The exact proportionality may vary slightly from one machine to the next, but can be readily determined for a given machine by serial dilution.

Fluorescence measurements can be carried out only on very dilute solutions because of quenching and self-absorption. For *in vivo* measurements, the problem of particulate scattering is added. If any of these problems were important, a non-linear relationship between chlorophyll concentration and fluorescence would result. This would be most readily seen at higher chlorophyll concentrations on the lower sensitivity scales. The linearity of this relationship was tested by the serial dilution of natural phytoplankton populations with PH Millipore® filtered seawater.   It is apparent (Fig. 1) that neither quenching, self-absorption nor particle scattering is a serious problem for *in vivo* measurements with this instrument from the linear relationship between chlorophyll concentration and fluorescence which was observed.

During the cruise off Baja California oceanic conditions prevailed and only a limited range of chlorophyll concentrations were encountered. Calibration curves could be constructed only for the two most sensitive scales. The calibration curve for the 30 × scale can be seen in Fig. 2. The regression line (a least squares fit) does not pass through the origin. Readings on PH Millipore® filtered seawater gave readings of 1, 3, 10 and 40 on the 1 ×, 3 ×, 10 × and 30 × sensitivity scales respectively. Similar readings were also obtained on distilled water. The possibility that other substances in seawater might interfere with this measurement seems slight since most natural substances fluoresce at much shorter wavelengths than chlorophyll (WILLIAMS and BRIDGES, 1964). The only group that might interfere are the porphyrins, of which chlorophyll is a member, but in view of the similar blank readings obtained on distilled water, these would not present a problem. The instrument is nulled at zero by placing an opaque piece of cardboard over the photomultiplier. In this manner, sensitivity scale changes can be made without any further adjustments other than changing the scale. Thus
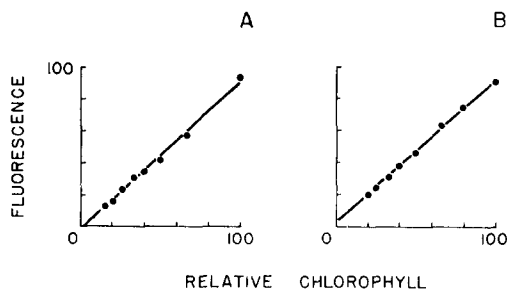
Fig. 1. Linear relationship between relative chlorophyll, serial dilutions of a single population with PH Millipore® filtered seawater, and *in vivo* fluorescence. Curve A, 1X sensitivity scale, and B 3X sensitvity scale.

the blank readings are probably the result of light scattering and/or light leakage through the color filters.
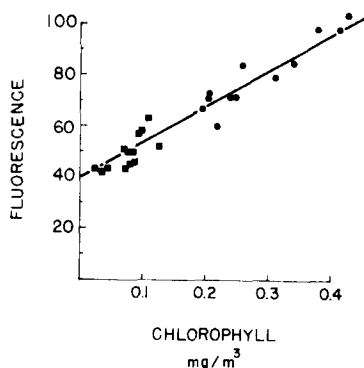

Fig. 2. Calibration curve for the 30X sensitivity scale. *In vivo* fluorescence plotted against extracted chlorophyll *a* values. Regression: $Y = 38 \cdot 6 + 146\,X$. Squares are from offshore and circles from nearshore.

DISCUSSION AND CONCLUSIONS

The instrument was operated continuously for the length of the cruise, 21 days, without any problems except minor mechanical faults in the paper recorder. The flow-through cuvette was removed and cleaned daily with alcoholic KOH even though fouling was not seen to occur. Most of the area covered by the cruise showed low and quite uniform surface chlorophyll concentrations. Patchiness on the order of < 1 km was generally not observed, and a change in chlorophyll concentration was usually associated with a change in surface temperature. Superficially, higher chlorophyll concentrations were found either in areas of colder water, or at the boundaries between water masses as indicated by thermal gradients. Fig. 3 shows a segment of the trace extending from 24° 55·0′N, 115° 4·8′W to 26° 9·3′N, 112° 45·0′W, a distance of about 230 km. Unfortunately, data on salinity or inorganic nutrients were not obtained in the same detail and any further discussion on cause and effect would be pure speculation.

Since all sensitivity scales on a fluorometer equipped with the present photomultiplier and filter combination appear to be linear, it would seem possible to measure continuously chlorophyll from about 0·04 to between 10 and 15 mg m^{-3}. The upper limit of chlorophyll concentration which can be measured may be raised somewhat by decreasing the sensitivity of the instrument, either by using a less intense lamp, a smaller cuvette or by placing a neutral density filter over the photomultiplier. However, the inherent problems of fluorometry must be kept in mind that such alterations may cause non-linearity of response at higher chlorophyll concentrations. Actually, the range of the instrument equipped as discussed above would probably be adequate for all areas of the open ocean.
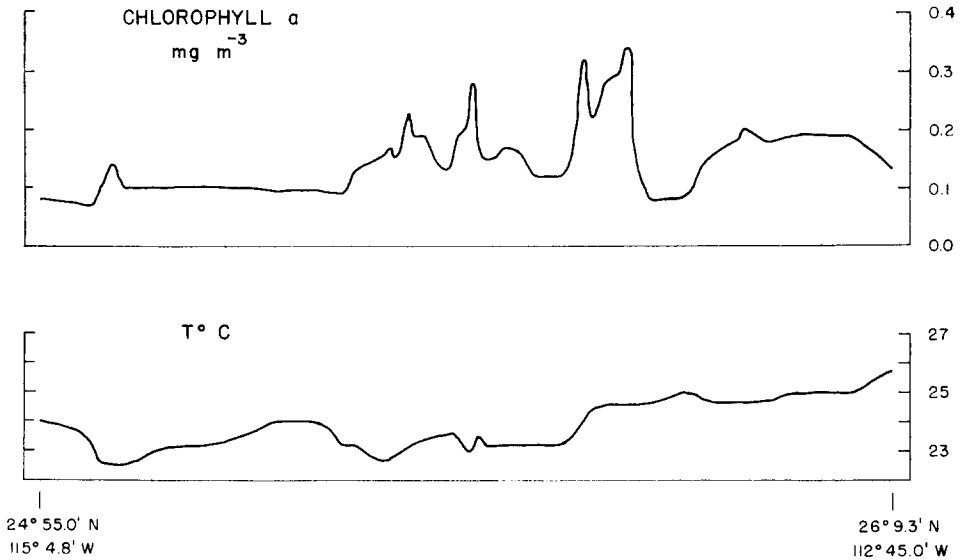
Fig. 3. A portion of the trace obtained on cruise TO–65–1 showing variations in chlorophyll *a* concentrations and temperature as the ship proceeded from 24° 55·0N′–115° 04·8′W to 26° 09·3′N 112° 45·0′W.

In vivo fluorescence can best be related to chlorophyll plus phaeophytin content since it is impossible to separate the fluorescent contribution of each in seawater. Phaeophytin normally is not found in phytoplankton cells (PATTERSON and PARSONS, 1963), but is produced as a result of zooplankton grazing (CURRIE, 1962). Apparently, surface waters contain rather small quantities of phaeophytin in relation to chlorophyll since 94% of the fluorescence of discrete samples ($n = 91$) collected on the present cruise can be attributed to chlorophyll, the remaining 6% being attributed to phaeophytin, a weight ratio of chlorophyll to phaeophytin of 8·5.

Fluorescence measurements are somewhat affected by temperature (WILLIAMS and BRIDGES, 1964). In solution, chlorophyll fluorescence decreases with increasing temperature at the rate of 0·3% per 1°C in the range 0–35°C. *In vivo* fluorescence is affected to a greater degree, with a decrease in yield of 1·4% per 1°C increase over the range of 12–30°C. This was determined by taking a natural population of phytoplankton of undetermined taxonomic composition and cooling and warming it to get the desired temperature range. Rapid temperature changes may adversely affect the cells, but this data indicates that temperature changes are not overwhelming. During the cruise, temperature ranged from 21–29°C, so that the error from this source is probably less than 13%.

Other errors which may contribute to the scatter in Fig. 2 are discussed in the following paragraphs. Statistically, the relationship of fluorescence and chlorophyll is very significant ($r = 0.96$). The regression line can be described by : $Y = 38.6 + 146\ X$.

The validity of *in vivo* chlorophyll measurements might be altered by factors affecting the fluorescence yield per unit weight of pigment in addition to the two mentioned above.

Different species or taxonomic groups might exhibit different efficiencies of fluorescence. This may be the case, but normally oceanic phytoplankton, especially in the lower latitudes, are fairly diverse (HULBERT, 1963), so that this effect probably is minimal. Also, the use of the large cuvette (20 ml) enables the instrument to integrate the sample since many cells are being sampled at the same time. The effect of taxonomic groups on this technique would result primarily from the presence of pigments other than chlorophyll *a* since calibration is based on chlorophyll *a* content. The present combination of color filters, primarily the red secondary filter, almost eliminates the effect of chlorophyll *c* since it is practically opaque to light below 650 mμ thus filtering out fluoresced light from chlorophyll *c*. Chlorophyll *b* on the other hand could be expected to interfere, but only chlorophyll *a* and *c* are found in the Chrysophyceae, Bacillariophyceae and Dinophyceae (STRAIN, 1951) and these groups probably constitute the major bulk of phytoplankton in oceanic regions.

The instrument could also be used for obtaining vertical profiles in conjunction with a pump. As in the case with horizontal profiles, the instrument would measure both chlorophyll and phaeophytin, essentially the measurement obtained with a spectrophotometer and called " chlorophyll *a*," but at depth the phaeophytin content is higher than in the surface (LORENZEN, 1965) and at times may form a significant fraction of the pigment present.

The data collecting system as described above has one significant feature, that being the commercial availability of the components. Automation can be carried even further by using a recorder or digital encoder with limit switches so that a selenoid or stepping motor can be actuated to change sensitivity scales automatically.

Thus it can be concluded that continuous *in vivo* chlorophyll *a* measurements by this method are valid and should prove to be an extremely useful tool in broad ecological programs carried out at sea.

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