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A Proposed Reference Standard for *in vivo* Chlorophyll *a* Fluorometry

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A PROPOSED REFERENCE STANDARD FOR *IN VIVO* CHLOROPHYLL *A* FLUOROMETRY

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Abstract

A major problem associated with *in vivo* chlorophyll *a* fluorometry is the lack of an unimpeachable standard with which to define units and which can be used to ascertain instrumental fidelity. A substance, Corning 3486 (colour standard 3-69) filter glass, has absorption and fluorescence emission properties which adequately mimic *in vivo* chlorophyll *a* fluorescence, and is proposed as the standard. Examples of its use with two commonly used fluorometers are described. It is proposed that the primary standard described herein forms the basis for intercalibration of fluorometers used in *in vivo* chlorophyll *a* fluorometry and also for standard units for reporting of data.

INTRODUCTION

The use of fluorescence from *in vivo* chlorophyll *a* as an estimator of phytoplankton biomass has become popular since its introduction by Lorenzen (1966). Recent refinements, namely the use of Diuron to maximise fluorescence, have disclosed the possibility of *in vivo* chl *a* fluorescence being an indicator of plant vitality and environmental opportunities (Samuelsson and Öquist 1977; Slovacek and Hannan 1977; Samuelsson *et al.* 1978; Cullen and Renger 1979). There are several attendant problems, both instrumental and biological. Before the biological problems can be evaluated it is necessary to distinguish between instability of instrumental accuracy (meaning changes in bias as defined by Cochran and Cox 1966) and fluctuations in the properties of the phytoplankton population. It is equally desirable to report and exchange data, in the first instance, in standardized units. A solution to these problems is to find and adopt an unimpeachable reference standard for *in vivo* chl *a* fluorescence and to define standard units of measurements. Such a reference standard is proposed and examples of its use are presented in this report.

THE *IN VIVO* CHLOROPHYLL *A* REFERENCE STANDARD

The proposed reference standard is Corning filter glass, type 3486 (Colour standard 3-69, Corning Glass Works, U.S.A.) which has a broad blue-green absorption spectrum (Corning, 1970) and a fluorescence spectrum between 560 and 774 nm, peaking at 685 nm (Turner, 1973). In Fig. 1 these characteristics are compared with absorption spectra of living marine phytoplankton (Lorenzen, 1972), and fluorescence spectra of *Laminaria*, peaking at 681 nm (Goedheer, 1972) and of *Chlorella*, peaking at 684 nm (Papageorgiou, 1975). Other examples may be found

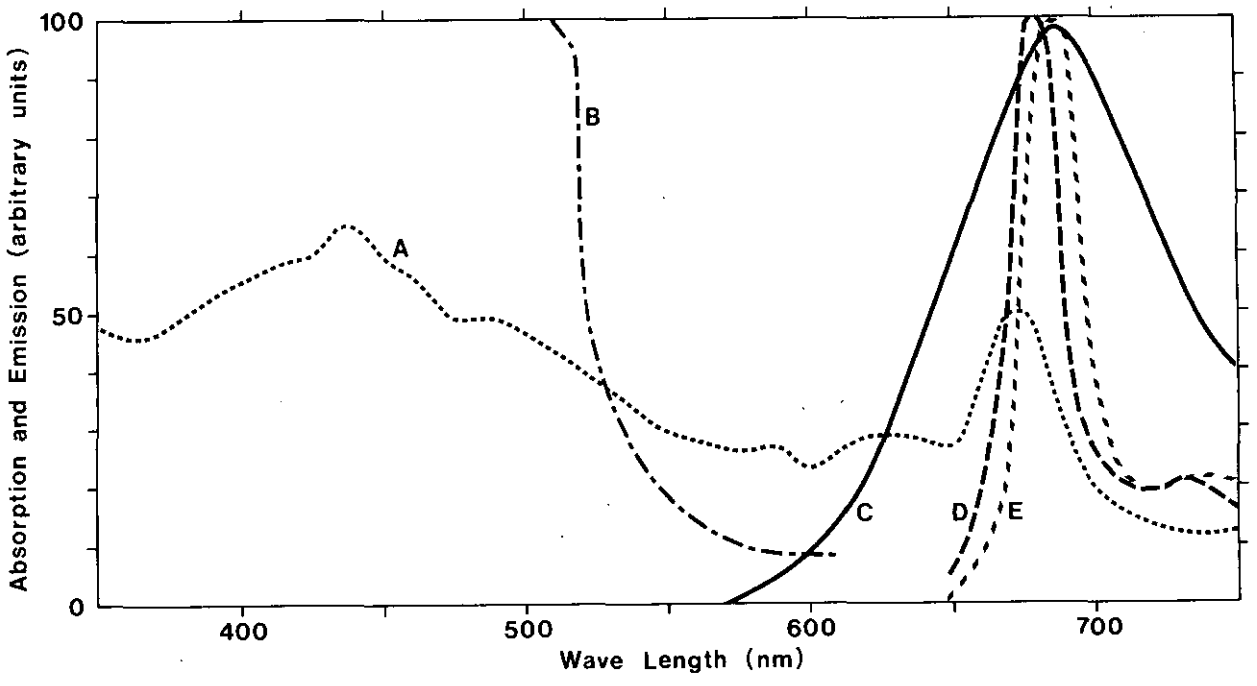

Fig. 1. Comparison of relative absorption and fluorescence spectra of living algae with those of Corning 3486 filter glass. A. absorption spectrum of living marine phytoplankton in suspension (redrawn from Lorenzen, 1972). B. absorption spectrum of Corning 3486 filter glass (redrawn from Corning, 1970). C. fluorescence emission spectrum of Corning 3486 filter glass excited with 440 nm light (redrawn from Turner, 1973). D. *in vivo* fluorescence spectrum of brown algae *Laminaria* (redrawn from Goedheer, 1972). E. *in vivo* fluorescence spectrum of the green algae *Chlorella* (redrawn from Papageorgiou, 1975).

in Yentsch & Yentsch (1979) with peak emissions said to be at 680 nm. Quite clearly, Corning 3486 filter glass mimics the excitation and fluorescence characteristics of the living chloroplasts adequately to serve as a reference standard.

EXAMPLES OF APPLICATION

Two types of fluorometers were used in this study: A Model 10-005 R flow-through fluorometer manufactured by Turner Designs, U.S.A., and Variosens I, an underwater instrument manufactured by Impulsphysik, W. Germany.

The absorption of light passing through a solution is governed by the Beer-Lambert law and, in the case where the absorbing molecules fluoresce,

$$F = \phi_F I_o (1 - e^{-\alpha c l}) \qquad (1)$$

where:

- F denotes the number of quanta fluoresced,
- ϕ denotes the fluorescence quantum efficiency of the absorbing molecule,

I_o denotes the number of quanta within the absorption spectrum of the fluorescing molecule which are transmitted by the solvent in the absence of the pigment.

α denotes the specific absorption coefficient of the pigment, per unit c and l,

c denotes the concentration of the pigment and

l denotes the pathlength of light through the solution.

Where $\alpha cl \leq 0.1$, as is usually the case in *in vivo* chlorophyll fluorescence work, eqn (1) may be approximated by

$$F \simeq \phi_F I_o \alpha cl \qquad (2)$$

so that F is a linear function of c where $\phi_F I_o \alpha l$ are held constant as is assumed in *in vivo* chl *a* fluorometry. F is equally a linear function of the exciting light where $\phi_F \alpha cl$ is held constant.

The Turner Designs instrument employs a continuous reference system which constantly removes bias due to fluctuations in voltage or efficiency of lamp or photomultiplier. We have experienced instability in the bias control (zero adjust) and high fidelty is essential in studies utilizing paired fluorometers for the calculation of the relative fluorescence increase in response to Diuron treatment (Cullen and Renger, 1979). Frequent reference to a reliable standard is desirable. The output of the fluorometer is linear within several levels of fluorescence emission, the change in levels being either manual or automatic. The problem of a reference standard was solved with a device providing a repeatable and constant intensity fluorescence emission when introduced into the optical geometry of the fluorometer.

A piece of Corning 3486 filter glass was fitted in a holder (Fig. 2) which replaced the cuvette with the instrument, after zeroing with iron-free distilled water, is adjusted to read 22 752 units (72 x 3.16 x 100). However, the fluorometer must be opened in order to check this adjustment, a tedious procedure with attendant danger of contamination and alteration of the filter alignment under field conditions.

Replacements for the upper and lower end fittings of the high volume continuous-flow cuvette system were manufactured (Fig. 3). These allowed easy access to the cuvette from the outside. A double 2-way valve (Fig.3) was made and fitted to allow isolation of the sample or replacement and flushing of the sample with distilled water. Pieces (about 0.2 g) of Corning 3486 filter glass were finely ground in a mortar and added to distilled water. After centrifuging for five minutes at 3600 RPM, the supernatant was decanted, a small amount of formalin and gelatin added, heated and mixed. This treatment provided a stable suspension of very fine particles of fluorescing glass. Sub-standards were made by sealing aliquots of this suspension in borosilicate glass tubing which could be inserted into the flow-through cuvette containing distilled water. The response values of these sub-standards were read with the fluorometer adjusted to the primary standard. Using distilled water for an instrument blank and these sub-standards, the fluorometer may be quickly and accurately calibrated in the field with minimum disruption to data aquisition and without disturbance of the optical alignment.

Variosens is described by Frünge1 and Koch (1976) and has been evaluated by Herman (1975). The output of the silicon photodiode is "logarithmically" amplified to produce a signal of 0 - 1 V, covering a span *in vivo* chl fluorescence from about

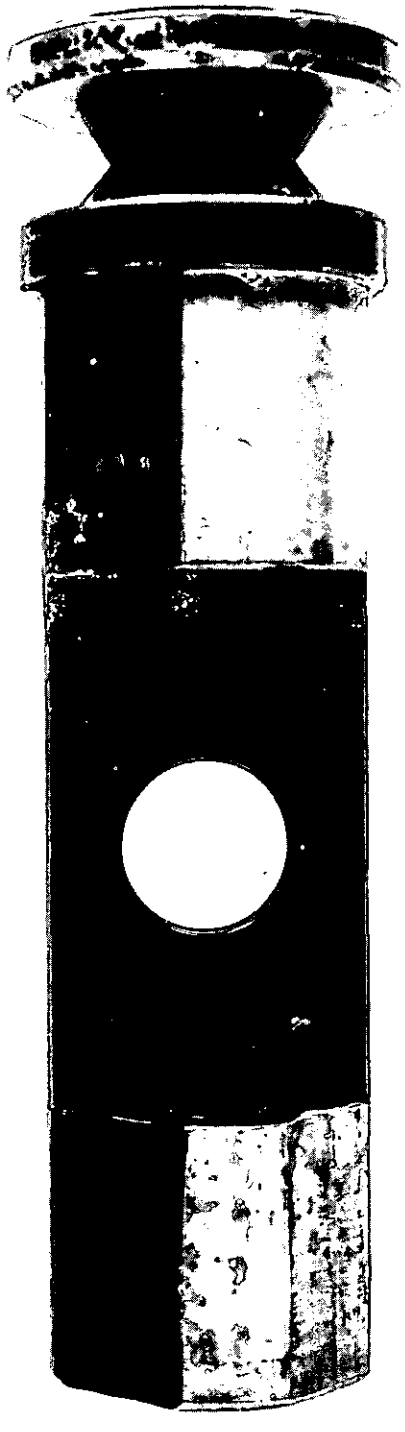

Fig. 2. Photograph of the primary standard proposed as the basis for *in vivo* chlorophyll *a* fluorescence measurements in this paper.

0.5 to 50 mg ℓ^{-1} of extractable chl *a*. The instrument is provided with adjustments for bias and sensitivity (span). However, these are internal and adjustments under field conditions necessitate breaking watertight seals and should be avoided. The Variosens employs no constant reference so its stability is uncertain; we have experienced drift in the bias control. Frequent reference to a reliable standard is desirable.

The "logarithmic" transformation of the photodiode output was checked by feeding known voltages into the circuitry and plotting the modified signal strength (x) on an arithmetic/log. scale grid. The results were not linear, as previously shown in graphs provided by the manufacturer and those of Herman (1975) and of Herman and Denman (1977). No simple mathematical curve was found which fitted these data without significantly increasing the error of the estimate. Accordingly, we decided to ascertain a linear fluorescence index (designated F_v as units were unspecified) using tabulated empirical equivalents for values of the pseudo-log. output.

Neutral density filters (Corning Glass Works, U.S.A.) were calibrated for the exciting light spectrum of the Variosens as follows. The spectral energy transmission of the blue filter (Jena Glaswirk, W. Germany) was measured, using a Cary Model 14 spectrophotometer, and integrated within 10 nm wide wavelength cells. These values were multiplied by the equivalent 10 nm wide wavelength increments of relative energy emitted from a xenon lamp (Fukuda and Sugiyama, 1961, Table 1) to give the relative energy distribution of the Variosens exciting light. Spectral transmittance of the neutral density

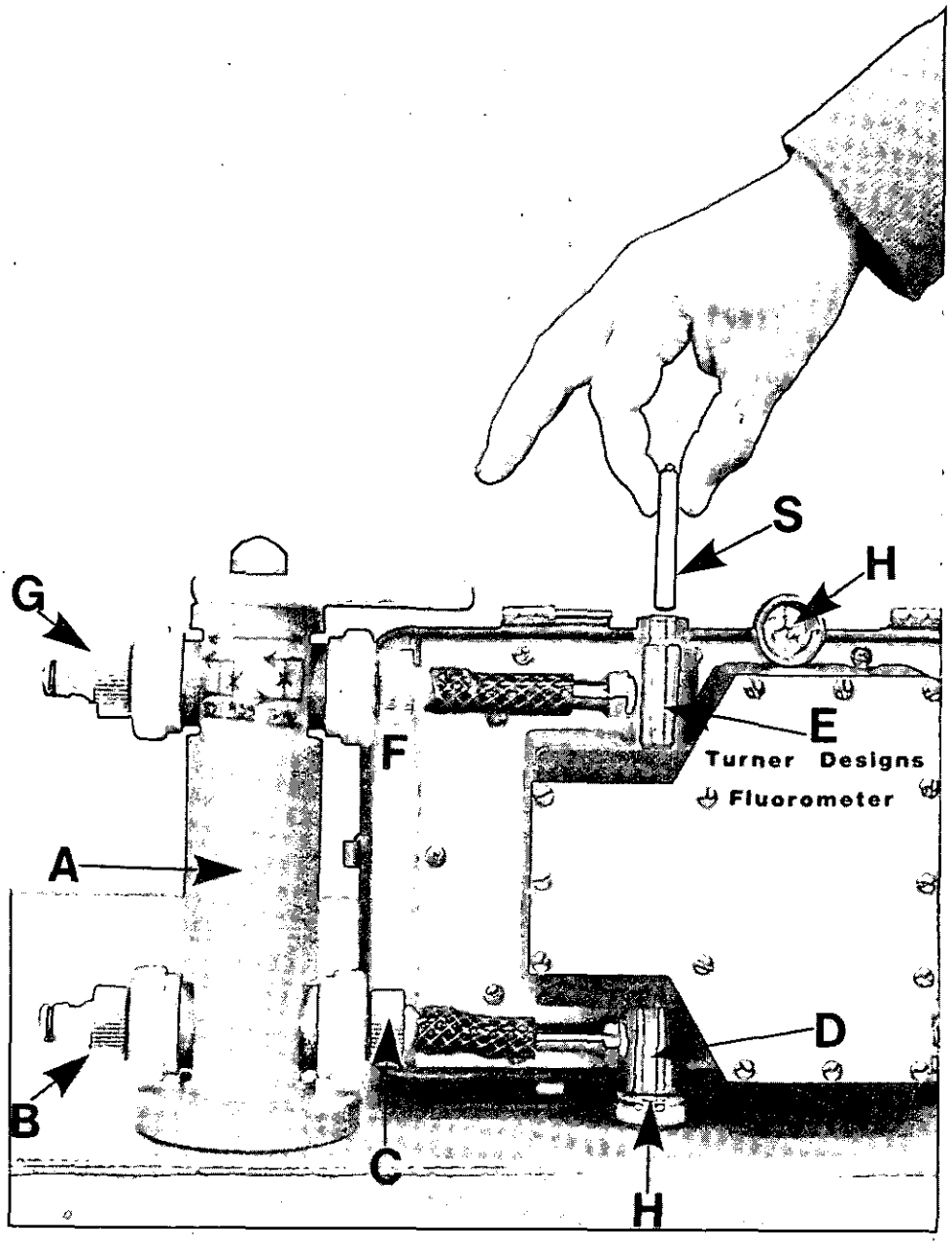

Fig. 3. Flow diagram and modifications to piping of fluorometer to allow access to cuvette for cleaning and calibration. The water supply stream enters the bypass valve (A) at B. When the valve is open (as shown) the water passes from B to C, through the modified intake fitting (D), through the cuvette (fluorescence chamber), through the modified exhaust fitting (E) to F and then to G. When the valve is closed water flows from B to G directly and the water trapped in the fluorometer from C to F is isolated. This may be drained by removing both knurled caps (H) and the cuvette may be cleaned with a test tube brush. The system may then be flushed with several rinses of distilled water and then zeroed on the last rinse using the blank adjust knob. The standard (S) is then inserted through the modified exhaust fitting, light excluded, and the instrument is then calibrated using the span adjust knob to achieve the standard's value.

filters, used singly and in combination, were likewise measured and averaged within 10 nm wide wavelength cells. These data were then used to estimate the decimal fraction of Variosens exciting light transmitted by each of 18 filter combinations.

A device was constructed to hold a sheet of 3486 filter glass which allowed repeatable precision positioning in the Variosens light path geometry, as shown in Fig. 4. Provision was made for holding the neutral density filters in the light path to reduce the intensity of the exciting light reaching the fluorescing glass. With this instrument a series of response values at 19 excitation light intensities, ranging from 0.0008 to 1.0000 of maximum, each replicated 27 times, was obtained. From these data the mean outputs ($\bar{x}$) and standard deviations (σ_x) were calculated.

Since a curve connecting these means (Fig. 5) cannot easily be described mathematically, any statistical estimation of the variance associated with predicting F from x would imply shape assumptions known not to be true. We used the simple expedient expedient of drawing linear interpolations between succeeding values of $\bar{x} \pm \sigma_x$ and measured the horizontal distance between the right and left enclosing σ_x envelopes (Fig. 5, insert). One half of this distance was taken as an estimate of standard deviation associated with the tabulated prediction of F_v from measured x. The empirically derived set of fluorescence indices, together with coefficients of variation ($\sigma_x/\bar{x}$) are given in Table 1. However, it is emphasized that the tabulated equivalents are valid for this particular instrument only.

The maximum coefficient of variation (C) is 0.091 at x = 250 mV. Over much of the response curve C is less than 5%. This suggests that the maximum instrumental precision is gained by reading the output to the nearest 20 mV interval, and conversely changes in the signal greater than 20 mV reflect changes in either ϕ_F or c rather than instrumental instability. The manufacturer's specifications give an upper signal limit at 1000 mV. We have found that readings up to 1300 mV are repeatable within the precision envelope described above.

With this device the fluorometer can be recalibrated to a specified scaling, using high and low transmittance filters. Fidelity of the instrument can be checked frequently in the field and corrections made, if necessary, to the raw data.

DISCUSSION

Substances usually used in calibrating fluorometers, e.g. ethidium bromide, quinine sulphate, Rhodamine B, etc., do not fluoresce at the same wavelengths as chlorophyll *a*. Their use would necessitate either replacement of the red filter, or use of the residual low transmittance outside its pass-band. In our instruments the filters are internal, necessitating breaking the watertight seals with attendant danger of contamination or alteration of optical geometry. For this reason, replacement of the red filter could perhaps be justified in the laboratory but would seem incautious as a standard procedure for field use.

The use of pure chlorophyll *a* intuitively would seem appropriate and is used by many workers (Lorenzen, 1966; Yentsch and Yentsch, 1979). The fluorescence spectrum of *in vivo* chl *a* (Papageorgiou, 1975; Goedheer, 1972; Yentsch and Yentsch, 1979) lies 14 to 18 nm toward the red from that of pure chl *a* dissolved in ether (Goedheer, 1966). Consequently the peak fluorescence emission of pure chl *a* is blocked by the Corning 2-64 and Schott AL673 nm filters

Table 1. Fluorescence indices and coefficients of variation corresponding to output signal of Variosens I (mV). Values are adjusted relative to 1.0 at 1300 mV.

Output reading (mV) 100's	Tens 00	10	20	30	40	50	60	70	80	90
0		.0015 .047	.0018 .065	.0020 .062	.0022 .062	.0025 .063	.0027 .055	.0029 .054	.0031 .054	.0033 .054
1	.0035 .054	.0036 .062	.0039 .065	.0042 .066	.0044 .065	.0047 .064	.0049 .064	.0052 .066	.0054 .074	.0057 .078
2	.0060 .087	.0063 .087	.0066 .088	.0069 .089	.0072 .090	.0076 .091	.0079 .088	.0082 .084	.0085 .074	.0088 .068
3	.0091 .061	.0094 .059	.0096 .057	.0099 .055	.0102 .054	.0105 .053	.0108 .052	.0110 .050	.0113 .049	.0116 .055
4	.0119 .059	.0124 .063	.0128 .068	.0132 .064	.0137 .061	.0142 .058	.0146 .056	.0150 .053	.0155 .051	.0160 .049
5	.0164 .047	.0169 .050	.0173 .055	.0180 .059	.0187 .061	.0194 .057	.0201 .054	.0208 .051	.0215 .048	.0222 .046
6	.0229 .043	.0236 .041	.0243 .039	.0250 .037	.0257 .035	.0264 .033	.0271 .033	.0279 .034	.0289 .040	.0300 .038
7	.0310 .036	.0320 .034	.0330 .032	.0340 .031	.0350 .029	.0361 .028	.0371 .026	.0381 .024	.0392 .026	.0405 .033
8	.0422 .034	.0439 .032	.0457 .030	.0473 .027	.0490 .026	.0508 .024	.0525 .022	.0542 .020	.0560 .023	.0585 .027
9	.0610 .027	.0634 .027	.0659 .027	.0683 .026	.0708 .026	.0731 .026	.0756 .026	.0780 .026	.0806 .047	.0871 .057
10	.0935 .052	.0999 .047	.106 .042	.113 .038	.119 .034	.126 .030	.132 .027	.138 .024	.145 .022	.151 .033
11	.165 .033	.179 .045	.193 .044	.208 .043	.221 .048	.243 .056	.267 .049	.290 .043	.314 .035	.344 .047
12	.378 .041	.415 .036	.450 .032	.498 .043	.555 .045	.612 .043	.668 .059	.770 .065	.873 .054	.976 .045

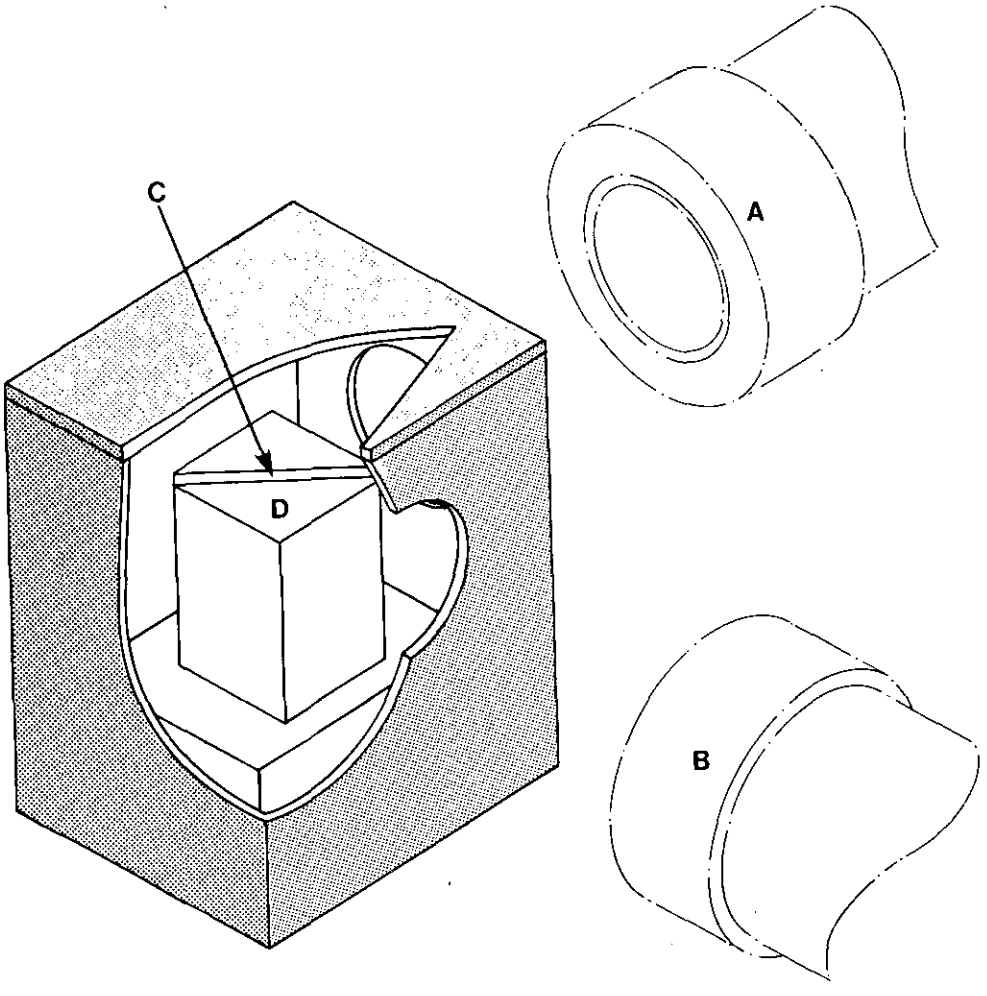

Fig. 4. Device constructed to employ fluorescing glass as a standard to calibrate Variosens. The box is made so that, in use, it fits tight against the lens cap of Variosens transmitter (A) and receiver (B) units and the exciting light focuses on the centre of the fluorescing glass (C) cemented between optical glass prisms (D). Provision was made for insertion of neutral density filters inside the box between the exciting light and the fluorescing glass.

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used in our instruments. These relationships are shown in Fig. 6 and reference to Fig. 1 suggests that Corning 3486 glass is a more appropriate reference standard.

The use of living phytoplankton cells is subject to large and uncertain variation in fluorescence quantum efficiency (ϕ_F in eqns 1 and 2), and would prove difficult, if not impossible, to standardize. It is assumed that the basic Beer-Lambert absorption relationships governing fluorescence emissions of pigments in solution described by eqns (1) and (2) hold for *in vivo* photosynthetic systems within the light-dependent portion of a Production/Irradiance (P *vs* I) curve. A major difference is that light absorption is governed by the concentration and exposure of chl *a* plus accessory pigment molecules, while *in vivo* fluorescence is almost entirely from excitation passed on

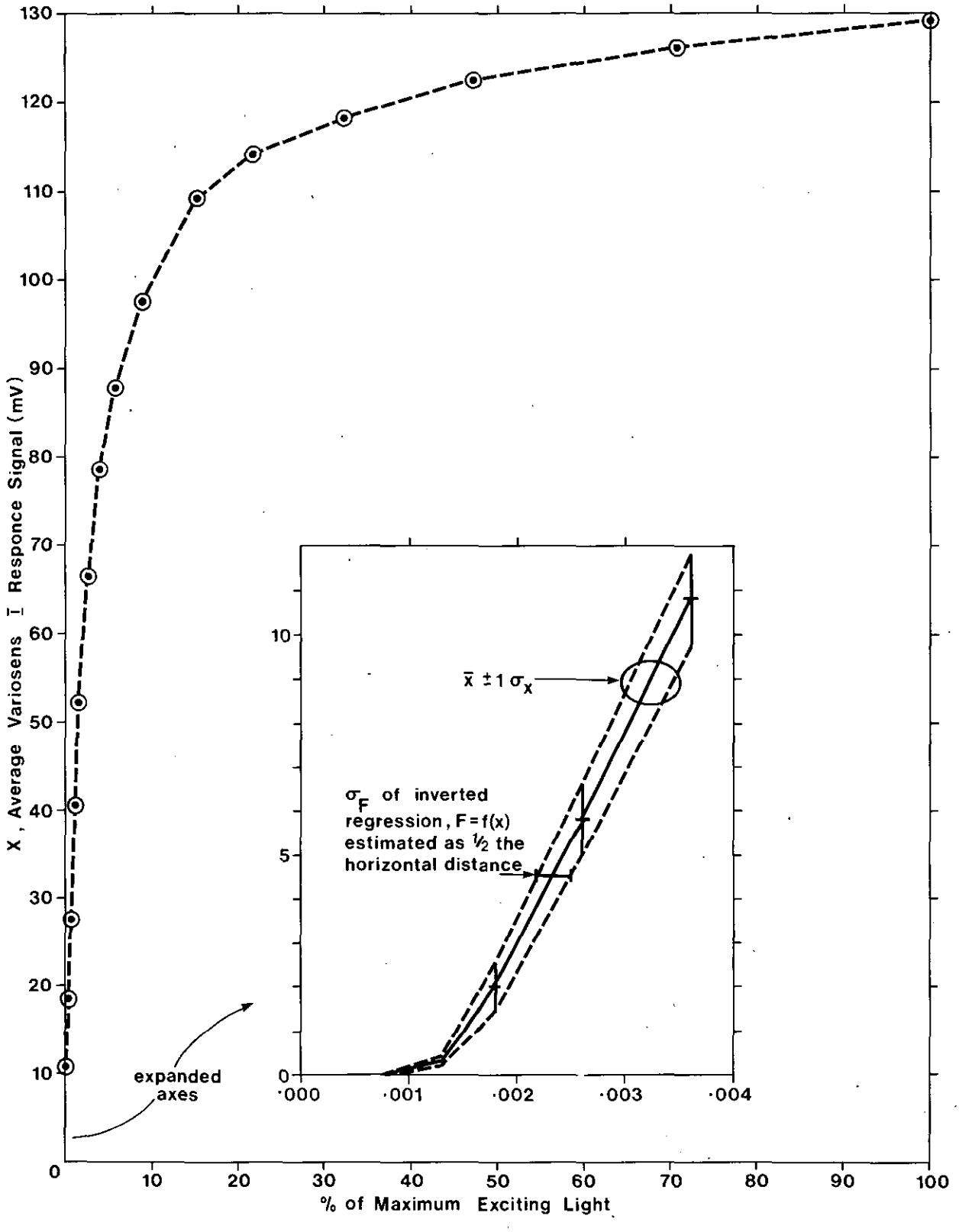

Fig. 5. Illustrating the shape of curve obtained for converting Variosens output to linear fluorescence response units and the estimation of the precision of the predicted values.

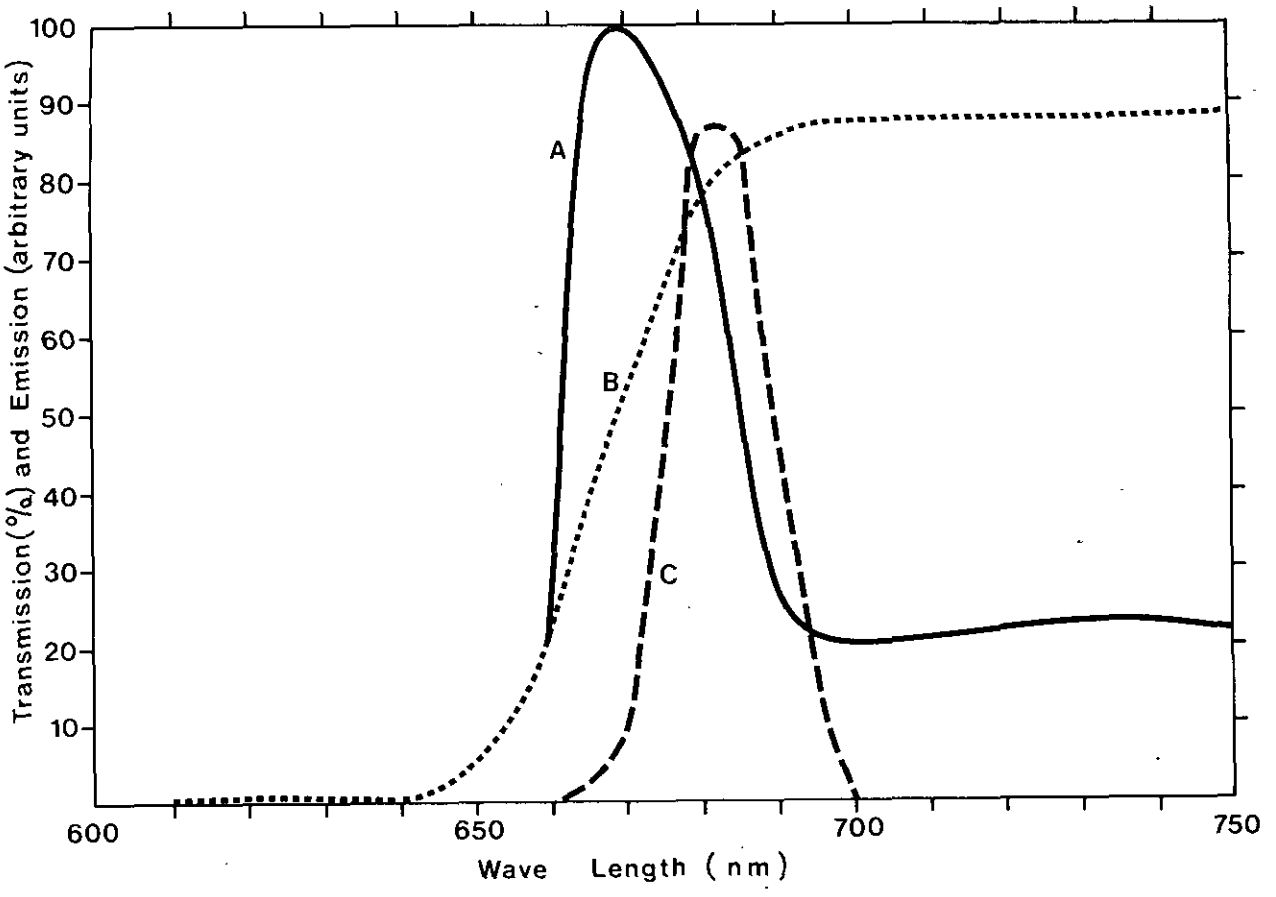

Fig. 6. Comparison of the fluorescence emission spectrum of pure chlorophyll *a* with the transmission spectra of red filters used in the fluorometers. A. fluorescence spectrum of pure chlorophyll *a* (redrawn from Goedheer, 1966). B. Corning CS 2-64 and C. Schott AL 673 as measured in Cary 14 spectrophotometer.

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from the light-harvesting pigments to particular chl *a* molecules located in Photosystem II (Govindjee and Govindjee, 1975). *In vivo* fluorescence is essentially excitation left over after the demands of photochemistry have been satiated. For this reason ϕ_F is a variable number, governed by the *in vivo* system responding to environmental attributes. In nature, this leads to a large spatial and temporal variation in the F: extractable chl *a* relationship. However, this is a

biological problem and not the subject of this paper. The point here is that the fluorescence spectrum of Corning 3486 filter glass adequately mimics that of *in vivo* chl *a* and provides a $\emptyset_F$ αcl value stable in time. Thus it is a preferred nominee to serve as a reference standard.

A similar standard is feasible for the 560-570 nm phycoerythrin fluorescence emission of blue-green prokaryotics and cryptomonads using Corning No. 3060 (CS 3-75) or No. 3391 (CS 3-74) filter glass. Such use would clearly separate instrumental and biological variation, important to the separation of blue-green bacteria from diatoms and dinoflagellates as suggested by Yentsch and Yentsch (1979).

The problem of *in vivo* chl *a* fluorescence standard units remains. While it is usual practice to convert *in vivo* fluorescence data into mg ℓ^{-1} of extractable chl *a*, the practice appears to convert a relatively precise measurement into an imprecise standard unit. Furthermore, the chl *a*: biomass relationship is variable (Banse, 1977) and where there is little intrinsic interest in chl *a per se*, there is little to recommend this intermediate conversion. It is, at least from a statistical viewpoint, more appropriate to treat *in vivo* fluorescence measurements as independent variables, measured without error, and to evaluate their precision and accuracy as objective predictors of preferred biomass units, e.g. into carbon, calories, volumes, etc.

The devices described above provide convenient and presumably stable references for the quantity $\emptyset_F$ αcl of eqn (2), thus providing a convenient means of checking the fidelity of or rescaling the fluorometers. We propose a specific chlorophyll *a* fluoroescence unit (SCAF) based on our Turner Design 10-005 R instrument employing our primary standard as described above. It would seem appropriate to exchange sub-standards among agencies in order to achieve standardization of reported and exchanged data.

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