INSTRUMENTS AND METHODS

A method for the rapid determination of chlorophyll plus phaeopigments in samples collected by merchant ships

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Abstract—A method is described for the estimation of the concentration of chlorophyll plus phaeopigments in seawater samples collected by the crews of merchant ships. Samples are filtered through Millipore® HA filters. The filters are stored dry and in the dark for later analysis. The measurement is without extraction: the filters are arranged on a thin-layer chromatography glass plate and processed by a fluorometer fitted with an automatic TLC scanning door. A 20-day delay is necessary before the measurements, during which the chlorophyll is transformed into stable degradation products; after 20 days, if the filters are kept dry and in darkness, the fluorescence remains nearly constant for several months.

The relation between the fluorescence of the filters and the chlorophyll plus phaeopigments concentration is linear up to concentrations as high as 6.6 mg m^{-3}. The error of the determinations is 60%, so the method is mainly of interest for collecting many data from wide areas by ships of opportunity.

INTRODUCTION

THE LABORATORY of the Office de la Recherche Scientifique et Technique Outre-Mer (Orstom) in Noumea (New Caledonia) conducts a programme for the study of the tropical Pacific Ocean based upon sea-surface sampling by the crews of merchant ships (DONGUY and HENIN, 1980). The programme uses about 20 ships sailing from Noumea to Sydney, Auckland, Panama, San Francisco, Hawaii, Japan, and Hong Kong. Excellent relationships between the scientists and the crews encouraged us to undertake other observations, more difficult than temperature observations and sampling for salinity. In 1977, filtrations for chlorophyll determinations were added to the list of routine operations.

Our attempt to monitor phytoplankton abundance is not the first; the best known are probably the continuous plankton records of HARDY (1939). For more than 40 years, many 'impressions of the quantity and extent of the phytoplankton' (RAE, 1952) have been gathered, obtained by comparing the silk that collects the plankton and a colour scale.

Except for fluorometric estimates chlorophyll measurements usually require a filtration immediately after sampling, storage of the filters, and later measurements in the laboratory after the ship calls in port. For each step, the collaboration of merchant ship crews presents problems, making necessary simple new technics presented below. The expense of placing fluorometers aboard the ships and their operation would be prohibitive.

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MODE OF OPERATION

Two rules govern the programme of the Centre Orstrom de Noumea. (1) The crews co-operate freely and gratuitously in the programme; any procedure too long or too troublesome will be rejected. (2) The co-operating ships also make regular meteorological observations, every 4 to 6 h; the oceanographic observations are made at the same intervals, and by the same observer so as to cause as little additional work as possible.

Filtration

Aboard most of the ships the sea-surface temperature is observed by means of a bucket thermometer, as commonly used in meteorology. When the temperature has been read, most of the water from the bucket is poured into a flask for later salinity determination. The volume of the remaining water is generally <100 cm^3. Consequently, the standard volume of seawater to be filtered has been fixed at 20 cm^3; filtration is by means of a syringe and a filtering cartridge, swinnex type, with a membrane filter HA type, 13 mm in diameter (Millipore products). Syringes (50 cm^3) with a core 2.6 cm in diameter are used to avoid too high pressures. The cartridges, each fitted with a filter, are provided to the ship to facilitate sample handling.

Storage of filters

Storage is the most critical point. The best way would be to place the dry filters immediately in a deep freezer, and we attempted this early in the work. But, unless a refrigerator was in the wheelroom, it was impossible to be sure that the transfer was undertaken immediately after the filtration. Thus, storage at ambient temperature has been adopted, as it means easier operation and can be repeated under identical conditions. Following filtration, the filter is removed from the swinnex cartridge, placed into a small numbered vial, and dropped into a jar with silica gel; the jar is kept in a dark place in the wheel room. The procedure destroys the 'active' chlorophyll *a*, and the measurements are made on a stable degraded form, pheophytin or pheophorbide.

Measurements

Existing methods can hardly measure the chlorophyll pigments from 20 cm^3 of (in most cases) oligotrophic seawater, especially when long storage of the filters makes difficult the acetone extraction of the chlorophyll from the cells (STRICKLAND and PARSONS, 1968). Methods without extraction have been described by YENTSCH (1957) and KONOVALOV and BEKASOVA (1969); they use a spectrophotometer where the filter is introduced in the optical path instead of cuvettes containing the chlorophyll solution. This idea is used and adapted to the fluorometer (Fig. 1). First, the filters are stuck in horizontal lines on a glass plate (20 × 20 cm, commonly used for thin-layer chromatography) with a glue stick. Once the TLC glass plate is filled with filters, it is placed in an automatic scanning door as for thin-layer chromatography. The fluorescence of the filters is recorded on a paper strip chart while the filters pass in turn in front of the optical system of the fluorometer (Fig. 2). The signal used for the computation is the difference in the fluorometer reading between a filter in new condition and the filter to be measured.

The fluorometer is a Turner model 111 fitted with a blue lamp and red-sensitive photomultiplier R 446. The secondary red optical filter is a 2-64 one; the primary one is com-
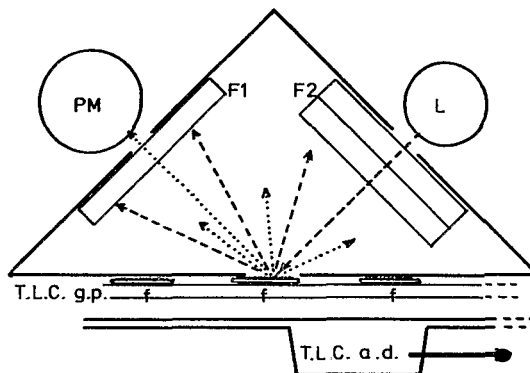
Fig. 1. Measurement system. PM, photomultiplier; L, lamp; F1, red filter 2-64; F2, blue filter 5-60; T.L.C. a.d., thin-layer chromatography automatic door; T.L.C.g.p, thin-layer chromatography glass plate; f... f... f, Millipore filters HA type, diameter 13 mm, holding phytoplankton; dashed line, blue light; dotted line, red light.

posed of two 5-60 ones. In this way much of the blue light is reflected towards the photomultiplier by the white Millipore filter, so the blue light needs to be free of red wavelength. The 5-60 blue filter allows about 1% of the incident red light to pass through, so two 5-60 blue filters allow only 0.01%, which avoids random variations of the zero value.

For routine analyses lamp window No. 30 of the fluorometer is used, with the slit of the TLC automatic scanning door opened to its maximum width. As adopted, the method presents two problems: What is the effect of the long-term storage of the filters at ambient temperature (20 to 90 days) and what is the precision and sensitivity.

EFFECT OF THE STORAGE OF THE FILTERS

Some merchant ships call in Noumea about every 20 days, while others call in every three months. Each time, the used filters are recovered to be measured in the laboratory, and new cartridges are supplied.
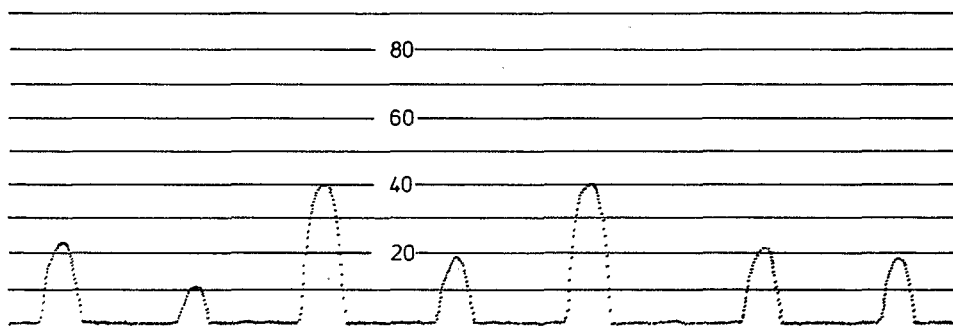

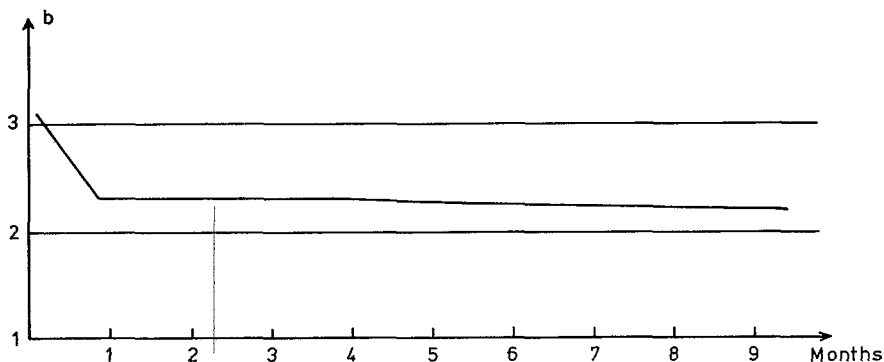Fig. 2. An example of a record of the fluorescence of a series of filters.

Fig. 3. Long-term conservation of the fluorescence of the filters. A set of filters corresponding to various filtered volumes stored over 10 months; b is the slope of the regression fluorescence function of filtered volume.

A series of filters corresponds to a whole voyage, so that the last filters have been stored only a few days, while the first ones have been stored 20 to 90 days. It would be possible to extend the storage to 90 days for all the filters, but this represents a long delay. Several experiments show an initial rapid decrease of the fluorescence followed by a long steady period during which the decrease is negligible (Fig. 3).

One set of filters has been examined at short time intervals for 33 days following filtration (Fig. 4). Some filters reached a steady state as early as 14 days after filtration, but 20 days seemed necessary for the others. Therefore, all the routine measurements are processed after a 20-day wait, the filters being stored in a dark and dry place.
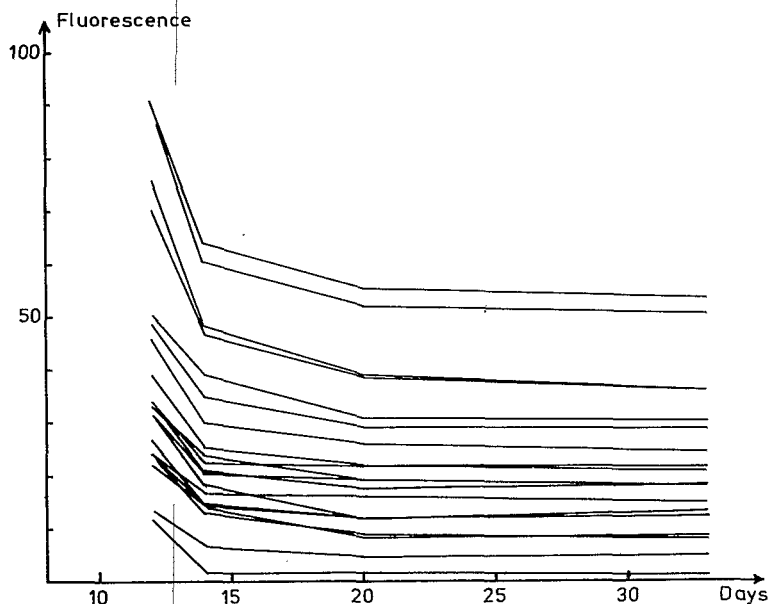

Fig. 4. Short-term changes of the fluorescence of the filters. 19 filters were measured after 12, 14, 20, and 33 days. After 20 days, their fluorescence reached nearly constant values.

The decrease of fluorescence during the first days of storage corresponds to a nearly complete disappearance of the chlorophyll a; all the filters brought back by a ship that had sailed around New Caledonia were recovered after the routine measurements and dissolved in 5 cm^3 of acetone during 2 h in a refrigerator. The fluorescence of the solutions was then determined before and after acidification with one drop of HCl (5%). The differences between the two values did not differ significantly from the normal decrease due to the volume of the drop, meaning that no active chlorophyll a was present in the extract. An oceanographic cruise (HYDROTHON 06 aboard R.V. *Coriolis*) showed for the same period and the same area, a ratio of chlorophyll a:chlorophyll a plus phaeopigments of 84%, indicating that the decrease in fluorescence due to the long storage at ambient temperature corresponds to the transformation of chlorophyll a into phaeopigments.

LIMITS OF APPLICATION

Experiences with seawater from the lagoon off Noumea have shown that in normal conditions the fluorescence of the filters is proportional to the filtered volume. A determination of the maximum load of chlorophyll on the filters above which the ratio of fluorescence to chlorophyll decreases (Fig. 5) showed that the relation of fluorescence to volume filtered is linear up to a filtered volume of 200 cm^3, corresponding to 0.133 µg of chlorophyll plus phaeopigments. Such a load, when 20 cm^3 of seawater are filtered, corresponds to waters with 6.6 mg m^{-3}. Such concentrations may occur in the network of co-operating merchant ships, in particular across the Equator in the eastern Pacific. Such concentrations are infrequent, but they would be underestimated by the present method.
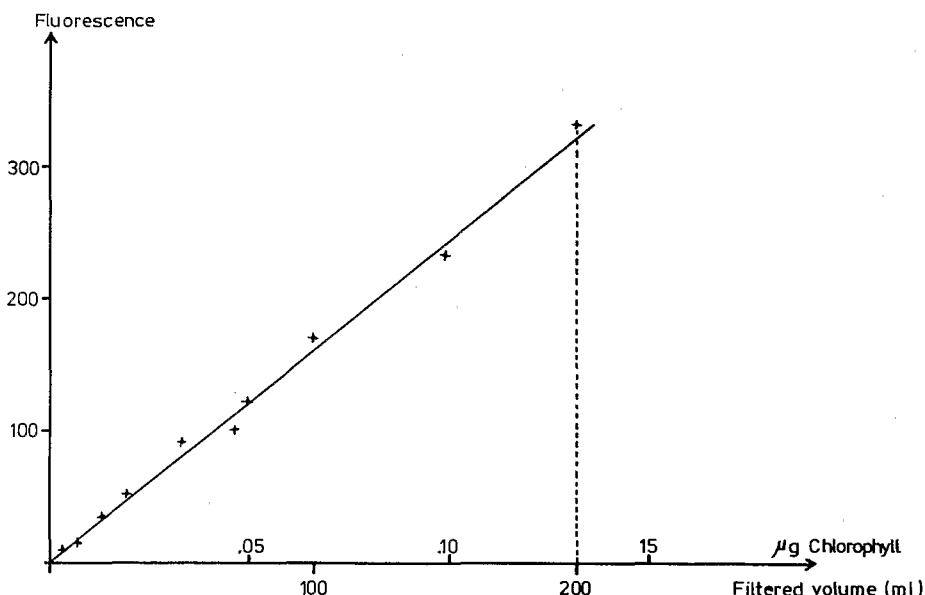

Fig. 5. Range of application: the fluorescence of filters corresponding to various filtered volumes of the same sample of seawater is proportional to the filtered volume up to 0.132 µg chlorophyll plus phaeopigments. If the filtered volume is 20 ml, this amount corresponds to a concentration of 6.6 mg m^{-3}.

RELATION BETWEEN THE FLUORESCENCE ON THE FILTERS AND VARIOUS SIGNALS RELATED TO THE CONCENTRATION OF CHLOROPHYLL PIGMENTS

A series of calibrations was carried out during the cruise HYDROTHON 06 aboard R.V. *Coriolis* by the Centre Orstom de Noumea. During the cruise, 14 samples of surface seawater were analyzed for chlorophyll pigments by five different procedures: (a) filtration of 20 cm^3 on Millipore HAWP filters, 13 mm in diameter, storage of the filters in a dry and dark place for 15 days, and measurement of the fluorescence of the filters according to the present method (the results are represented by Ff, Table 1); (b) measurement of the *in vivo* fluorescence of the sample by the method of LORENZEN (1966) immediately after the sampling (Fv); (c) measurement of the *in vivo* fluorescence of the sample after an addition of 5×10^{-6} mol l^{-1} of DCMU, according to the method described by SLOVACEK and HANNAN (1977) (Fdcmu); (d) filtration of 150 cm^3 on glass fiber filters Gelman type A, mechanical grinding of the filter in 5 cm^3 acetone for 20 s, extraction in the refrigerator for 10 h, and measurement of the fluorescence of the extract (Fo); and (e) following (d), addition of one drop of 5% HCl to degrade the chlorophyll *a* into phaeopigments (HOLM-HANSEN, LORENZEN, HOLMES and STRICKLAND, 1965), and then measurement of the fluorescence of the extract (Fa).

The results are presented in Table 1.

Table 2 indicates the correlation coefficients between all the signals. The correlation coefficients between Fv, Fdcmu, Fo, and Fa are also given.

Table 1

Sample number	1	2	3	4	5	6	7
Filtration of 20 cm^3: Ff fluorescence of the filters	16	11	40	12	7.5	9	15.5
In vivo fluorescence: Fv	43	41	97	32	60	24	24
In vivo fluorescence with DCMU: Fdcmu	96	101	201	81	101	73	60
Fluorescence of acetone extracts: Fo	272	160	572	238	246	221	300
Fluorescence of acetone extracts + HCl: Fa	167	98	340	166	136	147	176
Estimation Ff/49.17	0.33	0.22	0.81	0.24	0.15	0.18	0.32
Chlorophyl + phaeopigments* (mg m^3)	0.28	0.17	0.57	0.28	0.23	0.25	0.30
Error %	18	29	42	−14	−35	−28	−7

	8	9	10	11	12	13	14
Filtration of 20 cm^3: Ff fluorescence of the filters	10.5	15.5	14	33	21	34	27.5
In vivo fluorescence: Fv	48	64	77	73	117	153	133
In vivo fluorescence with DCMU: Fdcmu	85	117	97	153	218	330	270
Fluorescence of acetone extracts: Fo	235	374	279	373	552	763	698
Fluorescence of acetone extracts + HCl: Fa	165	245	170	229	297	458	407
Estimation Ff/49.17	0.21	0.32	0.28	0.67	0.43	0.69	0.56
Chlorophyl + phaeopigments* (mg m^3)	0.28	0.41	0.29	0.39	0.50	0.77	0.69
Error %	−25	−22	−3	72	−14	10	−19

*This concentration is calculated from Fa and from a calibration coefficient of the fluoremeter for phaeopigments.

Table 2. Correlation coefficients between the five signals of the calibration experiment

	Ff	Fv	Fdcmu	Fo
Fv	0.72			
Fdcmu	0.77	0.96		
Fo	0.81	0.93	0.96	
Fa	0.81	0.91	0.95	0.99

The signals that exhibit the closest correlation with the fluorescence of the filters (Ff) are those given by the fluorescence of the extracts ($r = 0.81$) before or after acidification (Fo or Fa).

As the chlorophyll a on stored filters is completely degraded, it appeared more consistent to relate the fluorescence of the filters to the concentration of chlorophyll a plus phaeopigments. The identical relation between Ff and Fa is due to a small phaeopigment content of the seawater during the cruise (16% as a mean), so that the total chlorophyll a plus phaeopigments was closely related to the amount of chlorophyll a.

The mean ratio Ff:chlorophyll a plus phaeopigments is $k = 49.17$. This value depends upon the equipment used and must be determined by calibrations at regular intervals. Table 1 indicates the concentrations of chlorophyll a plus phaeopigments estimated from Ff values and the corresponding errors in percentage. The number of values (14) hardly allows statistical calculations, however, if one assumes that the errors have a normal distribution, 95% of the errors are <60%.

DISCUSSION

The present method allows the measurement of chlorophyll pigments in the sea. It yields approximations because it does not distinguish active chlorophyll a from the other chlorophylls or their degradation products, and furthermore, the precision of the measurements is only 60%. So it would not be used when other methods can be applied, but it can be used to obtain results opportunistically. Chlorophyll determinations by oceanographers aboard research vessels are far more precise, but they are expensive and they are limited to restricted areas and times; furthermore, the results from various authors and institutions appear to vary widely (DANDONNEAU, 1979).

The present method provides data from areas where no oceanographic cruises are scheduled, and it also makes it possible to obtain data for long time series. Variations by a factor of 20 are common at the surface of the ocean, so a precision of 60% may be acceptable to describe large areas month after month, even if such a precision may not be useful for the detailed study of one phenomenon or one restricted area. The aim of such monitoring on the oceanic scale is mainly to describe the variations in size and intensity of phenomena already generally known, such as equatorial upwelling or the blooms corresponding to the winter cooling in subtropical areas.

It proved to be most reliable to subject the filters to fluorometry without extraction. Handling is reduced to a minimum, necessary because only very small amounts of chlorophyll from only 20 cm^3 are available. Moreover if the measurement reveals an interesting feature, microscopic examination of the filter is possible by clearing the undamaged filter with cedar oil (GOLDBERG, BAKER and FOX, 1952); however such examination may not be easy, due to

the remaining salt and poor preservations of non-armored phytoplankton cells. The present method is also quick; 300 measurements can easily be made by one person in a day.

Other ways can provide data from very wide areas: (1) It would be possible to place fluorometers aboard ships of opportunity and to record the *in vivo* fluorescence of the surface waters, but it would be costly, requiring equipment and maintenance. (2) Satellite observations are promising. Daily images of the chlorophyll content of the surface would enhance our knowledge of the relations between the large-scale oceanic features and the fertility of the sea, even if the precision were worse than for the present method. But offshore, ground truth is difficult to obtain; perhaps the present method will be of some use in providing it.

REFERENCES

DANDONNEAU Y. (1979) Concentrations en chlorophylle dans le Pacifique tropical sud-ouest: comparaison avec d'autres aires océaniques tropicales. *Oceanologica Acta*, **2**, 133–142.
DONGUY J. R. and C. HENIN (1980) Surface conditions in the eastern equatorial Pacific related to the intertropical convergence zone of winds. *Deep-Sea Research*, **27**, 693–714.
GOLDBERG E. D., M. BAKER and D. L. FOX (1952) Microfiltration in oceanic research. I. Marine sampling with the molecular filter. *Journal of Marine Research*, **11**, 194–204.
HARDY A. C. (1939) Ecological investigations with the continuous plankton recorder: object, plan and methods. *Hull Bulletin of Marine Ecology*, **1**, 1–57.
HOLM-HANSEN O., C. J. LORENZEN, R. W. HOLMES and J. D. STRICKLAND (1965) Fluorometric determination of chlorophyll. *Journal du Conseil, Conseil permanent international pour l'exploration de la Mer*, **30**, 3–15.
KONOVALOV B. V. and O. D. BEKOSOVA (1969) Method of determining the pigment content of marine phytoplankton without extraction. *Oceanology*, **9**, 717–725.
LORENZEN C. J. (1966) A method for the continuous measurement of *in vivo* chlorophyll concentrations. *Deep-Sea Research*, **13**, 223–227.
RAE K. M. (1952) Continuous plankton records: explanation and methods, 1946–1949. *Hull Bulletin of Marine Ecology*, **3**, 135–155.
SLOVACEK R. E. and P. J. HANNAN (1977) *In vivo* fluorescence determinations of phytoplankton chlorophyll *a*. *Limnology and Oceanography*, **22**, 919–925.
STRICKLAND J. D. H. and T. R. PARSONS (1968) *A practical handbook of seawater analysis*. Fisheries Research Board of Canada, Bulletin, 167, 311 pp.
YENTSCH C. S. (1957) A non extractive method for the quantitative estimation of chlorophyll in algal cultures. *Nature*, **179**, 1302–1304.