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A novel phytoplankton chlorophyll technique: toward automated analysis*

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Abstract. Interest in the < 1 μm picoplankton fraction of natural waters, especially the oligotrophic oceans, has generated a trend toward the use of smaller and smaller porosity membrane filters for particulate analyses. The controversy concerning the suitability of glass fiber *versus* membrane filters for retaining chlorophyll-containing particles is re-examined and the inadequacies of filtration discussed. Previous comparisons in the literature fail to resolve this controversy as tests were performed in waters of > 1.0 μg/l chlorophyll. A novel phytoplankton chlorophyll technique for fresh and marine waters is described which eliminates the need to concentrate plankton on filters. Two commercially available instruments have been modified to permit fluorescence measurements of whole water extracts. The lower limit of detection is 0.1 μg/l *in situ* concentration or 0.02 μg/l extract concentration. The technique lends itself well to automation with particular applications to *in situ* fluorometry, flow cytometry and continuous chlorophyll determinations.

Introduction

The extraction of chlorophyll into a solvent to measure phytoplankton standing crop began in the early 1930s. Kreps and Verjbinskaya (1930) collected plankton from the upper 25 m of the Barents Sea with a 64 μm net, extracted pigments into 96% alcohol and measured extract color spectrophotometrically. Harvey (1934) used a similar net and 80% acetone extracts which he compared to an artificial standard. However, Bigelow *et al.* (1940) found nets collected only 1–10% of the phytoplankton present in the Gulf of Maine, and soon after, Graham (1943), also concerned with the inadequacies of nets, stated six aspects essential to quantitative chlorophyll measurements: (i) use of a small quantity of water; (ii) rapid filtration; (iii) removal of at least 95% of the phytoplankton; (iv) use of a small amount of solvent; (v) rapid reading of chlorophyll concentration; and (vi) use of portable gear.

Figure 1 presents the general method as three basic steps listing the major differences and controversies associated with each step. While many established methodologies for the efficient extraction and quantification of chlorophyll from natural waters exist (Yentsch and Menzel, 1963; Holm-Hansen *et al.*, 1965; Lorenzen, 1967; Strickland and Parsons, 1972; Jeffrey and Humphrey, 1975; Jeffrey, 1981; Gieskes and Kraay, 1983) each relies on the efficiency of cell harvesting for a resultant chlorophyll estimate.

Glass fiber filters have traditionally been used to concentrate plankton for less sensitive spectrophotometric measurements. Membrane filters of varied composition and smaller nominal pore size have been suggested (Li *et al.*, 1983; Platt *et al.*, 1983), particularly since the discovery of small < 1 μm photosynthetic cyanobacteria (Waterbury *et al.*, 1979). The desire to retain the smallest organisms has raised doubts as

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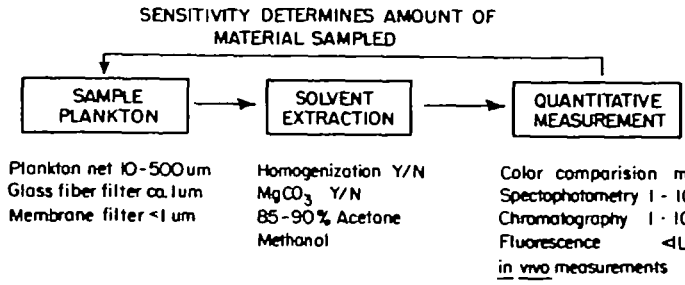

Fig. 1. The basic chlorophyll method, summarizing diversions in techniques.

to the suitability of glass fiber filters for removing all particles of interest from marine waters (Yentsch, 1983). Comparisons of glass fiber and membrane filters for marine seston (Sheldon, 1972; Hickel, 1984), particulate organic carbon (Salonen, 1979) and chlorophyll (Long and Cooke, 1971; Holm-Hansen and Riemann, 1978; Smith *et al.*, 1981) have failed to provide a consistent preference. Additionally, the inconsistencies concerning solvent, the need for $MgCO_3$ buffer or homogenization cause further complication.

In the present work, we re-examine the ability of filters to retain the smallest chlorophyll-containing particles in the light of Graham's criteria, and describe a novel phytoplankton chlorophyll technique which eliminates the need for sample filtration.

Methods

Filter comparisons

Seven glass fiber filters were compared to homogenized and unground Millipore HA 0.45 μm cellulose acetate membranes. A large volume natural sample was subsampled for each filter tested. Care was taken to ensure homogeneity of the volume before subsampling. Twenty liters of surface water were collected in a polyethylene carboy. Air pumped through the carboy facilitated mixing while sample water was circulated continuously by a peristaltic pump. Temperature and chlorophyll concentration were monitored at 15-min intervals until values equilibrated. Large changes were found to occur in the carboy system during the first 2 h.

Four replicate samples were drawn for each filter. All filtrations were performed under 5 in Hg vacuum. Millipore HA filters were used first and last to ascertain changes during the sampling interval. Immediately upon completion of filtration, filters were placed in glass vials containing 2 ml 85% acetone in the dark at 4°C until all samples were filtered. Total cold extraction time for all filters was 4 h.

Filters were homogenized for 1 min in a glass/Teflon tissue grinder with 3 ml 85% acetone and 0.2 mg $MgCO_3$. Homogenate was decanted to screw-cap test tubes and the volume brought to 12 ml. The extract was centrifuged for 5 min at 3000 r.p.m., supernatant removed and fluorescence before and after acidification measured using a Turner Model 111 fluorometer (Yentsch and Menzel, 1963). The fluorometer was calibrated using chlorophyll *a* from spinach (Sigma Chem. Co.).

A non-grinding technique was used in some data sets where HA filters were added directly to 12 ml 85% acetone and merely vortexed causing the filter to dissolve. Samples were cold-extracted in the dark at 4°C for 4 h before centrifugation and fluorescence measurement. $MgCO_3$ was not added.

Field data comparing a glass fiber filter and Millipore HA membranes were accumulated in the process of routine measurements at sea. Glass fiber filters were used to obtain particulate spectral signatures (Yentsch and Yentsch, 1979; Yentsch and Phinney, 1982) while HA filters were used for total chlorophyll determinations in support of continuous *in vivo* fluorescence monitoring. Seawater from the surface and at depth was collected with Niskin bottles or submersible pumping system into 2 l polyethylene jugs. The volume was completely mixed before subsamples of 100 ml for HA and 1000 ml for glass fiber filtrations were drawn. Samples were filtered, and fluorescence measured as above.

Whole water technique

Two commercially available fluorometers, Turner Model 111 and Turner Designs Model 10-005 have been modified to achieve the increased sensitivity required for this method. A high sensitivity R928 phototube (Hammamatsu Corp., Middlesex, NJ), output impedance 36 mA/W at 675 nm, replaced the R446 S20 response phototube currently supplied by manufacturers of these instruments for chlorophyll analysis. This represented a 3-fold increase over the 12 mA/W output of R446 phototubes. Further, a high sensitivity door was used on the Model 111 (6- to 10-fold increase), while a 40–60% neutral density filter was placed in the rear light path of the 10-005, increasing sensitivity 2-fold. The intensity of light reaching the photomultiplier tube *via* the rear light path determines sensitivity in a 10-005 fluorometer. The actual sensitivity realized is instrument specific; the neutral density filter selected should allow reagent blanking on the highest sensitivity scale.

One liter of seawater was pre-screened through 100 μm Nitex mesh and continuously stirred. Replicate 1.5 ml samples were placed in screw cap test tubes and 8.5 ml 100% acetone added. The sample was vortexed, a precipitate formed and extract volume was lost (~3%). No precipitate formed when the sample was fresh water. Samples were placed in the dark for 6 h after which they were clarified or precipitated by filtration though Whatman GF/F filters. High speed centrifugation may be employed. However, several replicate samples can be clarified using one GF/F filter which was found to be more convenient than centrifugation. Following clarification, extract fluorescence was measured as above.

Results

In laboratory experiments, averages of four replicate samples were subjected to Student's *t* tests to determine statistically significant differences among estimates. In Figure 2, solid bars indicate that of the glass fiber filters, only Whatman GF/F filters estimated statistically similar chlorophyll concentrations as HA filters within the range of concentrations measured. Unground HA filters also gave similar results. However, as the concentration of chlorophyll decreased below 1 μg/l, the retention of glass fiber filters other than GF/F also decreased. A trend of retention efficiency among filters was

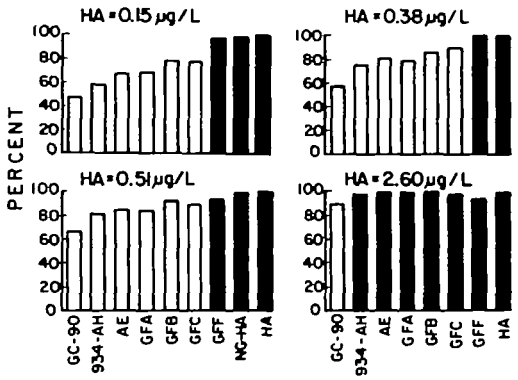

Fig. 2. Laboratory comparisons of chlorophyll estimates by seven glass fiber filters to Millipore HA filters for natural population samples from four oceanic regions. Bar heights represent the average of four replicate samples of Toyo GC-90, Reeve Angel 934-AH, Gelman AE, Whatman GF/A, GF/B, GF/C and GF/F glass fiber filters as a percentage of chlorophyll retained by homogenized HA filters. Solid bars indicate no statistical difference between the estimates at 95% confidence level.

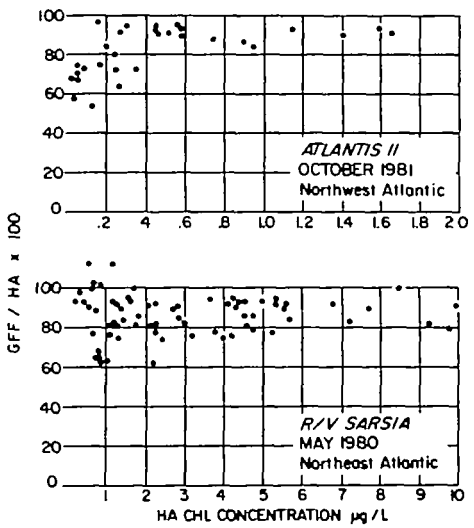

Fig. 3. Field comparisons of Whatman GF/F filters to Millipore HA membranes as a function of HA concentration.

generally found to correspond to the rated nominal pore size stated by manufacturers (AE = 1.0 μm, 934-AH = 1.5 μm, GF/A = 1.6 μm, GF/B = 1.0 μm, GF/C = 1.2 μm and GF/F = 0.7 μm).

Filtration of four replicates per sample is hardly practical when considering the number of samples taken during a typical cruise. Figures 3 and 4 present field comparisons of routine measurements at sea. Figure 3 shows the performance of GF/F filters, judged equally efficient to HA filters in laboratory experiments, over the range of concentrations encountered in two distinctly separate ocean regions. These examples suggest that even the most efficient glass fiber filter may retain only 60% of the chlorophyll

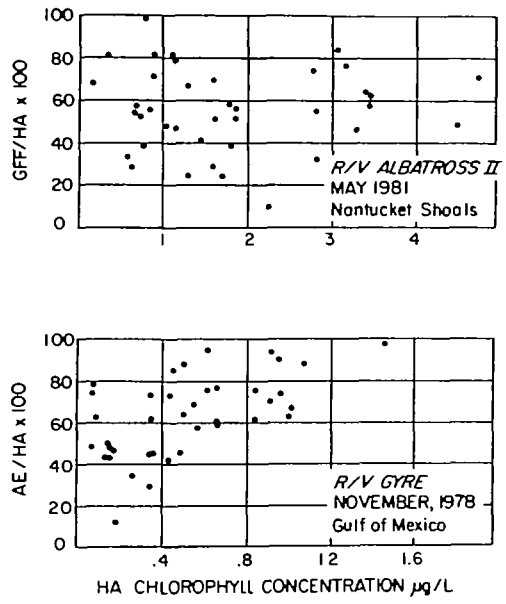

Fig. 4. Field comparisons of Whatman GF/F and Gelman AE filters to Millipore HA membranes as a function of HA concentration.

present in low concentration samples. Figure 4 shows the inadequacy of GF/F and AE filters in field experiments, with GF/F filters displaying variable retention characteristics over the range 0 – 5 µg/l chlorophyll. Attempts to improve retention by clogging glass fiber filters with $MgCO_3$ or to demonstrate a loading curve behavior at higher sample volumes failed. We feel these data provide strong evidence that glass fiber filters are not suitable in areas where low concentrations of phytoplankton prevail.

A precipitate of unknown composition formed upon addition of a marine sample to acetone. The amount of precipitate appeared to be related to sample salinity. Fresh water samples produced no precipitate. However, some fresh water samples with high concentrations of humic substances were found to possess high background fluorescence after filtration through a 0.2 µm Nucleopore filter. Marine samples treated in this manner were indistinguishable from an 85% distilled water:acetone blank. The presence of dissolved substances which contribute to sample fluorescence will certainly affect the accuracy of a chlorophyll estimate.

Complete extraction was obtained after 6 h for samples held in the dark at room temperature. Figure 5 shows this to be a considerable improvement over cold-extracted samples which require at least 30 h.

Table I contains data from ten replicate samples of three aqueous solutions of acetone for a natural sample. Precision of the method was comparable to that of traditional filter techniques. Total chlorophyll estimates for marine samples by whole water extraction averaged 5 – 10% more chlorophyll than HA estimates, the maximum difference measured was nearly 30%. Comparisons of HA filters and 48-h cold-extracted whole water samples suggested degradation products contribute to a majority of the additional chlorophyll as acid ratio values were always lower for whole water samples.

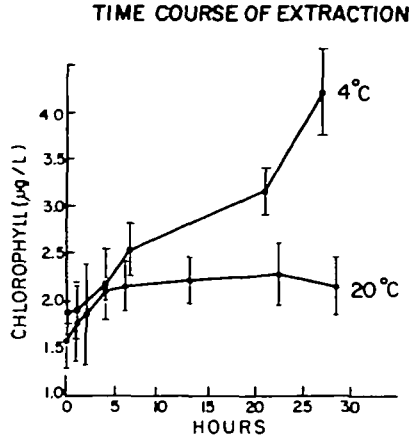

Fig. 5. Extraction efficiency with time, whole water technique at room temperature and 4°C.

Table I. Chlorophyll estimates (µg/l) of ten replicate samples for 85, 75 and 65% aqueous acetone.

Sample no.	Aqueous acetone (%)		
	85	75	65
1	1.94	1.60	1.33
2	1.57	1.48	1.35
3	1.70	1.43	1.28
4	1.85	1.46	1.18
5	1.75	1.33	1.23
6	1.73	1.39	1.18
7	1.63	1.35	1.32
8	2.36	1.61	1.24
9	1.79	1.57	1.32
10	1.67	1.43	1.33
Mean value	1.80 ± 0.16	1.47 ± 0.07	1.28 ± 0.05

Discussion

The routine measurement of chlorophyll as a phytoplankton biomass indicator has led to the diversification of thought as to the correct method. An inherent truism to any method is that sensitivity of the instrumentation determines sample volume. An apparent aspect of all methods is the need to concentrate phytoplankton on filters. Spectrophotometric methods require concentration of material on glass fiber filters or with nets for subsequent pigment extraction into a small volume of solvent. This aspect of chlorophyll methodology has been universally accepted and has led to the use of many commercially available filters. Additionally, standardized methods prevent the critical assessment of the performance of a particular filter.

As the importance of <1 µm autotrophs emerges, filters of differing composition and smaller porosity are being utilized to include this fraction. This is particularly true in subtropical and tropical regions where Li *et al.* (1983) found 25–90% of biomass

as chlorophyll passes a 1 μm filter and is retained by a 0.2 μm membrane in the Eastern Tropical Pacific. Also, Platt *et al.* (1983) state that 44% of the total chlorophyll biomass passes a 1 μm filter and is retained by a 0.4 μm membrane in the Tropical Atlantic. Similarly, 20–80% (Li *et al.*, 1983) and 60% (Platt *et al.*, 1983) of primary production are reported as passing a 1 μm filter. These results support the data in Figures 2–4 which demonstrate the inadequacies of most glass fiber filters for retaining all particulate chlorophyll.

We propose a working limit of 1 μg/l chlorophyll below which glass fiber filters fail to retain a significant portion of the population. If we extend this argument to membrane filters, we suggest that this phenomenon affects their performance at extremely low concentrations as well. Filtration, in general, will exclude some small fraction of a natural sample, only a non-filtering, whole water technique can guarantee complete particle capture. Unfortunately, comparisons in the literature have been made in areas where chlorophyll concentrations were much higher than 1 μg/l. Long and Cooke (1971) compare GF/C, GF/A and HA filters using a spectrophotometric method on lake samples between 8 and 40 μg/l chlorophyll. Holm-Hansen and Riemann (1978) compare Reeve Angle 984-AH, GF/C and HA filters with a fluorescence method in a tropical lagoon where >4 μg/l chlorophyll was measured. These studies judged glass fiber filters preferable, considering decreased filtration time and cost per sample. Our data suggest that at these high concentrations no statistical difference would be discerned among estimates by most of the filters tested. However, as the concentration of chlorophyll decreases below 1 μg/l, the efficiency of glass fiber filters decreases, presumably due to the small pigmented cells which dominate these samples.

Keeping Graham's six essential aspects in mind, we find that the desire to capture smaller and smaller particles introduces a compromise between filtration time and a filter's ability to remove 95% of the phytoplankton. Glass fiber filters permit fast filtration of large volumes but are inadequate for retaining the small cells present in oligotrophic phytoplankton populations. Sub-micron membranes retain picoplankton but require considerably longer filtration times even for small volumes. We suggest that filtration of any kind will not solve this dilemma, that filtration is simply a carry-over from earlier methods and given the increased sensitivity of fluorescence measurements (several orders of magnitude) chlorophyll can be measured on unfiltered, whole water samples.

During filtration, phytoplankton are removed from sample water by concentration on a filter. The filter is then placed in an aqueous solvent, such as acetone, for pigment extraction. Why not allow the sample water to occupy the aqueous fraction of the solvent? Figure 6 (redrawn from Schertz and Merz, 1928) shows the extraction efficiency with increasing aqueous fraction of three solvents for chlorophyll from stinging nettles. Our investigations (solid symbols ▲, ■) confirm that 85% acetone permits introduction of the largest volume of water to a solvent while maintaining 100% extraction efficiency.

The advantage of a whole water technique lies primarily in avoiding many of the pitfalls associated with filtration: no filtration time is required, no mechanical damage of cells under vacuum stress (Platt *et al.*, 1983) and no sample fraction is missed. Phytoplankton are sampled and extraction begins immediately, manipulations are minimized and losses due to transfer of extract, such as with homogenization, are avoid-

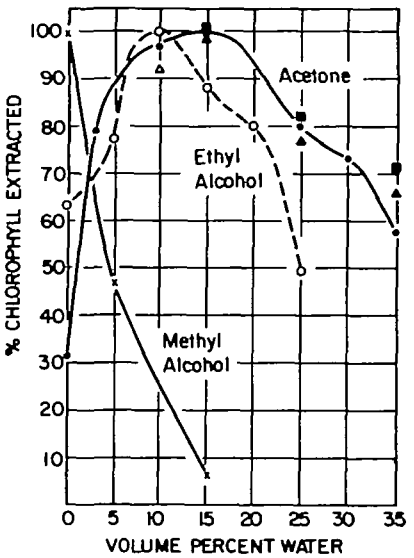

Fig. 6. Extraction efficiency of three organic solvents with increasing percent aqueous fraction. (Redrawn from Schertz and Merz, 1928). Solid symbols (▲,■) represent whole water technique in acetone.

ed. Automated chlorophyll analysis has been hindered by the complexity of multiple filtration, removing this cumbersome step greatly simplifies the design of an automated system.

There are, of course, limitations to the method. By allowing the sample water to occupy the aqueous portion of the solvent, restrictions are placed on the amount of sample used. Figure 6 shows that 85% acetone provides maximum extraction efficiency and sample size. Methanol is not suitable for the present method, as introduction of an aqueous fraction reduces extraction efficiency. Thus, sample size can be only 15% of the extract volume. While absolute sample and solvent volumes can be increased, extract concentration remains constant as cost per sample increases. We have chosen a 1.5 ml sample volume to minimize cost and physical sample dimensions. However, such a small volume introduces sampling problems for large cells of less than a few thousand per liter. We have found that the variance of natural population samples can be greatly reduced by pre-screening replicate samples through 100 μm Nitex mesh.

When measuring extremely low fluorescence levels, treatment of the extract is particularly important if maximum precision is to be realized. Large extract temperature changes, the presence of any scattering particles (i.e., precipitate) or unmatched cuvettes, can cause large changes in fluorescence signal.

Two commercially available fluorometers have been modified to obtain a lower detection limit of 0.1 μg/l *in situ* concentration (0.02 μg/l extract concentration). Therefore, this method is usable in all but the most oligotrophic regions. As oligotrophic samples are most affected by the inadequacies of filtration, increasing the sensitivity of fluorometers is necessary. The low excitation energy supplied by the coated fluorescent lamps used in filter fluorometers is probably responsible for the detection level. Instruments using mercury arc lamps or laser sources are capable of measuring the

fluorescence of a single cell. Advancing the optical design could improve the sensitivity of fluorometers by a factor of ten such that whole water extraction would be suitable for all regions. Nusch and Koppe (1981) describe a submersible fluorometer equipped with a high energy flash lamp excitation source which provides high sensitivity and linearity over a wide range of chlorophyll concentrations. Other similar instruments have been developed (G.Kullenberg, personal communication).

The introduction of sophisticated optical instruments to aquatic sciences has created the need for development of microtechniques capable of exploiting new avenues of interest. A flow cytometer need sort only $100-1000$ cells within the range 2×10^{-12} -2×10^{-13} g Chl *a* cell^{-1} into a 10 ml extract to meet the detection limit of the present technique. Continuous automated measurement of extracted chlorophyll in a manner similar to an auto-analyzer is also possible, coupled with *in vivo* fluorescence, nutrients, temperature and salinity. The amount of material needed for traditional bulk measurements prohibits their use with these types of systems.

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