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Improved Fluorescent Microscopy for Measuring the Standing Stock of Phytoplankton Including Fragile Components

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Abstract

Fluorescent microscopy has been used to separate autotrophic from heterotrophic microorganisms in environmental studies. We report on an improvement on this method for measuring standing stock of phytoplankton. Our method employs a membrane filter and can measure numbers of living phytoplankton including fragile flagellates and size of phytoplankton cells. This method was successfully used in Tokyo Bay and Kuroshio regions.

Introduction

Data of standing stock and composition of phytoplankton are vital for the study of aquatic ecosystems. Direct observations under a microscope have routinely been carried out to enumerate and identify phytoplankton population. However, there are some technical difficulties still to be solved in this method. One of the major problems is to distinguish phytoplankton from zooplankton and organic detritus. Another is that existing methods often destroy fragile flagellates in samples; it is well recognized that fragile flagellates play important roles in phytoplankton communities in tropical and subtropical area (Tsuji and Adachi, 1979).

Wood (1956, 1962) and Wood and Oppenheimer (1962) applied fluorescent microscopy in counting marine plankton by staining living organisms with acridine orange according to Strugger's method (1948). Chlorophyll-bearing organisms were easily detected by autofluorescence of chlorophylls. Therefore, their method was excellent for the purpose of discriminating photosynthetic microorganisms from non-photosynthetic microorganisms and organic detritus. Though this method was promising, it had some short-comings: (1) The danger of destroying fragile phytoplankton was hardly considered; (2) Preservation of a sample was impossible; (3) Samples of low phytoplankton density were difficult to study. After their studies, several authors (Coulon and Alexander, 1972; Brock, 1978) also adopted the fluorescent microscopy for the counting of phytoplankton. However, their methods still had some shortcomings, such as in a preservation of chlorophyll fluorescence of samples.

Our new method of fluorescent microscopy solves these three problems and has the following advantages: (1) Samples can be easily handled in the field, such as on a ship. (2) Samples can be observed not only by fluorescent microscopy (either transmitted or epi-type) but also by optical, phase contrast and Normarski's differential interference contrast (DIC).

Materials and Methods

Pre-examination of the Procedure

Cultivation of Pavlova lutheri. A small (6 to 10 μm in diameter) Haptophycean microorganism *Pavlova lutheri* (Droop, 1953, Green 1975) was used. Since this organism has very fragile membrane systems, the effects of the fixation should be clearly observed. The organism was cultured under a fluorescent lamp at 20 °C in a 100 ml Erlenmeyer flask. An enriched seawater medium (CS medium; Blankley, 1971) was used for culture and tests.

Examination of Fixatives. Among fixatives examined, glutaraldehyde and paraformaldehyde (both of them for electron microscopy) were found to be suitable for fluorescent microscopy, since they did not cause

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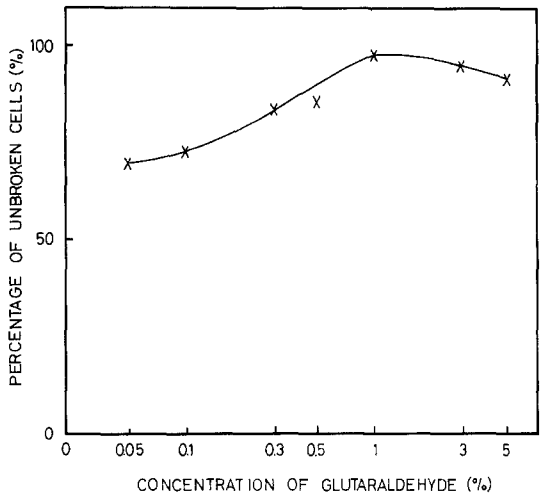

Fig. 1. *Pavlova lutheri.* Percentage of unbroken cells plotted on concentration of fixative. Neutral glutaraldehyde was added to the cells and stirred for 20 s with a tube buzzer. After a 60-min incubation, the numbers of unbroken cells were enumerated twice or three times on a Thoma haemacytomter under a microscope

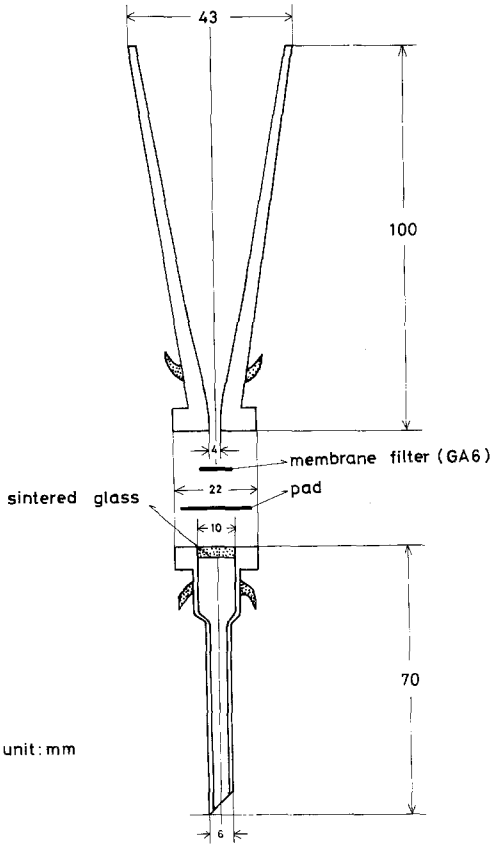

Fig. 2. The filtering apparatus of pyrex glass. Sintered glass was used to obtain a homogenous distribution of plankton on the filter. The pad was a Nuclepore (pore size: 8.0 μm) or Sartorius (pore size: 8 μm, SM 11301) membrane filter

phytoplankton cells to lose fluorescence of chlorophylls. The mixture of these 2 fixatives was investigated. The percentage of unbroken cells after an addition of mixture of fixatives was used as an index for the evaluation of the mixing ratio of these 2 fixatives. One percentage (V/V) glutaraldehyde (Nakarai Co. LTD, Kyoto, Japan) gave the maximal percentage of unbroken cells (96%) in both *Pavlova lutheri* (Fig. 1) and *Prorocentrum triestinum.* When paraformaldehyde (Taab Lab., Reading, England), whose concentration is between 0.03 and 1% was further added to 1% glutaraldehyde solution, the maximal percentage of unbroken cells approached 100%.

Filtration of Fixed Samples. The filter should be densely covered by cells, because a statistically sufficient number of cellular images (at least 300 cells) must be in one frame of the film. We devised a filtering apparatus (Fig. 2) so that the plankton samples could be collected in a very small circular area ($\phi = 4$ mm) on the membrane filter (Gelman GA 6). Usually, 4 ml of sample water from eutrophic regions and 30 ml from oligotrophic regions were sufficient.

Cleaning the Membrane Filter. Plankton cells were filtered on a membrane filter after fixation. For microscopic observation, the membrane filter should be transparent so as not to disturb the light path. We found that the combination of Gelman triacetate metricel filter (GA 6: pore size = 0.45 μm; refraction index: 1.47) and glycerol was the best for this purpose. One advantage of the use of glycerol was that it did not require the drying procedure of the filter prior to its application; therefore, the cells were not destroyed by drying.

Mounting the Filter. Glycerine jelly was used as a mountant. The following preparation was found to be the best: twenty ml of deionized water is added to 10 g gelatine (gelatine white extra fine DAB 7, Merck) and heated to 80 °C. After dissolving the gelatine, 50 ml of hot glycerol is added and mixed. All steps should proceed slowly in a hot incubator (ca 80 °C) to prevent air bubbles from being caught in the mountant. The preparation should be stored in a refrigerator until use.

Discrimination Between Living and Dead Phytoplankton. Chlorophyll fluorescence of phytoplankton decays gradually after their death. Therefore, we can discriminate living and dead phytoplankton according to the intensity of their chlorophyll fluorescence. Three algal strains were used to measure the velocity of the decay of the fluorescence. After the culture medium was replaced by seawater, cells were killed by dipping culture flasks into a 50 °C water bath for 2 min. Fig. 3 shows the time-course of the intensity of chlorophyll fluorescence after the kill. Though the fluorescence of phytoplankton decreased about 30% just after death, it might be difficult to recognize this reduction visually. The reduction of 70% which occurred 24 h after the

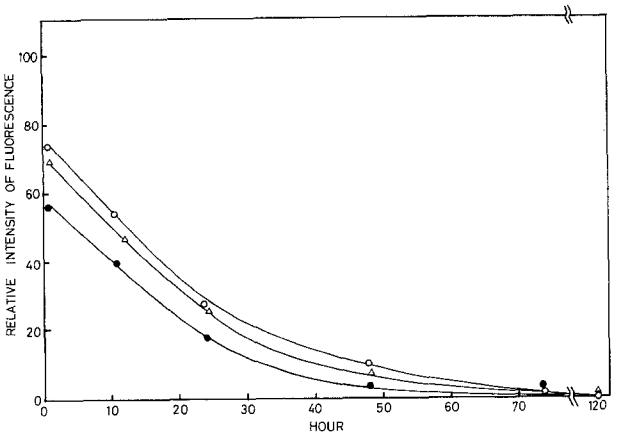

Fig. 3. *Pavlova lutheri* (o), *Chaetoceros* sp. (Δ; Bacillariophyceae), and *Syracosphaera carterae* (•; Haptophyceae). Decay of fluorescence by heat-killed suspended cells (relative intensity before heat treatment is 100). The intensity was measured by a fluorescence spectrophotometer (Hitachi 204S) at the wave length of 683 mμ (the excitation wave length: 410 mμ)

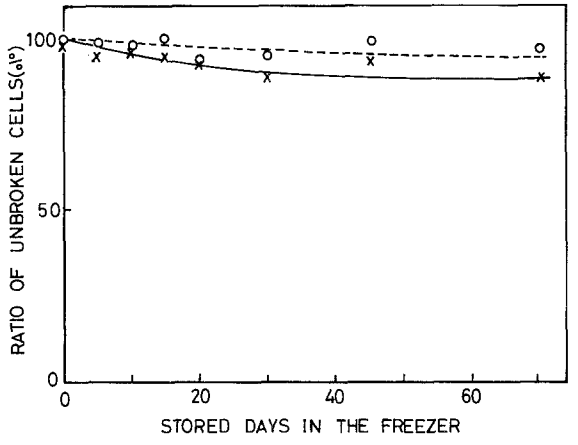

Fig. 4. *Pavola lutheri.* Decrease in the cell number having red chlorophyll fluorescence (x) and in the intact cells (o) in the preparation after storage at −20 °C. Value at the beginning of the storage is 100%

kill, however, must be much easier to notice. Therefore, the 70% decrease in the fluorescence was used in this method as a criterion to distinguish dead phytoplankton from living ones.

Preservation of the Preparation. A test was carried out to time the process of cell destruction in the preparation during the preservation at −20 °C (Fig. 4). The cells which lost chlorophyll fluorescence during two-month-storage were only about 10%. Frequent freezing and thawing of the preparation did not damage the cells; the glycerol might act as a protective reagent (Lusena, 1955). Thus, it was shown that the preparation can be stored for at least two months at −20 °C in a desiccator. Further, preservation of the preparation at −20 °C for nearly half a year did not cause a further decrease in the fluorescent cell number, therefore, preservation for up to half a year was thought to be safe.

Procedure of Measuring Standing Stock of Phytoplankton

Based on above experiments the following procedure was established.

Preparing the Microscopy. One gram of paraformaldehyde powder is dissolved in 50 ml of distilled water at 65 °C by continuous stirring. To clear the solution, 1 to 3 drops of 1N NaOH solution is added while stirring. The solution may still be a little cloudy; however, further addition of NaOH should be avoided. The solution is cooled and then mixed with 40 ml of 25% glutaraldehyde, and the pH is adjusted to 7 with NaOH or HCl. Finally, the volume of the mixture is adjusted to 100 ml by the addition of water. The concentrated fixative mixture is filtered by a membrane filter (GA6) and should be stored in a refrigerator. This mixture of one ninth of the volume of a sample is added to the sample and shaken. After incubation of > 1 h in a refrigerator, the sample is filtered on a Gelman triacetate metricel filter (GA6). A small lump (volume. ca 1 mm^3) of the solid mountant is put onto a cover glass and melted on a hot plate at 50 °C. The membrane filter with the plankton is put on the cover glass with the plankton side facing the glass and cooled to normal temperature. After placing 1-2 drops of glycerol on the reverse side of the membrane filter, it is dried completely in a desiccator at −20 °C for at least 3 d; water in the mountant and in the glycerol is completely eliminated, and the membrane filter becomes transparent. The desiccator can be stored in a freezer below −20 °C in the dark for up to half a year. The cover glass with a membrane filter is put on a glass slide (for fluorescent microscopy) for microscopic observation with the filter side down, and the edge of the cover glass is pitched with a mixture of paraffin and vaselin (volume. 1 : 1) or with plastic paste, if necessary.

Taking Microphotography. The desiccator for preserving the preparation is warmed to normal temperature (ca 20 °C). The preparation is put under a fluorescent microscope of a transmitted type. In our case, Nikon FL was adapted by changing its half-mirror for optical observation to a normal mirror. However, other types of fluorescent microscopes including epi-fluorescence types are suitable, if they can also be used as an optical microscope in transmitted light. As a fluorescent microscope, a 200W ultrahigh pressure mercury lamp is the light source. The filters at the light source are a BV exciting filter (wavelength of >50% transmission: 370-430 mμ) and a heat absorption filter, and the filter at the oculars in Y-50NF (wavelength of >50% transmission: more than 500 mμ). As an optical micro-

Table 1. *Pavlova lutheri.* Comparison of cell number measured by this method and Coulter counter technique

Series	Coulter counter (A)	This method (B)	A/B × 100
	cells ml^{-1}	cells ml^{-1}	%
1	23.0×10^4	19.3×10^4	120
2		19.7×10^4	117

A: The average value of 20 time measurements of the same sample
B: The average value obtained from 5 different visual fields on the same filter
The actual number of cells counted was 8 258 in Series 1 in our method and was 8 231 in Series 2. *P. lutheri* was cultured in CS medium pre-filtered by an HA millipore filter

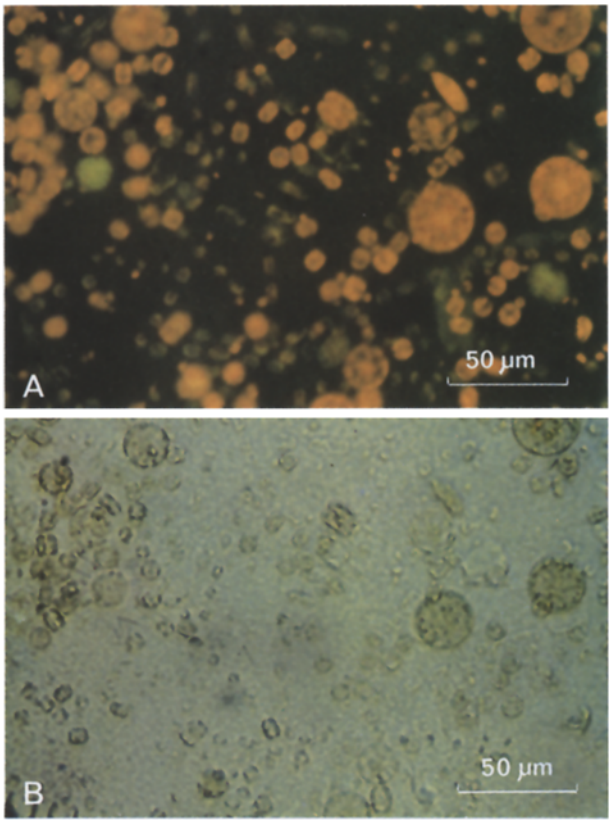

Fig. 5. Phytoplankton population from Tokyo Bay taken by fluorescent (A) and optical (B) microphotographs in the same visual field. The red fluorescence shows the living phytoplankton cells and the green fluorescence shows organic detritus or dead phytoplankton

scope, a 30W tungsten lamp is used for the light source with a color compensating filter. Two photographs are taken from the same visual field: one is a fluorescent photograph and the other an optical photograph. The two photographs are taken by turns of one roll of film (Ektachrome High Speed ASA 160, Eastman Kodak Company, Rochester, NY, USA). Generally, two or three pairs of visual fields are taken on one filter. Final amplification of the microphotograph is ordinally fifty times (objective: x10, ocular: x8, intermediate lens: x1.25, camera: x0.5). All of these procedures can be done at sea by putting an air bag under the microscope, since the exposure time for the fluorescent microscopy is short (2-3 s).

Counting. Pictures of fluorescent and optical microscopies are enlarged on a ground glass plate by a projector. In the fluorescent photograph, the color of phytoplankton is red or orange, while those of zooplankton and organic detritus are green. Therefore, phytoplankton is easily distinguished from zooplankton and organic detritus. The optical (phase contrast, or Nomarski DIC, if necessary) photograph enables us to see clear shapes of phytoplankton. Thus, comparing the fluorescent photographs with the optical photographs, it is possible to enumerate the cell number of phytoplankton, measure their sizes, and identify species of phytoplankton.

Results

The cell number of cultured *Pavlova lutheri* measured by Coulter counter (Type ZbI, Coulter Electronics, Inc., Hialeah, Florida, USA) technique was slightly greater than that measured by this method (Table 1). The difference might have resulted from dead cells and detritus in the sample. In fact, we could observe such materials in the preparation with a microscope. However, it was impossible to count them because most of them were amorphous. It might be estimated from the microscopic observation that their number was more than one tenth of the cultured phytoplankton. Thus, it is not probable that many phytoplankters are lost using our method.

This method was used in Tokyo Bay, Sagami Bay, and the Kuroshio Regions. Frames of both fluorescent and optical microphotographs were taken for individual samples (Fig. 5A, B). When necessary, Nomarski DIC and phase contrast microscopy were used. Cell number, cell length, cell width, and dominant species of phytoplankton were obtained from these microphotographs. We applied this method for freshwater samples from Lakes Naka-numa and Ashino-ko, and found that it was also applicable for freshwater samples.

Discussion and Conclusions

The component of the fixative in our method was mainly selected by using cells of *Pavlova lutheri.* Further, this fixative was found to be the best for dinophycean microorganism *Prorocentrum triestinum.* According to our application of this fixative to more than a few hundred samples from various fields, this fixative was effective for not only *P. lutheri* and *P. triestinum,* but also for very wide range of phytoplankton species including ultraplankton smaller than 5 μm.

In case of freshwater samples the addition of 0.01M calcium (final concentration, calcium chloride form) to the fixative prevented the cells from being fuzzy and made their membrane distinct under a microscope. According to Tooze (1964), the addition of calcium to a final concentration of 0.01M in the fixative completely suppressed haemoglobin extraction from erythrocytes. This addition seems to be unnecessary in case of seawater samples, for 0.01M calcium is originally present in seawater.

For the purpose of making a completely transparent preparation, it is most important to use completely dried silica-gel as a desiccant. If there is even a slight quantity of water in the glycerol, the refraction index of glycerol does not match that of the filter (Gelman GA 6). Further more, when very clear images of plankton cells are essential and when changing cell position on the membrane filter does not disturb the observation, glycerol alone can be used.

According to Watt (1971), Zeiss phase contrast mounting medium W15 could make an HA millipore filter transparent without drying it. He used this technique for the observation of phytoplankton. We tried his technique and found that it often damaged the structure of cells and the fluorescence of chlorophylls. Probably some materials in the cells dissolve in that medium. On the other hand, we could observe such an undesirable effect for concentrated glycerol. This observation is also supported by Cassel's result (1976) in which Ehrlich ascites tumor cells could be held in recoverable state in glycerol at $-60\,^{\circ}C$ for 1 year.

By adapting the photography, we could solve many problems. First, we could clearly recognize green-fluorescent cells difficult to see with the naked eye without staining with fluorochromes, such as acridine orange. The staining of cells containing RNA with acridine orange brought about such difficulties that those cells were hard to separate from chlorophyll bearing cells, for both of them fluoresced red. Second, by the photographic technique and the storage of samples the counting of cells soon after the sampling became unnecessary. Third, decay of chlorophyll fluorescence during the observation could be almost eliminated.

The chlorophyll fluorescence of heat-killed phytoplankton decayed to 30% after 24 h. Thus, we could avoid over-estimation of the cell number by eliminating dead phytoplankters observing the strength of chlorophyll fluorescence. On the other hand, even very fragile phytoplankton such as *Pavlova lutheri* could be enumerated by improving the fixative and by avoiding drying the cells while cleaning the filter. Thus, the main cause of underestimation could be eliminated.

This method was shown to have many advantages. Standing stock of phytoplankton including very fragile species could be measured by eliminating dead cells, even though population density was very low. That the preservation of samples is possible makes the method very easy to use in field. Preparation by this method was observed by many kinds of microscopy, which facilitate identification of species.

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