Hustedt and *Detonula confervacea* (Cleve) Gran. Can. J. Microbiol. **8**: 229–239.

HARGRAVES, P. E., AND R. R. L. GUILLARD. 1974. Structural and physiological observations on some small marine diatoms. Phycologia **13**: 163–172.

HASLE, G. R. 1969. An analysis of the phytoplankton of the Pacific Southern Ocean: Abundance, composition, and distribution during the "Brategg" Expedition, 1947–1948. Hvalradets Skr. **52**: 1–168.

HENTSCHEL, E. 1932. Die biologischen Methoden und das biologische Beobachtungs-material der "Meteor"-Expedition. Dtsch. Atl. *Exped. "Meteor"* 1925–1927. Wiss. Ergeb. **10**: 274 p.

HUSTEDT, F. 1927. Die Kieselalgen. *In* Rabenhorst's Kryptogammen Flora. Leipzig Akad. Verlagsges. **7**(1). 920 p.

LARRANCE, J. D. 1964. A method for determining volume of phytoplankton in a study of detrital chlorophyll *a*. M.S. thesis, Univ. Wash. 107 p.

MENZEL, D. W., E. M. HULBURT, AND J. H. RYTHER. 1963. The effects of enriching Sargasso Sea water on the production and species composition of the phytoplankton. Deep-Sea Res. **10**: 209–219.

MYERS, J. 1953. Growth characteristics of algae in relation to the problems of mass culture, p. 37–54. *In* J. S. Burlew [ed.], Algal culture from laboratory to pilot plant. Carnegie Inst. Wash. Publ. 600.

RILEY, G. A. 1941. Factors controlling phytoplankton populations on Georges Bank. J. Mar. Res. **6**: 54–73.

———, H. STOMMEL, AND D. F. BUMPUS. 1949. *Quantitative ecology of the plankton of the* western North Atlantic. Bull. Bingham Oceanogr. Collect. **12**(3): 169 p.

RYTHER, J. H., AND C. S. YENTSCH. 1957. The estimation of phytoplankton production in the ocean from chlorophyll and light data. Limnol. Oceanogr. **2**: 281–286.

SCHILLER, J. 1930. Coccolithineae. *In* Rabenhorst's Kryptogammen Flora, 2nd ed. **10**(2): 89–273.

STEELE, J. H., AND D. W. MENZEL. 1962. Conditions for maximum primary production in the mixed layer. Deep-Sea Res. **9**: 39–49.

STEEMANN NIELSEN, E., AND A. JENSEN. 1957. Primary oceanic production. Galathea Rep. **1**: 49–136.

STRATHMANN, R. R. 1967. Estimating the organic carbon content of phytoplankton from cell volume or plasma volume. Limnol. Oceanogr. **12**: 411–418.

THOMAS, W. H. 1970. Effect of ammonium and nitrate concentration on chlorophyll increases in natural tropical Pacific phytoplankton populations. Limnol. Oceanogr. **15**: 386–394.

WAILES, G. H. 1939. Canadian Pacific fauna. 1. Protozoa le. Mastigophora. Univ. Toronto.

Submitted: 25 November 1974
Accepted: 21 April 1975

A fluorometric technique for measuring sinking rates of freshwater phytoplankton[1]

Abstract—A recording fluorometer provides a rapid, automatic way to estimate sinking rates of freshwater phytoplankton, the determinations taking from 15 min for populations sinking at 1.0 m day^{-1} to 2 h for populations sinking near the limit of detection, 0.1 m day^{-1}. The shape of the fluorescence output provides information on the distribution of sinking rates about the mean. Measured sinking rates of *Asterionella formosa* Hass. agree well with published data. Sinking rates of fluorescent micronic beads at various temperatures agree with Stokes' law. The technique is applicable to marine phytoplankton, reducing the time needed for a determination two to five times over existing methods and so increasing the sensitivity that measurements may be directly possible for natural water samples.

[1] This study was supported by National Science Foundation grant GB-41315 to P. Kilham.

Most phytoplankton are slightly more dense than lake water, sinking at a measurable terminal velocity in still water. This terminal velocity, termed sinking rate, is a function of the density difference between a cell and its medium, and the size, shape, and orientation of the cell. Lund (1959) provided field evidence for the importance of sinking in the ecology of several species of phytoplankton. The population size and vertical distribution of *Asterionella formosa* Hass. in Windermere, for example, is influenced by its sinking rate and the degree of turbulence in the lake. Steele and Yentsch (1960) demonstrated that the vertical distribution of chlorophyll in natural waters can be explained if sinking rate decreases as phytoplankton reach dark, nutrient-rich

waters. Since sinking rate is affected by the physiological state of a phytoplankter (Eppley et al. 1967; Smayda and Boleyn 1965, 1966; Smayda 1974), the relationship between physiological state, nutrient status, and sinking rate of a species is of ecological importance. For these to be elucidated, controlled laboratory experiments are needed in which sinking rate is determined as the physiological state of a population change.

Eppley et al. (1967) reported a rapid fluorometric technique for estimating sinking rates of marine phytoplankton. They diluted their sample with distilled water, placed this fluid of lower density on seawater medium in a settling chamber, and were able to obtain a stable upper layer from which cells in their sample could settle. As cells settled, the increase in fluorescence was recorded until a plateau was reached. The layering method does not work for freshwater samples. Attempts to layer freshwater samples without using a density difference often result in mixing, which obscures sinking and causes great variability among replicates. Steele and Yentsch (1960) allowed uniformly mixed samples to settle and estimated relative sinking rates from the decrease in optical density. A combination of these two techniques, reported here, allows a uniformly mixed sample of cells to settle in a fluorometer. The decrease in fluorescence as cells sink permits an estimate of sinking rate.

The main advantages of fluorometric sinking rate measurements are high sensitivity and the rapidity and ease with which determinations are made. Eppley et al. (1967) listed the assumptions and disadvantages, including the inability of the fluorometric technique to ascribe variability of sinking rate to individual cell or colony morphological features. Both their method and the one described here provide some information on the variability of sinking rate about the mean. The visual counting technique used by Smayda and Boleyn (1965) for marine phytoplankton and by Smayda (1974) for freshwater diatoms permits a better estimate of this variability and how it can be attributed to differences in cell size and shape but requires the periodic attention of the investigator for 2 to 16 h. Smayda (1970) reviewed various methods of estimating sinking rates.

I wish to thank D. S. Wethey for assistance in the development of this technique and D. S. Wethey, S. S. Kilham, and P. Kilham for comments on the manuscript.

A 10-mm-ID by 75-mm-long fluorometric cuvette is used as the settling chamber. The inner diameter is 2.5× that used by Eppley et al. (1967) and wall effects should be insignificant. The bottom 17 mm and the top 38 mm of the cuvette are painted black; changes in fluorescence caused by sinking are monitored in the 20-mm clear region. The cuvette is held in the temperature stabilized door of a Turner model 111 fluorometer, its temperature maintained by an external water bath regulated to ±0.1°C. Thermal regulation is important since changes in temperature may cause convective mixing in the settling chamber and change the viscosity of the medium. A Turner number 7-60 (blue) is the primary filter; the secondary filter is a Turner number 25 (red). These are standard filters for chlorophyll analysis. The 30, 10, or 3× aperture is used to adjust initial fluorescence readings to approximately full scale; the 1× aperture does not provide full illumination of the settling chamber and could bias results. A sample (about 3 ml) of an algal culture is placed in the settling chamber so that the bottom of the meniscus is level with the upper edge of the open region. The cuvette is placed in the temperature stabilized sample holder for about 5 min to allow it to reach thermal equilibrium. It is then removed, gently inverted several times to suspend the cells uniformly in the medium, and replaced in the fluorometer. Fluorescence is recorded on a chart recorder until it falls to at least half the initial reading. The resulting traces for *Scenedesmus quadricauda* and *Tabellaria fenestrata* are shown in Figs. 1 and 2.

Two quantities need be known for an estimate of sinking rate by this method. One is the average distance through which

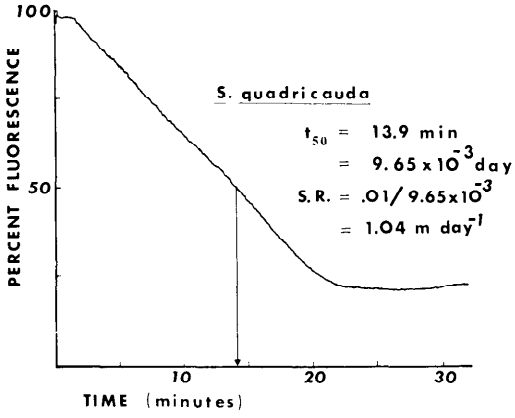

Fig. 1. Fluorescence output produced by an axenic, phosphate limited batch culture of *Scenedesmus quadricauda* (Indiana Culture Collection No. 76) grown at 20°C in a 14 h–10 h light–dark cycle under fluorescent light and allowed to settle at 20°C. Height of the open region of the settling chamber is 0.020 m, giving an average distance cells sink of 0.010 m.

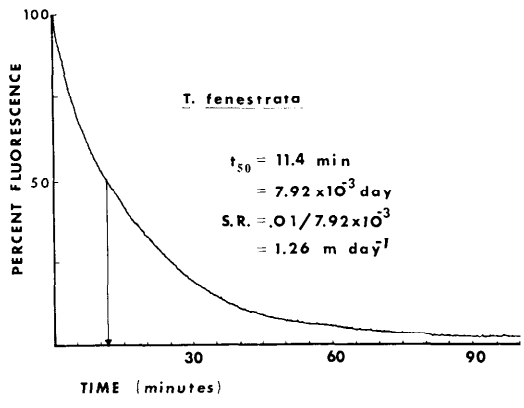

Fig. 2. Fluorescence output produced by an old batch culture of *Tabellaria fenestrata* (S. S. Kilham's clone NHTAB4) with the same conditions as for *Scenedesmus quadricauda* (Fig. 1).

cells sink in the monitored region of the settling chamber. Since the cells are uniformly suspended at the start, that distance is half the height of the monitored zone, or 0.010 m (Figs. 1 and 2). The other quantity is the time needed for half of the cells to sink this average distance. If fluorescence is proportional to cell concentration (an assumption made by Eppley et al. 1967), this can be approximated by the time for fluorescence to fall to 50% of the initial value and is designated t_{50} (Figs. 1 and 2). Sinking rate (S.R.), usually expressed as m day^{-1}, is

$$\text{S.R.} = (\text{average distance cells sink})(t_{50})^{-1} = (0.010 \text{ m})(t_{50} \text{ day})^{-1}.$$

The height of the open region of the settling chamber can be varied. Heights of 10 to 35 mm provide results comparable to those for 20 mm. The 10-mm distance will halve the time needed for a determination but will double the error caused by variability in filling volume; the chamber can be filled with a constant volume to eliminate this variability. The volume used should be such that the bottom of the meniscus is at or above the top of the open region of the cuvette. If the open region is of height X and the bottom of the meniscus is of height Y above the top of the open region, sinking rate is

$$\text{S.R.} = (0.5X + Y)(t_{50})^{-1}.$$

The mean sinking rate of a population is calculated from two measurements: initial fluorescence at time zero and 50% of the initial value at time t_{50}. If two populations have the same mean sinking rate their fluorescence outputs will pass through these two points. However, populations with the same mean sinking rate may differ greatly in the spread of sinking rates of cells about this mean. That difference may be of biological importance since the sinking rate of a particular cell will influence its vertical positioning (Lund 1959) and may influence its growth rate (Munk and Riley 1952).

To investigate how the spread of a sinking rate distribution influences the fluorometer output, I produced a computer model of sinking in the settling device described above. The model accepts as input any distribution of sinking rates and stores it as a histogram with up to 1,000 sinking rate increments. It is assumed that all cells of a particular sinking rate sink at exactly that rate at all times. The only allowance for size, shape, density, and orientation effects is included in the original distribution of sinking rates. The model generates theo-

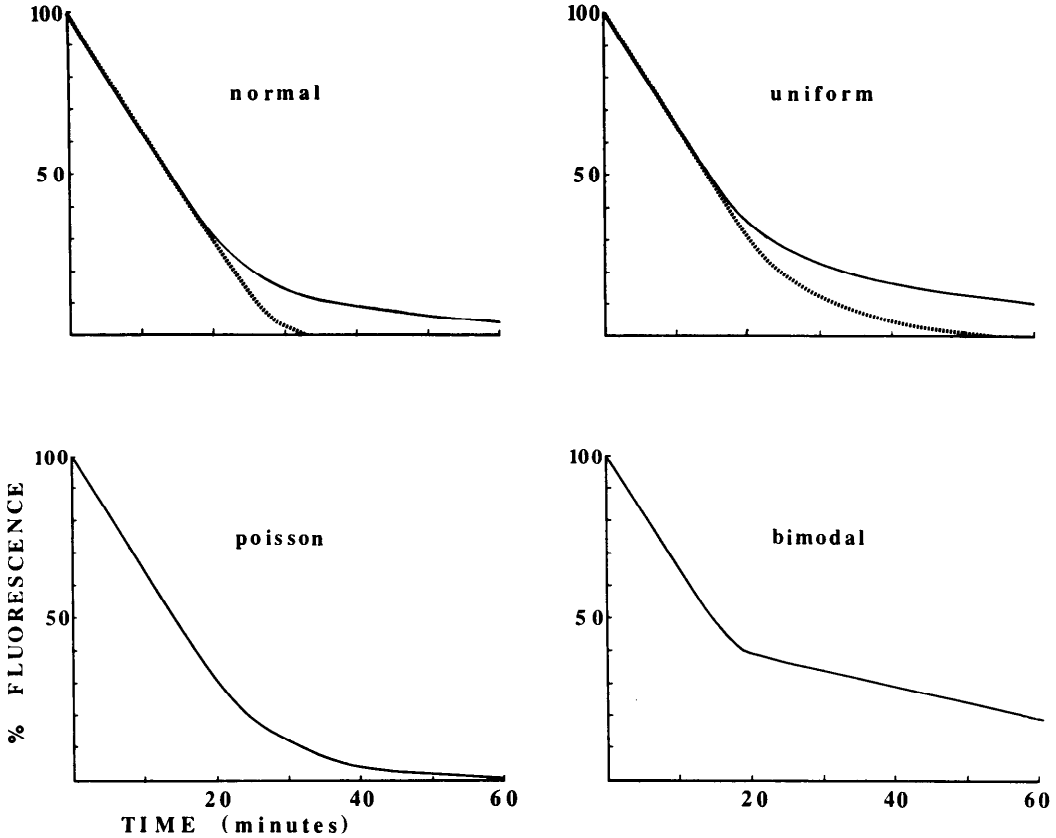

Fig. 3. Theoretical fluorescence outputs produced by a computer model for sinking of populations with the same mean sinking rate (1.0 m day^{-1}) but different distributions and variances (σ^2, m^2 day^{-2}). Normal cases—upper, solid curve has $\sigma^2 = 0.16$; lower, broken curve has $\sigma^2 = 0.02$. Uniform cases—upper, solid curve has $\sigma^2 = 0.32$; lower, broken curve has $\sigma^2 = 0.11$. Poisson case—$\sigma^2 = 1.0$. Bimodal case—two normal distributions, both with $\sigma^2 = 0.14$ and mean sinking rates of 0.30 and 1.70 m day^{-1}.

retical fluorescence outputs for any given sinking rate distribution.

Six such outputs for populations with the same mean sinking rate, 1.0 m day^{-1}, but with different distributions about this mean, are shown in Fig. 3. All these curves have a t_{50} of 14.4 min, corresponding to a mean sinking rate of 1.0 m day^{-1}. The shape of the fluorescence output depends on the distribution used and its spread (variance). In the two cases shown in which a normal distribution is used, the upper curve (from a population with a variance of 0.16 m^2 day^{-2}) diverges from the lower curve (variance of 0.02 m^2 day^{-2}) beyond time t_{50}. The same is true for the two cases shown in which populations had uniform sinking rate distributions; the upper curve is from a population with a higher variance (0.32 m^2 day^{-2} vs. 0.11 m^2 day^{-2}). The Poisson-like distribution with variance of 1.0 m^2 day^{-2} illustrates that fluorescence outputs from populations with the same mean and different variances cannot necessarily be compared if their type of distribution is not known. The fluorescence output produced by a bimodal distribution of sinking rates is qualitatively different from those produced by other types of distributions: it has two characteristic linear regions with a transition zone between. In the case shown, each of the normal distributions contains 50% of the population and the transition zone occurs in the region of 50%

fluorescence. If a population consists of two normal distributions with different modes, the proportion in each distribution and the mean sinking rate of each distribution can be estimated. (Details on request.) I have found several cases in which an algal population had two regions of linear fluorescence with a transition zone which may be caused by a bimodal distribution of sinking rates.

Although the sinking rate distribution of a population determines the shape of the fluorescence output, the mapping of a particular output back to the original sinking rate distribution is only possible if one assumes a particular type of distribution (normal, uniform, bimodal normal, etc.). I have no a priori reason to make such an assumption. If independent evidence indicates that a particular type of distribution is likely, the variance of the sinking rate distribution can be estimated by an iterative curve matching process. If one assumes that the same species will always have the same type of distribution, outputs can be ranked by the variance of the sinking rate distribution. Since the fluorescence outputs from populations with the same mean but different variances differ more the longer a run proceeds, experiments to estimate variance should continue until fluorescence is almost zero, as in the results for *T. fenestrata* (Fig. 2). This greatly lengthens the time needed for a determination.

The fluorometric technique has been used to measure sinking rates of various species of diatoms and green algae under differing physiological and environmental conditions. It has also been used to measure buoyancy in blue-green algae (Fig. 4). The results for *A. formosa* Hass. (Lund's clone "L262") are reported for comparison with those of Lund (1959), Smayda (1974), and Fritz (1935) (Fig. 5). Mean sinking rate calculated for exponentially growing cultures ($n = 6$) of *A. formosa* at 20°C is within the range reported by Smayda (1974) for all cultures at 15°C, but is significantly lower than that reported by Fritz (1935) at 20°C. The mean sinking rate for stationary cultures ($n = 7$) at 20°C is not significantly different from the rate reported by Fritz (1935) at 20°C. The significantly higher sinking rate of *A. formosa* in the stationary than in the exponential growth phase reported here agrees with a general trend of increased sinking rate with increased culture age, reported by Smayda (1974), Eppley et al. (1967), Smayda and Boleyn (1965, 1966), and others for marine and freshwater species. The apparent increase in sinking rate from 0°C to 20°C can be partially explained by temperature dependent changes in the viscosity and density of water. Since the measurements were made on different clones of *A. formosa* under different physiological conditions with different methods, it is not

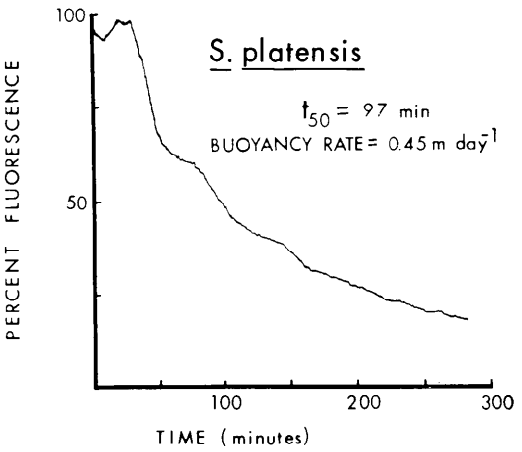

Fig. 4. Measurement of buoyancy rate for the blue-green alga *Spirulina platensis* (Nordst.) Geitl., clone OPL-1, isolated from L. Nakuru, Kenya, by S. S. Kilham. The cuvette used had the lowest 21 mm painted black, the middle 18 mm open, and the top region painted black. The cuvette was filled to more than 10 mm above the open region to provide a region in which buoyant cells would not be sensed by the fluorometer. Removal of the top 10 mm of the medium in the settling chamber at the end of the experimental run showed numerous cells present. Agitation of medium remaining in the settling chamber followed by measurement in the fluorometer indicated that essentially all the decrease in fluorescence was due to buoyancy, not sinking of the blue-green algae. The average distance cells rise in the chamber is (0.5) (18) + (21), or 0.030 m. This distance divided by t_{50} gives the buoyancy rate.

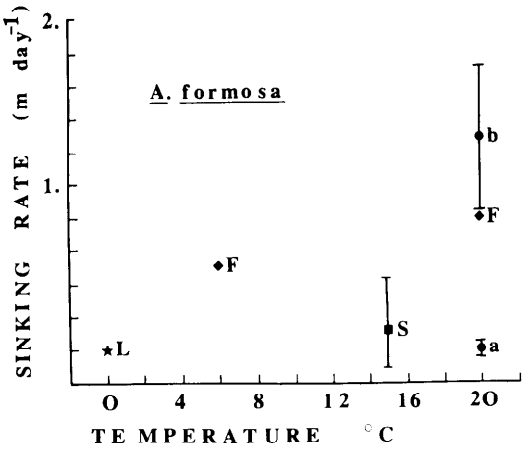

Fig. 5. Reported sinking rates of *Asterionella formosa* compared with my work. Data are from Lund 1959 (L), Fritz 1935 (F) and Smayda 1974 (S). Smayda's data are represented as mean ($n = 22$) and range. My data are mean (a) and standard error for exponentially growing cultures ($n = 6$) and mean (b) and standard error for stationary phase cultures ($n = 7$).

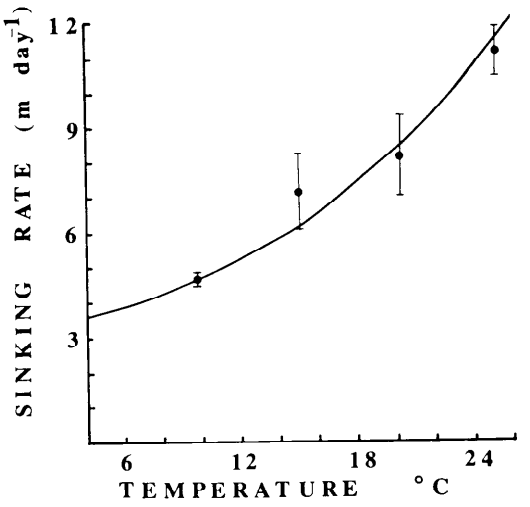

Fig. 6. Comparison of the theoretical relationship between sinking rate and temperature for micronic beads (solid curve) with actual measurements shown as mean and standard error. At each temperature, replicate determinations of sinking rate of the fluorescent micronic beads were performed with the fluorometric technique. For 10°C, $n = 4$; 15°C, $n = 3$; 20°C, $n = 5$; 25°C, $n = 4$.

possible to determine the relationship between sinking rate and temperature from these data.

As a further check on the technique, I measured sinking rates of fluorescent (pink) micronic beads (Ionics Inc., Watertown, Mass.) in the 20–30-μ range at four temperatures in distilled water and compared them with the theoretical relationship obtained from Stokes' law using the viscosity and density of water at those temperatures. Micronic beads were used because their density (estimated 1.04 g cm^{-3}) should not be significantly affected by temperature in the range studied (10–25°C) and because their spherical shape avoids problems of differing form resistance. There is close agreement between the theoretical and actual results (Fig. 6), indicating that the fluorometric method can be used to study the relationship between temperature and sinking rate for algal populations.

The method described here is the only rapid, automatic one reported for freshwater phytoplankton and is directly applicable to measurement of sinking rates of marine phytoplankton. The method reported by Eppley et al. (1967) requires that fluorescence be recorded until all cells have settled 33 mm, the entire height of the settling chamber. For cells sinking at exactly 1.0 m day^{-1}, 48 min are required. If there is any spread of sinking rates around the mean, the time required will increase: if the range about the mean of 1.0 m day^{-1} were from 0.5–1.5 m day^{-1}, about 95 min would be required for the plateau to be reached. With the method reported here, the determination of mean sinking rate would take 15 min regardless of the spread of sinking rates about the mean of 1.0 m day^{-1}. Even if the height of the settling chambers used in these two techniques were the same, the method of Eppley et al. (1967) would require at least twice as long. This technique is also more sensitive than theirs since the entire settling chamber may be filled with phytoplankton. If the highest sensitivity setting on the fluorometer (30×) and the maximal height of the open region of the settling chamber (ca. 35 mm) were used, it might be possible to make sinking rate determinations on natural freshwater and marine samples.

David Titman

Department of Zoology
University of Michigan
Ann Arbor 48104

References

EPPLEY, R. W., R. W. HOLMES, AND J. D. H. STRICKLAND. 1967. Sinking rates of marine phytoplankton measured with a fluorometer. J. Exp. Mar. Biol. Ecol. **1**: 191–208.

FRITZ, F. 1935. Über die Sinkgeschwindigkeit einiger Phytoplanktonorganismen. Int. Rev. Gesamten Hydrobiol. **32**: 424–431.

LUND, J. W. G. 1959. Buoyancy in relation to the ecology of freshwater phytoplankton. Brit. Phycol. Bull. **7**: 1–17.

MUNK, W. H., AND G. A. RILEY. 1952. Absorption of nutrients by aquatic plants. J. Mar. Res. **11**: 215–240.

SMAYDA, T. J. 1970. The suspension and sinking of phytoplankton in the sea. Oceanogr. Mar. Biol. Annu. Rev. **8**: 353–414.

———. 1974. Some experiments on sinking characteristics of two freshwater diatoms. Limnol. Oceanogr. **19**: 628–635.

———, AND B. J. BOLEYN. 1965. Experimental observations on the flotation of marine diatoms. 1. *Thalassiosira* cf. *nana*, *Thalassiosira rotula* and *Nitzchia seriata*. Limnol. Oceanogr. **10**: 499–509.

———, AND ———. 1966. Experimental observations on the flotation of marine diatoms. 2. *Skeletonema costatum* and *Rhizosolenia setigera*. Limnol. Oceanogr. **11**: 18–34.

STEELE, J. H., AND C. S. YENTSCH. 1960. The vertical distribution of chlorophyll. J. Mar. Biol. Assoc. U.K. **39**: 217–226.

Submitted: 2 December 1974
Accepted: 17 April 1975

Direct counts of aquatic bacteria by a modified epifluorescence technique[1]

Abstract—Various excitation and emission filters, microscope lamps, membrane filters, and field storage techniques used for direct counting of bacteria by epifluorescence microscopy have been compared. A rapid, simple modification of the Acridine Orange procedure using a new light filter combination, Sartorius cellulose membrane filters, and distilled water rinse gave the brightest cell fluorescence, darkest background, least fading, and highest counts for both estuarine and freshwater samples. Field fixation with Formalin permits storage of bacterial samples for 1 to 2 weeks without changes in bacterial numbers.

Direct counts are still the only reliable way to estimate the numbers and biomass of bacteria in natural waters. The usual method is to concentrate the bacteria onto membrane filters, stain them, clear the filter, and then count by phase contrast microscopy (Sorokin and Overbeck 1972). However, it is difficult to distinguish small coccoid bacteria from other particles and much experience is needed to obtain good precision. Much better resolution of the small bacteria is obtained by using fluorescent stains and viewing with epifluorescent illumination (Francisco et al. 1973).

Fluorescence techniques have been developed empirically without a complete understanding of fluorochrome function. As Jones' (1974) comparison of stains demonstrates, the techniques are sensitive to small changes in procedure. To maximize both cell counts and counting reproducibility, we have compared various microscope lamps, excitation and emission filters, membrane filters, and sample storage techniques, using both freshwater and estuarine samples.

The basic method is that of Francisco et al. (1973) as modified by Jones (1974). Briefly, the bacteria are stained with Acridine Orange in solution, filtered onto a membrane filter low in background fluorescence, and counted while still moist. This has the advantage that many very small (0.2–1.0 μm), faintly fluorescing bacteria can be counted, most of which are not seen at all when white membrane filters are dried, cleared, and viewed in transmitted light.

We are grateful to M. Nahmacher and D. Gann for advice and for the use of the instruments and facilities of E. Leitz, Inc., and to S. Jasper for discussions and technical assistance.

[1] This research was supported by NSF grant GV3912 (U.S.I.B.P. Biome Program) and Environment Canada (Kamloops Lake Study).