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Evaluation of an Automatic Technique for Counting Unicellular Organisms[1]

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ABSTRACT

The Coulter counter, originally designed for high-speed counting of blood cells, was used in this study for quantitative estimation of several species of unicellular algae. Tests of the instrument's reliability and the reproducibility of its counts have given satisfactory results. The instrument is also well suited for use in the study of growth curves of unicellular algae. The instrument's limitations and sources of error are discussed.

INTRODUCTION

The procedure of enumerating unicellular organisms has usually employed one or more of several methods, most of which involve counting small subsamples under a microscope; in the case of red and white blood corpuscles, cell numbers were estimated by using the haemocytometer. Such techniques are time consuming and of limited accuracy. Several attempts to reduce time and labor have been made by using simple techniques such as relative attenuation of light by cell suspension, or complicated systems such as "flying-spot" re-

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cording microscopes; in general, the former have lacked adequate accuracy, and the latter have proved too expensive.

A recently designed electronic device—the Coulter counter—seems to provide accurate counts, with considerable economy of time. This instrument was originally designed for high-speed counting of blood cells (Coulter, 1953). Its efficiency as a blood-corpuscle counter has been well established (Brecher *et al.*, 1956; Mattern *et al.*, 1957; Richar and Breakell, 1959; Grant *et al.*, 1960). The suitability of the counter in determining the number and size of algal cells has been shown by Maloney *et al.* (1962), and its usefulness in the counting and sizing of unicellular marine organisms has been indicated by Hastings *et al.* (1962).

The purpose of this study is to evaluate the Coulter counter as a research tool in counting unicellular organisms and to define its limitations.

MATERIALS AND METHODS

The Coulter Counter. The Coulter counter (Model A) is based on the principle that cells, compared to a saline solution, are poor electrical conductors. The cells are suspended in an electrically conductive medium, and as they pass through a small aperture, each cell displaces its own volume of saline solution and causes a drop in the voltage due to an increase in the aperture impedance. The larger the cell, the greater is the voltage drop. The resulting impulses are amplified, recorded on a decade counter, and visualized on an oscilloscope. The flow of the suspension through the orifice is controlled by an external vacuum and a mercury manometer. When the vacuum is cut off, the mercury is forced to return to its original level; thus a constant flow of the suspension is maintained, and the counter is successively activated or stopped by contact with two electrodes embedded in the manometer tube (Fig. 1). These contacts are so placed that the cells in 0.5 ml of suspension are counted. The counting period is approximately 15 sec. Only when two or more cells pass through the aperture at the same time is correction necessary. The coincidence loss is reduced by dilution to such a point that coincidence is negligible. Mattern *et al.* (1957) have indicated that the coincidence loss due to doublets, triplets, etc., can be expected to follow the Poisson distribution. Correction for the various aperture sizes is made by reference to the correction tables supplied with the instrument.

Cultures Used. Several algal species and one dinoflagellate were used in this study: *Amphora exigua*, *Chlamydomonas* sp., *Chlorella pyrenoidosa* (fresh water), *Chlorella* sp. (marine), *Nitzschia closterium*, *Platymonas* sp., *Porphyridium* sp., *Prasinocladus* sp., *Scenedesmus quadricauda*, *Peridinium trochoideum* (a dinoflagellate). Cultures were grown in a pure unialgal condition; purity was tested by inoculation into bacteriological medium and by microscopic examina-

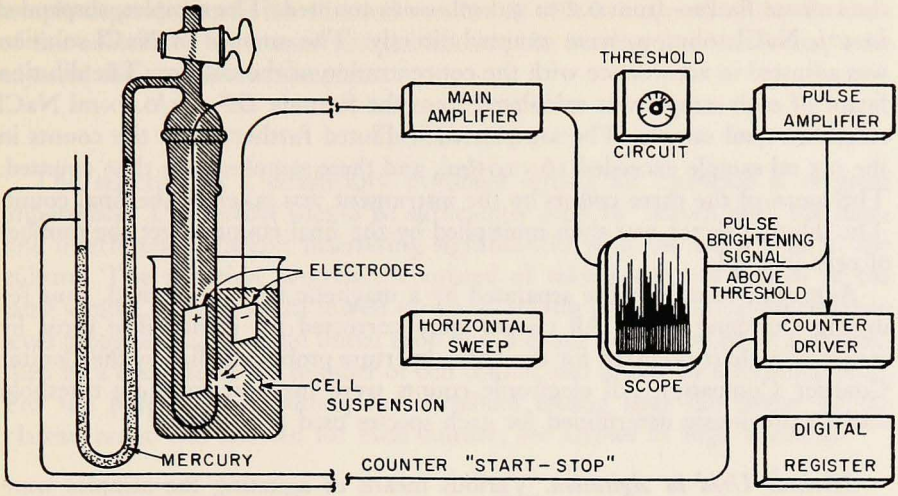

Figure 1. Schematic diagram of the electronic particle counter.

tion. Sterilization was carried out by autoclaving at 121°C for 15 minutes at 15 pounds of pressure. Each Erlenmeyer flask of 125 ml contained 50 ml of the growth medium with 2.0% inoculum of an actively growing culture; this was incubated at a constant temperature of 17°C and under continuous fluorescent lighting of 200 ft-c intensity. The culture flasks were agitated by means of a rotating table shaker.

For the growth curve experiments, 150-ml aliquots of culture medium were placed in 250-ml Erlenmeyer flasks. At zero time, 1.0 ml of the old culture was aseptically pipetted directly into each of the eight flasks of fresh medium. This division permitted agitation under the same conditions described above. All flasks were treated identically. At predetermined time intervals, aliquots of approximately 30 ml were withdrawn aseptically from one flask. Only four such samples were taken from any one flask so as not to reach a minimum concentration of the medium.

In the experiments reported herein, a 100-μ diameter aperture was used. The counting medium was isotonic sodium chloride, which gave a low background due to the absence of countable particles.

Counting Procedure. Samples drawn from culture flasks were enumerated by using both the haemocytometer and the electronic counter. With the haemocytometer, algal samples were counted in the five groups of 16 small squares. In most cases the counts were made at a magnification of 430X. When the counts exceeded 105 cells/ml, the samples were appropriately diluted and counted.

With the Coulter counter, a measured amount of culture withdrawn from

the culture flasks—from 0.2 to 5.0 ml—was counted. The samples, suspended in 1% NaCl solution, were counted directly. The amount of NaCl solution was adjusted in accordance with the concentration of the culture. The dilution factor of each sample was calculated from the formula $DF = 1/0.5 \times$ ml NaCl solution $\times 1/$ml sample. The samples were diluted further when the counts in the 0.5 ml sample exceeded 16×10^4/ml, and these samples were then counted. The mean of the three counts by the instrument was taken as the final count. The dilution factor was then multiplied by the final count to get the number of cells in 1 ml.

Aggregate samples were separated by a magnetic stirring method, thus reducing clumping error. All counts were corrected for coincidence error by means of a chart prepared for the 100-μ aperture probe (supplied by the Coulter Counter Company). All electronic counts were made at optimum threshold settings previously determined for each species used (see below).

Methods Used in Agitation. Various means of agitating the samples were tested. If an electric stirrer was used, the agitator shaft had to be of an insulating material to avoid electrical noise pick-up from the shaft. Also, the electric motor should be a brushless one with a grounded housing. Even with these precautions, there is the possibility that some electrical interference may produce false pulses.

Agitation with a mechanical stirrer gave satisfactory performance. The stirrer was made of an agitator shaft with a cylindrical turbine, the top of which was turned by compressed air from an oxygen tank. A constant flow of air was regulated so that the agitator shaft maintained a constant speed, this being adjusted to a minimum in order to prevent introduction of an excessive amount of dissolved air or air bubbles that might give false counts. The agitator shaft performed best when located on the outer edge of the sample beaker. By using compressed air to turn the agitator shaft, the possibility of electrical interference was removed; thus the operation of the counter was not impaired by the presence of a bulky motor next to it.

Difficulties Encountered in Counting Procedure. Clumping of particles and blockage of the aperature by lint and dust particles caused considerable trouble. Numerous tests showed that if a time-lapse was permitted between the preparation of the sample and its counting, the algal cells tended to clump. Also, lint and air particles in the sample caused blockage of the aperture during counting. However, by placing a sample under the probe immediately after agitation, much of this difficulty was avoided.

The use of Tween to lessen the surface tension on various samples was tested, but with no great success. Immediate preparation of the sample together with magnetic stirring and continued agitation seemed to be the best method to overcome the difficulties of clumping. The speed necessary for stirring the

sample depended upon the size and number of cells in suspension. As the sizes of the unicellular algae used became known, the stirring speed and time were adjusted for the various samples.

RESULTS

The selection of a satisfactory threshold setting for counting is of great importance. The setting should be sufficiently high to "screen out" the base-line interference without interfering significantly with the small cells in the culture. The selection procedure consisted of taking repeated counts of the same sample, starting at the lowest threshold setting and increasing the response level progressively. A rapid initial drop in the count was followed by a plateau, then by a decrease in the number of cell counts, and finally by a terminal drop. For the purpose of counting, the threshold setting near the center of the plateau range was selected for each culture; see arrows in Figs. 2 and 3.

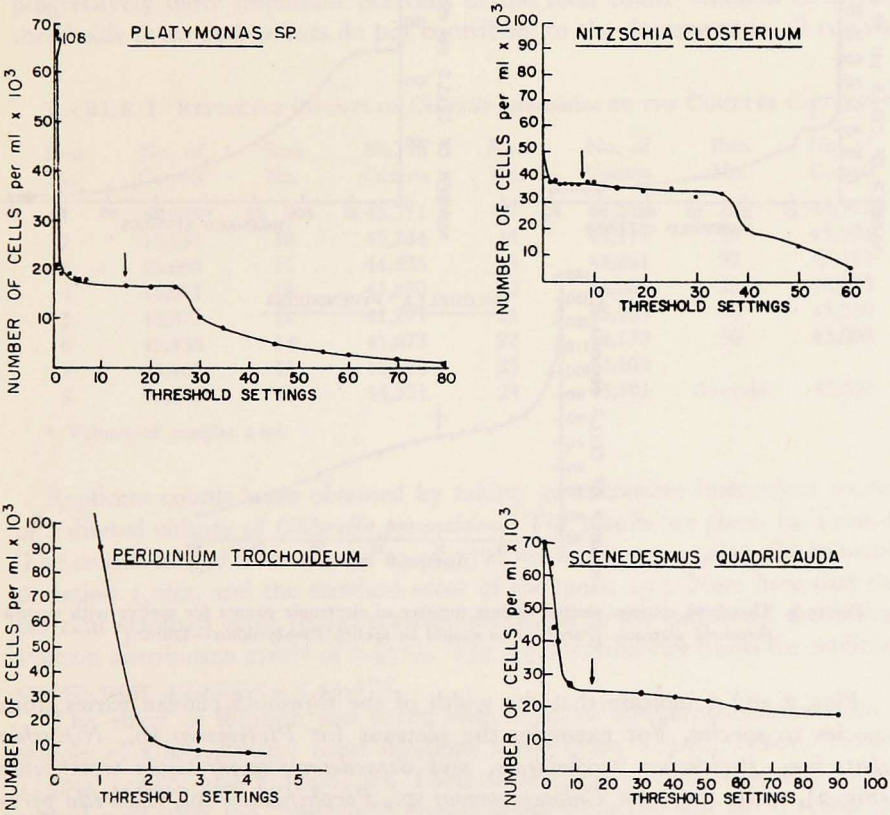

Figure 2. Threshold settings plotted against number of electronic counts for species with wide threshold plateaus.

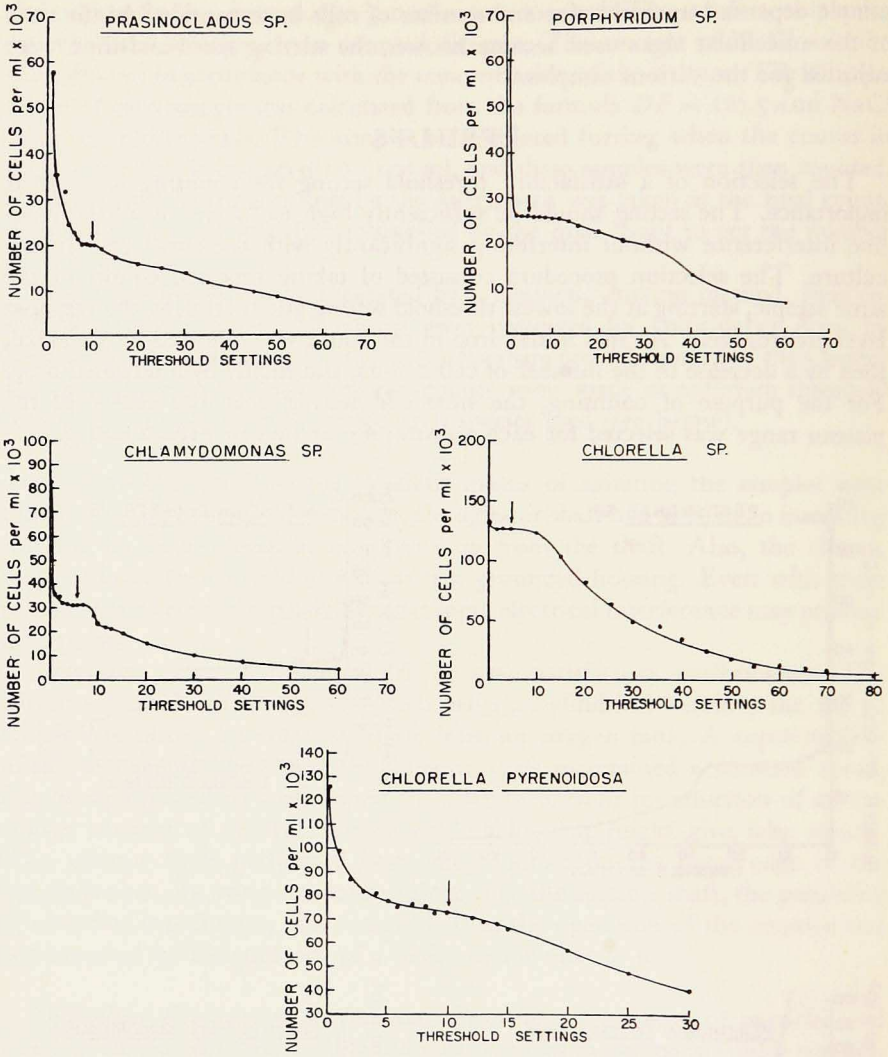

Figure 3. Threshold settings plotted against number of electronic counts for species with narrow threshold plateaus. [*Porphyridium* should be spelled *Porphyridium*—Editor.]

Figs. 2 and 3 indicate that the width of the threshold plateau varies from species to species. For example, the plateaus for *Platymonas* sp., *Nitzschia closterium*, *Peridinium trochoideum*, and *Scenedesmus quadricauda* were wide (Fig. 2), while those for *Chlamydomonas* sp., *Porphyridium* sp., *Chlorella pyrenoidosa*, *Chlorella* sp. (marine), and *Prasinocladus* sp. were narrow (Fig. 3). Further, the rate of numerical decline of counts after the plateaus had been

reached also showed some variations. For *Porphyridium* sp., *Chlamydomonas* sp., and *Chlorella* sp. (marine), readings dropped off with progressively greater rapidity as the threshold was increased beyond the plateau level, while for *Peridinium trochoideum*, *Nitzschia closterium*, and *Scenedesmus quadricauda* the rates of decline were less rapid. The sharp drops in counts are due to the "screening out" of background noise and possibly of dust particles that are below the size of the smallest cells. The slower drop-off in numbers at higher threshold settings is due to removal from the count of smaller countable cells. Figs. 2 and 3 show that, if the plateau is broad, the small cells do not overlap the background noise, but if the plateau is short, or nonexistent, this is unlikely to occur. Comparison of the nine graphs in Figs. 2 and 3 shows the differences between the "background noise" in the different cultures. One assumes that small extraneous particles are more important than line interference; the latter should be consistent in all samples if it is responsible for the noise at low threshold settings. It seems that, at lower counts, large dust particles become a progressively more important per cent of the total count whereas at higher thresholds these dust counts do not contribute to the decrement in all counts.

TABLE I. REPLICATE COUNTS OF *Chlorella pyrenoidosa* BY THE COULTER COUNTER*.

Run No.	No. of Counts	Run No.	No. of Counts	Run No.	No. of Counts	Run No.	No. of Counts
1	43,283	9	45,771	17	44,522	25	45,772
2	45,634	10	43,284	18	43,111	26	45,393
3	43,663	11	44,335	19	43,861	27	43,153
4	44,061	12	41,970	20	43,004	28	43,873
5	43,875	13	41,271	21	45,105	29	43,330
6	43,933	14	41,873	22	44,170	30	43,809
7	43,401	15	43,220	23	44,503		
8	43,061	16	44,271	24	43,501	Average	43,800

* Volume of sample: 2 ml.

Replicate counts were obtained by taking 30 successive instrument counts of a diluted culture of *Chlorella pyrenoidosa*. The results are given in Table I. The counts ranged from 41,271 to 45,772; the mean was 43,000, the standard deviation 1,062, and the standard error of the mean 193. Note here that the per cent standard error (0.44) corresponds closely to the calculated theoretical Poisson distribution error[2] of 0.47%. The 95% confidence limits for replicate counts were $43{,}423 < \mu < 44{,}177$.

The effect of dilution was tested with a set of dilutions obtained from a sample of *Chlorella* sp.; these dilutions were 1:20, 1:100, 1:200, 1:250, 1:500, 1:750, and 1:1000; see Table II and Fig. 4. The observed counts agreed well with the "expected" counts taken from the best fit to a straight

[2] Derived from $100/\sqrt{n}$, where n is the mean number of cells counted.

TABLE II. EFFECT OF DILUTION ON THE ELECTRONIC COUNTS OF *Chlorella* SP.*

Dilution	No. of Counts (cells/ml)
1:20	272,052
1:100	95,441
1:200	45,312
1:250	31,608
1:500	13,859
1:750	9,538
1:1000	7,539

* Volume of sample: 5 ml.

line, derived by the "least-square" method. Table II shows that the number of cells estimated by the electronic counts decreases with increase of dilution up to 1:500; however, with higher dilution, it was found that the electronic count does not vary appreciably with the dilution at which the count was made.

For comparison, several pairs of electronic and haemocytometer counts were performed on several unicellular organisms. The data for both counts are given in Table III for cultures of: *Chlorella pyrenoidosa, Chlorella* sp. (marine), *Nitzschia closterium*, *Platymonas* sp., and *Porphyridium* sp.

To test the correlation between the counts performed by the two methods,

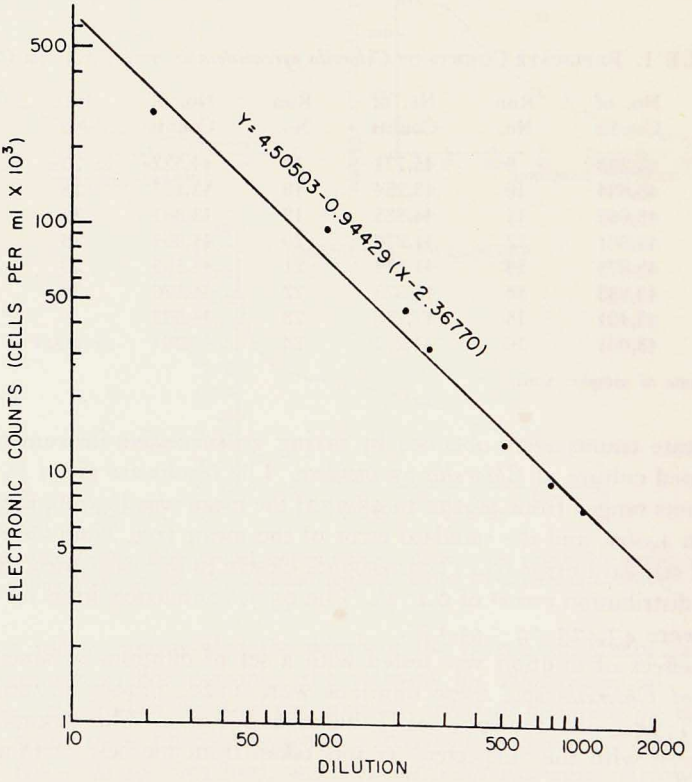

Figure 4. Effect of dilution on the electronic counts of *Chlorella* sp.

TABLE III. COMPARISON OF HAEMOCYTOMETER (HAEM.) AND ELECTRONIC (ELEC.) COUNTS (CELLS/ML × 10^5) FOR VARIOUS UNICELLULAR ALGAL SPECIES.

Species	Haem. Counts	Elec. Counts	Volume of Sample (ml)	Threshold	Dial
Chlorella pyrenoidosa	87.50	87.00	0.1	10	6
	26.25	25.60	0.1	10	6
	50.50	49.40	0.1	10	6
Chlorella sp. (marine)	57.81	55.91	0.2	5	7
	26.40	32.08	0.5	5	7
	55.68	55.98	0.5	5	7
Nitzschia closterium	8.00	6.26	5.0	8	4
	18.25	16.30	2.0	8	4
	23.75	23.00	1.0	8	4
	21.75	23.50	0.5	8	4
	16.50	20.70	1.0	8	4
	43.75	39.20	1.0	8	4
Platymonas sp.	14.50	17.00	1.0	15	4
	15.50	13.60	1.0	15	4
	22.85	23.80	1.0	15	4
	12.37	9.35	1.0	15	4
	16.00	14.80	1.0	15	4
	19.00	14.00	0.5	15	4
	63.60	64.10	1.0	15	4
	19.75	19.20	1.0	15	4
	13.50	14.75	1.0	15	4
Porphyridium sp.	30.50	24.45	2.0	5	4
	2.00	1.72	5.0	5	4
	14.40	13.95	5.0	5	4
	39.00	39.85	1.0	5	4
	36.00	35.60	1.0	5	4
	36.75	38.00	1.0	5	4
	131.25	166.40	1.0	5	4
	85.50	84.40	1.0	5	4
	74.80	71.40	1.0	5	4
	47.20	50.00	0.5	5	4
	96.75	100.00	0.5	5	4
$\bar{x}$ =	36.46	37.16			

the coefficient of correlation, r, was calculated for those species for which enough data had been collected. The regression-line equations derived by the least-square method, together with the values of r, are given in Table IV. Fig. 5 shows the electronic counts plotted against haemocytometer counts for *Porphyridium* sp. and *Nitzschia closterium*.

For the combined data in Table III, r was 0.9847, and the regression line fitted to the data (Fig. 6) was derived from the equation $Y = 37.135 + 1.1304\ (X - 36.464)$. Thus Table IV shows that the regression coefficients for the

TABLE IV. CORRELATION COEFFICIENTS AND REGRESSION LINE EQUATIONS FOR COUNTS BY THE HAEMOCYTOMETER AND COULTER COUNTER.

Species	Correlation Coefficient r	Regression Line Equation
Chlorella pyrenoidosa	0.991	$Y = 54.0 + 0.995\ (X - 54.6)$
Chlorella sp. (marine)	0.994	$Y = 48.0 + 0.629\ (X - 46.8)$
Nitzschia closterium	0.893	$Y = 21.50 + 0.796\ (X - 22.0)$
Platymonas sp.	0.951	$Y = 21.18 + 0.987\ (X - 21.9)$
Porphyridium sp.	0.996	$Y = 45.95 + 1.01\ (X - 46.32)$

electronic and haemocytometer counts were significantly high. Note in Table III that the mean values for the electronic and haemocytometer counts (36.46×10^5, and 37.13×10^5, respectively) correspond remarkably well.

Mean cell counts throughout a period of algal growth were compared with both the electronic counter and the haemocytometer. Growth studies of *Chlorella pyrenoidosa* over a period of a few days, using both methods, are given in Table V and Fig. 7. Fig. 7 shows that the growth curves obtained by the two methods approximate each other very closely, with the haemocytometer counts showing slightly higher cell counts than those given by the Coulter counter. The growth curve for *Chlorella pyrenoidosa* has the characteristic S-shape that shows a slow rise, a steady increase during the first eight days, and finally the beginning of a leveling off towards the end of its exponential growth phase.

TABLE V. NUMBER OF CELLS COUNTED WITH HAEMOCYTOMETER (HAEM.) AND ELECTRONIC (ELEC.) COUNTERS (CELLS/ML $\times 10^4$) DURING THE GROWTH PERIOD OF *Chlorella pyrenoidosa*, IN MAY 1962.

Date	Time	Haem. Counts	Elec. Counts	Date	Time	Haem. Counts	Elec. Counts
V/18	8 pm	2	2	V/25	8 am	1700	1640
V/19	8 am	15	12		8 pm	1800	1760
V/20	8 am	38	33	V/26	8 am	1850	1880
	8 pm	51	49		8 pm	1980	2030
V/21	8 am	150	125	V/27	8 am	2050	2070
	8 pm	240	225		8 pm	2150	2120
V/22	8 am	500	418	V/28	8 am	2200	2230
	8 pm	620	592		7 pm	2300	2270
V/23	8 am	875	820	V/29	8 am	2280	2270
	8 pm	1120	1100	V/30	8 am	2280	2290
V/24	8 am	1320	1300				
	8 pm	1520	1510				

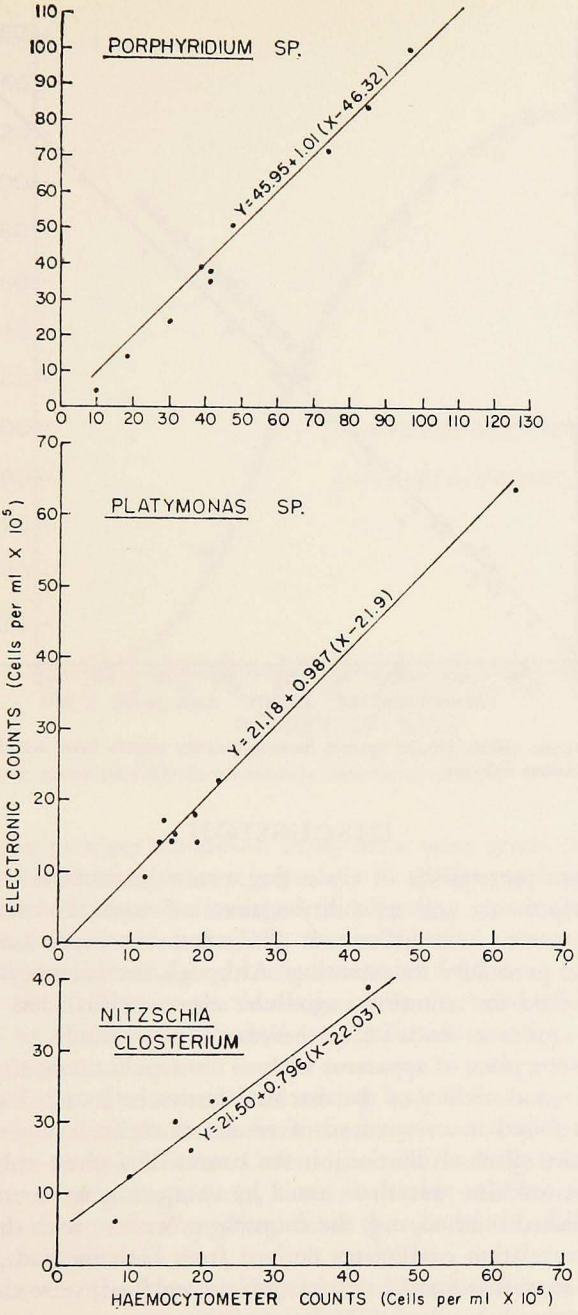

Figure 5. Electronic counts plotted against haemocytometer counts for *Porphyridium* sp., *Platymonas* sp., and *Nitzschia closterium*.

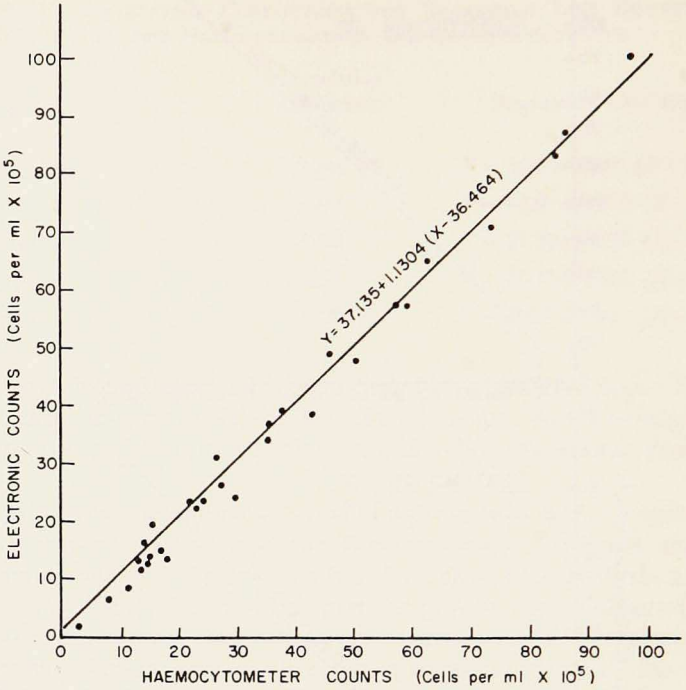

Figure 6. Electronic counts plotted against haemocytometer counts based on the combined data for various cultures.

DISCUSSION

An important prerequisite in evaluating a new instrument is a demonstration of its performance and reliability against a "standard" method, but in a study of this nature it is difficult to determine what constitutes a standard instrument and procedure for counting. Although the haemocytometer is not ideal as a standard for counting unicellular algae, nevertheless the lack of a more suitable apparatus made its use necessary in this study.

In evaluating a piece of apparatus such as the Coulter counter, it is imperative that the reproducibility of the machine counts be tested. Excellent reproducibility was found in comparison of results of replicate counts and in observations of the effect of dilution on the counts of a given culture. The reliability of the machine was then tested by comparing its counts with those from an established method, e.g. the counting procedure with the haemocytometer. The correlation coefficients derived from both methods, for the individual species as well as for the several species combined, were close. Although the correspondence between the Coulter and haemocytometer counts was good, in a few instances we noticed significant discrepancies. Several factors could

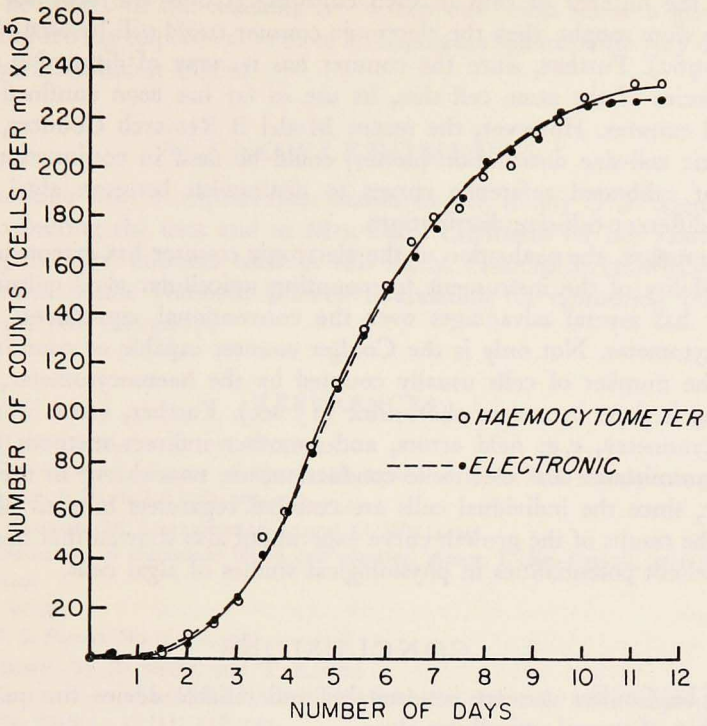

Figure 7. Growth curves for *Chlorella pyrenoidosa*, using haemocytometer and electronic counts.

have contributed to these anomalies. First, there were errors in the dilution procedure that could be significant, especially in the counts from the Coulter counter. Great care must be exercised in preparing the suspension and in determining the dilution factor (DF). Secondly, there were errors inherent in the standard chamber counting method, namely: field error, chamber error, and pipetting error. These were investigated by Berkson *et al.* (1940) and more recently by Richar and Breakell (1959) for blood cells.

The errors from the electronic counter were not as numerous as those from the haemocytometer. Once the initial mixing and dilution has been accomplished, the only source of error is from the actual counting by the machine; this was found to be insignificant. Counting errors resulting from coincidence losses do not pose serious problems, since correction can be made, as noted previously. However, errors resulting from the use of the wrong threshold setting could be significant.

Although the Coulter counter showed excellent performance in counting pure unialgal cultures, we have found that in the case of aggregate forms, e. g. *Scenedesmus quadricauda*, the instrument was of no practical value in deter-

mining the number of cells in such cultures. If only the volumes of these cultures were sought then the electronic counter could still be used (Maloney *et al.*, 1962). Further, since the counter has no way of discerning different algal species of the same cell size, its use so far has been confined to pure unialgal cultures. However, the recent Model B Research Counter, with its automatic cell-size distribution plotter, could be used in conjunction with a series of calibrated reference curves to distinguish between algal cultures having different cell-size distributions.

In summary, the evaluation of the electronic counter has demonstrated the dependability of the instrument for counting unicellular algal cultures. The counter has several advantages over the conventional equipment, e.g. the haemocytometer. Not only is the Coulter counter capable of counting many times the number of cells usually counted by the haemocytometer, but the counting is done in a very short time (15 sec). Further, errors inherent in haemocytometry, e.g. field errors, and in other indirect methods based on light transmittance and electronic conduction, are nonexistent in the Coulter counter, since the individual cells are counted regardless of their shape and size. The results of the growth curve experiment also showed that the counter has excellent potentialities in physiological studies of algal cells.

CONCLUSIONS

1. The Coulter counter is a suitable and reliable device for quantitative estimation of several unicellular algae.

2. The instrument has many advantages over conventional optical counting; of special significance are its speed and accuracy.

3. A test of the counter's reproducibility by taking replicate counts, and a test of its reliability by comparing its counts with those made by a standard procedure (e.g. haemocytometer) have proved further its suitability and reliability.

4. There is an optimum threshold setting for each culture at which the total cell counts are made.

5. The best counts are obtained when the cell concentrations do not exceed 10^5/ml.

6. Problems arising from cell clumping were solved by developing a mechanical stirrer that was used continuously during the counting procedure.

7. The instrument is not particularly suitable for counting algal species where long chains of cells are encountered.

8. The instrument is well suited for studying the growth curves of unialgal cultures. This makes its use very desirable in physiological studies.

9. The counter has great potentialities in aquatic research. With proper size of aperture, the instrument could also be used to count several species of microplankton and marine bacteria.

10. The ability of the machine to "screen out" cells under a given size, by means of setting response at various levels, makes the machine very desirable in cell-size distribution studies.

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