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COMPARISON OF THE DIFFERENT METHODS OF ESTIMATING NANOPLANKTON

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An assessment of the abundance of nanoplankton in the sea cannot be made until a reliable method for counting has been found. The object of the present investigation was to compare the efficiency of a number of methods. The organisms concerned range in size from less than 1 to about 10 μ, most being less than 5 μ, and belong to the classes Chrysophyceae, Cryptophyceae, Dinophyceae and Chlorophyceae.

The larger planktonic diatoms and armoured dinoflagellates, which occur in the plankton in numbers insufficient to give accurate results with the methods under comparison, have been omitted. Utermöhl's (1931) sedimentation method gives the best results for these larger forms (Nielsen, 1933); but it is not suitable for naked nanoplankton, since no adequate fixative has been discovered which will leave these organisms in a state recognizable for identification. A second method, suggested by Goldberg, Baker & Fox (1952), of filtering samples through a molecular filter, is convenient for treating all size-ranges of organisms at sea. Counts may then be made in the laboratory without any prior treatment of the sample, but the majority of the naked forms up to 5 μ are unidentifiable.

Some means of concentrating the sample is usually necessary in most counting methods since the depth of the water film which would be required to give any figure at all from a raw sample is too thick to allow an accurate count. This statement does not apply to Dr R. H. Millar's technique of counting under dark-ground illumination.

The methods compared here are the use of either a centrifuge or filters for concentrating the sample, followed by counting; a counting method using dark-ground illumination without previous concentration of the sample; and the utilization of dilution techniques.

My most sincere thanks are due to Dr M. Parke, of Plymouth, for her many helpful suggestions during the course of this investigation; to Dr R. H. Millar, of Millport, to whom I am indebted for an account of his method for counting flagellates under dark-ground illumination; and to Dr E. D. Goldberg, of the Scripps Institution of Oceanography, California, for a description of the use of the molecular filter, and for a supply of these filter membranes.

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Methods

Much has been said in the past about the use of a centrifuge for concentrating nanoplankton samples, and many different methods have been used. Gran (1929) states that 'test experiments with the centrifuge method show that the source of error involved by vortex formation in the tubes, as mentioned by Wulff (1926) and Utermöhl (1927), can be avoided altogether if care is taken first to ensure that the motion is not too suddenly checked, and tubes with a narrow bottom part used, and the clear liquid drawn up cautiously with a pipette'. The buoyancy of certain species (Kofoid, 1897; Gross & Zeuthen, 1948), due to a specific gravity less than that of water, causes selective action with the centrifuge, but the majority of such species are diatoms or water-bloom-forming organisms such as blue-green algae, which can be estimated with the sedimentation method after fixation.

The method of centrifugation adopted here is based upon that of Bruce, Knight & Parke (1940). 10 ml. samples are centrifuged for 15–30 min. at 1500 r.p.m., a relative centrifugal force of 358 × gravity. The centrifuge is then stopped very slowly, to avoid currents developing in the tubes, and the top 9 ml. are withdrawn by means of an upturned pipette attached to a pump. The use of the pump and a pipette with an upturned end enables the water to be drawn off evenly, and any suction currents which may develop will be only at the surface of the water. The remaining 1 ml. of water is then thoroughly mixed, and counts made in a haemacytometer, objectives up to $\frac{1}{6}$ in. being used if necessary. Average values, based on counts from four tubes, were recorded for each sample.

Several experiments have been carried out to determine the speed and duration of centrifugation which gives the maximum results (Table I). In these experiments no attempt was made to count all the organisms in the concentrate, as Lohmann (1908) did, but an average figure was obtained for each sample.

Nielsen & v. Brand (1933–34) describe the use of a precipitate to aid in the sedimentation of preserved cells while centrifugation is in progress, and it was thought that this principle might be applicable to the centrifugation of living cells. Accordingly, before centrifugation, 0·05 ml. of a 1% solution of potassium aluminium sulphate crystals was added to the 10 ml. sample of sea water (Atkins & Parke, 1951). The flocculent precipitate thus formed does not kill the organisms, nor does it hinder subsequent counting. Further treatment of the precipitate is therefore unnecessary. The results from this

method using the floc, compared with results from untreated samples (labelled 'normal') are found in Table I, Sample 1, p. 134, and Table IV, samples 2 and 3, p. 137.

As a check on the sedimentation of living organisms during centrifugation 10 ml. samples were centrifuged and split up immediately into three sections (i.e. the top 5 ml., middle 4 ml. and bottom 1 ml., the latter being the section usually used for estimating the numbers). These separate sections were then placed in dilute Erdschreiber[1] culture solution and left to grow. Never did more than two different organisms appear in the flask containing the top 5 ml. of the centrifuged sample, or more than three in the flask containing the middle 4 ml., and the time taken for these cultures to develop was comparable with that for cultures started from a single-cell inoculum at the same period of the year. The flask containing the bottom 1 ml. of the sample, however, produced a thick mixed culture in a short time. This evidence, together with that obtained from centrifuging cultures (Table II), proves that the bulk of living nanoplankton organisms will settle during centrifugation.

The second method of concentrating sea-water samples was by means of the 'Stefi' filter, using a collodion membrane having an average pore diameter of 0·5 μ (Cole & Knight-Jones, 1949). With this apparatus samples were concentrated to one-fiftieth of their original volume, and samples of the concentrate were counted in a haemacytometer.

To facilitate the counting of a large number of motile organisms under direct illumination, fixation with osmic vapour is necessary. This treatment leaves the organisms in a recognizable state, but cannot be used for large volumes of water. The percentage of motile and non-motile forms in each size-group can be determined later if required.

The third method tested for concentrating the nanoplankton was by means of the molecular filter suggested by Goldberg *et al.* (1952). This filter-membrane is composed of incompletely cross-linked high polymer molecules of partially substituted cellulose acetate and cellulose nitrate. The filters used in this work had an average pore-diameter of 0·5 μ. Following the method of Goldberg *et al.* all solutions used in the preparation of the sample were molecular-filtered before use to remove any particles. Organisms in the sample were then fixed by means of a saturated solution of iodine in potassium iodide, at a concentration of 40 ml. of fixative to a litre of sea water. Throughout, samples of 50 ml. were used. After fixation the sample was filtered, and the sea water was gradually replaced by distilled water by washing with a series of sea water/distilled water mixtures. 20 ml. of Fast Green were added to the filter, which was then disconnected from the pump, and left for 30 min. The Fast Green was then drawn through, and the membrane was dehydrated with ethyl alcohol and cleared with cedar oil.

[1] Dilute Erdschreiber culture solution (Plymouth formula): sea water 1 l., $NaNO_3$ 0·15 g., $Na_2HPO_4 . 12H_2O$ 0·015 g., soil extract 25 ml.

The membrane was then removed from the filter, cut in half, and each half mounted in balsam on a microscope slide. If the number of diatoms and larger dinoflagellates in the sample is required, the entire area of the filter is used. These organisms can usually be easily identified on the membrane. To estimate the numbers of nanoplankton four fields only were counted, each being taken at random from the filtering area of the membrane. The mean of these counts was multiplied by $83 \times 95/50{,}000$ to bring the figure to numbers per cubic millimetre of the original sample. This correction factor must be calculated for each filtering apparatus and microscope used when counting.

The next method (unpublished), designed and used by Dr R. H. Millar of Millport, for the counting of flagellates, makes use of the highly refractive nature of the nanoplankton organisms. The details of this method have been communicated to me by Dr Millar, for use in these comparative studies. In his method a drop of water is placed in the cell of a counting slide (Thoma haemacytometer), and, with a phase-contrast microscope adjusted to give dark-ground illumination with a low-power objective, all the moving flagellates are rapidly counted in the whole area of the cell, which is scanned by means of a mechanical stage. Any microscope fitted with dark-ground equipment can be used for this method, but the advantage of a phase-contrast microscope is the ease with which it can be altered to give either direct illumination, or actual phase contrast, so that particular organisms can be observed more clearly. The count must be made as soon as possible after the drop of water is placed on the slide, as many organisms settle very rapidly.

The next two methods tested for estimating nanoplankton involve the use of cultures. The first was proposed by Allen (1919) and the second by Knight-Jones (1951). Allen's method consists simply of adding 0·5 ml. of the sea-water sample to 1·5 l. culture solution, shaking thoroughly, and distributing this into a large number of flasks, which are then left in a north light for the organisms to grow. It is then assumed that every species which grows in each flask was started from a single cell, thus giving a minimal count. Knight Jones's method utilizes the serial dilution technique used by bacteriologists, with Erdschreiber culture solution as the nutrient medium. Three series of five tubes are used in each estimation, each series containing one-tenth of the volume of the sample that is present in the previous series. The tubes are placed in a north light and left to grow for 1–2 months in the summer, and longer in the winter. On the basis of the number of tubes in each series which show growth the final results are worked out from Swaroop's (1938) tables of the most probable numbers in the original sample. This serial dilution method has been used with sea-water samples, where the numbers obtained have been compared with counts after concentrating with the membrane filter and centrifuge, and also with actively growing cultures, where the number of cells was counted prior to dilution.

Results

Centrifugation

(Tables I, II, IV sample 2, VIII, and X sample 2)

The experiments to test the efficiency of centrifugation at different speeds and for different periods of time were carried out using both samples treated with potassium alum and untreated ('normal') samples. The results of these experiments are shown in Table I and the figures from which they were obtained are in Table VIII (p. 143).

Sample 1 shows that there is no significant difference between untreated samples and those which have been treated with potassium alum. This conclusion is confirmed by results shown in Table IV (p. 137), and from other results not given here, including some from experiments using cultures.

Results of centrifugation for different periods at 1500 r.p.m. (Sample 1) show that a 10 min. period does not give a maximum count, but from 15 min. onwards there is no significant increase. Increasing the centrifuge speed to 2000 r.p.m. does not improve the efficiency of sedimentation (Samples 2 and 3).

After the relative centrifugal force and time above which no further sedimentation occurs had been established, the actual efficiency of centrifugation was tested by the use of cultures. Cultures of a density that permitted an accurate count in a haemacytometer were centrifuged for 20–30 min., and counted. The results from these experiments are shown in Table II. The culture had usually to be diluted to one-tenth of its original strength prior to centrifugation, as the excessive number of organisms after concentration would otherwise have precluded an accurate count.

Throughout Table II the expected standard deviation (σ), based on the Poisson distribution, has been given, the actual standard deviation being unknown. Where thick cultures were used in this series of experiments a lower count than that obtained from the original sample resulted after centrifugation. This was particularly true with flagellate no. 3 (Plymouth collection) and *Hemiselmis rufescens*. In *Hemiselmis* the count from the centrifuged diluted culture is considerably larger than that from the centrifuged undiluted culture, and it seems possible that the density of the culture limits the efficiency of sedimentation during centrifugation.

These results strongly suggest that the centrifuge sediments the great majority of the nanoplankton, particularly in samples in which the organisms are not very numerous, as in raw sea water itself.

Centrifugation and membrane filter

(Tables III and IX)

Table III shows the comparison of results obtained by counting after samples had been concentrated with the membrane filter and the centrifuge.

TABLE I. COMPARISON OF SPEEDS AND TIMES OF CENTRIFUGATION

(Figures throughout refer to numbers per cubic millimetre, and results marked * are significantly different.)

Sample 1: 1500 r.p.m. (relative centrifugal force = 358)

Duration (min.)	'Normal' or 'Floc'	Mean	Standard deviation (s)	Difference between means	Standard error of difference
10	N	7·25	1·83		
	F	7·62	2·98	0·37	±1·24
15	N	10·25	3·18		
	F	9·9	3·04	0·35	±1·55
30	N	11·25	2·71		
	F	10·0	2·4	1·25	±1·28
60	N	10·0	2·83		

Treatments compared	'Normal' or 'Floc'	Difference between means	Standard error of difference
10 and 15 min.	N	3·0	±1·34*
	F	2·28	±1·51
15 and 30 min.	N	1·0	±1·475
	F	0·1	±1·42
30 and 60 min.	N	1·25	±1·38
10 and 30 min.	N	4·0	±1·15*
15 and 60 min.	N	0·25	±1·5

As there is no significant difference between 'normal' and 'floc' in any series, these two sets of figures have been treated together for each length of time, and the following results are obtained.

Duration (min.)	Mean	Standard deviation
10	7·44	2·36
15	10·1	3·0
30	10·62	2·56
60	10·0	2·83

Treatments compared	Difference between means	Standard error of difference
10 and 15 min.	2·66	±0·96*
15 and 30 min.	0·52	±0·99
30 and 60 min.	0·62	±1·19
10 and 30 min.	3·18	±0·87*
15 and 60 min.	0·1	±1·25

Sample 2: collected from Knap Buoy, 3 June 1952

Treatment	Mean	Standard deviation (s)
1500 r.p.m. for 15 min.	25·75	2·49
2000 r.p.m. for 15 min.	25·22	3·42
2000 r.p.m. for 30 min.	25·4	4·11

Sample 3: 15 min. at 1500 and at 2000 r.p.m. compared

Speed in r.p.m.	Mean	Standard deviation	Difference between means	Standard error of difference
1500	10·4	2·45		
2000	9·0	2·07	1·4	±1·12

N.B. At 2000 r.p.m. the relative centrifugal force is equal to 636 × gravity.

It will be seen that the theoretical Poisson standard deviation, the root of the mean, is frequently much less than the observed standard deviation for membrane-filtered samples, a fact which suggests that the organisms tend to aggregate when subjected to this treatment. χ^2 has then been calculated, and the probability found from Tables. This figure is often very low. This evidence

TABLE II. RESULTS FROM THE CENTRIFUGATION OF CULTURES

(Centrifugation at 1500 r.p.m. for 20–30 min. The results are expressed per cubic milli metre of the original culture.)

	Original count		Culture centrifuged		Culture diluted and centrifuged	
Organism	Mean	Theoretical standard error (σ)	Mean	Theoretical standard error (σ)	Mean	Theoretical standard error (σ)
Chlorophyceae						
Chlamydomonas sp. 1	4440	221	..	..	4300	208
Pyramimonas grossii Parke	220	47	..	..	200	44·7
Chrysophyceae						
Flagellate no. 3	3230	180	..	..	2760	167
Chromulina pleiades Parke	7770	279	..	..	7310	270
Cryptophyceae						
Flagellate no. 6	5300	230	..	..	4900	222
Hemiselmis rufescens Parke	3420	186	2800	53	3210	179
Dinophyceae						
Peridinium trochoideum (Stein) Lemm.	143	37·8	151	12·6	146	38·2
Gymnodinium sp. 2	180	42·5	..	..	190	43·2

of aggregation is rarely found in centrifuged samples. In all the results from centrifuged samples there is only one (Table III, sample 4) in which the probability is less than 0·05, a result well within chance expectations. The counts from which these figures are derived will be found in Table IX (p. 144).

The figures in Table III show little, if any, difference between the actual results from the two methods of concentration, except for the sample taken on 3 June 1952, which was full of detritus. The time involved in using the two methods is, however, very different. The centrifuge is simple to operate, and the counts can be made fairly rapidly, as there are only twenty or thirty organisms per field, whereas with the membrane filter great care must be taken to ensure that the membrane does not dry out completely, for in so doing a number of the naked flagellates would be destroyed. The number of organisms per field after this latter treatment is also large, and the time taken to count them is therefore much greater. The additional time spent on the method appears not to be justified by any greater, or more reliable, estimate of the number of organisms.

TABLE III. COMPARISON OF THE RESULTS OBTAINED BY COUNTING AFTER CONCENTRATING WITH THE MEMBRANE FILTER AND THE CENTRIFUGE

(All samples are from Knap Buoy.)

	Counts per haemacytometer field					Numbers per cubic millimetre of the original sample	
	Mean	Standard deviation (s)	Deviation (σ)	χ^2	Probability	Mean	s
Sample 1, 27. v. 52							
Membrane filter							
Total	101·7	20·95	10·1	21·5	< 0·001	20·3	4·19
Under 2 μ	64·6	15·95	8·04	19·6	0·01–0·001	12·9	3·19
2–under 5 μ	28·7	6·15	5·35	..	..	5·7	1·23
Over 5 μ	8·3	2·34	2·88	..	..	1·7	0·47
Centrifuge							
Total	20·13	4·375	4·58	..	..	20·13	4·375
Under 2 μ	9·19	2·4	3·03	..	..	9·19	2·4
2–under 5 μ	7·75	2·98	2·78	..	..	7·75	2·98
Over 5 μ	3·19	1·56	1·79	..	..	3·19	1·56
Serial dilution	..	..	..	..	..	3·9	2·1
Sample 2, 29. v. 52							
Membrane filter							
Total	89·7	16·8	9·7	15·7	0·01–0·001	17·94	3·36
Under 2 μ	53·9	9·7	7·35	..	..	10·78	1·94
2–under 5 μ	25·0	4·52	5·0	..	..	5·0	0·9
Over 5 μ	10·8	5·17	3·29	12·3	0·05–0·02	2·16	1·03
Centrifuge							
Total	22·0	4·76	4·68	..	..	22·0	4·76
Under 2 μ	11·12	2·25	3·34	..	..	11·12	2·25
2–under 5 μ	6·63	2·78	2·58	..	..	6·63	2·78
Over 5 μ	4·25	1·895	2·06	..	..	4·25	1·895
Serial dilution	..	..	..	..	..	2·0	1·0
Sample 3, 3. vi. 52							
Membrane filter							
Total	202	16·9	14·2	..	..	40·4	3·4
Under 2 μ	131	17·4	11·45	11·5	0·05–0·02	26·2	3·5
2–under 5 μ	44·3	18·5	6·66	38·6	< 0·001	8·9	3·7
Over 5 μ	26·7	5·46	5·17	..	..	5·3	1·09
Centrifuge							
Total	33·1	6·77	5·75	..	..	33·1	6·77
Under 2 μ	21·0	4·62	4·58	..	..	21·0	4·62
2–under 5 μ	6·7	2·33	2·59	..	..	6·7	2·33
Over 5 μ	5·4	1·96	2·32	..	..	5·4	1·96
Sample 4, 6. vi. 52							
Membrane filter							
Total	173·0	24·75	13·2	17·7	0·01–0·001	34·6	4·95
Under 2 μ	124·0	20·6	11·3	17·01	0·01–0·001	24·8	4·12
2–under 5 μ	26·0	6·32	5·1	..	..	5·2	1·26
Over 5 μ	23·0	5·43	4·8	..	..	4·6	1·1
Centrifuge							
Total	36·0	8·57	6·0	14·22	0·05–0·02	36·0	8·57
Under 2 μ	25·9	6·86	5·1	..	..	25·9	6·86
2–under 5 μ	4·5	2·0	2·12	..	..	4·5	2·0
Over 5 μ	5·6	2·51	2·37	..	..	5·6	2·51
Sample 5, 9. vi. 52							
Membrane filter							
Total	135·9	38·7	11·7	55·0	< 0·001	27·18	7·7
Under 2 μ	100·5	32·7	10·03	53·0	< 0·001	20·1	6·5
2–under 5 μ	23·7	5·27	4·87	..	..	4·74	1·05
Over 5 μ	11·7	3·88	3·42	..	..	2·34	0·78
Centrifuge							
Total	27·1	5·35	5·21	..	..	27·1	5·35
Under 2 μ	19·0	3·02	4·36	..	..	19·0	3·02
2–under 5 μ	4·7	2·45	2·17	..	..	4·7	2·45
Over 5 μ	3·4	1·65	1·84	..	..	3·4	1·65

N.B. This sample was full of detritus, and very difficult to count, and this fact may account for the divergent results.

Centrifugation, membrane filter, dilution techniques

(Tables III samples 1 and 2, IV, V, IX samples 1 and 2, X, and XI)

In Table III, samples 1 and 2 show a third estimate of numbers, obtained by the serial dilution method. Further figures obtained from sea-water samples by this method are shown in Table IV, in which the figures are derived from counts shown in Table X (p. 145).

TABLE IV. TABLE OF RESULTS FROM SAMPLES ESTIMATED BY (*a*) CENTRIFUGATION, FOLLOWED BY COUNTING IN A HAEMACYTOMETER, AND (*b*) BY DILUTION

(Numbers are given per cubic millimetre of the original sample.)

Method	Mean	Standard deviation	Difference between means	Standard error of difference
	Sample 1, 9. iv. 51			
Centrifuge	32·0	8·26	..	..
Serial dilution	0·79	0·45	..	..
Allen's dilution	0·204	..	..	..
	Sample 2, 2. iv. 52			
Centrifuge 'Normal'	8·12	1·46	0·88	±0·66
Centrifuge 'Floc'	9·0	0·815		
Serial dilution	2·1	1·0	..	..
	Sample 3, 9. iv. 52			
Centrifuge 'Normal'	9·5	2·93	2·0	±1·48
Centrifuge 'Floc'	11·5	2·83		
Serial dilution	1·3	0·7	..	..

Tables III and IV show that the estimates from the dilution methods are extremely low. It appears that this method of estimation cannot be generally applied until a more suitable culture solution and optimum cultural conditions for all the organisms are available—that is, if it is desired to estimate the total number of organisms in the phytoplankton. Dr H. A. Cole, of Conway (private communication), states that 'We have found that figures for the density of flagellates in our tanks, obtained by direct counts of concentrated samples using the membrane filter technique, are almost invariably between about four and ten times those obtained by serial dilutions.'

Further corroboration of this fact is found in Table V, which shows the results of serial dilutions set up using cultures. The daily counts from these cultures over the period when the dilutions were set up show them all to be in a state of active growth (see Table XI, p. 145).

In this series of experiments, as in those using sea-water samples, a suitable preliminary dilution was made before the serial dilutions were set up. This preliminary dilution varied, 1 ml. of the sample to 100 ml. culture solution for a sea-water sample, and 1 : 10,000 for cultures, except *Pyramimonas*, where the preliminary dilution was 1 : 1000.

With the exception of flagellate K, a chrysomonad with three flagella, the numbers obtained by the dilution method were very low throughout these experiments.

TABLE V. THE RESULTS OF THE SERIAL DILUTION TECHNIQUE USED ON CULTURES OF FLAGELLATES, COMPARED WITH THE KNOWN COUNTS FROM THE CULTURES PRIOR TO DILUTION

(Results are expressed per cubic millimetre of the original culture.)

	Count from culture		Dilution method	
Organism	Mean	Expected σ	Most probable no.	σ
Pyramimonas sp.	90	30	39	21
Gymnodinium sp. 2	200	44·8	63	37
Pseudopedinella sp. 1	180	42·5	18	9
Flagellate K	340	58·4	240	121
Flagellate no. 16	255	50·5	33	17

TABLE VI. COMPARISON OF THE RESULTS OBTAINED BY CONCENTRATING SAMPLES BY CENTRIFUGATION AND THE USE OF THE MOLECULAR FILTER

(All samples are from Knap Buoy, and counts are expressed per cubic millimetre of the original sample.)

	Centrifuge		Molecular filter	
Sample	Mean	s	Mean	s
13. xi. 52. Total	20·0	3·8	18·06	3·97
Under 2 μ	12·5	2·64	14·85	3·0
2–under 5 μ	4·3	1·38	2·09	0·32
Over 5 μ	3·2	1·74	1·12	0·223
19. xi. 52. Total	19·8	4·76	16·8	2·1
Under 2 μ	14·3	3·4	14·2	1·91
2–under 5 μ	3·5	1·35	1·85	0·395
Over 5 μ	2·0	0·95	0·75	0·199
21. xi. 52. Total	21·8	4·06	21·6	1·35
Under 2 μ	14·4	3·44	18·1	0·9
2–under 5 μ	4·9	1·6	2·6	0·58
Over 5 μ	2·5	0·975	0·9	0·25
28. xi. 52. Total	24·3	4·94	22·8	0·94
Under 2 μ	16·3	3·44	16·9	0·92
2–under 5 μ	5·8	1·83	4·55	0·47
Over 5 μ	2·2	1·475	1·35	0·42
4. xii. 52. Total	23·3	3·06	24·6	1·65
Under 2 μ	15·4	3·47	19·98	1·79
2–under 5 μ	4·8	1·34	3·0	0·56
Over 5 μ	3·1	1·2	1·62	0·152

Centrifugation, molecular filter

(Tables VI and XII)

Results from the use of the molecular filter are very similar to those obtained after centrifugation. The figures in Table VI showing these results are derived from counts given in Table XII (p. 146).

This method has certain advantages in that diatoms may be estimated in

the same sample as nanoplankton, and that the sample may be made into a permanent preparation which can be counted at a later date. Its disadvantages are two, namely that for routine sampling the time involved in the preparation of a sample for counting is about 3 hr., and the actual counting is rather difficult, as the naked forms tend to shrink after killing, and so make identification difficult, if not impossible.

Centrifugation, Dr Millar's method

(Tables VII and XIII)

The last method used in this comparative study is the use of dark-ground illumination for counting an unconcentrated sample. The Thoma cell used has a total volume of 8·66 cubic millimetres, and this is the volume of water in which organisms were counted. The figures for these counts, together with those from samples after centrifuging, will be found in Table XIII (p. 147).

The results from the counts of cultures, given in the first part of Table VII, show that when only motile cells were counted under dark-ground illumination a large number of the individuals, at least of the two species used, were missed, because they had become non-motile. In other samples when both motile and non-motile cells were counted, a figure was obtained which was very near to the count from the culture prior to dilution. The counting of non-motile cells does, however, need considerable care, because a speck of detritus the same size as an organism will produce an image very similar to that of a living cell under dark-ground illumination.

Throughout the estimations of sea-water samples by this method, the results of which are given in the second part of Table VII, both motile and non-motile forms have been counted, and it can be seen that the agreement between the results obtained by the two methods is very close.

Discussion

In considering the relative merits of the above six methods for estimating nanoplankton the ultimate aim of the investigation must be clearly defined. There is very little difference between the efficiencies of the four counting methods with regard to the final estimate of numbers given. If the purpose is to determine only the total number of organisms in a sample of sea water, then counting under dark-ground illumination is the most rapid method. Counts after concentrating with the centrifuge or filters will also give this figure. If, however, the aim is to count the organisms, place them in size groups, and assign them as far as possible to their systematic position, the centrifuge or membrane filter for concentrating living samples should be used. Centrifugation is better because of its speed, and the uniformity of the subsequent counts, as there is no evidence of aggregation of the organisms after this treatment, as there was with the membrane filter.

TABLE VII. COMPARISON OF COUNTS USING DARK-GROUND ILLUMINATION WITH COUNTS FROM CULTURES AND AFTER CONCENTRATION BY CENTRIFUGATION

(a) Results obtained from cultures

(Numbers are per 0·1 mm.³.)

Organism	Straight count in haemacytometer	Dark-ground counts: Motile only	Dark-ground counts: Motile and non-motile
Hemiselmis rufescens Parke	189	138	179
Chromulina pusilla Butcher	426	230	450

In both cases the cultures were diluted prior to the dark-ground count: the dilution was 1:50 in the case of *Hemiselmis*, and 1:100 in the case of *Chromulina*.

(b) Results obtained from sea-water samples

(Numbers per cubic millimetre of the original sample.)

Sample	Size-group	Centrifuge and count	Dark-ground count
10. xii. 52. West end of breakwater	Total	17·6	16·3
	Under 2 μ	9·9	10·3
	2–under 5 μ	4·5	3·7
	5–under 10 μ	2·3	1·5
	Over 10 μ	0·9	0·8
11. xii. 52.	Total	19·3	19·2
	Under 2 μ	10·1	11·7
	2–under 5 μ	6·1	4·5
	5–under 10 μ	2·2	2·08
	Over 10 μ	0·9	0·92
12. xii. 52. Tank water from the Aquarium	Total	6·4	7·75
	Under 2 μ	4·3	5·15
	2–under 5 μ	1·2	1·62
	5–under 10 μ	0·5	0·64
	Over 10 μ	0·4	0·34
15. xii. 52. Knap Buoy	Total	11·4	10·2
	Under 2 μ	7·4	6·2
	2–under 5 μ	2·5	2·2
	5–under 10 μ	1·1	1·16
	Over 10 μ	0·4	0·64

Sample		Straight count	Diluted to 1/10 prior to dark-ground count
Mixed culture of diatoms and flagellates	Total	11·6	13·4
	Under 5 μ	7·9	8·9
	5–under 10 μ	2·6	3·1
	Over 10 μ	1·1	1·4

In this last sample the numbers are given per 0·1 mm.³.

The dilution methods give very low estimates of the numbers of nanoplankton in sea water, and the time elapsing between setting up the dilutions and the end of the period required for the organisms to give a reasonably thick culture may be several months. This makes the method cumbersome if a large number of samples are to be estimated.

The importance of cultures of nanoplankton should not, however, be underestimated, in view of the numbers of hitherto undescribed species which occur in sea water. It is very necessary to pick out organisms and grow them in species-pure culture in order to study them more closely. The two dilution methods can also be of great use in isolating certain organisms, as they save the tedious labour involved in isolating individual organisms by means of a micropipette.

Summary

Six methods for estimating the numbers of nanoplankton organisms in sea water have been compared. Of these, centrifugation of a living sample, followed by counts of the numbers of organisms in the concentrate, appears to be the most satisfactory method from all points of view. The method is rapid, simple to operate, and gives a result which is strictly comparable with results from counts made under dark-ground illumination of unconcentrated samples, and from counts after concentrating with filters. By this method the organisms counted can usually be assigned to the class and order to which they belong, even if their systematic position cannot be determined more definitely.

The use of species-pure cultures is a necessary adjunct to the direct examination of sea-water samples when studying the distribution, abundance, and annual fluctuations of nanoplankton, as so little is known of the systematics of these organisms.

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TABLE VIII. THE COMPARISON OF DIFFERENT SPEEDS AND TIMES OF CENTRIFUGATION USING BOTH 'NORMAL' SAMPLES AND THOSE TREATED WITH POTASSIUM ALUM ('FLOC')*

Sample 1

	'Floc'								'Normal'							
1500 r.p.m. for 10 min. (R.C.F. = 358)																
Total	5	10	12	7	5	6	5	11	5	10	8	8	9	7	5	6
Under 2 μ	..	4	3	3	3	3	3	5	2	5	3	4	4	5	3	4
2–under 5 μ	5	4	3	2	1	2	2	4	1	3	2	3	4	2	2	1
Over 5 μ	..	2	6	2	1	1	..	2	2	2	3	1	1	..	..	1
1500 r.p.m. for 15 min. (R.C.F. = 358)																
Total	7	15	7	11	10	11	12	6	15	11	9	12	8	13	9	5
Under 2 μ	4	8	3	5	4	5	3	3	7	4	2	6	5	5	3	..
2–under 5 μ	2	4	2	3	1	3	5	3	5	4	4	4	2	6	4	4
Over 5 μ	1	3	2	3	5	3	4	..	3	3	3	2	1	2	2	1
1500 r.p.m. for 30 min. (R.C.F. = 358)																
Total	8	11	9	6	11	14	11	10	11	12	14	11	10	6	11	15
Under 2 μ	3	5	4	4	6	4	4	7	6	2	4	3	4	4	3	5
2–under 5 μ	4	5	3	2	4	6	5	3	3	4	5	4	4	2	5	7
Over 5 μ	1	1	2	..	1	4	2	..	2	6	5	4	2	..	3	3
1500 r.p.m. for 1 hr. (R.C.F. = 358)																
Total									12	9	13	12	8	7	6	13
Under 2 μ									5	4	6	6	4	5	2	6
2–under 5 μ									6	4	5	5	3	2	4	5
Over 5 μ									1	1	2	1	1	..	..	2

Sample 2, Knap Buoy, 3. vi. 52

(All counts made with untreated samples)

1500 r.p.m. for 15 min. (R.C.F. = 358)								
Total	24	22	25	29	29	24	26	27
Under 2 μ	13	4	15	18	18	18	17	17
2–under 5 μ	6	8	4	4	9	4	5	5
Over 5 μ	5	10	6	7	2	2	4	5
2000 r.p.m. for 15 min. (R.C.F. = 636)								
Total	25	24	19	30	24	28	24	28
Under 2 μ	16	13	14	19	16	18	13	24
2–under 5 μ	3	3	3	6	4	2	3	2
Over 5 μ	6	8	2	5	4	8	8	2
2000 r.p.m. for 30 min. (R.C.F. = 636)								
Total	24	21	26	23	23	23	33	30
Under 2 μ	12	15	15	17	16	14	22	19
2–under 5 μ	4	4	5	2	4	3	5	7
Over 5 μ	8	2	6	4	3	6	6	4

Sample 3

(Both untreated samples, i.e. 'Normal', and only the totals are given)

1500 r.p.m. for 15 min.	12	14	10	10	12	7	11	7
2000 r.p.m. for 15 min.	11	5	7	10	11	9	9	10

* Throughout these tables (VIII–XIII), unless otherwise stated, the figures given refer to one field of the Thoma haemacytometer, i.e. they are equivalent to 1 mm.^{3} of the original sample in the case of a centrifuged sample and 5 mm.^{3} of a filtered sample.

TABLE IX. COUNTS FROM THE SAME SAMPLES AFTER CONCENTRATING TO 1/10 WITH THE CENTRIFUGE, AND TO 1/50 WITH THE MEMBRANE FILTER

(All samples are from Knap Buoy)

Sample 1, 27. v. 52

Centrifuge																
Total	18	16	17	32	27	19	22	24	17	18	16	22	19	17	19	19
Under 2 μ	8	7	9	14	13	9	11	11	6	7	6	10	7	11	10	8
2–under 5 μ	6	7	6	16	12	8	8	10	5	7	7	8	9	5	6	4
Over 5 μ	4	2	2	2	2	2	3	3	6	4	3	4	3	1	3	7
Membrane filter																
Total	130	122	106	88	82	82										
Under 2 μ	78	86	72	54	48	50										
2–under 5 μ	40	30	28	26	26	22										
Over 5 μ	12	6	6	8	8	10										

Sample 2, 29. v. 52

Centrifuge																
Total	19	15	30	24	24	25	25	19	26	24	22	29	15	22	17	16
Under 2 μ	10	9	14	12	10	13	13	10	13	12	13	14	9	11	8	7
2–under 5 μ	4	3	13	7	10	8	8	6	8	8	8	6	4	7	3	3
Over 5 μ	5	3	3	5	4	4	4	3	5	4	1	9	2	4	6	6
Membrane filter																
Total	63	95	104	90	78	108										
Under 2 μ	40	55	64	60	44	60										
2–under 5 μ	18	30	28	22	24	28										
Over 5 μ	5	10	12	8	10	20										

Sample 3, 3. vi. 52

Centrifuge																
Total	21	45	39	29	34	37	35	29	41	35	40	31	34	22	25	33
Under 2 μ	11	31	20	18	22	26	23	21	23	23	25	19	21	14	19	21
2–under 5 μ	7	6	12	8	6	7	6	4	10	7	6	6	9	5	2	6
Over 5 μ	3	8	7	3	6	4	6	4	8	5	9	6	4	3	4	6
Membrane filter																
Total	222	186	213	205	180	205										
Under 2 μ	124	110	119	145	130	157										
2–under 5 μ	70	50	60	30	32	24										
Over 5 μ	28	26	34	30	18	24										

Sample 4, 6. vi. 52

Centrifuge								
Total	31	48	49	32	37	36	25	30
Under 2 μ	22	36	35	23	25	29	19	18
2–under 5 μ	4	4	7	2	8	4	4	3
Over 5 μ	5	8	7	7	4	3	2	9
Membrane filter								
Total	126	181	191	186	165	188		
Under 2 μ	86	138	124	138	118	140		
2–under 5 μ	26	22	38	26	24	20		
Over 5 μ	14	21	29	22	23	28		

Sample 5, 9. vi. 52

Centrifuge										
Total	26	39	24	23	24	27	25	33	21	29
Under 2 μ	16	24	17	16	20	20	16	21	17	23
2–under 5 μ	7	10	4	4	2	4	6	5	3	2
Over 5 μ	3	5	3	3	2	3	3	7	1	4
Membrane filter										
Total	80	169	100	172	161	133				
Under 2 μ	51	130	74	128	124	96				
2–under 5 μ	20	20	18	32	26	26				
Over 5 μ	9	19	8	12	11	11				

TABLE X. TABLE OF COUNTS FROM CENTRIFUGED SAMPLES FROM WHICH SERIAL DILUTIONS WERE ALSO SET UP

Sample 1, 3 miles south of Looe Island, 9. iv. 51

Total	30	39	43	22	39	24	27
Under 2 μ	16	9	8	8	17	12	13
2–under 5 μ	9	11	24	12	11	9	11
Over 5 μ	5	19	11	2	11	3	3

Sample 2, 2. iv. 52

	'Floc'				'Normal'							
Total	9	9	10	8	7	10	9	8	7	10	8	6
Under 2 μ	7	5	7	5	5	5	5	3	5	4	4	3
2–under 5 μ	2	4	3	3	2	4	4	4	2	3	3	2
Over 5 μ	..	..	..	..	..	1	..	1	..	3	1	1

Sample 3, 2 miles south of Rame, 9. iv. 52

	'Floc'								'Normal'							
Total	15	12	10	9	16	8	12	10	9	13	15	9	7	8	7	8
Under 2 μ	7	3	5	6	10	4	7	4	4	6	6	4	2	4	3	3
2–under 5 μ	6	4	3	3	5	4	4	5	2	4	5	4	2	3	3	3
Over 5 μ	2	5	2	..	1	..	1	1	3	3	4	1	3	1	1	2

TABLE XI. TABLE OF COUNTS TO DETERMINE GROWTH-RATES OF CULTURES USED TO SET UP SERIAL DILUTIONS

Days after inoculation	*Pyramimonas* sp.		*Gymnodinium* sp. 2		*Pseudopedinella* sp. 1	
	Mean	σ (theor.)	Mean	σ (theor.)	Mean	σ (theor.)
0	..	..	59	24·3	75	27·4
1	..	..	74	27·2	82	28·6
2	20	14·3	87	29·5	118	34·4
3	36	19·0	110	33·2	131	36·2
4	61	20·47	152	39·0	162	40·3
5	90*	30·0	200*	44·8	180*	42·5
6	..	..	292	54·0	260	51·0
7	400	63·3	..	..	..	..
8	710	84·4	..	..	..	..

	Flagellate K		Flagellate 16	
	Mean	σ (theor.)	Mean	σ (theor.)
0	200	44·7	200	44·7
1	213	46·2	220	47·0
2	..	..	255*	50·5
3	250	50·0	..	..
4	340*	58·4	290	54·0
5	520	72·0	310	55·7

The standard deviation given here is theoretical, based on the Poisson Distribution, and equal to $\sqrt{(\text{Mean of counts})}$.

* Indicates the density of the culture at which the serial dilution was set up. Numbers are given per cubic millimetre of the culture.

TABLE XII. COUNTS OBTAINED FROM THE SAME SAMPLE AFTER CONCENTRATION TO 1/10 WITH THE CENTRIFUGE AND TO 1/63 WITH THE MOLECULAR FILTER

(The figures for the counts on the molecular filter refer to one field of the microscope. All samples are from Knap Buoy.)

	Centrifuge										Molecular filter			
Sample 1, 13. xi. 52														
Total	20	17	25	27	19	16	21	16	22	17	132	140	89	97
Under 2 μ	11	10	18	15	12	12	14	9	13	11	112	117	70	78
2–under 5 μ	3	4	5	7	6	3	4	3	4	4	15	15	11	12
Over 5 μ	6	3	2	5	1	1	3	4	5	2	5	8	8	7
Sample 2, 19. xi. 52														
Total	21	19	26	17	13	12	19	23	25	23	117	119	94	96
Under 2 μ	14	13	19	13	9	10	13	16	18	18	100	101	79	80
2–under 5 μ	6	3	5	3	3	1	3	4	4	3	12	15	9	11
Over 5 μ	1	3	2	1	1	1	3	3	3	2	5	3	6	5
Sample 3, 21. xi. 52														
Total	25	24	24	18	25	18	25	15	26	18	149	134	129	135
Under 2 μ	15	12	18	11	19	12	19	10	16	12	121	115	107	115
2–under 5 μ	7	8	5	4	4	4	4	3	6	4	21	13	18	14
Over 5 μ	3	4	1	3	2	2	2	2	4	2	7	6	4	6
Sample 4, 28. xi. 52														
Total	30	33	20	19	29	26	23	20	20	23	149	137	150	144
Under 2 μ	20	17	15	12	22	20	15	14	12	16	111	101	114	105
2–under 5 μ	7	10	3	5	5	5	6	5	6	6	33	26	28	28
Over 5 μ	3	6	2	2	2	1	2	1	2	1	5	10	8	11
Sample 5, 4. xii. 52														
Total	27	25	22	23	24	18	20	26	27	21	163	152	165	143
Under 2 μ	17	18	15	16	16	11	10	18	21	12	138	118	135	115
2–under 5 μ	5	5	4	4	3	5	6	6	4	6	16	24	19	17
Over 5 μ	5	2	3	3	5	2	4	2	2	3	9	10	11	11

TABLE XIII. COUNTS OBTAINED FROM THE SAME SAMPLE BY (*a*) CONCENTRATING TO 1/10 WITH THE CENTRIFUGE AND (*b*) COUNTING UNDER DARK-GROUND ILLUMINATION

(Numbers here are equivalent to 0·1 mm.³ of the original sample for a straight count, 1 mm.³ for a sample concentrated by centrifuging and 8·66 mm.³ in the dark-ground count.)

(*a*) *Counts of cultures*

Organisms	Straight count				Culture diluted: dark-ground counts: Motile cells		Motile and non-motile cells		
Chromulina pusilla Butcher	386	480	412		190	206	419	359	
Hemiselmis rufescens Parke	184	170	212	190	243	234	327	268	332

(*b*) *Counts of samples*

	Centrifuge and count										Dark-ground count		
Sample 1, west end of the Breakwater, 10. xii. 52													
Total	16	17	19	19	19	13	20	15	18	20	107	190	124
Under 2 μ	8	9	10	11	12	8	11	10	9	11	65	138	64
2–under 5 μ	5	4	6	3	3	4	6	3	6	5	21	36	39
5–under 10 μ	3	2	3	2	3	0	3	1	3	3	14	11	14
Over 10 μ	0	2	0	3	1	1	0	1	0	1	7	5	7
Sample 2, 11. xii. 52													
Total	20	22	16	17	21	20	17	19	22	19	182	150	
Under 2 μ	13	11	10	8	12	11	8	9	10	9	113	89	
2–under 5 μ	5	6	4	5	6	8	5	7	8	7	40	38	
5–under 10 μ	1	3	2	3	2	0	3	2	3	3	19	17	
Over 10 μ	1	2	0	1	1	1	1	1	1	0	10	6	
Sample 3, aquarium tank water, 12. xii. 52													
Total	8	6	5	7	6	4	8	7	6	7	62	72	
Under 2 μ	5	4	4	5	4	4	4	4	5	4	41	48	
2–under 5 μ	1	1	1	1	2	0	2	2	1	1	12	16	
5–under 10 μ	1	0	0	1	0	0	1	1	0	1	5	6	
Over 10 μ	1	1	0	0	0	0	1	0	0	1	4	2	
Sample 4, Knap Buoy, 15. xii. 52													
Total	12	10	14	9	12	14	10	8	13	12	96	80	
Under 2 μ	10	7	10	6	7	7	6	6	7	8	56	51	
2–under 5 μ	1	2	3	2	2	3	3	2	4	3	21	17	
5–under 10 μ	1	1	0	1	2	3	0	0	2	1	13	7	
Over 10 μ	0	0	1	0	1	1	1	0	0	0	6	5	

	Straight count										Dark-ground count after dilution to 1/10	
Sample 5, mixed culture of diatoms and flagellates												
Total	11	13	14	13	9	10	13	11	12	10	108	124
Under 5 μ	8	10	9	9	6	8	9	6	7	7	70	82
5–under 10 μ	2	3	3	4	2	1	2	3	4	2	29	27
Over 10 μ	1	0	2	0	1	1	2	2	1	1	9	15