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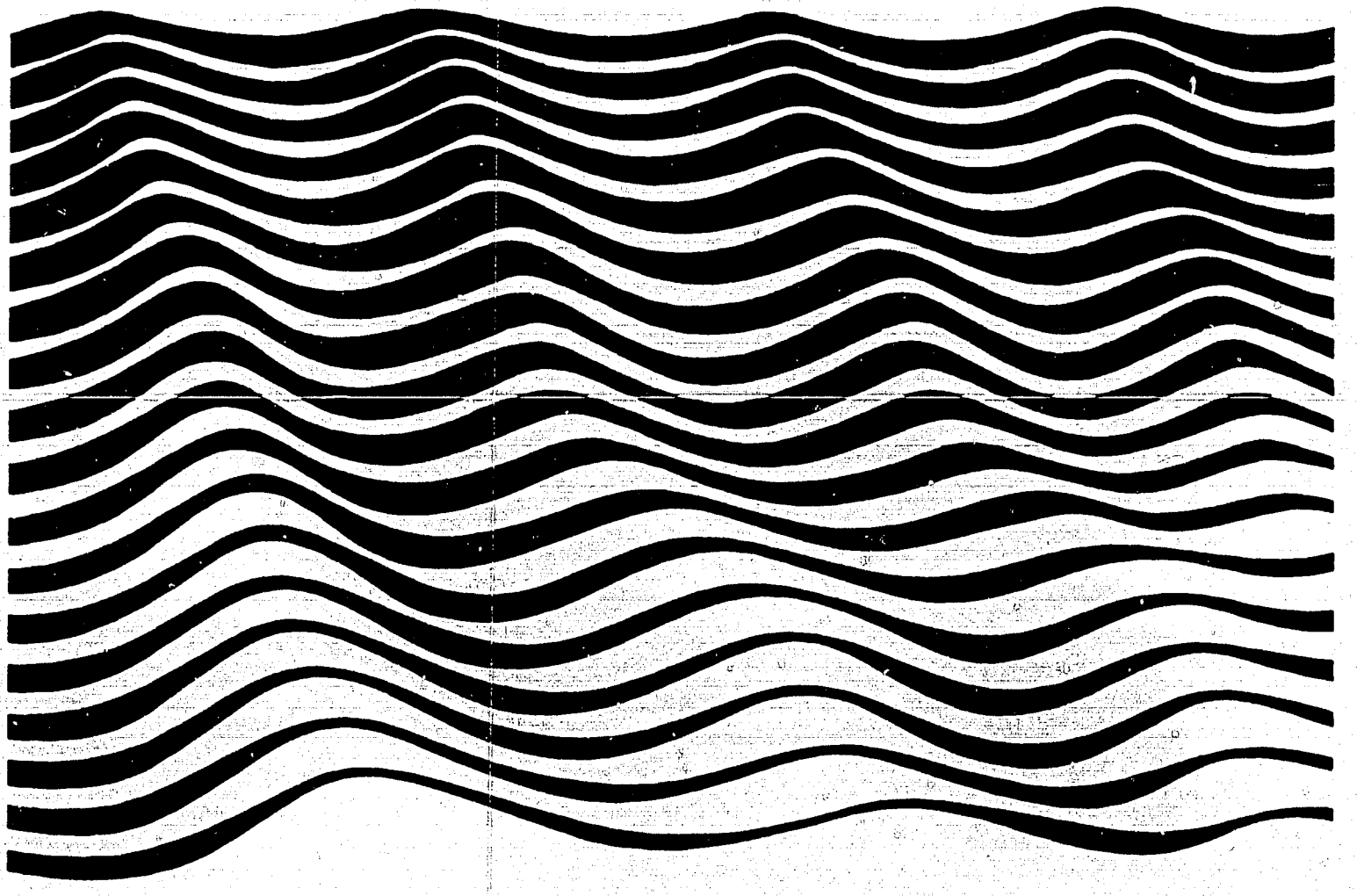
A review of methods used for quantitative phytoplankton studies

Final report of
SCOR working group 33



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PREFACE

This series, the Unesco Technical Papers in Marine Science, is produced by the Unesco Division of Marine Sciences as a means of informing the scientific community of recent developments in oceanographic research and marine science affairs.

Many of the texts published within the series result from research activities of the Scientific Committee on Oceanographic Research (SCOR) and are submitted to Unesco for printing following final approval by SCOR of the relevant working group report.

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I. INTRODUCTION

The Working Group on Phytoplankton Methods was established by the Executive Committee of the Scientific Committee on Oceanic Research (SCOR) of the International Council for Scientific Unions during its executive meeting of January 1969, held in Mexico City. During the meeting, Professor Braarud had pointed out the need for consideration of phytoplankton methods other than those involving pigment and other chemical analysis. Widely different methods are used in different countries and laboratories, and it is increasingly difficult to compare the results. The IOC Working Group on Training and Education has commented on the need for modern textbooks and manuals, and a review of phytoplankton methods could result in a manual on the subject (from SCOR Proceedings, 5 [1], p. 5, 1969).

The Executive Committee decided to establish Working Group 33 on Phytoplankton Methods with the following terms of reference:

(1) To review the methods now used for quantitative phytoplankton studies (exclusive of pigment and other chemical methods);

(2) To select the most satisfactory methods for various purposes, such as the description of species composition of communities, studies of special components, and biomass estimation;

(3) For the selected methods, to recommend detailed procedures for sample collection, preservation and laboratory examination;

(4) To prepare a report that might serve as a basis for a manual, including references to literature on taxonomy of the main groups and on methods for using quantitative phytoplankton data in ecological studies.

The members of Working Group 33 were: K. Banse, Seattle, USA (Chairman and Rapporteur); M. Bernhard, La Spezia, Italy; R.W. Eppley, La Jolla, USA; G.R. Hasle, Oslo, Norway; R. Marumo, Tokyo, Japan; G.A. Robinson, Edinburgh, UK; G.I. Semina, Moscow, USSR; and T.J. Smayda, Kingston, USA. One of the members was supported by the International Biological Program/Productivity Marine.

After considerable preparatory correspondence, the Working Group (minus one member) met at the University of Rhode Island, Kingston, Rhode Island, USA, on 1-3 December 1970, and discussed primarily items (1) and (4) of the Terms of Reference.

Unanimity on many methods and procedures was attained by the Working Group at Kingston. However, before recommending the most satisfactory methods, the Working Group wanted to compare widely used procedures for concentrating the counting of plankton samples. To save travel funds, samples of known composition, made up from algal cultures, were mailed from Seattle for study by the members in their own laboratories. Such samples were prepared by J.R. Postel and R.E. Munson. A. Dick,

Oslo, Norway; L.A. Hobson, Victoria, Canada; L. Rampi, La Spezia, Italy; F.M.H. Reid, La Jolla, USA; and K. Tangen, Oslo, Norway, also participated in the counting. The experiment took an unreasonable amount of time; in hindsight, it seems that a second meeting of the Working Group devoted to experimental investigations would have been more efficient and profitable.

Subsequent to the meeting at Kingston, various members of the Working Group met, as opportunities arose, both before and after the interim report was prepared. This report was sent to the SCOR Executive Committee early in 1972 (cf. SCOR Proceedings 8 [1], p. 31, 1972). The Final Report was achieved by correspondence with the members of the Working Group. It was submitted in May 1973, discussed by the SCOR Executive Committee at its meeting of May 1973, in Texel (Netherlands), and accepted (cf. SCOR Proceedings 9, p. 6, 1973, and Section VI, herein). The Working Group was discharged at that time.

The SCOR Executive Committee recommended that the report should be published following some changes in the way the counting experiments were reported. Thus, Section II of this presentation contains the main body of the Final Report which is a review and expression of preference of methods now used in quantitative phytoplankton research (Item (1) of the Terms of Reference). Section III deals with the content of a manual (Item (4) of the Terms of Reference). Section IV contains the formal recommendations arrived at by the Working Group; they are largely concerned with methodology. Section V gives the References cited. Sections II - V differ from the Final Report only in editorial details. The original Section I of the Report (Introduction), however, has been expanded slightly to include the history of the Working Group; also, the Appendix has been expanded. In addition, a review of developments since the acceptance of the Final Report is provided as a postscript (Section VI). The Chairman of the Working Group is responsible for the additional statements in Section VI and the Appendix.

II. REVIEW OF CURRENT METHODS USED FOR QUANTITATIVE ASSESSMENT OF PHYTOPLANKTON POPULATIONS

This section summarizes methods which will give unbiased results from counting of samples. It presupposes that the samples have been collected on the basis of a well-conceived research program which justifies the great expenditures involved in the counting of phytoplankton. Such a program would include the correct choice of types of samples taken (e.g., point vs. integrated samples); properly planned allocation of effort between taking of samples and processing them; and availability of environmental information without which the results cannot be meaningfully evaluated.

The members of the Working Group wish to emphasize at the outset that their own experience is in collecting (except collecting from the surface film, this section, 1.1.3.), subsampling, concentrating by gravity or centrifuging, and counting. They have no personal experience with many of the methods mentioned under other headings so that the review can serve here only as a guide to the literature. Critical experimentation must be undertaken by each investigator.

"Precision," as used herein, indicates the random or chance variability about the observed mean value for repeated measurements. "Accuracy" refers to the difference between the observed mean and the true value (also called systematic error, or bias).

1. Collecting

Quantitative collections of phytoplankton can be grouped into those which will be used for estimates of cell numbers or biomass, and those which will be used for assessments of all specimens and species, for investigations of geographic distribution or diversity. Different collecting methods have to be employed depending on the aims.

1.1. Collecting for determination of cell numbers or biomass

The investigator must collect the abundant forms of all sizes, as well as the rare, large cells which, because of their size, may make important contributions to the biomass. The entire size range, however, cannot be sampled with a single procedure. One small sample suffices for the small abundant species but a much larger sample must be taken for the rare, large forms.

Among the species less than 5 - 10 µm, naked algae, i.e., unarmored forms, very often dominate. Even in the open sea, they can contribute a considerable part of the biomass (e.g., about 50%, cf. Bernhard et al., 1967) but have often been overlooked because of their small size or because of losses incurred during or after fixing the water samples. The effect of fixation and preservation should be checked by comparing counts of preserved subsamples with those of live subsamples, or with the results of autoradiography (e.g., Maguire and Neill, 1972; Watt, 1971). Comparisons of biomass estimates from cell counts with photosynthetic rates in other subsamples are also useful. If quantitative assessments of the naked forms that cannot be reliably preserved are required, live counting or serial dilution

(see below) must be employed. Both procedures, as well as autoradiography, require nontoxic sampling bottles. To determine the nontoxicity of the plastic and rubber material involved, either a determination of photosynthetic rates or culturing is necessary; in the latter case, the sampling gear must be sterile.

1.1.1. Point sampling. The small sample (this section, 1.1), which may vary between 25 and 500 ml for depths within the upper 100-200 m, is best taken with a water bottle. Modifications of the samplers introduced by Niskin (1962) or Jannasch and Maddux (1967) are suitable as sterile, nontoxic samplers.

The large sample, which in the upper 100-200 m should be between 5 and 10 liters depending on the abundance of phytoplankton, can also be taken by a water bottle. Hose and pump give greater flexibility in sample size but water bottles can be used at any depth, in any weather. We have no experience on the possible adverse effects of a pump on naked or otherwise fragile species.

It is advantageous to concentrate the large sample on board ship, for example, by straining through a fine-meshed net (cf. Hentschel, 1941, Fig. 2), of a mesh size considerably smaller than the organisms to be retained (cf. 1.1.2).

Because of the high concentrations of plankton and other particulate matter in the surface film (cf. 1.1.3), the so-called "surface sample" in a series of vertical point samples should not be taken by a bucket but by a water bottle or pump at 1 m depth. With either method, contamination of samples from the upper few meters, by benthic forms growing on the ship, should be taken into consideration and sampling on the leeward side of the ship avoided.

1.1.2. Integrated sampling. It is sometimes desirable to obtain a mean concentration over a certain distance. This may be accomplished in the vertical direction by a net or a hose; the latter may be closed on the upper end before retrieving it, or may be used with a pump. Integrating in the horizontal or oblique direction may be accomplished by towing the intake of a pump, or a net (the latter only for the large forms). Depth should be monitored by a depth recorder. Especially in oblique tows, it must be ascertained that the gear sampled all depths equally. The net must be metered to determine the volume of water filtered. General information on design and use of nets, calibration problems of flow meters, etc., is given by Unesco (1968). Special attention is drawn to the loss of spherical bodies seemingly large enough to be retained (Heron and Kerr, 1968; Vannucci, 1968), and thin, long species (Clarke et al., 1943). Although smaller mesh sizes are now available, it does not seem advisable to build conical nets with mesh sizes smaller than approximately 60 to 70 µm (equivalent to bolting cloth No. 25) because of the danger of rapid clogging in most situations.

Pooling of a number of point samples may provide an integrated sample if volumes from the individual samples are weighted according to the distance between sampling points (cf. Bernhard and Rampi, 1967; Riley, 1957). Samples from time

series from a station can also be pooled to reduce counting efforts (cf. Jensen and Sakshaug, 1970).

1.1.3. Surface film sampling. Owing to the physical properties of the surface, and chemical and biological processes associated with it, the uppermost millimeter of the sea contains very high concentrations of dead particulate matter and plankton which may include flagellates, diatoms, Trichodesmium, etc. Qualitative sampling of the uppermost 0.1 mm with a screen has been described by Jarvis et al. (1967), and with a glass plate by Harvey and Burzell (1972). Contamination from the ship or boat must be avoided.

1.2. Collecting for distributional studies

In contrast to investigations aimed at estimates of biomass of a plankton population, evaluation of specific composition must also include rare, small species which cannot be sampled with a fine net. Although much of section 1.1 applies here, larger volumes must be studied than are needed for counting the common, small species. The number of species found depends on the sample size, i.e., the frequency of specimen (cf. Hasle, 1959).

It is advisable to collect and preserve samples of 1-liter size, and to take quantitative samples by a net (cf. 1.1.2) or a continuous flow centrifuge (cf. 3.3.2) for scanning of the content of larger volumes of water. Even in 1-liter samples only a few species may be found that can be collected by towing a coarse net (170 - 180 µm mesh size; Semina, 1962). The net will, however, grossly distort the numerical composition of the samples because of loss of small species. As a less desirable alternative, many small samples, as are often available from surveys, may be pooled for searching for rare species (cf. 1.1.2 on pooling of samples).

1.3. Subsampling from collecting devices

If plankton is collected by water bottles and only a part of the contents is to be used, subsamples should be withdrawn as soon as possible. It is advisable to randomize the contents of the water bottles by mixing in order to destroy original, uneven distribution of the plankton, and to circumvent the effects of sinking or upward swimming (or floating) of organisms after enclosure in the bottles. It should be pointed out that during the mixing, some chains of cells may be broken (cf. 3.1).

2. Treatment of Live Samples and Storage of Preserved Samples

2.1. Live samples

Counts should be made in the first 1 or 2 hours after collecting. Storage in dim light (or in the dark if taken at night) at a temperature close to the in situ value is preferred. We are not aware of published experiments showing that refrigeration is advisable.

2.2. Preserved samples

2.2.1. Preservatives. A universal preservative which fixes and

preserves naked algae, armored dinoflagellates, and the calcareous organisms is not yet known (see also Recommendation No. 2). At the present time, fixing and preservation of phytoplankton in water samples with buffered formaldehyde seems most preferable. To insure good mixing, the filtered stock solution should be put into the sample bottles first, and the sample should be added rapidly. Dilution of the sample by the preservative should be taken into consideration in the calculation of cell concentrations.

There is no agreement among the Working Group on the preferred strength and pH of the formaldehyde solution for storage (preservation). The Plankton Section of the International Council for the Exploration of the Sea (Anon., 1922, p. 54) recommended a concentration of 0.5 to 0.6% of formaldehyde (1.2 to 1.5% of 40% formalin) but we do not know of the data on which the recommendation was based. We are not aware either of newer systematic studies with solutions of varying strength on fixing and preserving of phytoplankton. Several members of the Working Group prefer concentrations of 0.2 to 0.4% formaldehyde; some use 0.8 to 2.0%. We have learned that SCOR/Unesco Working Group 23 (Zooplankton Laboratory Methods), after extended experimentation, will recommend somewhat lower concentrations of formaldehyde than are now commonly used in zooplankton work. For Protozoa, a concentration of 1% formaldehyde with the addition of a bactericide and fungicide is being discussed for fixing and preserving.

There is agreement that samples with very low concentrations of fixative can deteriorate soon after opening of the bottles so that addition of some formalin is useful at that time (cf. 2.2.2). For the same reason, formalin may be added when such samples are being concentrated in settling chambers.

Buffering of the formalin is essential when calcareous organisms are to be preserved. Sodium acetate or hexamine (urotropine, i.e., hexamethylentetramine) are preferred as buffering agents (see also Recommendation No. 3). The pH of the preserved sample should be between 7.5 and 8.0 (Hasle) or at 8.5 (Bernhard). We are aware of the statement of Working Group 23 that storage of animal tissue at a pH of 8.0 and higher is not advisable. Acid-base indicators (dyes, including pH paper) cannot be used for measuring the pH of the samples because the electrolyte concentration is not known.

Another commonly used fixative and preservative is Lugol's solution (10 g potassium iodide neutrale, 5 g iodine, 5 g sodium acetate [without water] in 70 g water; a few drops to 100 ml of sample; Utermöhl, 1958). These samples must be kept in the dark. This preservative is useful in that even blue-green algae sink readily in settling chambers; however, it is necessary to compare with live material to learn the identification of the strongly stained algae. Also, the effect of long storage in acetate-buffered Lugol's solution has not been studied adequately (Utermöhl, 1958).

For phytoplankton collected by nets, preservation in 2% buffered formaldehyde is preferred. The volume of the supernatant preservative should at least equal the settled volume of the plankton.

2.2.2. Containers and storage. Storage in bottles made of soft glass with screw caps is preferred. Because surface water is usually undersaturated in respect to silicate, storage in bottles of high-quality glass (e.g., Pyrex) which does not release much silicate, or plastic bottles may result in slow solution of delicate frustules or spines of diatoms. This may happen in plastic bottles in one to a few years (see also Recommendation No. 4). It may be noted that use of glass of very low quality may result in precipitates. Storage in cool, dark rooms is recommended.

It is highly desirable, even for laboratories without depository functions, to keep samples after subsamples for counting have been removed, primarily for subsequent checking of identification of specimens. If space presents a problem, pooled samples can still be very useful. The appropriate fixative must be added after opening of the containers when material is preserved in weak formaldehyde (cf. 2.2.1), or periodically (as indicated by the loss of color) when Lugol's solution is used.

3. Preparation for Counting

3.1. Subsampling from storage containers

Thorough shaking of bottles, especially those that have been stored for a long time, is required before subsampling. Shaking of the samples tends to break up chains. Although it would be desirable to keep the chains intact, many also are broken up during collecting (Banse), fixation or storage. Thorough mixing of the sample, however, is of overriding concern for reduction of subsampling errors, a major source of possible bias. Subsampling after mixing may involve pouring of a large volume into a settling chamber or removal of a small amount of water by a piston (stempel) pipette (e.g., Frolander, 1968).

Each investigator should check his subsampling technique for absence of bias at the start of, and at fixed intervals during, his study by counting some species in subsamples taken successively from the same storage bottle (e.g., Hasle, 1969; cf. 6).

3.2. Chambers for counting dense populations

Only very high natural plankton concentrations can be counted directly with a compound microscope using a haemocytometer (cf. medical-technical handbooks for their use; also Lund et al., 1958), a counting cell such as the type proposed by Lund (1959, 1962) or Palmer and Maloney (1954), or, for low magnification, a Sedgwick-Rafter cell (Biological Methods Panel, 1969). These cells are filled by means of pipettes, which does not ensure random distribution of organisms. Therefore, counting of only parts of the chambers may lead to serious errors (cf. McAlice, 1971).

3.3. Concentrating of plankton

Usually, concentration of the plankton is required before microscopical counting.

3.3.1. Concentrating by gravity. Settling of preserved plankton from water samples in measuring cylinders and removing much of the supernatant fluid may concentrate material sufficiently for counts under a compound microscope by one of the methods outlined under 3.2. Preconcentration may occasionally also be desirable for the study of very sparse populations by the inverted microscope technique (Hasle, 1959). The procedure may be repeated with the first concentrate in another, narrower tube. The procedure thus is flexible regarding the concentration factor but involves considerable handling of the samples. This may introduce dust from the air, or organisms from the distilled water used for washing. Also, it can lead to reduction of numbers of certain species due to hanging-up on the walls (Paasche, 1960).

The settling chambers used with the inverted microscope are usually filled directly from the storage bottles. Hence, no further handling of the subsample of water is involved and there is little danger of loss of plankton or contamination. The method (Lund et al., 1958; Uehlinger, 1964; Utermöhl, 1958) is, therefore, highly preferred for the study of preserved samples. A small settling chamber (1 cm deep) has been described by Throndsen (1970). Divided (compound) chambers (Dawson, 1960; Lovegrove, 1960; Utermöhl, 1958) should be employed for settling larger volumes (>10-20 ml) because the high cylinders necessary for sufficient concentration of plankton do not permit favorable illumination during counting. In the settling chambers used with the inverted microscope method, individual algal cells can be turned over, or picked out for study under a compound microscope without disturbing the other cells (Haller-Nielsen, 1950; Throndsen, 1969a).

In any sedimentation method, some plankton may settle very slowly and reach the bottom of tall cylinders only after one or two days (cf. Hasle, 1969). Some species may not settle at all. Live counts or filtration of the supernatant fluid (cf. 3.3.3) should be used to check the adequacy of the procedure.

Methods for preparing permanent mounts from samples settled in such chambers, which moveover can be studied with ordinary compound microscopes, have been proposed by Coulon and Alexander (1972), Dickman (1968) and Sanford, Sands and Goldman (1969). The procedure by Coulon and Alexander provides for easy removal of sea salt before final mounting. For the study of diatoms, mounting media with high refractive indices (1.6 to 1.7) are preferred.

3.3.2. Concentrating by centrifugation. Centrifugation is a rapid method for concentration of live or preserved plankton, as in 3.2, and for counting can be used on board ship. The method is prone to bias when quantitative work is being done (cf. Javornický, 1958): Tender forms can be damaged by centrifuging, and several species may not settle at all, or (with cup-type centrifuges) may be resuspended upon slowing down of rotation (Steemann Nielsen, 1933); living plankton is more affected than fixed material. Checking of losses by comparison of counts with those obtained from preserved samples settled

by gravity (cf. 3.3.1), or by filtration of the supernatant (cf. 3.3.3), is mandatory.

The tips of the conical tubes in the cup-type centrifuges should be pointed instead of rounded, and the supernatant siphoned off rather than decanted. Construction and use of continuous flow centrifuges is described by Hartman (1958) and Kimball and Wood (1964). When reporting on the technique, the centrifuge should be described in terms of acceleration (g) instead of number of revolutions per unit time.

3.3.3. Concentrating by filtration. Live or preserved plankton can be concentrated by filtering the water sample at low suction pressure over membrane-type (collodium, etc.) filters. Because complete resuspension of material on the filter for transfer into a counting chamber is difficult, the plankton is usually stained and counted on the filter after the water and air in the matrix of the filter have been replaced by immersion oil or another neutral substance of the same refractive index as the matrix of the filter (1.5 in nitrocellulose) (Biological Methods Panel, 1969). The preparations can serve as a permanent mount (see also Recommendation No. 5). This method can be used on board ship.

By filtration of live samples, organisms may be collected that cannot be preserved otherwise. However, many naked algae, or thin-walled diatoms, may be deformed or broken. Therefore, the method is more suitable for counting of species known to the investigator than for identification of unknown ones. In any case, the investigator must compare the counts with those from another method to check the suitability of the mounting medium, even for intermediate-sized organisms, and estimate the loss of cells (cf. Javornický, 1958, for losses).

4. Instruments and Procedures for Counting

4.1. Microscopic equipment

The instrument must allow magnification of up to 500 x for routine counting. It is convenient to have objectives staggered by a factor of 2 x to 2.5 x. For use with white light, achromatic objectives are sufficient but phase contrast is a much more efficient way of counting, especially with live material. The ocular must contain a grid or be provided with hairlines or threads (Lund et al., 1958; Utermöhl, 1958) which facilitate scanning of the central part of the field of view. Thus, all organisms counted during traversing (as contrasted to counts of randomly chosen fields) are in view for an approximately equal period of time. For use with the inverted microscope, as well as with a haemocytometer on a thick slide, a condensor with a long working distance is preferred.

4.2. Use of fluorescence

Illumination with ultraviolet light can be used to distinguish chlorophyll-containing algae from pigment-free algae, or to facilitate distinction of organisms from organic detritus (Wood, 1962; Wood and Oppenheimer, 1962). In live samples the autofluorescence of chlorophyll can be used for the first purpose, and staining of protoplasm with acridin orange or similar

dyes for the latter. Identification of organisms (as contrasted to mere counting of cells) is difficult because the outlines of the cells are hard to see.

The procedure for counting with ultraviolet light, as described by Wood (cf. Biological Methods Panel, 1969) requires 4 - 5 liters of water when dealing with sparse populations.

4.3. Use of narcotization

Live counts are often facilitated by narcotizing the organisms with drops of isotonic magnesiumchloride solution or one drop of saturated uranylacetate to the water under the cover slip. Alternatively, the viscosity of the water may be increased by adding methyl cellulose.

4.4. Notes on microscopical procedures

Counting of unnecessarily large numbers of cells should be avoided because of the other errors inherent in plankton work (Gillbricht, 1962; Holmes and Widrig, 1956; Lund et al., 1958; McAlice, 1971; Uehlinger, 1964; cf. 6). Often, therefore, the entire area of the chamber would not be enumerated. However, unless the plankton is randomly distributed in a chamber (cf. the above references; also, Sanford et al., 1969), the enumeration effort must be distributed uniformly (for example, by counting in cylindrical chambers along every second, third, or fourth parallel traverse). Random distribution of chains is unlikely for certain species which do not sink vertically; they tend to be deposited preferentially around the edges of tall settling chambers. To avoid the necessity of counting of only a portion of the bottom area of a chamber for common forms, yet to have large volumes settled for counting of rare species, it is useful to fill a series of chambers of different volumes and bottom areas (for example, 1, 5, 25, 100 ml) from each sample and enumerate each in full. It may be noted that there will be a differential effect on the variance of the overall result when the counts are standardized to a common volume (cf. 6).

In enumerating along traverses rather than in randomly chosen fields, small forms must be counted with a magnification higher than is needed for their recognition while the chamber is at rest.

4.5. Use of serial dilution culture

This method may yield large numbers of small flagellates not found in preserved samples. The principle of the approach is described in every bacteriological manual (e.g., Rodina, 1972). Growth of diatoms in such cultures can be specifically suppressed by adding germanium oxide (Lewin, 1966). The precision depends on the number of replicates but is in practice low because the degree of replication is limited. Serial dilution may only be used as a supplement to other methods because all species cannot be cultured on a given medium so that serious distortions of the specific composition of the plankton assemblage will be observed. Recent marine applications are given by Bernhard et al. (1967) and Throndsen (1969b).

4.6. Electronic counting

With the available electronic (automatic) counters, it is possible to enumerate and size particles, and to discriminate between chlorophyll-containing and other material. Electronic counters can be applied to enumerating species if cultures of known specific composition or plankton blooms with strong dominance of algae are studied which do not overlap in size with other particles. If size (volume) of algae is to be determined, live samples must be used; preserved cells register considerably smaller volumes in instruments which estimate volume from change of conductance (Eppley). Descriptions of various counting procedures for marine biological purposes using the Coulter counter are given by Sheldon and Parsons (1967).

Automated estimation of volume of phytoplankton in natural seawater by incubation (see Eppley, 1969) has been proposed by Cushing et al. (1968). The approach assumes a constant growth rate of all species during this period.

5. Expression of Results

While reporting names of species, it should be noted that the rules of nomenclature differ for the Plant and Animal Kingdoms. To permit later checks of identification, efforts should be made to deposit representative samples, preferably as permanent mounts, in large museums. Also, the deposition in a recognized data center of a copy of the tabulated raw data that cannot be published adds greatly to the usefulness of an investigation (see also Recommendation No. 7).

Whereas counting of cells provides the distribution of specimen numbers over the species encountered, many problems require a knowledge of the sizes of the organisms. Computation of surface area has been suggested for reducing the apparent predominance of the small forms when numbers are being considered and of the large forms when volume or weight is calculated (e.g., Paasche, 1960; Smayda, 1965). When turnover rates are needed as provided by the ratio of rate of carbon uptake over concentration of carbon, or efficiencies of food conversion are wanted, the carbon content of phytoplankton can be computed from measurements of cell dimensions according to Eppley, Reid and Strickland (1970)*, Strathmann (1967) and Zeitzschel (1970). Other mass estimates often employed are cell volumes, which, however, include vacuoles as inert spaces (Lohmann, 1908). For any of such computations, only cell dimensions should be used which have been determined for the season and geographic area under study. (For computations, see also Recommendation No. 7).

6. Determination of Errors

Whereas faulty methods lead first of all to systematic errors, random errors in the results stem from the variability of plankton distribution in space or time, the procedure of sampling from the populations to be studied, the subsampling from the collecting devices and the storage bottles, and the counting

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*The paper contained a misprint: the formula actually used for diatoms was $\log C = 0.76 \log V - 0.352$ (Eppley).

itself (effect of low numbers). The systematic errors can be nearly avoided. The random errors can only be kept small; they do not cancel each other. To the contrary, patchiness affects precision and accuracy of plankton sampling (Wiebe and Holland, 1969). Unless very large differences (orders of magnitude) are investigated, ignorance of the resulting overall error prevents profitable discussion of differences between observations.

Generally speaking, the goal of studies of distribution in space or time is the determination of representative values (usually means) of concentrations of organisms and the associated variability, rather than the determination of concentrations in single samples. As a minimal requirement for determining the variability, statistical tests should be applied after conclusion of the field collection to indicate the statistical significance of the results. Replicate samples and replicated steps in the procedures are the usual raw material for such tests. It is often overlooked, however, that replicates in space do not measure the variability in time which is needed for the evaluation of time series.

Much more advisable than a study-after-the-fact is to make a before-hand investigation of variability. If cell concentrations in point samples taken almost simultaneously several tens or hundreds of meters apart, or on the same spot hours or days apart, are found to differ by a factor of ten (larger variability is known, e.g., Bernhard and Rampi, 1966), a study of the seasonal cycle based on weekly collection of point samples is not worth beginning unless the seasonal range of concentrations may be expected to be much larger than tenfold; this statement, of course, assumes that the number of samples that can be taken on any date, and counted, is limited. The solution to the problem may be a scheme of pooling point samples or of taking integrated samples (cf. 1.1.2), which may require approximately the same ship time but reduces the cost of counting.

An initial study of natural variability (microdistribution) and procedural errors is mandatory when a before-hand estimate of the effort (for example, number of samples) needed for a result with given confidence limits is wanted. This also permits rational--rather than intuitive--decisions on the allocation of effort (for example, more emphasis on field collection or on laboratory enumerations, stratified sampling, pooling of samples, short-cuts, etc.). Given limited funds and time for completion of an investigation, proper strategy is the difference between success and failure.

The specifics of the sampling design will depend on the objectives of the work. Introductions to general methods for planning field sampling are available in Cassie (1968), Cochran (1963), Greig-Smith (1964) and Raj (1968). Guides for estimating errors of plankton counting in the laboratory are given by Holmes and Widrig (1956), Gillbricht (1962), Lund et al. (1958) and Uehlinger (1964). The contributions of subsampling errors to total variance of a sample, and suggestions for trade-offs of error sources are treated by Venrick (1971; see also Kelley and McManus, 1969). Most of the procedures recommended in these papers are based on parametric statistics and necessitate normalizing of the counts before application. Often, nonparametric methods (e.g., Tate and Clelland, 1957), which do not require normality, are adequate.

III. A MANUAL FOR METHODS USED FOR QUANTITATIVE ASSESSMENTS OF PHYTOPLANKTON POPULATIONS

Item 4 of the Terms of Reference was interpreted as asking for suggestions for a list of content of a manual.

We believe strongly that a manual on methods of phytoplankton research is needed that emphasizes the investigation of distribution of phytoplankton in space and time, and stresses the determination of concentrations instead of rates. The treatments of these subjects by the Biological Methods Panel (1969) and by the IBP Handbook edited by Vollenweider (1969), useful as they are, do not give enough details for guiding the person who enters the field with only general training in biology or in a related discipline. For the content of such a manual, the general ecological background for putting a research plan into perspective, some rationale for choosing among different methods, and detailed descriptions of procedural steps and pitfalls seem warranted. The manual should not be restricted to microscopical study of phytoplankton but should also treat briefly pigment analysis and related measurements because specific composition is only one aspect of a plant community, physiological parameters like pigment content being another one. The treatment of rate measurements, however, including their connection with experimental work in the laboratory, would presumably expand the scope of such a manual too much, even though concentrations of organisms and the rates of change are equally important aspects in ecological research.

1. Subjects to be Treated

1.1. Goals of investigating the distribution of phytoplankton in space and time

(Essentially a short referenced guide to phytoplankton ecology, which is needed to provide scope to studies of distribution, and which would range from food chain research through speciation to history of the oceans. The role of the experimental approach to studies of this kind should at least be mentioned and references for obtaining and analyzing collateral samples, e.g., for determination of chlorophyll and nutrient salts, should be included.)

1.2. Sampling design

(A treatment ranging from the choice of sampling depths in the presence of discontinuity layers to the optimization of sampling and effort on the basis of studies of variability. Studies of variability will in turn have to be explained.)

1.3. Instrumentation and methods for the field

(Also including details on the minimum requirements for the design and performance of water bottles, especially for sterile sampling, instructions for their use, and samples of protocol sheets.)

1.4. Instrumentation and methods for the laboratory

(Again, detailed instructions should be provided. If

a new working group on microscopical methods in phytoplankton studies is established [see Recommendation no. 1], the results of the experimental work should be taken into consideration.)

1.5. Evaluation of observations

(May also involve brief explanations of methods like computation of diversity indices.)

1.6. Guide to the literature for identification
(An annotated bibliography)

Such a manual would obviously require several authors; however, there should be a managing editor responsible for cohesiveness among chapters. We believe that, generally speaking, a biological oceanographer (ecologist) would be preferable to a taxonomist.

2. Suggestions for Interim Aid to Investigators

The writing of a manual of methods of quantitative assessments of phytoplankton populations will take considerable time. Therefore, some members of the Working Group believe that, in the interim, reprinting 15-25 previously published key articles on methods as a paperback book would be useful for investigators wanting to start the study of phytoplankton. In addition to individual investigators, many marine stations with small libraries would be interested in obtaining these books. Such compilations, as well as taxonomic studies, which were photographically reproduced, have been commercially successful in other fields.

Examples of papers on methods that might be included are those by Braarud (1958), Lund *et al.* (1958), Palmer and Maloney (1954) and Utermöhl (1958). Papers on well-executed distributional studies might also be added. Recent papers in widely distributed journals and purely taxonomic papers should be omitted, but references to pertinent papers should be included.

Some members of the Working Group, however, hold that most of the references which are likely candidates for inclusion have been reviewed in the IBP Handbook, edited by Vollenweider (1969), and the booklet by the Biological Methods Panel (1969). They believe that making certain taxonomic papers more widely available is at least as important as reprinting papers on methods as outlined.

IV. RECOMMENDATIONS

1. Microscopical Methods in Phytoplankton Studies (New Working Group)

Working Group 33 did not answer items 2 and 3 of the Terms of Reference concerned with selecting the most satisfactory phytoplankton methods and providing detailed procedures. The need for such recommendations is underlined by the results of counting experiments by members of Working Group 33 (see Appendix). Since the participants in the experiment had experience in phytoplankton work, it is likely that subtle variations in procedures for concentrating or counting may lead to grossly varying results anywhere. The implications for field work, especially in cooperative studies, are plain.

In our opinion, there is no chance that electronic pattern recognition and automated counting will replace the human eye in identification of phytoplankton species in oceanographic laboratories in the foreseeable future.

It is recommended, therefore, that a new working group be set up with the following terms of reference:

> To prepare detailed procedures for the concentration and the microscopical counting of phytoplankton from sea water samples. The work should involve the experimental checking, if needed, of specific points in published methods and the experimental comparison of the chosen procedures.
>
> The procedures should comprise:
>
> (a) concentration by gravity
> i) in suitable containers for transfer into chambers to be used under a compound microscope or into chambers as in ii.
> ii) in chambers to be used in the inverted microscope procedure.
> (b) concentration by centrifuge
> i) cup-type
> ii) continuous-flow
> (c) concentration by membrane-type filters, including the preparation of permanent mounts
> (d) procedures for counting (also applicable to samples that have not been pre-concentrated), including the statistics of subsampling from containers and settling chambers (partial counting).

It seems advisable to draw on the limnological experience and invite one or two limnologists to become members of the Working Group. Because considerable experimental work can be expected, one or two members of the Working Group should be knowledgeable in experimental design. It would also be useful for the editor of the handbook (section III) to be a member.

2. Preservatives (General)
(Cf. II.2.2.1)

The search for more satisfactory fixatives and/or preservatives for phytoplankton is of utmost importance. The main problems are the fixation of naked flagellates and the effect of long-term storage of quantitative samples. The study of storing quantitative samples for periods of 5 to 10 years is required to facilitate current work on nannoplankton, and of 100 to 200 years for monitoring of species composition over very extended periods.

It is recommended that work by an interested biochemist or histologist, cooperating with a phycologist, be financed similarly as was done in SCOR/Unesco Working Group 23.

3. Buffered Formalin
(Cf. II.2.2.1)

In the absence of a better fixative and preservative, buffered formalin is widely used for fixing and preserving of phytoplankton. Its pH is important because of the desirability of avoiding solution of $CaCO_3$ (coccoliths) and the adverse effect of alkaline media in dissolving proteins and diatom frustules. Among the buffering agents often used, sodium bicarbonate and calcium carbonate tend to result in precipitates. Hexamine was employed very widely for zooplankton preservation during the International Indian Ocean Expedition, but bad decay of Crustacea has been reported by the Indian Ocean Sorting Center and Working Group 23.

It is recommended that the buffering agents to be used with formalin in phytoplankton work be tested. The desirability of counting small zooplankton in the same samples should be considered.

4. Storage of Diatoms in Aqueous Solution
(Cf. II.2.2.2)

Net hauls can be transferred after fixing into a suitable medium (like alcohol) for preservation. This is not possible with plankton in water samples. However, delicate diatom frustules dissolve, especially in alkaline water, during storage. Also, members of the Working Group have conflicting experience on the utility of plastic bottles which on shipboard are preferable to glass bottles.

It is recommended that long-term studies be initiated to investigate the preservation of diatoms in general, and the utility of plastic bottles in particular.

5. Permanent Microscopical Mounts of Quantitative Samples
(Cf. II.3.3.1 and II.3.3.3)

Even when the problem of storing of water samples with phytoplankton can be solved, permanent mounts are of great importance when samples have to be studied several times, for

example, in connection with court proceedings on pollution or with monitoring of the oceans. Whereas samples on cleared membrane filters provide for this in respect to the large forms with some skeleton, recognition and even preservation of small forms is difficult.

> It is recommended that studies be commissioned on the preparation of whole mounts of phytoplankton samples which maintain the quantitative character of the samples and also allow recognition of forms of the size of about 5 µm.

6. Phytoplankton Course for Experienced Participants

Many workers in the field of phytoplankton studies are self-trained. After some time spent in such investigations, they realize how difficult the subject is, both in regard to sampling (including sampling design) and in regard to identification. For such scientists, a short visit to a laboratory specializing in phytoplankton research can result in enormous advancement. Many realize this and individually approach institutions which may offer help. The needs of such individuals cannot easily be incorporated in the daily routine of a small laboratory, although personnel may have considerable experience in advanced instruction of the type needed.

The Working Group believes that an effective improvement of the situation might be obtained through the arrangement of specialized courses for people with some experience in phytoplankton work. As a first step, a course might be conducted with the University of Oslo responsible for the teaching program.

A tentative plan for a course of this type would be:

(1) training in identification and counting of marine planktonic algae (3 weeks)
(2) establishment of unialgal cultures for quantitative and qualitative studies (1 week).

Three instructors should be involved. The optimal number of visiting scientists is 8-10.

> It is recommended that SCOR consider the advisability of suggesting such a course, including the best way of financing it, and perhaps approach the International Association for Biological Oceanography/International Union of Biological Sciences for further guidance.

7. Catalogues of Computer Programs

Converting cell counts to numbers per units of volume or surface, computing cell volumes, etc., is time consuming. The calculations can be easily computerized, as is the case in calculating diversity indices, etc. Many programs are already available, but it is difficult for the individual worker or laboratory to learn about them. Considerable saving of time for the scientists, and of funds for programmers, would be derived

from having catalogues which list for each program the name, author, language, purpose and/or capability, machine needs, and the availability (cost) of copies.

> It is recommended that SCOR consider the utility of having catalogues which list computer programs useful in biological oceanography periodically prepared.

8. Deposition of Raw Data in National Data Centers (Cf. II.5)

Few scientific journals accept raw data for publication. Also, journals discourage the duplication of the content of figures in tables even if numerical values cannot be accurately read from the former. It seems advantageous to have original biological data preserved and made available, even when reporting forms or units have not been standardized, as long as some assessment of quality is possible.

> It is recommended that SCOR encourage biological oceanographers to deposit their data in national data centers in a form that can be copied; also, SCOR should urge the national committees to find ways to cover the costs to the authors arising from such a procedure.

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VI. POSTSCRIPT

This section is concerned with developments since the submission of the Final Report. It is mostly based on minutes of the meetings of the SCOR Executive Committee in Texel (May 1973; cf. SCOR Proceedings 9) and Canberra (February 1974; yet unpublished), and on correspondence of W.S. Wooster while President of SCOR, and R.I. Currie, Secretary of SCOR, with K. Banse, Chairman of Working Group 33. The page references below refer to the present paper.

Manual (p. 13): Dr. A. Sournia, Muséum National d'Histoire Naturelle, Pêches Outre-Mer, 75005 Paris, has been invited to serve as the editor for a handbook with a content which largely follows the suggestions outlined in Section III. A small meeting will be convened during late 1974 in Oslo to discuss details of contents, choice of authors, and a schedule. SCOR has agreed to fund the attendance of four participants at the meeting.

Reprinting (p. 14): The SCOR Executive Committee discussed the reprinting of a collection of articles on methods and agreed that, although this might be done relatively quickly as an interim measure, it was preferable for the whole question to be considered further by Editorial Committee.

Recommendation 1 (New Working Group [p. 15]): The SCOR Executive Committee has asked the International Association of Biological Oceanography (IABO) to discuss the establishing of a working group charged with the study of the inverted microscope method, and to report on this discussion to the General Meeting of SCOR in September 1974.

Recommendations 2 and 3 (Preservatives: General; Buffered Formalin [p. 16]): The SCOR Executive Committee recognizes the need for working on these problems of long standing. Little can be done by SCOR, however, until investigators who are willing to undertake such studies can be found.

It was suggested that the question of buffered formalin might be already covered in the forthcoming manual prepared by SCOR/Unesco Working Group 23 (Zooplankton Laboratory Methods). The discussions within Working Group 33, however, clearly indicate that methods for fixing and preserving of ciliates or copepods might not be suitable for phytoflagellates.

Recommendations 4 and 5 (Storage of Diatoms; Permanent Microscopical Mounts [p. 16]): The SCOR Executive Committee wondered whether it would be appropriate to ask one of the Sorting Centers to undertake such studies. The chairman of Working Group 33 agrees that sorting centers or museums with appropriate talent among their staff might indeed best provide the continuity needed for such experimentation. The initial work on the methodology and the statistical design of the experiments, however, might better be performed by a working group in order to draw on a wider range of expertise. The experimental design is of utmost importance because it will be very difficult in these experiments to assess the operator errors when 10 or 20 years have intervened, i.e., to separate the questions of precision

and accuracy of the operators from the possible decay in the samples.

Recommendation 6 (Phytoplankton Course [p. 17]): The SCOR Executive Committee agreed to encourage the University of Oslo to establish a course with a content appropriate for experienced biologists. If such a course were offered, SCOR would ask its National Committees to designate suitable participants and would also request financial support from Unesco for selected scientists from developing countries who take the course.

Recommendations 7 and 8 (Catalogues of Computer Programs; Deposition of Raw Data [pp. 17, 18]): No special action was taken by SCOR beyond raising the question whether it would appeal to people in one of the Data Centers to maintain catalogues of programs.

Seattle, 1 May 1974 Karl Banse

APPENDIX

REMARKS ON COUNTING EXPERIMENTS

The intent of the experimental work by the Working Group was to see whether agreement could be obtained on the ranking of some commonly used methods for enumerating phytoplankton; the members of the Working Group attending the Kingston meeting did not hold a unanimous opinion on this key point.

The experimental design was to have the members apply their own methods and procedures, in their home laboratories, to two subsamples prepared by the University of Washington, Seattle, and mailed to each participant. One was to be drawn from a sample with approximately 50,000 cells/liter, and another one from a sample with 1 Mill. cells/liter, thus spanning a reasonable range of concentrations. The samples were to be made up from cultures of five or six easily recognizable species, and the participants informed of the species they would find. Two aliquots from each subsample were to be counted, and several aliquots were to be counted twice (to separate the counting error from the error arising from withdrawing the aliquot). The error from the subsampling of the original samples was not to be assessed.

It was hoped that several persons would use the inverted microscope method which is preferred by the greater number of the members of the Working Group, and that others would use centrifuges or membrane filters for concentrating and count on various types of counting slides or on membrane filters by means of a compound microscope. It was also hoped that errors--if any--introduced by the centrifuging could be assessed separately from those arising from the subsequent handling. As the scope of the experiment was ambitious, it was expected that members could evaluate data already available but not discussed during the Kingston meeting which would eliminate the need for some of this experimentation; this was, however, not the case.

Two experiments were attempted. In both, samples were prepared from pure algal cultures, the concentrations of the stock cultures were assessed by replicated counts with a haemocytometer, and then the appropriate volume of culture added to filtered sea water; in the second attempt, the stock cultures were fixed before determining cell numbers and diluting appropriately.

The first experiment was aborted, however, because of complaints that small cells could not be found; for the two non-diatom species, at least, preservation problems might have been the reason, but this could have not been so for the small diatom. Thus, only two colleagues made counts. They used the inverted microscope. For the second experiment, four scientists enumerated with the inverted microscope method, concentrating by gravity within the counting chambers; one centrifuged and counted on a suitable slide; and one used a direct count on ship board, without pre-concentration, for the high-density sample.

The results of the experiment confirm many statements in the

literature that the counting error (duplicate counting of an aliquot) is small, and that withdrawing aliquots from a storage bottle leads to a subsampling error of up to several ten per cents. The principal problem turned out to be, however, severe systematic errors which seem to arise from the methods as such and the skill in using them: the majority of the reported cell numbers (means for each species from two aliquots per operator) were off by several hundred per cent, being usually too low. The apparent loss of cells can be traced for some species and operators to the use of too low a magnification during enumeration, but other important sources of bias cannot be pinpointed. For both pairs of subsamples, the counts with the inverted microscope method as used in Oslo were closest to the expected values; it is noteworthy that the second pair was counted by a different person than the first one. Otherwise, however, gross systematic errors were incurred even within the inverted microscope method. No purpose would be served by recording the numbers of cells enumerated by the participants as it is clear that the goal of the experiment has not been attained.

The experiment showed that it can be difficult for persons with some experience to enumerate cells accurately even when preservation problems are minor (e.g., medium sized diatoms). Analogous problems have been experienced in other fields of oceanography with comparatively much simpler methods, e.g., the determinations of oxygen, phosphate or silicate concentration; however, the difficulties have been overcome by meetings of working parties to study the methods further, as well as by sending samples to various operators or institutions during cooperative investigations in order to intercalibrate. For plankton counting, intercalibration of procedures likewise seems mandatory at the present time when results from different operators must be compared. If this is to be accomplished by mailing of mock plankton samples as was done by Working Group 33, the following hints will be useful: Culture media should contain as little dissolved organic matter as possible. Species, or conditions of growth, should be chosen so that organic matter is not excreted during growth. Only algae with strong frustules should be used so that problems in fixing and preservation, including differing length of storage during the intercalibration work, do not cause variability. Shapes of algae should differ sufficiently so that errors in identification during enumeration will be excluded.