The Enumeration of Algae, Estimation of Cell Volume, and Use in Bioassays 1990

Methods for the Examination of Waters and Associated Material

This booklet contains a general introduction followed by three sections:

A Algal Cell Counts

B Algal Cell Volume

C Algal Bioassays

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About This Series

This booklet is part of a series intended to provide recommended methods for determining the quality of water and associated materials. In addition short reviews of the more important analytical techniques of interest to the water and sewage industries are included.

In the past, the Department of the Environment and its predecessors, in collaboration with various learned societies, have issued volumes of methods for the analysis of water and sewage culminating in 'Analysis of Raw, Potable and Waste Waters'. These volumes inevitably took some years to prepare, so that they were often partially out of date before they appeared in print. The present series is published as a series of booklets on single or related topics; thus allowing for the replacement or addition of methods as quickly as possible without need of waiting for the next edition. The rate of publication will also be related to the urgency of requirement for that particular method.

Although ideally, all methods published should be fully tested, this is not often possible without delay in publication. Furthermore, the limit of detection, range, precision and interference effects applying to instrumental methods can depend on the actual instrument used, as well as on sample type, reagent purity, operator skill, etc. Even methods tested in many laboratories have been known to acquire problems when a new domestic product appeared (introducing a new substance into effluents), changes in production methods altered reagent quality, or the method was used to analyse a new type of sample (despite apparent similarity to samples already evaluated). As a guide, the following categories have been given to methods: tested, usually in five or more laboratories

— no grade indicated;

tested in one to three or four laboratories

— Tentative;

evaluated, but not fully tested, but publication is urgently required

— Note;

tested and found to be satisfactory by several laboratories, but in the opinion of experts requires a high degree of skill or has some other difficulty such that the method would be replaced if a better method were discovered

— Provisional.

The aim is to provide as complete and up to date a collection of methods and reviews as is practicable, which will, as far as possible, take into account the analytical facilities available in different parts of the Kingdom, and the quality criteria of interest to those responsible for the various aspects of the water cycle. Because both needs and equipment vary widely, where necessary, a selection of methods may be recommended for a single determinand. It will be the responsibility of the users—the senior technical staff, to decide which of these methods to use for the determination in hand. Whilst the attention of the user is drawn to any special known hazards which may occur with the use of any particular method, responsibility for proper supervision and the provision of safe working conditions must remain with the user.

The preparation of this series and its continuous revision is the responsibility of the Standing Committee of Analysts (to review Standard Methods for Quality Control of the Water Cycle). The Standing Committee of Analysts is a committee of the Department of the Environment set up in 1972. Currently it has 9 Working Groups, each responsible for one section or aspect of water cycle quality analysis. They are:

1.0 General Principles of Sampling and Accuracy of Results
2.0 Microbiological Methods
3.0 Empirical and Physical Methods
4.0 Metals and Metalloids
5.0 General Nonmetallic Substances
6.0 Organic Impurities
7.0 Biological Monitoring
8.0 Sewage Works Control Methods
9.0 Radiochemical Methods.

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and the main committee. The names of those associated with this method are listed inside the back cover.

Publication of new or revised methods will be notified to the technical press, whilst a list of Methods in Print is given in the current HMSO Sectional Publication List No 5.

Whilst an effort is made to prevent errors from occurring in the published text, a few errors have been found in booklets in this series. Correction notes and minor additions to published booklets not warranting a new booklet in this series will be issued periodically as the need arises. Should an error be found affecting the operation of a method, the true sense not being obvious, or an error in the printed text be discovered prior to sale, a separate correction note will be issued for inclusion in that booklet.

L R PITTWELL
Chairman and Secretary

1 February 1990

Warning to Users

The analytical procedures given in this booklet should only be carried out by competent trained persons, with adequate supervision when necessary.

Local Safety Regulations must be observed.

Laboratory procedures should be carried out only in properly equipped laboratories.

Field Operations should be conducted with due regard to possible local hazards, and portable safety equipment should be carried.

Care should be taken against creating hazards for one's self, one's colleagues, those outside the laboratory or work place, or subsequently for maintenance or waste disposal workers. Where the Committee have considered that a special unusual hazard exists, attention has been drawn to this in the text, so that additional care might be taken beyond that which should be exercised at all times when carrying out analytical procedures. Reagents of adequate purity must be used, along with properly maintained apparatus and equipment of correct specifications. Specifications for reagents, apparatus and equipment are given in manufacturers' catalogues and various published standards. If contamination is suspected, reagent purity should be checked before use.

The best safeguard is a thorough consideration of hazards and the consequent safety precautions and remedies well in advance. Without intending to give a complete check-list, points that experience has shown are often forgotten include: laboratory tidiness, stray radiation leaks (including ultra violet), use of correct protective clothing and goggles, removal of toxic fumes and wastes, containment in the event of breakage, access to taps, escape routes, and the accessibility of the correct and properly maintained first-aid, fire-fighting, and rescue equipment. Hazardous reagents and solutions should always be stored in plain sight and below face level. Attention should also be given to potential vapour and fire risks. If in doubt, it is safer to assume that the hazard may exist and take reasonable precautions, rather than to assume that no hazard exists until proved otherwise.

There are numerous handbooks on first aid and laboratory safety. Among such publications are: 'Safe practices in Chemical Laboratories' and 'Hazards in the Chemical Laboratory', issued by the Royal Society of Chemistry, London: 'Safety in Biological Laboratories' (Editors Hartree and Booth), Biochemical Society Special Publication No 5, The Biochemical Society, London, which includes biological hazards; and 'The Prevention of Laboratory Acquired Infection', Public Health Laboratory Services Monograph 6, HMSO, London.

It cannot be too strongly emphasised that prompt first aid, decontamination, or administration of the correct antidote can save life; but that incorrect treatment can make matters worse. It is suggested that both supervisors and operators be familiar with emergency procedures before starting even a slightly hazardous operation, and that doctors consulted after any accident involving chemical contamination, ingestion, or inhalation, be made familiar with the chemical nature of the injury, as some chemical injuries require specialist treatment not normally encountered by most doctors. Similar warning should be given if a biological or radiochemical injury is suspected. Some very unusual parasites, viruses and other micro-organisms are occasionally encountered in samples and when sampling in the field. In the latter case, all equipment including footwear should be disinfected by appropriate methods if contamination is suspected. If an ambulance is called or a hospital notified of an incoming patient, give information on the type of injury, especially if poisoning is suspected, as the patient may be taken directly to a specialized hospital.

Safety while Sampling

Prior consideration must be given, especially when sampling in confined spaces or where access is difficult, to guard against suffocation, drowning, falls, and poisoning or infection by ingestion, inhalation, or skin contact.

Good Laboratory Practice

The Department of Health issues a booklet entitled: Good Laboratory Practice; the United Kingdom Compliance Programme, 1989.
This can be obtained by writing to that Department in London. It deals chiefly with toxicity studies, but much can be applied to analytical chemistry.

The enumeration of algae, estimation of cell volume, and use in bioassays 1990

Introduction

Algae are natural constituents of nearly all surface waters, both lotic and lentic. As such they play a vital role and may be responsible for the major part of the primary production in aquatic ecosystems. When nutrients and light are plentiful large growths may occur which can give rise to water quality and engineering problems such as colouration of the water, production of tastes and odours and the clogging of filters. Some species produce toxins, which have been associated with fish and other wildlife mortalities. Thus a knowledge of their abundance, estimated quantity and growth potential is of great interest to water scientists and engineers. The purpose of this booklet is to set down the basic methods for counting cells, describe the measurements and calculations required for determining cell volume and finally to describe the procedures for using certain species as bioassay organisms in order to estimate the nutrient status or potential toxicity of a water body.

Section A Algal Cell Counts

A1 Introduction

In most field studies of algae there is a requirement to determine cell numbers or biomass. This may be achieved in direct or indirect ways (eg light microscopy counts, dry weight, organic carbon, cell volume, chlorophyll *a* content). Among these long-established methods only microscopy allows the worker to identify the species present, although it is likely that other methods for identifying species will soon become widespread.

By estimating changes in cell numbers in a succession of samples understanding is gained of the dynamic aspects of species composition in a community. From these numbers it is also possible to estimate by indirect calculations the amount of algal biomass present at any given time.

This part of the booklet describes various methods available for estimating algal cell numbers in sufficient detail to allow the reader to choose those methods most suited for the populations in their samples.

The main topics considered are:

Sample preparation

Microscopic enumeration

Electronic enumeration.

No single method for estimating algal numbers is ideal for all species because of the wide range in cell size (< 1 to >200 μm) and the great variety of morphology (single cell, colony, filament etc). Two groups of methods are available, direct and indirect.

Direct methods

Counting by direct microscopical examination provides a quantitative assessment of the numbers and species. In contrast methods of counting, using electronic particle counters, provide an estimate of the total number of algal and other particles (isolated cells or colonies) in a known volume of water.

Indirect methods

In these methods the numbers of cells present may be inferred or estimated from determinations of another variable. These may involve passing light through a sample (light scattering nephelometry), light attenuation, *in vivo* fluorescence, chemical methods etc.

Only direct methods are considered in this booklet; indirect methods are described elsewhere in the SCA series.

A2 Preparation of samples

A2.1 Preservation

All samples, with the exception of those used for certain indirect methods such as *in vivo* fluorescence (but see A4.3.2) or chlorophyll extraction, should be fixed in a suitable preservative immediately after collection. The choice of preservative can pose problems with some of the more delicate forms, especially those with flagella. The most widely recommended preservative is Lugol's iodine solution. This may be prepared by dissolving 20 g potassium iodide in 200 ml of distilled water and then adding 10 g iodine. The solution must not be supersaturated with iodine since this can result in crystal formation with consequent interference in counting. Supersaturation can be tested by diluting 1 ml of stock solution to 100 ml with distilled water. If iodine crystals appear after standing, more potassium iodide (approx 5 g) should be added

and the test repeated. If no crystals appear, 20 ml glacial acetic acid is mixed in and the solution is ready for use. It should be stored in a darkened bottle out of the light. Lugol's iodine is used at a concentration of 1–2 ml per 100 ml sample. A note should always be made of the exact volume of preservative used and the volume of the sample, if counting is to be done later. Iodine has the disadvantage of staining both the algae and organic detritus, possibly making them difficult to distinguish. It can also distort or disrupt more delicate cells. When such delicate forms are present it may be preferable to use an alternative fixative such as formaldehyde neutralised with calcium carbonate. This can be used as 5 ml 100 ml^{-1} sample, but must be used with caution, avoiding inhalation and contact with skin.

Buffered glutaraldehyde (take care!) is required if electron microscopy is to be used later.

Sample preservation is best carried out immediately after collection, but, if samples are to be used for chlorophyll estimation or certain other indirect methods, no preservative should be used.

If identification from fresh material is needed, either take a larger sample and subdivide, or keep a duplicate sample.

A2.2 Sample pre-concentration

When algal cell densities are very low, the sample may need to be concentrated.

A2.2.1 Concentration by sedimentation

Concentration by sedimentation is slower than other methods, but is gentler and, according to Lund *et al.* (1958), is quantitative. All species can be sedimented if conditions are right. Difficulties may be experienced, however, with certain buoyant species such as *Microcystis, Aphanizomenon* and *Anabaena*. If preliminary observations indicate the presence of such buoyant species, collapse of the gas vacuoles prior to preservation is recommended. This may be achieved by filling completely a soft-walled polythene bottle with the sample and firmly screwing on the cap. The bottle is then dropped from a height of about 1 m on to a hard surface. The impact suddenly increases the pressure inside the vessel thus collapsing the gas vacuoles in the algae. The algae should then sink. If enough algae are present a change in colour may also be noted due to changes in light refraction, although no visible difference will be apparent if cell densities are low. The process may need to be repeated several times.

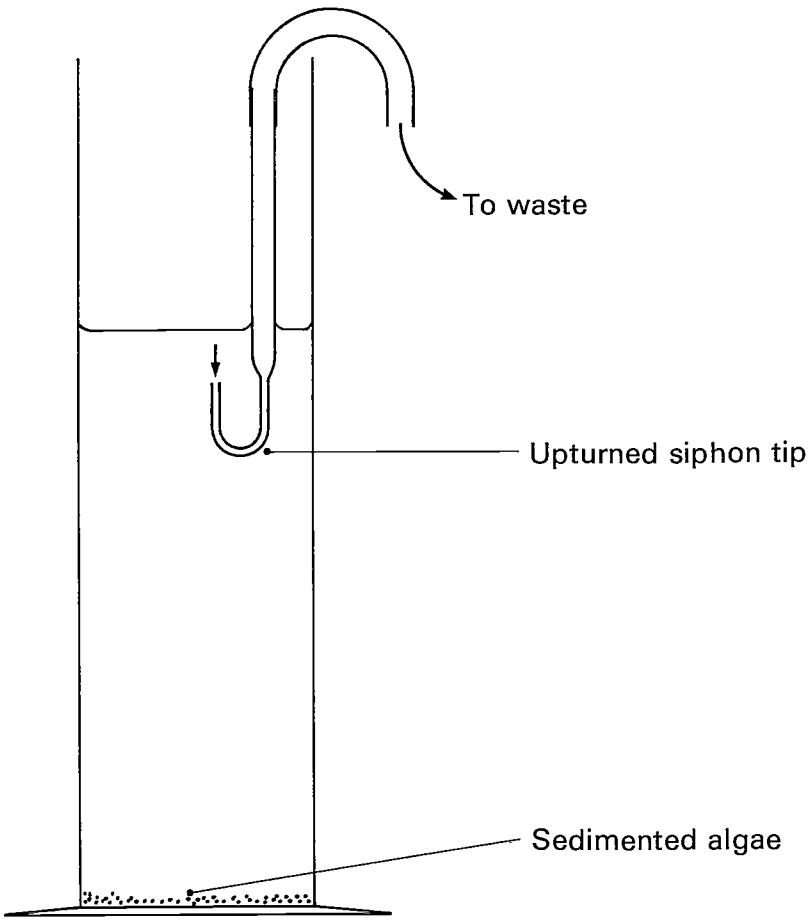
Sedimentation of cells is enhanced if they are preserved in Lugol's iodine. Algae absorb iodine to a greater or lesser extent with the formation of polyiodine species within the cells (Teitelbaum *et al.* 1978) and this increases cell density, aiding sedimentation. Convection currents can be generated and cause problems if counting chambers are too tall (Nauwerk 1963): a height: diameter ratio of not more than 5 is advised. The time allowed for settling is also critical because different species settle at different rates. Furet and Benson-Evans (1982) suggest that a settling time of 48 h in a 16-cm vertical column at 20°C is acceptable for phytoplankton fixed in Lugol's iodine. If small diatoms are present, however, a settling period of 96 h is recommended. Once settlement has occurred the supernatant is siphoned off carefully. A minimum of 10% of the liquid should be left in the cylinder to avoid disturbance of the sedimented algae. Siphoning is carried out gently by placing the siphon end (Fig. A1) just below the liquid surface and moving it down carefully as the liquid is removed until 10% is left. The siphon is then broken by lifting the tip above the liquid surface. A modification to reduce further the chances of entrainment of sedimented cells is to use a pipette tip which has been bent into a U-shape, so that the orifice points upwards (Fig. A1). The orifice is again placed just below the surface during siphoning, but it ensures that liquid is drawn from above, not directly below the siphon tip.

A2.2.2 Concentration by centrifugation

Centrifugation is a rapid and convenient method of concentrating a sample, especially if the species present are large and robust. A speed of 1,500 rpm (360 g) with a minimum time of 15 min for sample volumes of 10–20 ml is recommended (Ballantine

Figure A1 Concentration by sedimentation

Following sedimentation the supernatant is carefully siphoned off.



1953). Errors can arise if the centrifuge is slowed too rapidly. It is thus essential to allow a gradual slowing down. Once stopped the top 90% is removed using an upturned pipette (A2.2.1) and a siphon action, taking great care not to disturb the deposited algae. The remaining liquid must be mixed very thoroughly to resuspend the cells evenly and avoid clumping. Care must be taken, however, during the resuspension to avoid damage to cells, filaments or colonies. Centrifugation may be aided by the addition of a precipitating agent, such as potassium aluminium sulphate (1% solution), which is added at a concentration of 0.05 ml per 10 ml sample. Buoyant cells cannot be centrifuged. Although centrifugation can sometimes be used to concentrate living flagellate forms, damage is almost inevitable with the more delicate species.

Continuous centrifugation has been described for separating seston from large volumes of water (Vollenweider 1974) although an efficiency of only 80–90% removal is quoted.

A3 Enumeration

A3.1 Direct methods

A3.1.1 Membrane filtration

This method does not require pre-concentration, because the required concentration can be obtained by adjusting the volume of sample filtered.

A suitable volume of sample (1 to 200 ml per cm^2 of filter surface (Vollenweider 1974) is passed through a filter of 0.2 or 0.45 μm pore size. Either cellulose-based or polycarbonate membranes can be used. Care must be taken when adding the sample to the filter holder to prevent water currents leading to an uneven distribution of cells

on the filter. To ensure that this does not occur, allow the sample to stand for 2–5 min in the filter holder before applying the vacuum. Only gentle suction should be used. The sample may be preserved before filtration or stained subsequently. If an iodine preserved sample is used, the filter should be washed with a small amount of distilled water to remove excess iodine, with care taken not to affect the distribution of cells on the filter. If there is a risk that the introduction of the distilled water could disturb the filtered cells this stage should be omitted. Washing is essential for sea water samples in order to remove any residual salt.

When all the sample and wash water have passed through, gently draw air through filter for a further minute. One of the following three procedures may then be followed:

(i) The algal cells are now dehydrated by passing volumes of 10–15 ml of progressively more concentrated ethanol through the filter. If this procedure is followed, *it is essential to use a filter pad which is unaffected by ethanol.* After dehydration the cells are stained with alcoholic fast green (0.1% in 95% ethanol) by allowing the solution to stand over the filter for 20 min before drawing through. The filter is finally washed in ethanol (Vollenweider 1974).

(ii) A drop of either immersion oil or good quality cedar wood oil is placed on a microscope slide†. The membrane filter is then placed, face up, on the drop of oil on the slide, which may then be stood in a dust free atmosphere for 24 h to clear or for quicker processing placed in an oven at not more than 45°C*. The oil moves up through the filter replacing any air or water rendering the filter transparent. Whether the process takes place at room temperature or in a warm oven the filter should be left until it is visibly clear. Cedar wood oil, although cheaper than immersion oil, is darker in colour and so partially obscures further microscopy. When clear, the filter is then covered with a cover slip. To avoid trapping air a small amount of oil may have to be placed on the upper surface of the filter; care is needed because algal cells are readily disturbed when the cover slip is lowered, thus affecting the random distribution over the surface.

(iii) The procedure is the same in principle as (ii) but is modified to preserve the random distribution of cells on the filter surface. To achieve this, the drop of immersion/cedar wood oil is placed on a suitably sized cover slip (large enough to take the filter pad) and allowed to spread. The filter is then placed face down on the oil and left at room temperature or in an oven at not more than 45°C. Then the cover slip and filter are carefully inverted onto a microscope slide.

Cell numbers can then be estimated by one of the following methods:

(i) Most probable number estimate (MPN)

This method is suitable for the more abundant species. Infrequent species may be missed and are best determined by an alternative technique.

MPN counts are based not only on the recorded presence of organisms but also on their recorded absence. The slide is first placed on a mechanical stage of a microscope and scanned using the low power objective to confirm visually that the cells are not clumped. Next, using suitable magnifications the most commonly occurring species are identified. (This should be done in conjunction with observations on live and pre-filtered preserved material.) Select an appropriate objective so that the most common species is present in the field of view approximately 80% of the time. If this cannot be achieved then concentration or dilution of the sample may be necessary. The slide is now moved and observed in a random manner, so that 50 such fields of view are observed and the number of them in which the most common species occurs is noted**. If the percentage occurrence is substantially less than 80% a larger field of view or preconcentration should be used; if it is much above 90% then a smaller one or sample dilution should be used.

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†The oil to be used should be tested for suitability with the particular type of membrane filter.

*Higher temperatures can lead to curling and distortion of the membrane.

**If the membrane is gridded so that each square may be numbered then a random number table may be used to select appropriate squares.

Cell counts are not necessary, only presence or absence. When records for 50 fields are complete the percentage frequency (F) is calculated for each species using the following formula:

$$F = \frac{\text{total number of occurrences of a species x 100}}{\text{total number of quadrats estimated}}$$

Assuming a random distribution of cells on the filter the percentage frequency may be converted into a theoretical average number of individuals per field observed. This is called the theoretical density d. Given the assumption of a random distribution the theoretical density may be calculated from the formula:

$d = \log_e [100/(100-F)]$

see Fracker and Brischle (1944).

Having estimated values for d, the number of each species per litre of original sample is calculated as follows:

$$\text{number of cells per litre} = \frac{d \times a_1}{a_2 \times (V_1 + V_2)}$$

where (d) = theoretical density (see above); a_1 = area of filter;
a_2 = area of field of view; V_1 = volume of sample filtered in litres, and
V_2 = volume of preservative added in litres.

The validity for the conversion of F to d is based on the premise that the cells are distributed randomly on the filter. This can be tested using the index developed by McGuinnies (1934)

where $K = D/d$

in which K is the deviation from randomness and D is the average number of organisms per field as determined by direct counting. If K = 1 then randomness is indicated. Values of 0.5 or less indicate that the organisms are more scattered than expected by chance and values of 2.0 or more indicate greater aggregation than expected by chance. Detailed examples are given in McNabb (1960).

(ii) Single field counts

As with MPN counts the filtration area of the membrane A and the field size for the observation a need to be known. It is also desirable to obtain randomness in the distribution of organisms across the filter surface but, as previously, this is not always possible, especially with larger cells and filaments.

With this technique a gridded filter pad is used and all organisms within a single grid area a are counted and the process repeated. The number of organisms per unit volume of sample N is given by

$$N = \frac{Y \times A \times d}{a \times v}$$

where Y is the mean count per grid area, A is the membrane filtration area, a is the grid area, v the sample volume, d the dilution factor for the sample.

A full description of this technique together with modifications for staining and fluorescence and statistical validation are given in Jones (1979).

A3.1.2 Utermöhl's inverted microscope technique and modifications

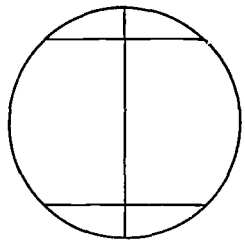
Utermöhl surveyed various counting techniques for algal cells and then devised his own counting chamber and counting technique using an inverted microscope. This method is most suited to larger algae although it can be used with smaller species (see Lund *et al.*, 1958); alternative methods for nanoplankton species are described later. A working description is included here. The sample is preserved in Lugol's iodine, placed in a sedimentation chamber and the algae allowed to sediment onto the glass bottom. They are then viewed upwards by means of a suitable microscope objective using an inverted microscope.

(i) The microscope

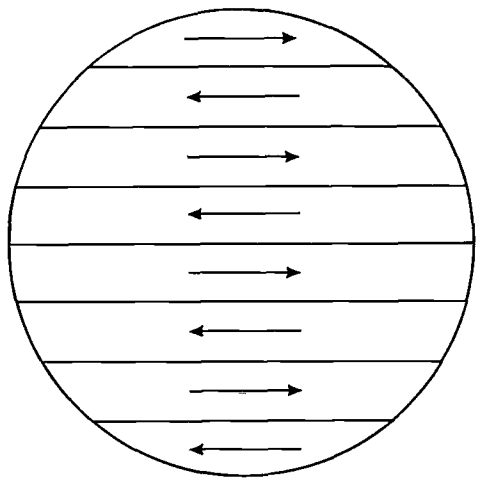
In order to count all algae that have sedimented, the whole of the bottom of the sedimentation chamber must be examined in a regular manner. This is facilitated by

Figure A2

(i) Microscope eyepiece hair–line marker



(ii) Systematic pattern used to scan the whole base of a sedimentation chamber



a hairline marker in the eyepiece and, using the mechanical stage, traversing the chamber as indicated in Fig. A2. As a traverse is made only organisms lying between the two parallel cross hairs are counted and then only as they cross the vertical line. Since some organisms will lie across the parallel hairs during a traverse, it is customary to count those across the upper one whilst ignoring those across the lower one. The latter will be counted on the next traverse. Normally the entire chamber is counted. If, however, species are present at high densities and are randomly distributed, then fewer transects may be viewed and suitable multiplication factors brought in, provided the user accepts that the exact confidence limits cannot be given.

(ii) Sedimentation tubes

Sedimentation tubes are either available commercially from optical and microscopic specialists such as E. Leitz, or may be constructed by the investigator. They consist of a suitable tube, the bottom of which is ground flat and then has a coverslip or glass disc fixed to it. The coverslip/glass disc fixing may be permanent or removable. The thickness of the coverslip depends on the working distance of the objectives used with the inverted microscope. Various types of tube are described below:

Fixed base tubes

The tube is cut from soda glass or perspex tubing of suitable internal diameter. This will vary, as will the height of the tube, depending upon the size of the samples and abundance of the species being counted. Sizes between 0.5 and 3.0 cm diameter and 2.5 cm to 6.5 cm height are normally used. One end is ground perfectly flat and smooth exactly at right angles to the long axis of the tube. A suitable size coverslip or glass disc of similar thickness to a microscope slide is then cemented into place using an adhesive such as Araldite. Care must be taken not to allow the adhesive to flow into the inside of the counting chamber. If the tube is glass, excess Araldite can be removed carefully by a mixture of conc. sulphuric acid/potassium dichromate which can be poured into

the chamber once the Araldite has been given sufficient time to harden. Care must be taken not to leave the cleaning mixture too long, because it will attach the seal as well as the excess. It is better to leave the acid for a shorter rather than a longer time. After the appropriate time the cleaning mixture is poured off and the excess material, which should now have softened, can be easily removed with a needle or a scalpel. This cleaning procedure cannot be used if the tubes are made of perspex. Once cleaned the tubes are thoroughly washed to remove acid. Whilst perspex is an easier material with which to work it does not produce such a durable cell as glass.

(ii) Split counting chambers

When fixed bottomed chambers are placed on a microscope for counting, the light has to pass through a considerable thickness of liquid before reaching the sedimented specimens. The optical conditions are thus far from ideal. This disadvantage may be partly overcome with a split tube technique. This uses a perspex tube in two sections which allows most of the supernatant water to be removed after sedimentation; the depth of liquid through which the light has to pass is thus greatly reduced and, hence the optical properties of the system markedly improved. The design of the split tube is given in Fig. A3.

The construction is as follows:

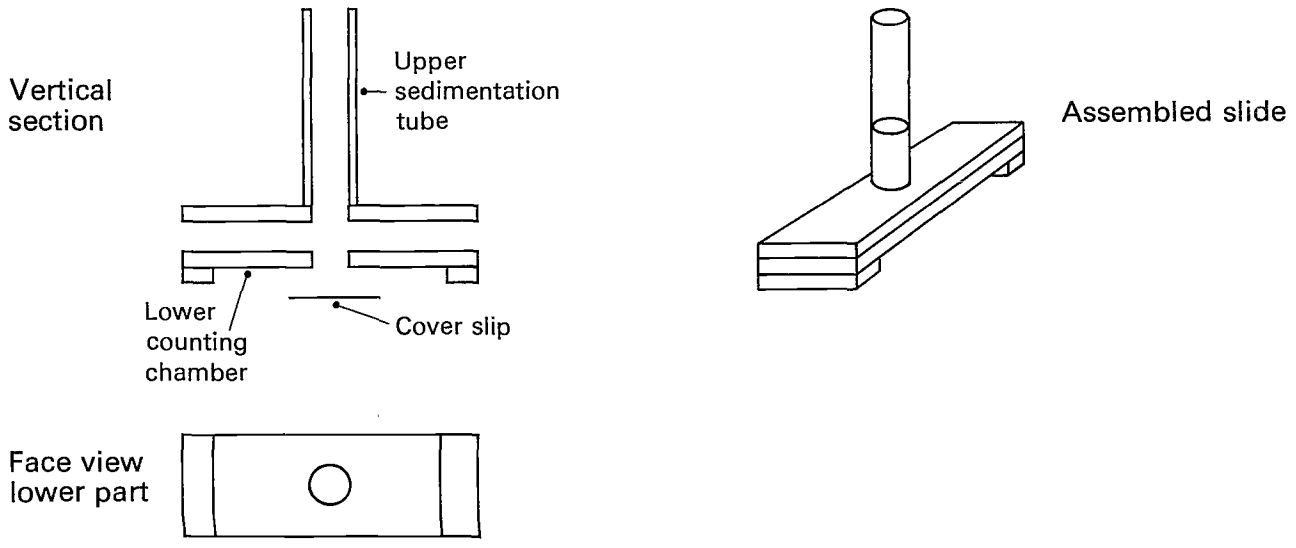
(i) Two plates the same size as a standard microscope slide are cut from a sheet of perspex (2–3 mm in thickness).

(ii) A suitable length of perspex tubing is cut for the sedimentation tube. The tube is generally between 4 and 8 cm long and between 0.5 cm and 1.0 cm internal diameter, depending upon the volume of sample to be sedimented (usually 1–5 ml). The end is ground smooth and care is taken to ensure that it is exactly at right angles to the long axis of the tube.

(iii) The two flat perspex plates are then firmly clamped together and a hole drilled through both of diameter equal to the internal diameter of the sedimentation tube that is going to be attached later. All of the cut and drilled edges are cleaned with a scalpel or fine file to remove any swarf.

(iv) To one perspex plate is cemented a sedimentation tube of equal diameter by means of Araldite or a similar adhesive. Care must be taken to ensure that the sedimentation tube is absolutely vertical and properly aligned with the hole and that it is completely sealed to the plate. Two small fillets of perspex are cemented to the second of the perspex plates to act as feet (Fig. A3).

Figure A3 Construction of a split-level chamber



The whole unit is assembled as follows:

A cover slip is attached to the underside of the bottom perspex plate (the one with feet) by means of either Araldite (for a permanent fixture) or with petroleum jelly (for a temporary fixture). Care must be taken to ensure that, although it is properly sealed, no sealant enters the counting chamber. The under side of the top plate and the top side of the bottom plate are then lightly smeared with petroleum jelly and squeezed together to seal the unit again ensuring no jelly gets into the counting chamber. If any does, it should be removed with a needle.

The assembled counting chamber is then ready for use. The chamber should be placed on a horizontal surface, a suitable volume of iodine preserved sample added and then allowed to stand for an appropriate time at a constant temperature in the dark to allow all cells to sediment onto the cover slip. The time for sedimentation depends upon the depth and volume of the chamber tube (A2.2.1). At least 3 h per cm depth of liquid should be allowed, but ideally chambers should be left overnight, or even longer if small cells are to be assessed by this method.

Following sedimentation, fixed chambers are now ready for immediate counting. For the split chambers the procedure is as follows. The chamber is carefully placed on a firm horizontal surface making sure that the contents are in no way disturbed. The upper section is then firmly slid sideways so that the lower part of the chamber is sealed off. The liquid in the upper part is then pipetted off. The upper part is then completely removed by sliding sideways, and a coverslip is then placed over the lower chamber (with the sedimented algae), making sure that no air bubbles are trapped underneath. The chamber is now ready for counting either on an inverted microscope or, if large celled algal species are present, easily visible under a low power objective, on a normal compound microscope viewing downwards.

A3.1.3 Haemacytometer

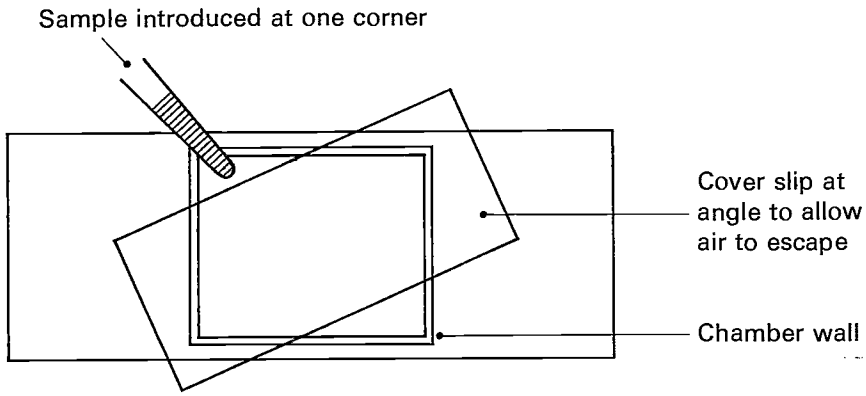
The haemacytometer consists of a specialized glass slide, part of the surface of which is carefully ground down to an exact and pre-determined depth. Around this area is a deeper area to allow the excape of excess sample fluid. The carefully ground area is provided with a ruled graticule for counting. The technique involves placing a drop of sample on the ruled area. The special haemacytometer cover glass is then placed on top and is supported by the slide. This also causes excess liquid to run into the deeper grooves/moat areas. The organisms in the ruled area are then counted and the total number per unit area determined. Knowing the area and depth of this ruled area the number of organisms per unit volume may be calculated. Typical chambers based on this design are the Fuchs-Rosenthal haemacytometer and the Helber chamber.

A3.1.4 Sedgwick-Rafter chamber

The Sedgwick-Rafter chamber (as described in Whipple *et al.*, 1927) consists of a microscope slide onto which has been built a rectangular chamber. On to the top of this chamber is placed a coverslip (see Fig. A4). The chamber is usually approximately 50 mm long, 20 mm wide and 1 mm deep. The total area of the bottom is approximately 1000 mm^2 and the total volume approximately 1000 mm^3. The exact dimensions of the cell should be determined before use. If necessary an accurate mean depth may be determined from the weight of water contained in the chamber with the coverslip in place.

The cell can be used with either living or preserved material. To fill the cell place the coverslip across the chamber top (Fig. A4). This allows air bubbles to escape during the filling procedure. The sample is then taken into a 1 ml wide-mouthed pipette and then carefully transferred to the chamber. Do not overfill the chamber, because the volume of sample in the chamber must be known exactly and the coverslip must not float free, but be held onto the cell walls by surface tension. During counting water may evaporate from the chamber. To prevent gas bubble formation, a small drop of distilled water may be placed on the slide outside the cell wall just touching the cell wall and coverslip. Before the cell count is made the Sedgwick-Rafter chamber should be allowed to stand for at least 15 min to allow the algae to settle to the bottom.

Figure A4 Filling a Sedgewick-Rafter chamber



The chamber is scanned quickly with a lower power objective to ensure a random distribution of algae on the bottom. With this criterion satisfied, one of two counting procedures may be used.

(i) If cell densities are not very high (less than 10 individuals per field of view) then a transect of the chamber may be counted, using either a ×10 or ×20 objective. Higher magnifications cannot be used satisfactorily, because of the depth of the chamber. One or more transects of the cell bottom (the area of which depends on the objective, but should be calculated exactly) are traversed and all algal cells present noted. When only one or two transects are counted, it is customary to traverse as near to the centre of the chambers as possible to avoid edge effects. To avoid counting the same individuals twice, a cross-hair eye-piece graticule (A3.1.2) is used. The density can then be calculated by multiplying the traverse count by the fraction of the total area which the strip represents. If the original sample was concentrated or diluted before counting, the result needs to be multiplied by an appropriate factor.

(ii) If the algal count exceeds 10 cells per field of view, an alternative technique may be used. At least 10 randomly chosen fields of view are counted. The exact area of each field of view must be known and the count then multiplied by an appropriate factor to calculate the numbers of algal cells per whole chamber. Again, if concentration or dilution of the original sample has occurred an additional correction factor must be used.

Buoyant species, such as *Microcystis*, do not settle to the bottom of the chamber, but float to the under surface of the coverslip. They may be deflated initially (3.1) or a separate count made by focussing the objective lens just below the coverslip (instead of on the base of the chamber). This count is then added to the chamber base count to give the total population.

A4 Fluorescence and epifluorescence

A4.1 Introduction

When algal cells are irradiated with blue-violet light (excitation wavelength about 450 nm) the chlorophyll re-emits the absorbed light at longer wavelengths as a red fluorescence. Thus fluorescence may be used to distinguish between cellular and detrital material and to distinguish algal cells that are small and more difficult to see, such as picoplankton (Sieburth *et al.*, 1978). It is however not necessary for the cells to be alive, because suitably preserved material also fluoresces (see below).

A4.2 Equipment

Any standard microscope fitted with suitable fluorescence (or for dark-ground, epifluorescence) attachments. Both blue and green excitation filters should be available and, if an oil-immersion objective lens is to be used, non-fluorescent immersion oil.

A4.3 Fluorescence

If samples require concentrating either centrifugation or filtration are recommended. If preservation is required, neutralised formalin to a final concentration of 2% should be used. The sample is then placed on a microscope slide and irradiated with light at approximately 450 nm. The cells exhibiting brick red fluorescence can then be counted. If required, a second count can be made of total cells after staining with acridine orange (final strength 1:5000 in water) which stains DNA and RNA. Cells then fluorescence green or orange red. By subtracting the first (brick red non-stained cells) count from the second (acridine orange) count, non-chlorophyll bearing cells may be estimated.

A4.4 Epifluorescence and phototropic phytoplankton

Special techniques are needed to obtain reliable counts of very small cells. The most widely used is epifluorescence microscopy, but laser flow cytometry is becoming increasingly important (Li & Platt, 1987). Detailed treatment of the latter method lies outside the scope of this booklet, but the separation stage described for epifluorescence microscopy is usually applied also for laser flow cytometry, even though in theory it is not essential.

For epifluorescence microscopy, very small cells need to be separated from larger cells and other particles. It is not possible to get a sharp separation with one particular size of filter. Therefore the filtration step needs to be followed by measurements of individual cells, if it is required to obtain an exact size class eg for picoplankton, which is commonly accepted to constitute those cells < 2 μm (Sieburth *et al.*, 1978).

The preserved sample (Kuppo-Leinikki & Kuosa, 1989; Hawley & Whitton, in press) is prefiltered under gravity through a 3.0 μm Nuclepore polycarbonate membrane. Between 5 and 15 ml of filtrate are drawn through a 25 mm 0.2 μm Nuclepore filter under partial vacuum (<100 mm Hg) that has been prestained for 20 minutes with irgalan black (Ceiba-Geigy, 2 g l^{-1} and 2% acetic acid). A glass-fibre filter eg Whatman GF/C is placed between the polycarbonate membrane and the filter holder and acts as a backing filter to create an even vacuum, so cells are drawn on to the membrane in a random fashion.

The membrane is then mounted on a glass slide that has been pre-chilled at 4°C for 20 min; the condensation formed on the slide ensures the filter attaches firmly to the slide. A drop of non-fluorescent immersion oil (eg Cargille type A) is placed on the filter and a coverslip is gently placed on top, sandwiching the oil evenly between filter and coverslip. Uneven pressure at this stage causes cells to move non-randomly on the filter. Preparations are ready for enumeration under an epifluorescence microscope using a $\times 100$ oil-immersion objective.

The autofluorescent pigments in picoplankton are best detected by green (ca. 500–550 nm) or blue (ca. 420–490 nm) excitation filter sets.

A total of 400 cells is counted to give a precision of ±10%.

Calculation

The multiplication factor equating the area of the ocular grid to the cross-sectional area of the filter chimney was calculated by measuring the inside diameter of the chimney (ca. 16 mm in a Millipore-25 mm filter holder) to get its cross-sectional area and by measuring the size of the ocular grid in the microscope with a stage micrometer to get its area (ca. 180×180 μm, using a $100\times$ oil-immersion objective). The factor of approximately 1×10^4 is calculated by dividing the cross-sectional area of the chimney by the area of the ocular grid.

The number of cells per millilitre of sample filtered is calculated from:

mean number of cells per field $\times 10^4$

A5 Electronic Particle Counting—The Coulter Counter*

A5.1 Introduction

Many different models of the Coulter Counter have been produced since the original. Although the basic concept has remained unaltered, the models range from the Industrial Model A and the 2B to the more recent, and more expensive, Model TAII/PCAI Accucomp system. Suspended particles displace an electrolyte liquid and can thus be sized and counted. The simpler and less expensive models available include the Model D which has replaced the obsolete industrial Model A. The Model TAII/PCAI is capable of dealing with a wider size range, is more versatile, computer-linked, and will produce data rapidly in tabular or graphical form. The Model D is adequate for simple routine size and count analyses and ideal as an introductory teaching or research instrument.

For counting and measuring algal cells in axenic, unialgal culture the Coulter Counter is quick, efficient and reliable. Algae may even be counted accurately in contaminated cultures, provided the algal cells are sufficiently numerous and distinct in size.

In dealing with aquatic samples from the field which will normally contain widely varying densities and sizes of non-algal particles, it is necessary to be more cautious and refer to the totality of suspended particles as seston. Sufficiently large and distinct populations of algae can often be selected from the overall size spectrum and their volumes compared with the results of other estimates of biomass.

A5.2 Operational Principles

The essential principles of operation of the Coulter Counter, are the same for all models. Detailed descriptions of the mathematical and physical aspects of the interpretation of electrical response to particle size may be found in Coulter Counter Manuals (Anon. 1984) or the various original publications upon which such manuals are based. A simplified version will be presented here and is sufficient for most operators. It is illustrated schematically in Fig. A5, which is based upon the Model D. In operation, the glassware is filled with an appropriate electrolyte solution (eg 0.5% NaCl) and, when the top tap is opened, mercury is drawn down the manometer tube to the pre-set vacuum level as indicated in Fig. A5(a). If the electrolyte in the sample beaker now being drawn through the aperture has been membrane-filtered, the signal generated on the oscilloscope screen display is flat and completely deviod of 'spikes'. If, however, there are particles in suspension in the electrolyte liquid, these will produce a visible display. The frequency of the spikes is a function of the particle concentration and their height is a function of their displacement volume. When the top tap is closed to release the vacuum, the mercury returns to its rest position, triggering as it does so the start-stop operation of the digital register. The volume of liquid drawn through the aperture may be pre-set by a switch on the manometer so that the number of particles counted can be related to a known volume of the liquid. Essentially each particle passing through the aperture causes a change in resistance between the electrodes. The electronic circuitry converts this change in resistance to a brief voltage pulse. By adopting a series of amplification and threshold settings, a series of counts on a cumulative basis can be recorded. As there is a direct relationship between particle size and the voltage pulse, pre-calibration of the instrument with particles of a known and essentially uniform displacement volume will allow particles of a mixed and unkown size to be counted and sized.

NB (1) A range of aperture tubes are available which differ from one another only in the diameter of the aperture. For freshwater seston/algal mixtures, a 200-μm diameter aperture tube is usually the most appropriate choice. (Fig A6 indicates typical sizes encountered).

(2) For marine or brackish water samples no additional electrolyte is required, while for fresh water it is best to make the samples up to a standard salinity. A simple method is to add sufficient 10% membrane filtered NaCl solution to produce a concentration of 0.5% NaCl in the final solution.

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*Available from Coulter Electronics Ltd, Northwell Drive, Luton, Beds, England

Figure A5(a) Schematics of the Coulter counter principle of operation

(with reference to the Model D)

(a) Glassware containing mercury and electrolyte

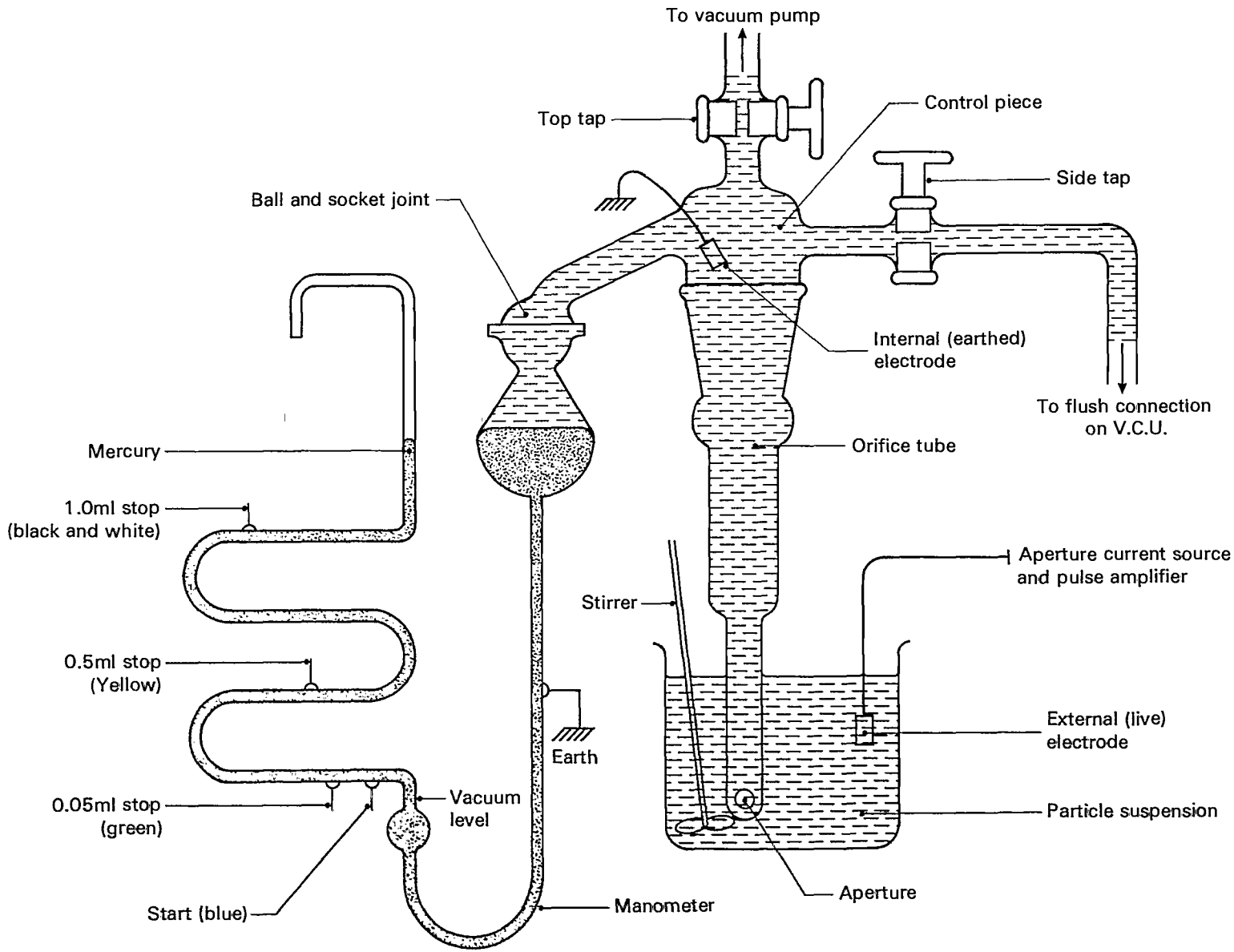
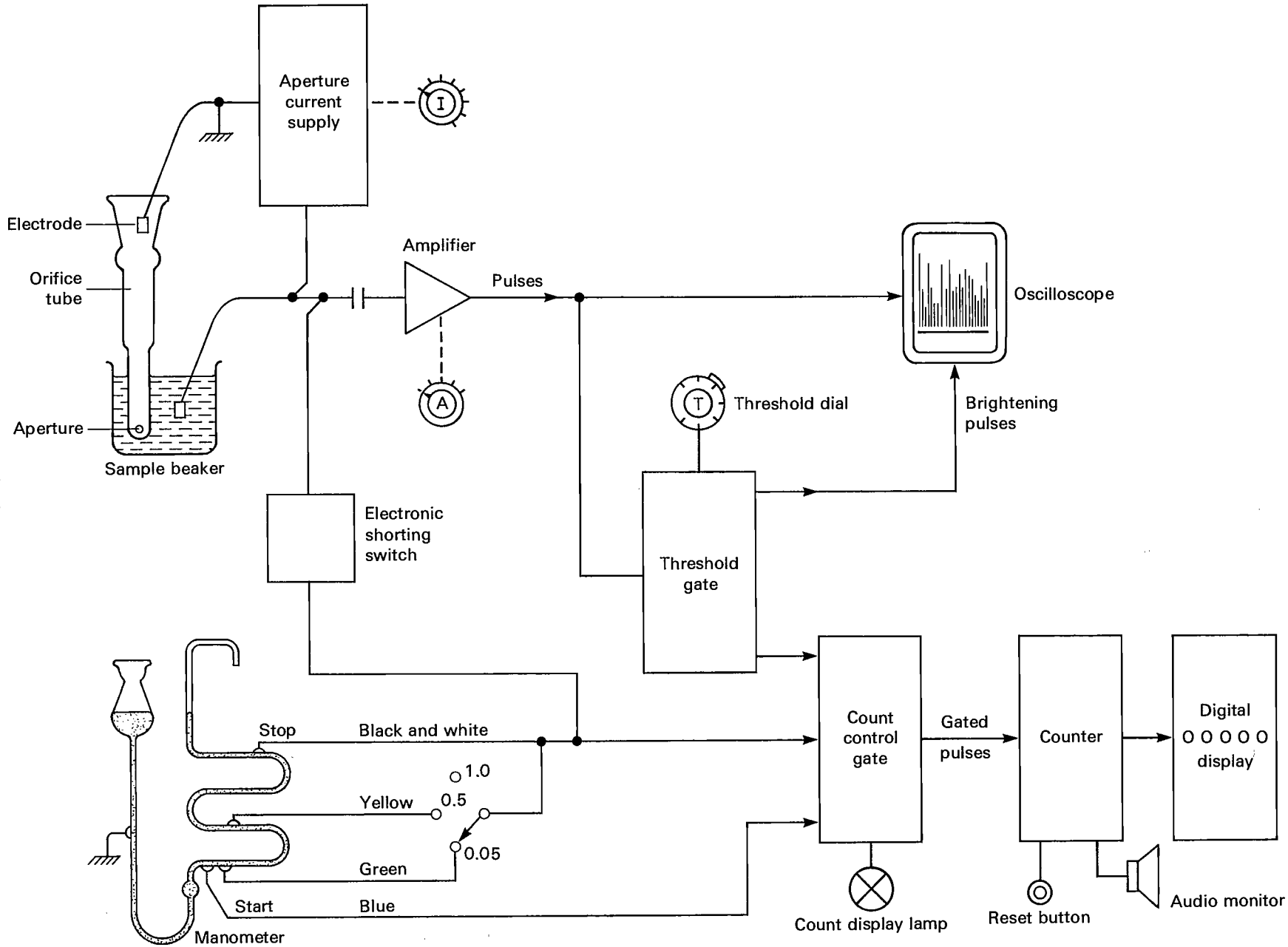


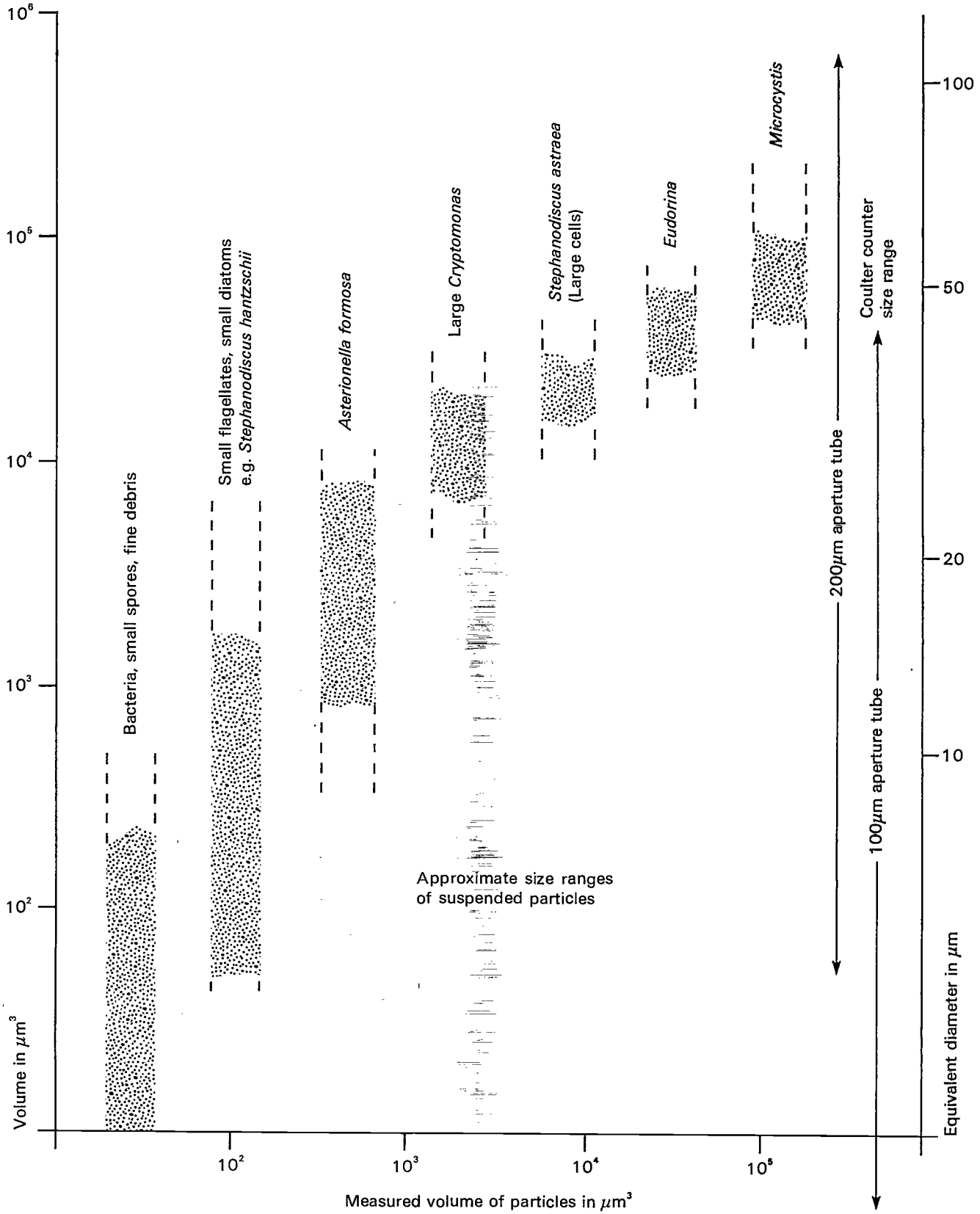
Figure A5(b) Simplified electronic block diagram



19

Figure A6 Chart to indicate overall size range covered by two selected aperture tubes useful for seston and algal analysis

Some examples are given of particles which generally fall within certain size ranges together with, on the horizontal axis, approximate values derived from microscope measurement and calculation. *Eudorina* is considered as coenobia, *Asterionella* and *Microcystis* as colonies, and the other algae as cells.



(3) With an increasing concentration of particles in the sample beaker, there will be an increasing chance of more than one particle entering the aperture at the same time. Corrections are made for this electronically in the more sophisticated models and by simple calculation in Model D. These are known as coincidence corrections.

A5.3 Calibration

Having selected an aperture tube and an electrolyte concentration appropriate for the samples to be analysed, it is necessary, if information about absolute size of particles is required, to calibrate the instrument. This may be done in a number of ways, but the most simple and reliable is the use of uniform sized particles and the 'half-count' technique. Spherical particles with a known diameter, and a wide range of sizes, eg *Lycopodium* spores, puff-ball spores, PDVB (polystyrene divinyl benzene) latex, can be obtained from Coulter Electronics Ltd, Northwell Drive, Luton, Beds, LU3 3RH. These are dispersed in the electrolyte to a concentration of several thousand particles per ml. The instrument switches (usually Attenuation, Threshold and Aperture Current) are set to their lowest levels. After opening the top tap, drawing the mercury down and (on the Model D) pressing the re-set button, pulses appear on the oscilloscope screen. By operating the attenuation and aperture current switches these pulses can be adjusted to a height of about 2 cm. The threshold setting is then increased until a shadow line approximates to the height of most of the pulses. The threshold dial reading is recorded as 'T'. A count is then recorded by closing the top tap—usually at the 0.5 ml volume setting. This count is repeated and a mean count noted from a series of counts. The threshold dial is re-set at 1/2T and a mean of 2 or 4 counts is recorded (N). The dial is gain a re-set, at 1.1/2T, and another mean count (N2) recorded. The mean of N1 and N2 should be quite close to the original count at setting T. If this is not so, the procedure is repeated, adjusting the threshold dial until the values are within two threshold divisions. The final setting is noted as t′, the attenuation switch as A and the aperture current switch as I.

The diameter calibration factor (k) can then be calculated from:

$$K = \sqrt[3]{\frac{d}{t' \times I \times A}}$$

and, as the instrument response is linear, any combination of switch settings can be calculated as diameters by:

$$d = K. \sqrt[3]{\frac{d}{t' \times I \times A}}$$

Therefore for any set combination of aperture tube and electrolyte type and concentration, a series of instrument settings can be related to a series of particle sizes.

For the routine monitoring of seston, including various possible phytoplankton components ranging from small single cells to quite large colonies and coenobia, it is advisable to decide upon a series of sizes (volumes) of particles and, from calculations, relate the switch settings to them. For most purposes, a series of sizes as indicated in the tables would be quite adequate.

This suggested range of sizes may either be simplified or further refined. By using a smaller aperture diameter tube (eg 140 μm), smaller particles may be analysed. To cover a wider range of particles sizes the 2 tube technique may be adopted (see Haffner & Evans 1974; appropriate Coulter Counter Operators Handbook) though this is unlikely to be needed frequently.

A5.4 Preparation of samples

Samples should be analysed as soon after collection as possible and preferably within two hours. If there is an unavoidable delay (overnight), samples should be stored in darkness at a low temperature, but not frozen. It is possible, but not advisable, to fix samples in buffered 4% formaldehyde solution for longer storage.

It is not routinely necessary to pre-filter samples for analysis, but if there are numerous large particles which would physically block the aperture, it is helpful to pre-filter the samples, using a coarse filter, such as zooplankton netting held in a funnel. Care should be taken that smaller particles are not lost.

Before analysing an unkown sample, it is advisable to run a membrane-filtered blank at the same salinity concentration as the test samples to establish the background count of particles. This ought to be very low, eg. not more than about 100 ml^{-1} with a 200 μm aperture tube and the minimum size setting. If the background count is much higher, the sample beaker should be rinsed with fresh, filtered electrolyte solution. Care should be taken that the outside of the beaker and the platform are clean and dry. Electrical interference can cause false counts and precautions should be taken to avoid this; the insertion of a one-to-one isolating transformer in the mains input to the instrument can be an inexpensive aid. The water samples are prepared for analysis as follows, although the quoted volumes and concentrations are only for guidance. To 15 ml of a membrane-filtered 10% NaCl solution in a 500-ml measuring cylinder is added a well-mixed sub-sample (to be counted) to make the volume 300 ml. This 0.5% NaCl sub-sample is then poured into the sample beaker and a count made at the lowest size setting of the size range to be analysed. If the count is within the acceptable upper limit for a 200 μm-aperture tube, which would be 10^4 ml^{-1} (5000 per 0.5 ml), the full count can proceed. If an excessively high count is recorded then the sub-sample will have to be diluted, with membrane-filtered NaCl of the appropriate concentration (eg 0.5%), until the count is reduced sufficiently.

A5.5 Full and partial sample counts

For regular routine analyses it is usual to carry out full counts of the samples. However, in some circumstances only partial counts may be needed. To deal with seston, including phytoplankton, from most freshwater habitats, a size range from 50 to 60,000 μm^3 (equivalent spherical diameters of 4.6 to 48.5 μm) in about 16 steps, as in Table A1, is adequate. The size discrimination from step to step is much better than can be achieved by normal optical microscopy. The usual procedure is to begin at the low end of the size range (highest number of particles) and work up the scale recording 2, 4 or more counts at each size setting, depending upon the numbers counted. As an approximate guide two counts are normally sufficient at >1000 particles per count and four counts at <500. The operator should aim at an agreement of counts well within 10% of one another at each size setting. Eventually, at the upper end of the size range, the counts will be reduced to very low, probably meaningless, numbers. As a rule it makes sense to ignore counts of <10 particles (or some other previously agreed 'cut off' point).

Partial counts can be achieved easily by omitting selected steps in the size range spectrum. An extreme version to count only at the lowest size setting which provides only a total count for the entire size range. This is a very rapid and useful technique for dealing with, for example, experimental uni-algal cultures, where the cells are relatively uniform in size.

A5.6 Data handling and interpretation

The more advanced Coulter Counters provide data already plotted in histogram or some other diagrammatic form. To understand such data, however, it is useful to have served some sort of apprenticeship with a more simple type of instrument (eg the Model D). For such instruments the data sheets (eg in Table A1), will have raw counts entered during the analysis of the sample and several calculations are necessary to process these raw count data. For each step in the size range a mean count ($\bar{n}'$) is calculated and if this exceeds 500, a coincidence correction must be carried out in which a coincidence factor (P) appropriate to the aperture size is applied. In some counters, notably the Z-series, such corrections can be carried out electronically by means of an 'add-on' coincidence correction device. The cumulative counts should next be transformed into counts within each step of the size range (Δn) by a series of simple step by step subtractions. The product of each n and V value will be the minimum total volume of all particles within each step of the size range (ΔnV) and the sum of all of these will

Table A1 Tabular layout for the operating parameters, outputs and subsequent calculations for a Coulter Counter

SAMPLE: DATE:

Aperture diameter 200μm	Manometer volume 0.5 ml	Coincidence Factor (P) 20	Calibration Factor (K) 14.6 (14.3.86)
Aperture resistance	Temperature 10 - 30°C	Electrolyte 0.5% NaCl (Max. count 5,000)	

t^1	I	A	n^1 (Raw counts) - cumulative				$\overline{n^1}$ Mean raw counts - cumulative	$n^1 = P\left(\frac{\overline{n^1}}{1000}\right)^2$ Coincidence correction	$n = \overline{n^1} + n$ Corrected mean raw counts - cumulative	Spare column - for surface area equivalents, or background counts etc.	d = μm	V $\mu m^3 \times 10^3$	Δn Number of particles (per 0.5ml) at each size step	Δnv Total volume of particles (per 0.5 ml) at each size step	$\Sigma \Delta nv$ Total volume of particles (per 0.5 ml) in cumulative steps
37	0.25	4									48.5	60			
31	0.25	4									45.7	50			
25	0.25	4									42.5	40			
18	0.25	4									38.5	30			
23	0.13	4									33.6	20			
18	0.25	2									30.0	15			
23	0.13	2									26.7	10			
18	0.13	2									24.2	7.5			
23	0.13	1									21.2	5			
28	0.066	1									17.4	3			
15	0.066	1									14.5	1.6			
15	0.033	1									11.5	0.8			
14	0.017	1									9.1	0.4			
13	0.0093	1									7.2	0.2			
11	0.0053	1									5.7	0.1			
10	0.0033	1									4.6	0.05			
Threshold	Aperture current	Attenuation													

be the total particulate volume (TPV) over the entire size range analysed. Some operators (and some manuals and handbooks) refer to an 'average' volume for each step in the size spectrum ie. mean of upper and lower values for each step. The assumption is that there is a normal distribution of volumes within each size interval but as this is unlikely it is better to base all calculations upon the minimum volume. There is then at least consistency throughout. If the manometer has been set to deal with steps of 0.5 ml, then this last value will have to be multiplied by 2 (and by any sample dilution factor) to provide a result for each ml of the original sample. It is helpful to shift the decimal place and express the results as $\mu m^3 \times 10^6$ ml^{-1}. Each completed data sheet, provides three main types of information:

(i) Number of particles within the size range analysed (En ml^{-1})

(ii) Total particulate volume (TPV, in $\mu m^3 \times 10^6$ ml^{-1})

(iii) Frequency distribution of the various size particles.

A5.7 Comparison with other methods

There have been many comparisons between techniques for estimating algal biomass (see Butterwick *et al.*, 1982; Stein, 1973; Vollenweider, 1974) and several comparison involving the Coulter Counter method (eg Evans & McGill, 1970). There is general agreement that a visual count, although slow, is the most reliable. Microscopy is essential to identify the algae and to check their condition. Chemical methods, especially those for carbon and chlorophyll analysis, can be informative but may be quite misleading where population numbers are low or cells unhealthy. The Coulter Counter technique only provides a reliable estimate of biomass if the seston consists largely of an algal population of distinctive size range. The best procedure, therefore, is to combine routinely several methods of biomass analysis:

(i) Examine a sub-sample by the sedimentation technique (A3.1.2), count suitable algal units and convert to a population density (Section B).

(ii) Chlorophyll *a* extraction in hot 90% methanol (see HMSO 1983).

(iii) Coultaer Counter analysis to derive seston data.

The results of (i) and (ii) can be used to help interpret those from (iii).

Section B Algal Cell Volume

B1 Introduction

Cell dimensions, and hence cell volumes, vary greatly from one species to another and even within the same species. A species of *Chlorella*, for example, with a mean diameter of 4 μm would have a mean cell volume of 30 μm^3 whereas *Stephanodiscus* with a mean diamter of 35 μm would have a cell volume in excess of 15,000 μm^3. Cell volume can be converted to cell carbon or cell mass to give a more realistic estimate of population biomass, although such estimates must be treated with caution.

Cell size and volume may also be of use in interpreting the growth and behaviour of algal populations. Nutrients must be absorbed from the surrounding medium to be utilized by the cell. A knowledge of the surface area available for absorption and hence the cell surface area to volume ratio can help in the interpretation of algal population dynamics (see Reynolds 1984).

B2 Measurement of cell size

Many algal cells approximate to simple geometric shapes. (Table B1).

Table B1 Approximate cell shape and key dimensions for various algal genera

Genus	Shape	Key Dimensions
Chlamydomonas	sphere	diameter
Chlorella	sphere	diameter
Spirogyra	cylinder	diameter, height
Mallomonas	ellipsoidal	length, diameter at centre
Oscillatoria	cylinder	diameter, height
Asterionella	cuboid	length, width, height
Cyclotella	cylinder	diameter, height
Melosira	cylinder	diameter, height
Stephanodiscus	cylinder	diameter, height

In many taxa the shape is more complex and can only be described by a combination of geometric shapes. In these cases the key dimensions are those required to describe each separate shape making up the whole. Examples of such complex combinations of geometric shapes are given in Table B2.

For most complex shapes it is advised to sketch a range of cells in different views in order to decide on the best combination of geometric shapes.

Table B2 Examples of algal species requiring combinations of geometric shapes to describe their shape

Species	Geometric Shape
Ceratium hirundinella	a sphere, one cylinder (apical horn) two or three cones (lateral horns)
Scenedesmus quadricauda	four cylinders (for a 4-celled colony)
Nitzschia acicularis	double cone
Rhizosolenia acuminata	cylinder with cone at either end
Staurastrum pingue	two cones with six cuboidal arms

Some taxa (eg the diatoms *Cymbella* and *Amphora*) cannot be described successfully even by means of combinations of geometric shapes. In these cases estimates have to be made by other means.

Measurements of cell dimensions should be made, where possible, on both fresh and preserved material. Whilst it is more convenient to make measurements on preserved material it is possible that delicate species may distort or change size. For diatoms, measurements made on cleaned frustules mounted in a suitable medium (eg Naphrax), are more accurate. As many cells from a population as possible should be measured but in any event the number should not be less than 25. If cell density is low in a sample the results from a number of samples may need to be combined to give a suitable average. With some cell shapes it can be difficult to make all appropriate measurements on the same cell because they may tend to settle in one plane. This is particularly true with some diatoms, which can settle showing either the valve or the girdle view uppermost. In such cases measurements must be made from the views available and averages taken.

B3 Calculation of cell volume

Because cell size can vary from one population to another, it is necessary to make original measurements, rather than transpose data from other locations Bellinger (1974). Having determined the linear dimensions of at least 25 cells, the cell volume can be calculated using the appropriate geometric formula or combination of formulae. If all the appropriate dimensions are available for each cell measured the volume for each cell should be calculated individually and only then calculate the average cell volume for the population. This reduces errors in calculation caused by averaging linear dimensions.

Some cells have shapes which do not conform to standard geometric formulae or combinations of formulae. In such cases either the best approximation should be used or plastic models may be made and the volume of liquid displaced measured to determine volume (Bellinger 1974). It should also be recognized that not only can variations in cell size and volume occur between individuals of the same species at different geographical locations, but also between individuals of the same species and location but at different times of year (Bellinger 1977).

Plasma volume and vacuoles

In many cells, especially larger ones, the body is not composed entirely of protoplasm, but also contains a vacuole. The latter sometimes occupies the larger portion of the total cell volume and it has been suggested that the plasma volume (ie the volume occupied by the protoplasm) is a better estimate of biomass.

It can be very difficult to measure the thickness of the protoplasmic layer, which is usually in a film around the outside of the cell (1–2 μm). Table B3, derived from Smayda (1965), gives suggested values for parietal protoplasmic layer thickness based upon various surface area to volume ratio. In addition there are strands traversing the cell forming protoplasmic bridges. The plasma volume (PV) can thus be calculated as either:

(i) Total cell volume – cell vacuole volume, or

(ii) Surface area (in μm^2) × parietal protoplasmic layer thickness.

An additional factor is added to account for the protoplasmic bridges (5% of the parietal protoplasmic volume) and also one to represent the nutritious portion of the cell sap (which is not all water and does contain dissolved nutrients). This latter has been taken arbitrarily to be 10% of the total cell volume (Smayda 1970).

Table B3 Suggested parietal protoplasmic layer thickness for cells of various surface area to volume ratios (from Smayda 1965)

Surface:volume ratio	>0.90	0.51–0.89	0.35–0.5	<0.35
Protoplasmic layer thickness (μm)	total vol	1.0	1.5	2.0

B4 Conversion of cell volume to cell carbon

Although total cell volume can provide a useful estimate of biomass it can be of more use to be able to estimate the cell carbon content of a population. Various workers have suggested equations for converting cell volume into cell carbon. Mullin *et al.* (1966) suggested:

$$\log_{10}C = 0.76(\log_{10}V) - 0.29$$

Strathman (1967) developed this to distinguish between diatoms and other phytoplankton to produce two relationships:

$\log_{10}C = 0.758(\log_{10}V) - 0.442$ .....diatoms

$\log_{10}C = 0.866(\log_{10}V) - 0.460$ .....other phytoplankton

Minor modifications to Strathman's formulae have been suggested by Eppley *et al.* (1970) and Taguchi (1976) but probably the most realistic estimate of cell carbon was found by Strathman to be based on plasma volume. Using a 1 μm protoplasmic peripheral layer thickness, he suggested the following relationship:

$\log_{10}C = 0.892(\log_{10}V) - 0.61$

Section C Algal Bioassays

C1 Introduction

When nutrients and light are plentiful, dense growths may give rise to water quality and engineering problems such as clogging of filters in water treatment works, colouration of the water or production of tastes and odours. Some species produce toxic substances which have been associated with fish and other wildlife mortalities (Collins 1978). Thus a knowledge of their growth potential in water is of value to water scientists, managers and engineers. Measurements of such growth potential gives some indication of the concentration of biologically available nutrients. If controlled quantities of specific nutrients are added to water samples, algal population size changes can indicate which, if any, of the nutrients were previously limiting. If nutrients and physical conditions are known to be adequate in a test sample but growth of a standard algal species is still limited, then the presence of toxic or inhibitory substances should be considered. However, it is not always possible to be certain whether nutrients and/or physical conditions are adequate.

Algal bioassays are based on the assumption that there is a direct relationship between the growth of the test species and the concentration of the nutrient in shortest supply (or, in the case of toxins, the concentration present). Whilst this concept is usually applied to a single limiting nutrient, it has also been extended to include physical as well as chemical variables (Shelford 1913; Odum 1959). Algal bioassays are often carried out with more than one nutrient simultaneously eg combined additions of nitrogen and phosphorous and the effect of the N:P ratio. The test can thus be used to (1) identify the substances which are limiting algal growth, (2) determine the biological availability of substances in the sample and (3) quantify the biological response to changes in concentrations of substances in the sample. These measurements are made in culture tests by adding a selected test alga to the water sample with or without addition of nutrients and determining algal growth at appropriate intervals. All physical conditions are kept constant throughout the test. A flow diagram summarizing the overall test procedures is shown in Fig C1.

C2 Test Algae

C2.1 Standard species

The recommended standard test alga is the green alga (Chlorophyta) *Selenastrum capricornutum* Printz*, a relative of the widespread green algae *Chlorella, Scenedesmus* and *Ankistrodeesmus*. *Selenastrum capricornutum* is a unicellular species dividing by endospores, although it may at times form clumps. It may be obtained as a standard culture from the Culture Centre of Algae and Protozoa†.

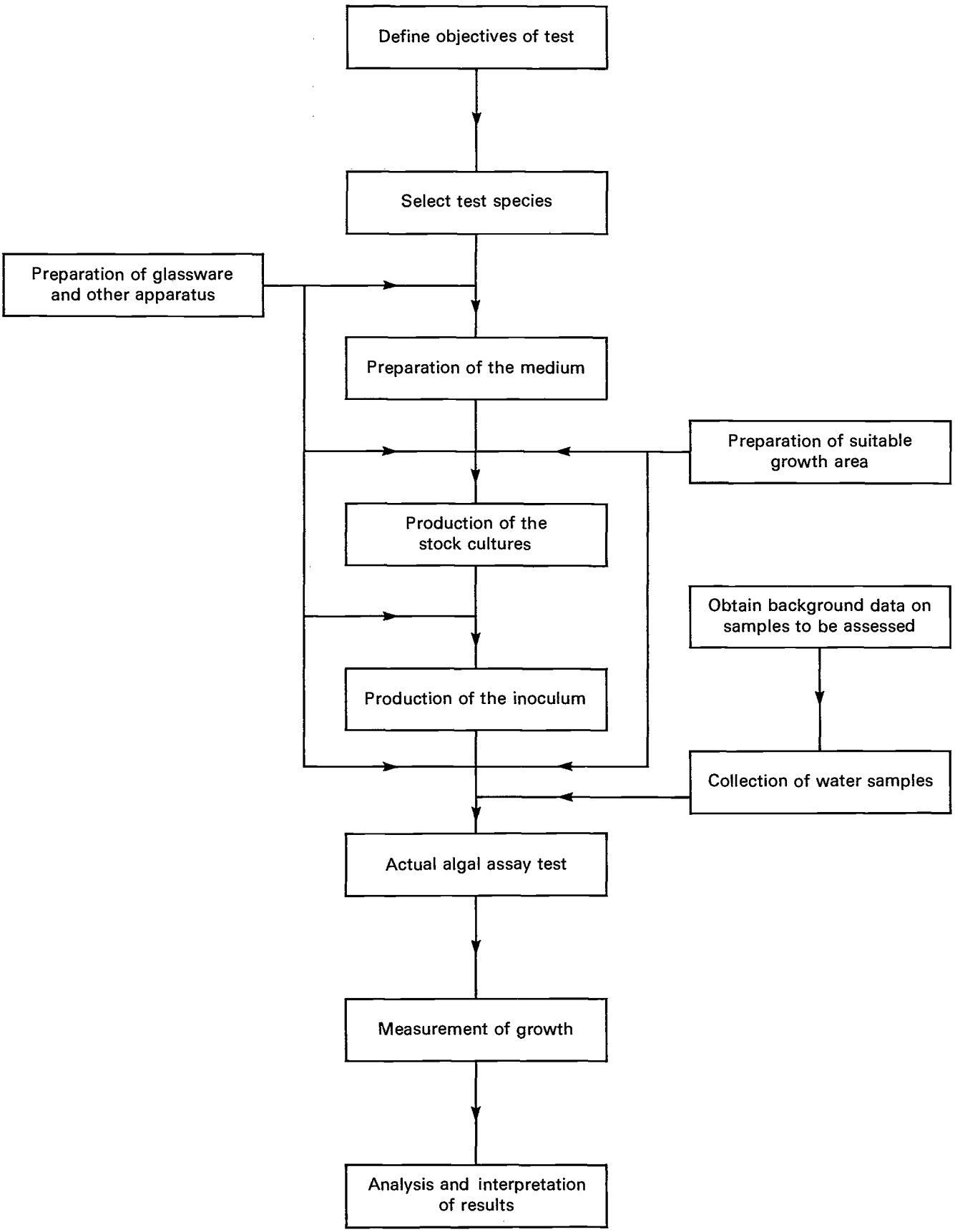
C2.2 Other species

Because of the varying requirements of different taxa, bioassays should also be carried out with at least one additional species in addition to *S. capricornutum*. This should be representative of the indigenous populations in the water body being tested. It should be realized that species within the same genus may vary widely in their requirements. Ideally this test alga will be obtained from a culture of a species isolated from the indigenous population. If this is impossible, the following list indicates strains from which additional test organisms may be selected from the Culture Collection of Algae and Protozoa.

**Monoraphidium* sp. and *Chlorella* sp. are acceptable and may be used with equal success. They are available from standard culture collections.

†CCAP, Institute of Freshwater Ecology, Windermere Laboratory, Far Sawry, Ambleside, Cumbria UK LA22 0LP

Figure C1 Flow diagram showing steps in the algal bioassay procedure



Cyanbacteria (Blue-green algae)	
Microcystis aeruginosa Kütz	CCAP 1450/4
Anabaena flos-aquae (Lyngb.) Bréb.	CCAP 1403/13C
Chlorophyta	
Scenedesmus quadricauda (Turp.) Bréb.	CCAP 276/4b
Bacillariophyta	
Asterionella formosa Hass.	(CCAP 1005/16

The inclusion of a species of *Anabaena* or other nitogen-fixing blue-green alga is desirable because their nitrogen requirements differ markedly from other species.

C3 Preparation of glassware and other apparatus

All apparatus used in the bioassay tests must be kept scrupulously clean. The procedure recommended is as follows.

C3.1 Experimental flasks

These should be of top quality borosilicate glass.

The flasks are first filled to the brim with a concentrated solution of saturated Na_3PO_4 for cleaning purposes and left overnight. They are then emptied, washed in tap water and then washed six times in de-ionised or distilled water. If phosphate limitation is suspected in the test water, nitrated sulphuric acid* can be used instead of the phosphate cleaning solution. The flasks are then filled to the top with 1:1 HCl (sg 1.18) : water and left for about 20 min. They are then emptied and again washed 6 times in de-ionised or distilled water. About 200 ml of de-ionised or distilled water is then boiled in each flask. They are then emptied, allowed to cool and then covered (with cellophane, 'cling film' or equivalent). Each flask should be numbered with a diamond pencil and that code number referred to in all future experiments. This allows the experimenter to note whether one particular flask consistently gives different results from the rest. If this happens that flask should be discarded. The flasks should be covered and stored in a clean environment until required. A somewhat similar procedure is used for other glassware, apart from pipettes and filtration equipment which require slight modifications.

C3.2 Pipettes

The pipettes are washed by total immersion in HCl (1:1 vol:vol) for at least 1 h. They are then removed, drained and totally immersed in six changes of de-ionised or distilled water, allowing the pipettes to stand for at least 5 min. in each change of water. If the pipettes are stored dry they should be wrapped in protective material such as cling film; if stored wet they can be left in dilute HCl, but must be washed before use.

C3.3 Filtration equipment

The fritted glass filter (Gallenkamp & Co, Millipore or Sartorius) is first cleaned in concentrated nitrated sulphuric acid. This is necessary in order to remove all possible contaminating material which might be adhering to the filter. This includes algae entrapped in the fritted glass from previous uses. The filter is then washed at least six times in distilled water. The whole filter apparatus is then oven dried at 100°C. Allow the filter to cool before using.

The Buchner receiving flask which collects the test water must be cleaned between each experiment as follows. It is first washed in concentrated Na_3PO_4, then rinsed in tap

*1 pt H_2SO_4 (conc): 1 pt HNO_3 (conc) mixture. Care must be taken when making up the mixture. Rubber gloves and safety glasses must be worn. Add the nitric to the sulphuric acid slowly, thoroughly mixing and cooling after each addition.

water, washed 6 times in de-ionised or distilled water and left to stand for at least one hour in the final rinse water. It is then washed in dilute HCl (1:1) and then 6 times with distilled or de-ionised water. The filtration apparatus and flask are then assembled and one litre of test water passed through a GF/C filter paper. The filtrate is swilled round the flask and also used to rinse the experimental flasks before discarding. The filter pad is discarded. A rinse at every stage involves swilling the water round and inverting the flask to drain. When not in use both the mouth and the side arm of the Buchner flask should be covered with cellophane/cling film.

C4 Maintenance of Stock Cultures

C4.1 Test species

Stock cultures of *Selenastrum capriconutum* should be maintained in a culture medium containing the following analytical reagent grade chemicals in de-ionized water:

Macronutrients

	concentrations in stock solution
$NaNO_3$	25.50 g l^{-1}
K_2HPO_4	1.04 g l^{-1}
$MgCl_2$	5.70 g l^{-1}
$MgSO_4.7H_2O$	14.70 g l^{-1}
$CaCl_2.2H_2O$	4.41 g l^{-1}
$NaHCO_3$	15.00 g l^{-1}

Micronutrients

One litre of a mixture of the following chemicals made up as follows:

H_3BO_3	185.52 mg l^{-1}
$MnCl_2$	274.26 mg l^{-1}
$ZnCl_2$	3.27 mg l^{-1}
$CoCl_2$	0.78 mg l^{-1}
$CuCl_2$	0.01 mg l^{-1}
$Na_2MoO_4.2H_2O$	7.26 mg l^{-1}
$FeCl_3$	96.00 mg l^{-1}
$Na_2EDTA.2H_2O$	300.00 mg l^{-1}

(ethlenediaminetetra-acetic acid, di-sodium salt)

For all components containing less than 100 mg l^{-1} it is advisable to make up stock solutions 100 times more concentrated. 10 ml of these are then added together with the remaining chemicals to about 800 ml deionized water and the whole made up to 1 litre to give the final concentration. For all solutions, care must be taken to avoid precipitation of any component on mixing. If this occurs and the precipitation does not redissolve on dilution a new solution should be made up without the precipitating compound which is then added only after dilution to 950 ml. The whole is then shaken and no precpitate should occur. It can then be made up to 1 litre.

C4.2 Preparation of medium

1 ml of each of the macronutrient stock solutions and 1 ml of the single micronutrient solution are placed in a flask containing 500 ml of distilled water, thoroughly mixed and the whole then made up to 1,000 ml with glass distilled water. This is then autoclaved at 10 kN m^{-2} (15 psi) at 121°C for 15 min in flasks sealed with sterile foam (or other gas-permeable) plugs. After cooling the medium should be allowed to equilibrate in air, but still sealed aseptically, to restore lost CO_2. Alternatively the test solution may be sterilized by filtration through a 0.45 μm pore size membrane filter which has been previously washed by passing 500 ml of de-ionised water through it; this is recommended in particular for $NaHCO_3$. Filtration should be carried out under reduced pressure (0.5 atmospheres or less) and the whole filtration unit sterilized before use. Sterilization is not required if the assay is carried out with non-axenic organisms.

The final growth medium contains the following concentrations of elements:

Element	Concentration
N	4.20 mg l^{-1}
P	0.186 mg l^{-1}
S	1.91 mg l^{-1}
C	2.14 mg l^{-1}
Na	11.01 mg l^{-1}
K	0.469 mg l^{-1}
Mg	2.90 mg l^{-1}
Ca	1.20 mg l^{-1}
B	32.5 μg l^{-1}
Mn	115.4 μg l^{-1}
Zn	15.4 μg l^{-1}
Co	0.354 μg l^{-1}
Cu	0.004 μg l^{-1}
Mo	2.87 μg l^{-1}
Fe	33.1 μg l^{-1}

The sterilized medium and the stock solutions should be stored in the dark at a temperature near 0°C to minimize photochemical changes.

C4.3 Culture media for other species

Different species of algae have different nutrient requirements. Various culture media are therefore required to maintain the stock cultures. Members of the Chlorophyta are maintained in the same medium as *S capricornutum*. Diatoms (Bacillariophyta) are grown in a modified Chu 10 (Chu 1942, modified by Lund *et al.* 1975).

This is made up to the following composition:

$Ca(NO_3)_2.4H_2O$	20.00 mg l^{-1}
KH_2PO_4	6.20 mg l^{-1}
$MgSO_4.7H_2O$	25.00 mg l^{-1}
Na_2CO_3	20.00 mg l^{-1}
Na_2SiO_3	25.00 mg l^{-1}
HCl (2N)	0.25 ml l^{-1}
Na_2EDTA	2.00 mg l^{-1}
$FeCl_3$	1.00 mg l^{-1}
H_3BO_3	2.48 mg l^{-1}
$MnCl_2.4H_2O$	1.39 mg l^{-1}
$(NH_4)Mo_7O_{24}.4H_2O$	1.00 mg l^{-1}
Vitamin B_{12}	0.01 mg l^{-1}
Vitamin B_1	0.001 mg l^{-1}
Biotin	0.001 mg l^{-1}

All compounds to be made as either × 100 or × 1,000 concentration stock solutions and diluted accordingly for final working solution.

Blue-green algae (Cyanobacteria) should also be maintained in a relatively dilute medium, such as the BG-11 of Stanier *et al.* (1971). A modified version BG-11_o is used when combined nitrogen is not required (Rippka *et al.* 1979). BG-11 is also suitable for many other fresh water algae, especially those from more nutrient rich environments (BG-11 contains a relatively high level of phosphate). The concentrations of chemicals in the two media are given below.

	Concentration (mg l^{-1})	
	BG-11	BG-11_o
$MgSO_4.7H_2O$	75	75
$NaNO_3$	1500	–
$K_2HPO_4.3H_2O$	40	40
$CaCl_2.2H_2O$	36	36
Citric acid	6	6
Ferric ammonium citrate	6	6

	Concentration (mg l^{-1})			
	BG-11		$BG\text{-}11_o$	
EDTA (disodium magnesium salt)	1		1	
Na_2CO_3	2		2	
Trace element stock solution	1	ml	1	ml
Deionized water to	1000	ml	1000	ml
Trace element stock solution	concentration in g l^{-1}			
H_3BO_3	2.86			
$MnCl_2.4H_2O$	1.81			
$ZnSO_4.7H_2O$	0.22			
$Na_2MoO_4.2H_2O$	0.39			
$CuSO_4.5H_2O$	0.079			
$Co(NO_3)_2.6H_2O$	0.0494			

Based on Kratz and Myers, 1955, with cobalt added. Ni is not included in this trace element mix, but is apparently required by many blue-green algae, especially nitrogen-fixers. In view of the purity of modern reagents, it is recommended that this element is included ($NiSO_4.7H_2O$ at 0.048 mg l^{-1}). Both blue-green algal and diatom media should be sterilized according to the methods for *S capricornutum* stock culture medium.

C5 Growth Conditions for Stock Cultures

C5.1 Physical Conditions

Cultures are maintained in an ambient temperature of 20°C±1°C under continuous illumination using daylight fluorescent tubes at a suitable distance to give about 100 μmol photon $m^{-2}\,s^{-1}$ Photosynthetically Active Radiation (PAR) at the culture vessel surface. Flasks should be moved at regular intervals to randomize their positions and thus eliminate the possibility of local variations.

C5.2 Subculturing

When the culture has reached the end of exponential growth, as determined by cell counts, nephelometry or chlorophyll measurements, it should be subcultured aseptically. The time chosen depends upon the growth rate of the particular strain. The volume of old culture used to start the new culture is not critical as long as enough cells are present to minimize the lag period eg. about 1 ml of inoculum in 100 ml of medium placed in a 500 ml conical flask. The age of the culture for experimental inocula varies with species. A routine for sub-culturing should be established in order to maintain a continuous supply of healthy cells.

C5.3 Inoculum

C5.3.1 Preparation of inoculum

The algal inoculum should have excess culture medium removed before adding to the test solutions. This may be achieved by filtration or centrifugation.

G5.3.2 Filtration

Larger algae, such as *Asterionella* or *Oscillatoria*, can be separated conveniently from the test solution by filtration as there is little chance of them either passing or becoming entangled in a suitable filter. To avoid the problem of entangling within the meshes of filter pads of materials such as glass-fibre, a fritted glass filter is recommended (Lund *et al.* 1971).

The required amount of algal culture is passed through the filter together with more sterile water. The culture fluid associated with the cells can be removed by passing at least 2.5 litres sterile water through the filter under pressure. To avoid damage algal cells must always be immersed in water, so filtration should be continuous, with constant addition of distilled water, and ceased before all the water has passed through the filter. The algae, together with a thin layer of water, are then resuspended into a

convenient volume of the sterile sample water to be tested. Sterile conditions must be maintained throughout the preparation of the inoculum.

G5.3.3 Centrifugation

For small species of algae, such as *Selenastrum*, filtration cannot be conveniently used because of problems of entanglement within the mesh of the filter pad. For these species the following centrifugation procedure is recommended. Place the stock culture into a centrifuge tube and spin at $10^3 \times g$ for 5 min. Decant the supernatant and then resuspend the cells in sterile distilled water. This procedure is repeated twice and the final cell suspension used to prepare the inoculum. Centrifugation can also be used for larger algae if required, although colonial forms, such as *Asterionella*, can be broken up so great care must be taken. It must also be recognized that centrifugation and resuspension in distilled water could shock the cells causing a lag in growth and even zoospore formation in some green algae.

G5.3.4 Inoculum concentration

The size of the inoculum depends on the species. For *S capricornutum* a final concentration of approximately 1×10^3 cells ml^{-1} should be used, whereas for blue-green algae, because of their slower growth, it should be approximately 50×10^3 cells ml^{-1}.

The concentration of cells in the resuspended, washed stock algal suspension is now determined (see C7) and the appropriate amount then added to each test flask. If, for example, the resuspended cell concentration for *S capricornutum* were 2×10^5 cells ml^{-1} and 250 – ml flasks containing 100 ml sample water were being used then 0.5 ml of that resuspended stock should be added to each flask to give a cell concentration of 1×10^3 cells ml^{-1}.

C6 Test Conditions

C6.1 Temperature

The temperature at which the bioassay flasks are kept will depend to a certain extent on the species concerned, the likely temperature prevailing at the test sample location and whether or not the algae are field specimens or laboratory cultured ones. For *Selenastrum* a temperature of 20°C±1°C is recommended. With *Asterionella* and other planktonic diatoms a slightly lower temperature of 18°C + 1°C is preferred. For field specimens a temperature close to their normal field growing temperature should be chosen.

C6.2 Illumination

Conditions of illumination may vary with the species. For *Selenastrum* and *Asterionella* use 25 μmol photon $m^{-2}s^{-1}$ PAR and for blue-green algae use 50 μmol photon $m^{-2}s^{-1}$ PAR continuous illumination should be used. For natural populations the recommended light regime is 100 μmol photon $m^{-2}s^{-1}$ PAR with alternating periods of 12 h light and 12 h dark; the cycle should commence with a light period.

C6.3 Agitation

The flasks should be shaken manually at least once daily or continuously on an orbital shaker at about 60–80 oscillations min^{-1}.

C6.4 Procedure and measurement

When all the equipment and algae have been prepared, the appropriate amounts of sample water and inoculum are added to each set of triplicate flasks and incubated under the appropriate conditions.

C7 Estimation of growth parameters

After the period of incubation (ie when the maximum crop has been achieved as assessed by optical density or *in vivo* chlorophyll *a*) the yield is estimated by measuring

the dry weight of the algae in the flask, the total particulate volume, the chlorophyll content or the total cell count.

Dry weight

A known volume of algal suspension is filtered through a 0.45 μm (or similar porosity) filter. The filters must be pre-dried at not more than 70°C and then cooled in a desiccator before weighing. A suitable volume of algal suspension is then filtered through the pre-weighed paper. The volume filtered will depend upon the cell concentration but as little as 10 ml could be sufficient. The sides of the filter funnel are rinsed with 50 ml distilled water from a wash bottle. Only gentle suction should be applied when filtering the algae. The filter pads are then dried to constant weight at 70°C, cooled in a desiccator and re-weighed.

Total particulate volume (see A5)

Chlorophyll *a*: *in vivo*

Whilst chlorophyll *a* measurements are generally quite sensitive and rapid they suffer from the disadvantage that the ratio of chlorophyll content to cell biomass varies considerably with growth conditions. Chlorophyll *a* comparisons are thus better restricted to a specific test rather than using them for conversion to the more universal measure of biomass. The full method for *in vivo* measurement of chlorophyll *a* is given in reference (HMSO 1983).

Direct microscopic enumeration (see A3)

C8 Analysis of Results

All results must be subjected to statistical analysis before interpretation.

C8.1 Rejection of outliers

Whenever a series of algal bioassays are carried out there is often a small number of flasks which give anomalous results. It is advisable to number each individual flask so that if one flask repeatedly gives results which are substantially different it may be discarded. Even then occasional anomalies do occur. Such outliers can be rejected but this should not be done automatically as they can provide useful information. If there is good reason to suspect that the outlier has been produced as a result of error then the following test may be applied although care must be taken if the number of replicates is small (ie 4). It should also be noted that this test assumes a normal distribution of data (taken from Miller *et al* 1978).

(i) Rank order the data in the group containing the suspected outliers (all observations in the group are supposedly treated alike).

$X_1\ X_2\ X_3 \ldots X_n$

(ii) Compute the appropriate criterion

if X_2 is the outlier

$C = (X_2 - X_1)/(X_n - X_2)$

if X_n is the outlier

$C = n - (X_n) - 1/X_n - X_1$

(iii) If C exceeds the critical value 'n' in Table C1—then the outlier is rejected.

Table C1 Critical values for determining outliers

n	critical values	
	$\alpha = 0.05$	$\alpha = 0.01$
3	0.941	0.988
4	0.765	0.889
5	0.642	0.780
6	0.560	0.698
7	0.507	0.637

Example:

The following standing crop dry weight measurements were made for a bioassay group: 7.9, 8.0, 7.6, 6.9, 8.2, 7.2, 7.5, 4.9 and 7.9. The value of 4.9 is suspected to be erroneous or an outlier.

First rank order the data:

X_1	X_2	X_3	X_4	X_5	X_6	X_7
4.9	6.9	7.2	7.5	7.6	7.9	8.2
			n = 7			

Calculate $C = X_2 - X_1$ $6.9 - 4.9$ 2.0

$= 0.606$

$X_n - X_1$ $8.2 - 4.9$ 3.3

Since n = 7 and $0.507 < C = 0.606 < 0.637$ there is less than a 5% and greater than a 1% chance that the value 4.9 is from the same normal distribution as the 'good' data. The experimenter might discard the value as an outlier with a 1%–5% chance that it was a valid measurement. If there is more than one suspected outlier eg X_1 and X_2 or X_1 and X_n the test should be repeated but always apply it to the most extreme case first. However, beware of the situation of two or more outlying low values perhaps due to a failure of algal growth for some unknown reason for which $C = (X_2 - X_1)/(X_n - X_1)$ would be misleadingly small and non-significant.

C8.2 Limits of confidence

Confidence limits, which should be presented for all results, should be based on at least 5 replicates unless the test rate has known characteristics and has previously varied little between replicates, in which case 3 replicates will suffice. The confidence limits are based on the estimates (s) of standard error where:

$$s = \sqrt{\frac{\Sigma x^2 - \frac{\Sigma (\bar{x})^2}{n}}{n(n-1)}}$$

$x_1, x_2 \ldots x_n$ are the replicates
$\bar{x}$ = mean of the n replicates

The confidence intervals are calculated in the usual way from the standard error s and students 't' t.

If the mean is $\bar{x}$ and s = standard error:

the upper limit = $\bar{x} \pm (s.t)$
the lower limit = $\bar{x} \pm (s.t)$

The values for t may be obtained from any standard statistical book but the more useful values for these tests are given below:

	degrees of freedom	95%	99%
n = 3	2	4.303	6.965
n = 4	3	3.182	4.541
n = 5	4	2.776	3.747

Table C2 Typical assay results expressed as cells $ml^{-1} \times 10^{-3}$

Replicate		1	2	3	average
Days	1	1.00	1.00	1.00	1.00
	3	1.11	1.12	1.19	1.14
	5	1.18	1.20	1.24	1.21
	8	1.38	1.38	1.45	1.40
	10	2.17	2.18	2.44	2.26

C9 Algal bioassays in nutrient studies and toxicity tests and their interpretation

In addition to using the algal bioassay test for assessing the biological growth potential of a particular water sample, it can also be used to give some idea of the effects resulting from a change in nutrient status, such as a new or changed discharge into a river.

C9.1 Assessment of nutrient limitation

This procedure is based upon the principle that growth of the test alga is probably limited by one or more nutrient substances in the water, so, if these are added in suitable quantities, then growth will be enhanced. Initially tests are made with additions of N, P and the chelating agent EDTA but further tests may be needed with other substances, such as trace metals, organics etc, using a number of strains.

C9.1.1 Typical experimental design for nitrogen, phosphorus and EDTA

If the background information on the sample is incomplete, the additions recommended are those in Table C3.

Table C3 Basic additions for initial nutrient limitation studies. P is added as K_2HPO_4 and N as $NaNO_3$

Control	no addition
Control	+0.05 mg l^{-1} – P
Control	+1.00 mg l^{-1} – N
Control	+0.05 mg – P and 1.00 mg l^{-1} – N (as above)
Control	+1.00 mg l^{-1} – Na_2EDTA
Control	+0.05 mg – P and 1.00 mg l^{-1} – Na_2EDTA
Control	+1.00 mg – N and 1.00 mg l^{-1} – Na_2EDTA
Control	+0.05 mg – P and 1.00 mg – N and 1.00 mg l^{-1} – Na_2EDTA

If the test sample comes from a highly eutrophic or oligotrophic source then the additions may need to be varied accordingly. Some background information on the sample chemistry is required.

The samples together with additions are then inoculated with the test algae and incubated as in C3 and C4.

C9.1.2 Interpretation of results

The results are expressed as maximum biomass measured in mg l^{-1} or if cell counts have been made cell number ml^{-1}. Typical data for growth response from a phosphorus-limited sample are given in Table C4.

Table C4 Typical results from a phosphorus-limited sample, using *Selenastrum capricornutum* as a test organism. P is added as K_2HPO_4 and N as $NaNO_3$.

Treatment	Max Standing Crop (mg l^{-1} dry weight)
Control	1.14
Control + 0.05 mg l^{-1} – P	3.13
Control + 1.00 mg l^{-1} – N	1.25
Control + 0.05 mg – P and 1.00 mg l^{-1} – N	12.98
Control + 1.00 mg – EDTA	1.12
Control + 1.00 mg – EDTA and 0.05 mg l^{-1} – P	2.63
Control + 1.00 mg – EDTA and 1.00 mg l^{-1} – N	1.40
Control + 1.00 mg – EDTA and 1.0 mg l^{-1} – N and 0.05 mg l^{-1} – P	13.03

The original water sample contained the following: total P 12.0 μg l^{-1}; ortho P 4.0 μg l^{-1}; total N 0.46 mg l^{-1}; NO_3.N 0.30 mg l^{-1}; NH_3-N 0.04 mg l^{-1}; NO_2-N negligible; pH 6.8. The total soluble inorganic nitrogen (TSIN) $(NO_3+NO_2+NH_3)=0.30+0.04=0.34$. Based on previous data (EPA) each μg P l^{-1} should yield 0.430 ± 20% mg dry weight l^{-1} and each mg N l^{-1} should yield up to 0.038 ± 20% mg l^{-1} dry weight assuming that no other factors are limiting. Using these yield factors the natural water sample yields should be for TSIN $0.34\times38=12.92$ ± 20% and for ortho P $0.004\times430=1.72$ ± 20%. The N:P ratio (TSIN–ortho P) is 85:1. For *S capricornutum* an approximate guide to a more suitable N:P ratio for optimum growth would be 16:1. Hence there is a strong possibility that phosphorus is the main limiting factor in the test sample.

C9.1.3 Assessment

Algal bioassays should only be used as a guide and in combination with other methods of assessment (eg chemical analysis of the water). The previous history of the algal cells, the possibility of luxury phosphate uptake by certain species and the inadvertent transfer of substances from the stock cultures to the algal assay flasks are but a few of the many possible sources of error. The preparation of the inoculum can itself lead to stress (C5.3.3).

C9.2 Algal assays for assessing the effects of discharges on receiving water quality

Algal bioassays have been used successfully to monitor the effects of discharge from sewage works into surface waters and to give an indication of the possible effect of a proposed discharge in that water. They have also been used to monitor the performance of sewage works and the quality of their effluents. A knowledge of the chemistry of the effluent, particularly the N and P concentration, and of the receiving water are required as well as the dilution factors anticipated for the effluent in the receiving water. It is advisable to simulate a range of dilutions in the bioassay in order to obtain as comprehensive a picture as possible. When interpreting the results it should be borne in mind the assay may not take into account adequately those chemical and physical interactions taking place with the effluent and the receiving water.

C9.3 Toxicity testing

Many effluents and discharges contain substances inhibitory to growth. A straight forward chemical analysis only indicates the concentration of such a substance, not its bioavailability or its bioeffect. It will also not reflect the range of chemical, physical and biological interactions taking place in the receiving water. The growth response of a test alga can give an idea of the biological effects of the substance, but the results should be treated with some caution as they only reflect the test conditions not the conditions prevailing in the field. The various levels of response of the test alga are given in Fig. C2.

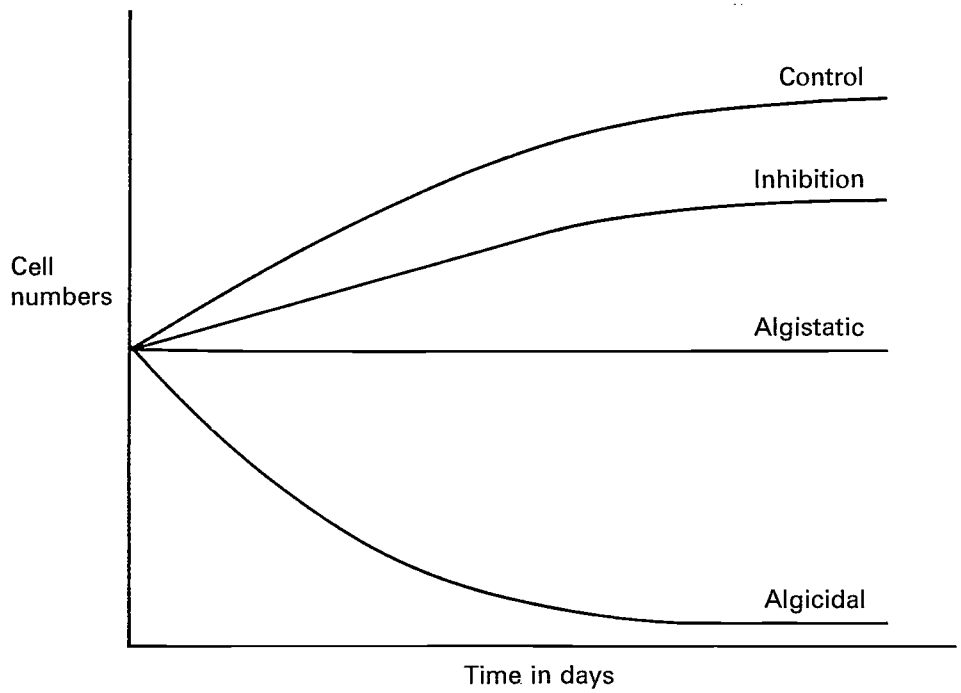
C9.3.1 Procedure

The procedure is similar to that outlined previously. Additions of the suspected toxin are added at a range of dilutions, together with EDTA, P and N individually or in combinations. The nutrient concentrations in the potential receiving water should also be known.

C9.3.2 Results

All results are expressed as % inhibition at day 14 calculated from changes in dry weight compared with the control. It may be found that EDTA forms unavailable complexes with substances such as bio-reactive metals, thus enabling the alga to reach maximum potential growth for the particular combination of nutrients present.

Figure C2 Types of response to toxic substances



C10 Bioassays using natural populations

C10.1 Introduction

Whilst it can be advantageous to use standard test species whose response to nutrients etc in solution may be well known, they may not be truly representative of the species present in the sample water. In order to overcome this objection an additional series of bioassays may also be carried out using natural populations.

Advantages

(i) If the test can be carried out properly the species present may reflect natural changes more accurately.

(ii) No acclimatization to the nutrient levels in the test water is needed for natural populations.

(iii) Diversity of species means that there is a good chance that at least one will be able to respond rapidly to changing conditions.

Disadvantages

There are several disadvantages of using natural populations of algae for bioassay testing. These may be summarized:

(i) Standard unialgal cultures are more reproducible in their response.

(ii) Natural populations may consist of dozens of different species and are constantly changing with regards to dominance, biomass etc.

(iii) The behaviour of many natural species in culture is unknown.

(iv) Preparation of a suitable inoculum for natural population bioassay tests can be difficult.

(v) Estimates using chlorophyll *a* will be less reliable with populations of mixed species.

(vi) Possible problems with parasites etc affecting growth in culture.

C10.2 Procedure—tentative

Natural populations in nature may not be sufficiently concentrated to use as a bioassay inoculum. If some form of concentrating procedure is necessary, gentle centrifugation,

filtering with minimal pressure, or the use of a net haul sample is advised even though the latter will markedly distort the species composition and balance of the population. The algae are then incubated in 12 h light 12 h dark at the temperature equivalent to that of the water from which they were collected. The procedure is as described in Sections C4, C6 and C7. The inoculum should be at least as dense as that used for unialgal cultures (10^3 cells ml^{-1}). An incubation time of between 7 and 14 days will be required. It should be borne in mind, however, that community change will occur during the period, so that a value judgment must be made as to how long is required to get a large enough change in standing crop, without too much change in the population structure.

C11 Additional reading

Useful references not included above are:

Clarsson and Forsberg (1980)
Greene *et al.* (1975)
Marvan *et al.*(1979)
Mitt. int. Verh. Limnol. (1978)
Nordforsk (1973)
Shubert (1984)
Skulberg (1966)
Toerien and Steyn (1973)

References

Anon 1984. *Fine particle application note for Coulter Counters.* Coulter Electronics Ltd, Luton, England.

Ballantine, D., 1953. Comparison of different methods of estimating nanoplankton. *J. Mar. Biol. Ass. UK.,* **32**, 129–147.

Bellinger, E G., 1974. A note on the use of algal sizes in estimates of population standing crops. *Br. Phycol. J.,* **9**, 167–161.

Bellinger, E G., 1977. Seasonal size changes in certain diatoms and their possible significance. *Br. Phycol. J.,* **12**, 233–239.

Butterwick, C., Heaney, S I. and Talling, J F., 1982. A comparison of eight methods for estimating the biomass and growth of planktonic algae. *Br. Phycol. J.,* **17**, 69–79.

Chu, S P., 1942. The influence of mineral composition of the medium on the growth of planktonic algae. I. Methods and culture media. *J. Ecol.,* **30**, 284–325.

Clarsson, A. and Forsberg, A., 1980. Algal assay studies of wastewater polluted lakes. *Arch. Hydrobiol.,* **89**, 108–224.

Collins, M., 1978. Algal toxins. *Microbiol. Rev.,* **42**, 725–746.

Eppley, R W., Reid, F M. and Strickland, J D H., 1970. The ecology of the phytoplankton of La Jolla, California, in the period April through September 1967 (Ed J D H. Strickland) Pt III Estimates of phytoplankton crop size, growth rate and primary production. In: *Bulletin Scripps Institute of Oceanography,* **17**, 33–42.

Evans, J H., 1972. A modified sedimentation system for counting algae with an inverted microscope. *Hydrobiologia,* **40**, 247–250.

Evans, J H. and McGill, S M., 1970. An investigation of the Counter in 'Biomass' determinations of natural freshwater phytoplankton populations. *Hydrobiologia,* **35**, 401–419.

Fracker, S B., and Brischle, H A., 1944. Measuring the local distribution of *Ribes*. *Ecology,* **25**, 283–303.

Furet, J E., and Benson-Evans, K., 1982. An evaluation of the time required to obtain complete sedimentation of fixed algal particles prior to enumeration. *Br. Phycol. J.,* **17**, 253–238.

Greene, J C., Miller, W E., Shiroyama, T, and Maloney, T., 1975. Utilisation of algal assays to assess the effects of municipal, industrial and agricultural wastewater effluents upon phytoplankton production in the Snake River system. *Water, Air and Soil Pollution,* **4**, 415–434.

Haffner, G D. and Evans, J H., 1974. Determination of seston size distribution with Coulter Counter models A and B and the 2 tube technique. *Br. Phycol. J.,* **9**, 255–260.

Hawley, S. and Whitton, B A., (in press) Survey of algal picoplankton from five continents. *Verh. int. Verein. theor. angew. Limnol.,* **23**.

HMSO 1983. *The determination of chlorophyll a in aquatic environments, 1980.* In the series 'Methods for the examination of waters and associated materials'. HMSO, London.

Jones, J G., 1979. A guide to methods of estimating microbial numbers and biomass in fresh waters. *FBA. Scient. Publs. No 39.* Ambleside: Freshwater Biological Association.

Kratz, W A. and Myers, J., 1955, Nutrition and Growth of Several Blue-Green Algae. *Am. J. Bot.* **42**, 282–287.

Kuppo-Leinikki, P. and Kuosa, H., 1989. Preservation of picoplankton cyanobacteria and heterotrophic nanoflagellates for epifluorescence microscopy. *Arch. Hydrobiol.,* **114**, 631–636.

Li, W K W. and Platt, T., 1987. Photosynthetic picoplankton in the ocean. *Sci. Prog., Oxford*, Oxford, **71**, 117–132.

Lund, J W G., 1959. A simple counting chamber for nanoplankton. *Limnol. Oceanogr.,* **4**, 57–65.

Lund, J W G., Jaworski, G H M. and Bucka, H., 1971. A technique for bioassay of freshwater, with special reference to algal ecology. *Acta Hydrobiol.,* **13**, 235–249.

Lund, J W G., Jaworski, G H M. and Butterwick, C., 1975. Algal bioassay of water from Blelham Tarn, English Lake District and the growth of plankton diatoms. *Arch. Hydrobiol., Suppl.,* **49**, *Algological Studies,* **14**, 48–69.

Lund, J W G., Kipling, C. and LeCren, E D., 1958. The inverted microscope method of estimating algal numbers and the statistical basis of estimation by counting. *Hydrobiologia,* **11**, 143–170.

McGuinnies, W G., 1934. The relation between frequency index and abundance as applied to plant populations in a semi-arid region. *Ecology,* **15**, 263–282.

McNabb, C D., 1960. Enumeration of freshwater phytoplankton concentrated on the membrane filter. *Limnol. Oceanogr.,* **5**, 57–61.

Marvan, P., Pribil, S. and Lhotsky, O., 1979. *Algal assays and monitoring eutrophication.* E. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart. ISBN 3 510 65091 3.

Miller, W E., Green, J C. and Shiroyama, T., 1978. The *Selenastrum capricornutum* Printz algal assay bottle test. US Environmental Protection Agency. Corvallis, Oregon, 97330. **EPA-600/9-78-018**, 126 pp.

Mitt. int. Ver. Limnol., 1978. Symposium: *Experimental use of algal cultures in limnology.* E. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart. 607 pp.

Mullin, M M., Sloan, F M. and Eppley, R W., 1966. Relationship between carbon content, cell volume and area in phytoplankton. *Limnol. Oceanogr.,* **11**, 307–311.

Nauwerk, A., 1963. The relations between zooplankton and phytoplankton in lake Erken. *Symb. Bot. Upsal.,* **17**, 1–163.

Nordforsk, 1973. Algal assays in water pollution research. *Proc. Nordic Symp. Oslo, Oct. 1972.* Nordforsk, Secretariat of Environmental Sciences, Publication 1973: 2.

Odum, E P., 1959. *Fundamentals of ecology.* Saunders, Philadelphia, USA 546 pp.

Reynolds, C S., 1984. Phytoplankton periodicity: form, function and environmental variability. *Freshwat. Biol.,* **14**, 111–142.

Rippka, R., Deruelles, J., Waterbury, J B., Herdman, M., and Stanier, R Y., 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.,* **111**, 1–61.

Shelford, V E., 1913. *Animal communities in temperate America.* University of Chicago Press, Chicago, USA.

Shubert, L E. (ed.), 1984. *Algae as ecological indicators.* Academic Press, 434 pp.

Sieburth, J McN., Smetacek, V. and Lenz, J., 1978. Pelagic ecosystem structure: heterotrophic components of the plankton and their relationship to plankton size-fractions. *Limnol. Oceanogr.,* **23**, 1256–1263.

Skulberg, O M., 1966. *Algal cultures as a means to assess the fertilising influence of pollution.* Third Int. Conf. on Water Pollution Research. Wat. Pollut. Cont. Fed., 1966, 1–15.

Smayda, T J., 1965. A quantitative analysis of the phytoplankton of the Gulf of Panama. II On the relationship between C-14 assimilation and the diatom standing crop. *Bull. inter-Am. trop. Tuna Commn.,* **9**, 467–531.

Smayda, T J., 1970. The suspension and sinking of phytoplankton in the sea. *Oceanogr. mar. Biol. A. Rev.,* **8**, 353–414.

Stanier, R W., Kunisawa, R., Mandel, M. and Cohen-Bazire, G., 1971. Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriological Reviews,* **35**, 171–205.

Stein, J R. (ed.), 1973. *Handbook of phycological methods: culture methods and growth measurements.* Cambridge Univ Press.

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

Taguchi, S., 1976. Relationships between photosynthesis and cell size of marine diatoms. *J. Phycol.,* **12**, 185–189.

Teitelbaum, R G., Ruby, S L. and Marks, T J., 1978. On the structure of starch-iodine. *J. Am. Chem. Soc.,* **100**, 2315–2316.

Toerien, D F. and Steyn, D J., 1973. Application of algal bioassays in eutrophication analyses. *South African Journal of Science,* **69**, 79–82.

Vollenweider, R A. (ed.), 1974. A manual on methods for measuring primary production in aquatic environments. *IBP. Handbook No. 12.* Oxford. Blackwell. 2nd. edn. pp. 225.

Whipple, G C., Fair, G M. and Whipple, M C., *The microscopy of drinking water.* 4th Edn. 1927. Chapman and Hall, London.

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