CULTURING ALGAE

A GUIDE FOR SCHOOLS AND COLLEGES

Culture Collection of Algae and Protozoa
NATURAL ENVIRONMENT RESEARCH COUNCIL

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A guide for schools and colleges

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and
Erica Swale

Culture Collection of Algae and Protozoa
(CCAP)

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Cover
Organisms in flasks and photomicrographs — from left to right — *Anabaena, Haematococcus, Euglena*

This booklet was written by Drs. H. Belcher and E. Swale at the Culture Centre of Algae and Protozoa (CCAP), Cambridge. During 1986 the cultures and activities of CCAP were transferred to the Freshwater Biological Association at Ambleside (freshwater algae and all protozoa) and the Scottish Marine Biological Association at Oban (marine algae). As a result of these changes the Centre has been renamed the Culture Collection of Algae and Protozoa but the acronoym remains CCAP.

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Contents

Foreword

In recent years a great deal of physiological, biochemical and morphological research has been carried out using cultures of the smaller algae, and their value in teaching is being increasingly realised. The purpose of this guide is to enable such cultures to be maintained and handled in schools and colleges, both for instruction and for student projects. Simple methods for isolation into unialgal (but not bacteria-free) culture are also given.

We would stress that this is not a manual for postgraduates taking up algal physiology, who are advised to seek help in more advanced publications such as those cited in the references. However, it may be of use to those working in other areas such as ecology, who wish to carry out a small amount of work with algae and who do not want to become involved in too much detail.

1. Introduction

"There is nothing, Sir, too little for so little a creature as man. It is by studying the little things that we attain the great art of having as little misery and as much happiness as possible." – Samuel Johnson.

We have received an increasing number of enquiries from students and staff of schools and colleges who want to maintain algae in culture and carry out projects involving simple growth experiments using them. There are books setting out to help the more advanced worker in this field, but there seems to be no published information for the inexperienced worker who has no contact with people skilled in the techniques involved. We have written this guide with such students in mind.

Successful work with algal cultures needs patience, care and skill (plus some luck!) and it must be stressed that any experiment decided upon should not be too ambitious in scope. If kept as simple as possible, there is much more likelihood of positive and meaningful results being obtained.

Bacteriological methods are in many ways similar to those for algal cultures. Advice from a bacteriologist on handling cultures would be helpful, remembering that algae differ from bacteria in growing far more slowly and in needing light for photosynthesis.

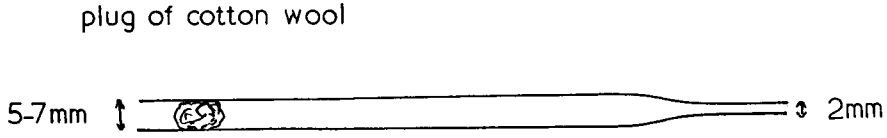
2. Preparation of medium

Distilled water and analytical reagent (AR) quality chemicals should be used if possible in making media. When a series of experiments is to be performed, it is often helpful to make up concentrated stock solutions of each of the compounds used. Medium once prepared should be kept in the dark in a flask with a cotton wool plug, and if the intention is to keep it for a week or more it should be sterilized either by autoclaving or by being brought to the boil in the flask on 3 successive days (to prevent the growth of bacteria).

3. Glassware and its cleaning

The most frequently used culture vessels are thick-walled test-tubes, preferably of Pyrex or other heat-resisting glass, about 150 mm by 16 mm. Boiling tubes or conical flasks can also be employed, while Petri dishes may be needed for certain purposes. (Failing these, other glass vessels may be pressed into service with a little ingenuity.) Glass graduated pipettes will be needed for the general handling of small volumes of solutions and for the quantitative sampling of cultures. For qualitative handling of liquids, pipettes pulled from Pyrex (best) or soda glass tubing will suffice (Figure 1).

Clean glassware is essential for culture work and it is worthwhile taking considerable care over this. Both new and used culture vessels should be



pipette pulled from glass tubing

length approximately 20 cm

cleaned with hot water and a brush and given a final rinse with distilled water. On occasion, it may be necessary to use detergents, but, as these are toxic to some algae, the tubes, etc, must be washed even more thoroughly, and it is advisable to let them stand in tap water overnight before the final rinse in distilled water. In hard water districts, calcium and magnesium salts become deposited on the walls of the vessels. These salts can be removed by soaking in dilute hydrochloric acid followed by multiple rinses in water.

All pipettes, flasks and other glassware used in preparing the media should also be thoroughly clean.

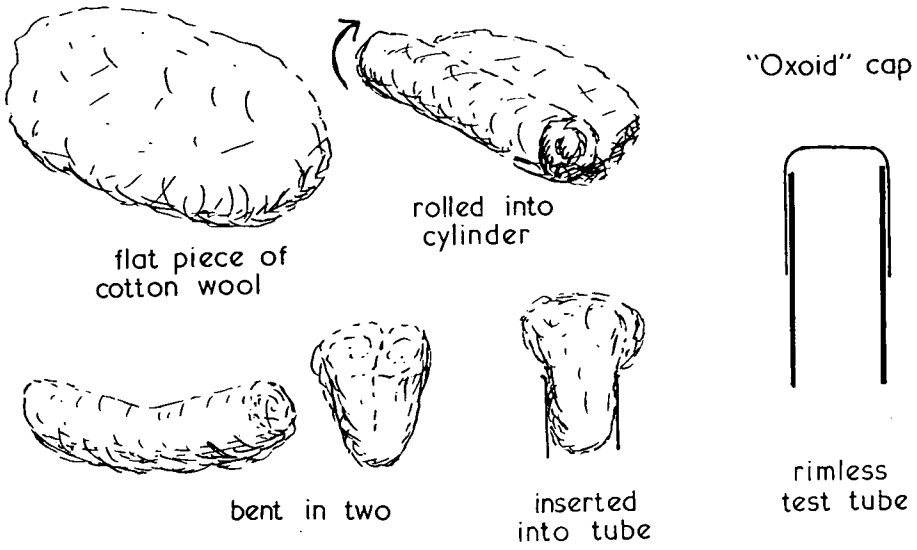
4. Plugs, tubes, etc

The purpose of a cotton wool plug in a culture or flask is to exclude dust and bacteria while allowing free diffusion of gases. Plugs should be made of non-absorbent cotton wool made for microbiological work. (The absorbent sort, as sold by chemists, gets damp easily and encourages the growth of bacteria and moulds.)

Plugs can be made by taking a small mass of cotton wool from the package, teasing it out into a flat rectangular shape, then rolling it lengthwise into a cylinder. This is folded over in the middle and the bent-over portion pushed into the neck of the tube (Figure 2). Practice is needed to judge how much cotton wool is needed, but a correctly made plug ought to be of such a tightness that the tube may be lifted by it, but loose enough to be replaced fairly easily with one hand (often the left for a right-handed person).

Instead of plugs, many laboratories now use loosely fitting caps of aluminium or plastic, such as "Oxoid" caps (Figure 3). Sterile disposable culture tubes of polystyrene can also be obtained. They are bought sterile and after a single use are either thrown away or used for something not requiring sterile technique. The screw caps of these tubes should be left loose after inoculation to allow for gas exchange.

All pipettes used in handling sterile solutions should also be plugged at the wide end with a piece of cotton wool about 10 mm inside the tube, to stop



2 making plugs

3

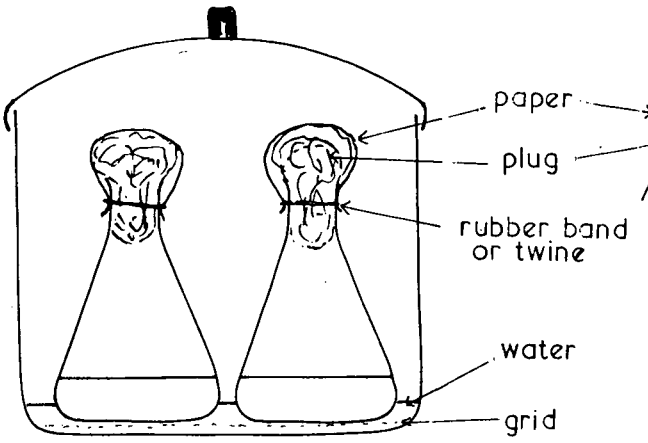
bacteria being carried in with the breath (Figure 1). A long pin or mounted needle with the end bent over into a tiny hook will be needed for removing plugs from the pipettes before they are washed.

5. Sterilization and flaming

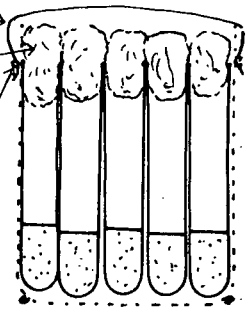
Ideally, all culture vessels (except disposable plastic ones), pipettes and media (whether in the culture tubes or separately) should be sterilized in an autoclave for 15 or 20 minutes at 15 lb pressure. Failing this piece of equipment, which few schools will have, sterilization can be done for the same length of time in a large pressure cooker (Figure 4). (NB Remember the necessary layer of water at the bottom of both this and the autoclave.) It is important to remember that boiling alone is not sufficient to kill all spores of bacteria and fungi.

Tubes are best autoclaved standing in a wire basket, but beakers may be used, when the time for sterilization should be increased somewhat.

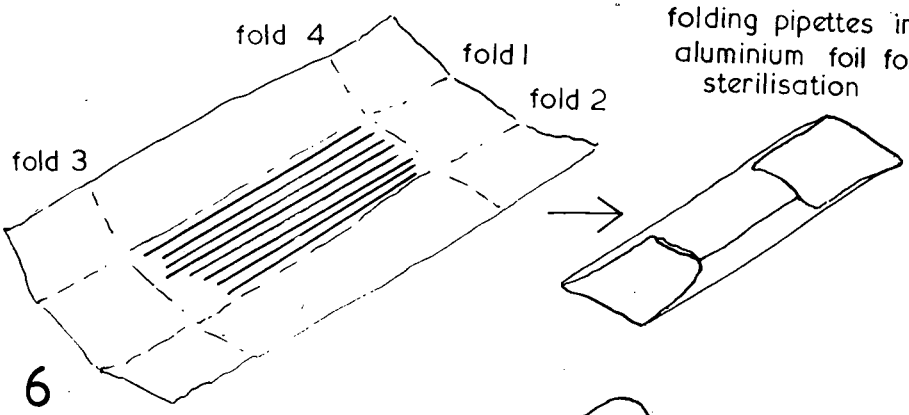
Before putting tubes or flasks into the autoclave or pressure cooker, all plugs must be covered with a piece of greaseproof paper held round the neck of the vessel by a rubber band. A piece of aluminium foil can be substituted for the paper. This cover prevents wetting of the plugs by steam or condensation water (Figures 4, 5).



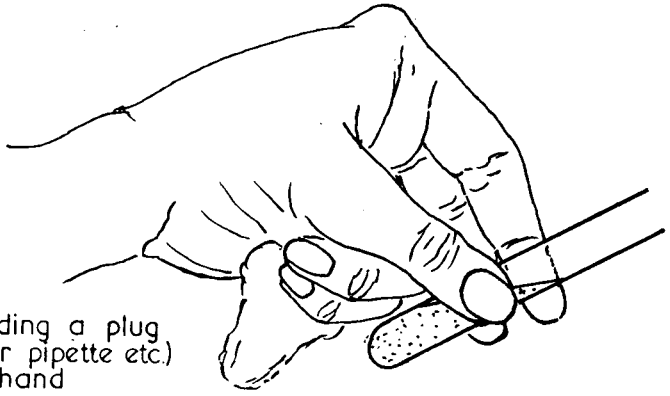
4 sterilisation in pressure cooker



5 tubes in wire basket



6 folding pipettes in aluminium foil for sterilisation



7 method of holding a plug and a tube (or pipette etc.) in the same hand

Pipettes and empty glassware, etc, may be sterilized by baking in an oven for 2 hours at 160°C (320°F). If a suitable long metal box (as sometimes sold for the purpose) is not available for holding the pipettes, they can be wrapped in aluminium foil, making a flat package with the ends turned over (Figure 6).

Short ungraduated pipettes, either pulled from glass tubing or bought as "disposable", can be baked in an oval Pyrex casserole with a lid, and kept in this container until needed.

Whenever anything is added to or removed from a culture, the neck of the tube (including the "donor" tube) should be passed through a flame immediately after the plug is removed and immediately before it is replaced, taking care not to flame the plug! This ensures that any dust particles with adhering bacteria settling on the lip of the tube are destroyed. With practice, it is possible to hold a tube in the left hand and remove the plug with the curled little finger of the right, while manipulating a pipette or wire loop with the remaining fingers and thumb of this hand. When pouring from one tube to another, the tubes are held between the forefinger and thumb of each hand, and the plugs withdrawn and replaced with the little finger of the opposite hand (Figure 7).

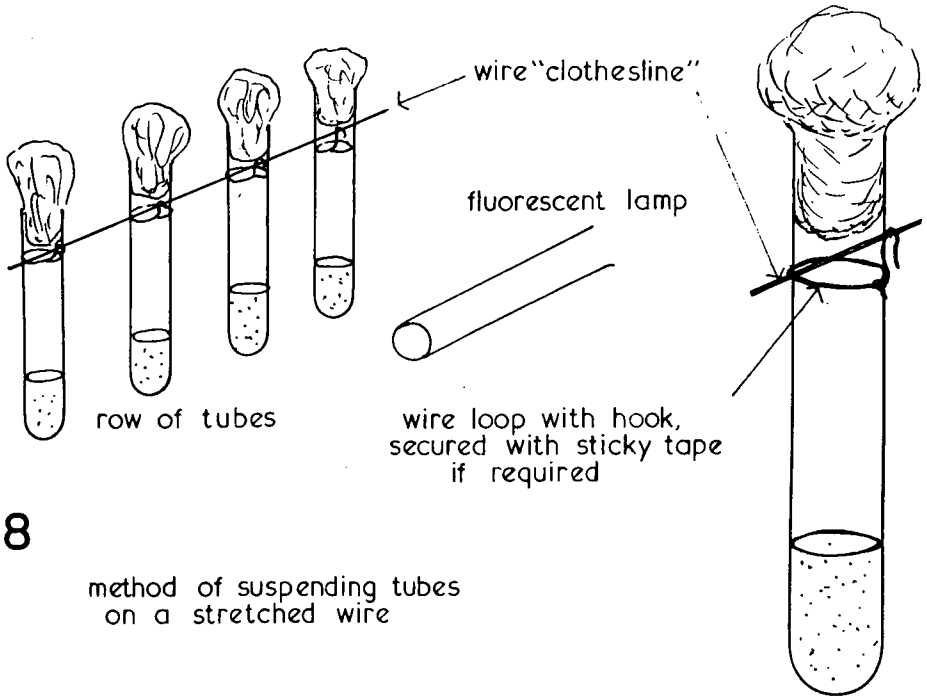
6. Lighting and temperature

Algal cultures need a light source, for which daylight may be adequate. The best position for the culture vessels is near a north-facing window, but, if a window facing another direction has to be used, care must be taken to ensure that direct sunlight never falls on the tubes. Greaseproof paper or tissue paper stuck on to the glass is an effective diffuser of light, as also is a coat of whitewash.

If constant illumination (or variable "day length") is required, either incandescent bulbs or fluorescent tubes may be used, but the light source must not heat the tubes. It is advisable to set up a dummy culture whose temperature can be measured. Pringsheim (1946) suggests how a bulb can be cooled.

Tubes may be stood in beakers, jars or racks, but more even illumination is achieved by suspending them with wire clips from a "clothes line" of stretched horizontal wire, or in a suitable rack (Figure 8).

The temperature for algal cultures should not exceed 25°C (roughly 78°F), but 20°C (c 68°F) is better. Room temperature is satisfactory for many algae, but may rise above 25°C in summer (especially when a building is unoccupied and the windows closed, as over a weekend). For constant temperature, the tubes are immersed in a thermostatically regulated water bath, either of glass or transparent plastic, or metal. If the latter, suitable top lighting must be arranged.



8

method of suspending tubes on a stretched wire

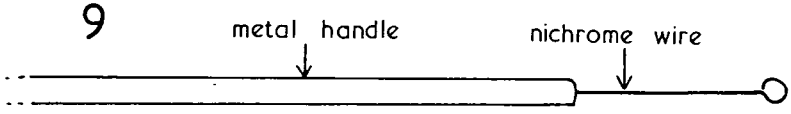
7. Types of culture

Liquid

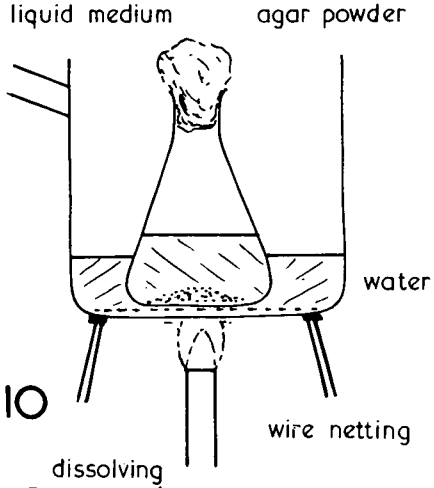
These are likely to be the most frequently used for experimental work. A small quantity of the organism is inoculated into the tube of medium, and there are various ways of doing this. A few drops of a previously existing culture may be poured into the new medium (remembering to flame the necks of both vessels); it may be added by pipette, or by a metal loop (Figure 9) (from either liquid or solid cultures).

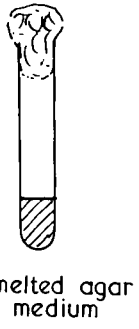
Solid (agar cultures)

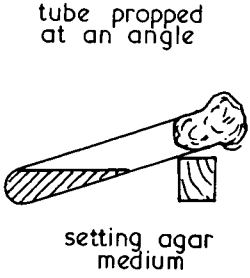
To produce a solid substratum, 1 or 1½% by weight of agar is mixed (no lumps) with medium in a flask and dissolved by heating either over a water bath or in water in a saucepan over a heat source (Figure 10). Liquids containing agar must not be heated over a direct flame or the mixture will scorch at the base (and probably break the flask). After the agar has dissolved, it must be thoroughly sterilized as in Section 5. The hot sterile solution is then poured into sterile Petri dishes (to form "agar plates") or flasks. Petri dishes should be filled to about half their depth and flasks to a level of about



inoculating loop







preparing agar slopes

1–2 cm. If the agar layer is too thin, the culture tends to dry out before the experiment is finished. When using tubes, these should be filled about a third full and then sterilized. When tubes are being cooled, they must be propped at such an angle that the surface of the agar forms a slope (hence the term "slope" for tubes of agar and the cultures on them) extending for about two-thirds of the length of the tube (Figure 11). They are generally inoculated using a wire loop (Figure 9).

8. Setting up experiments

Having prepared a number of similar tubes (or other vessels) containing the same quantity of medium, a growth experiment can be set up. The following points should be remembered:

i. It is advisable always to work in duplicate and average the results.

ii. Always have a pair of control cultures under standard conditions with nothing added or varied.

iii. All cultures in a given experiment must be under the same conditions, apart from the one variable factor which you are investigating.

iv. If testing the effect of a particular compound, add it to a duplicated series

of tubes in logarithmically increasing concentration, eg in ratios of 1, 2, 4, 8, 16 or 1, 10, 100, 1000, 10,000, etc.

The use of a logarithmic scale is preferable to an arithmetical one, because a much wider range of concentrations can be covered. In practice, it is often advisable to begin with the 1, 10, 100 series, then, when the approximate range has been found over which the effect is produced, to use the 1, 2, 4 series in subsequent experiments covering a narrower range of concentrations.

An arithmetical scale is sometimes useful, for instance when testing the effects of 2 media mixed together. If salinity tolerance were being measured, marine or freshwater media could be added in steps such as 10, 20, 30, 40, 50 %.

v. If testing the effect of omitting one of the standard components of the medium, leave out that component when makingup the medium, which can be used for the controls. The ingredient to be tested can then be added to a series of tubes as above.

9. Estimation of growth

Estimation may be either qualitative or quantitative. Qualitatively, it is often easy to tell by eye whether there has been much growth, little, or none. An approximate estimate is suitable for many purposes such as testing media or discovering the concentration above which a particular substance becomes toxic.

Quantitative measurements can generally be carried out only with liquid cultures of unicellular algae which can be dispersed evenly throughout the medium by shaking. Optical density may be estimated by some form of optical density meter, but a more accurate method of following changes in cell numbers is to make counts with a haemocytometer (blood counting slide) on samples removed at regular intervals. There are several forms and makes of haemocytometer, but the Fuchs-Rosenthal is one widely used. Here the central area of the thick glass slide bears an engraved rectangle of given size, subdivided by a grid of intersecting lines. When a drop of culture is mounted, the special coverslip is supported at a known height above the central grid by the slightly elevated ridges on either side (Figure 12).

The cells lying in a chosen number of small squares (defined by the engraved lines) are then counted. It is convenient to dilute the sample, if necessary, by a known amount of water so that about 100 cells can be counted in a reasonable time. A simple calculation gives the volume of the liquid whose cells have been counted, and the cell concentrations per ml of the original sample can then be worked out.

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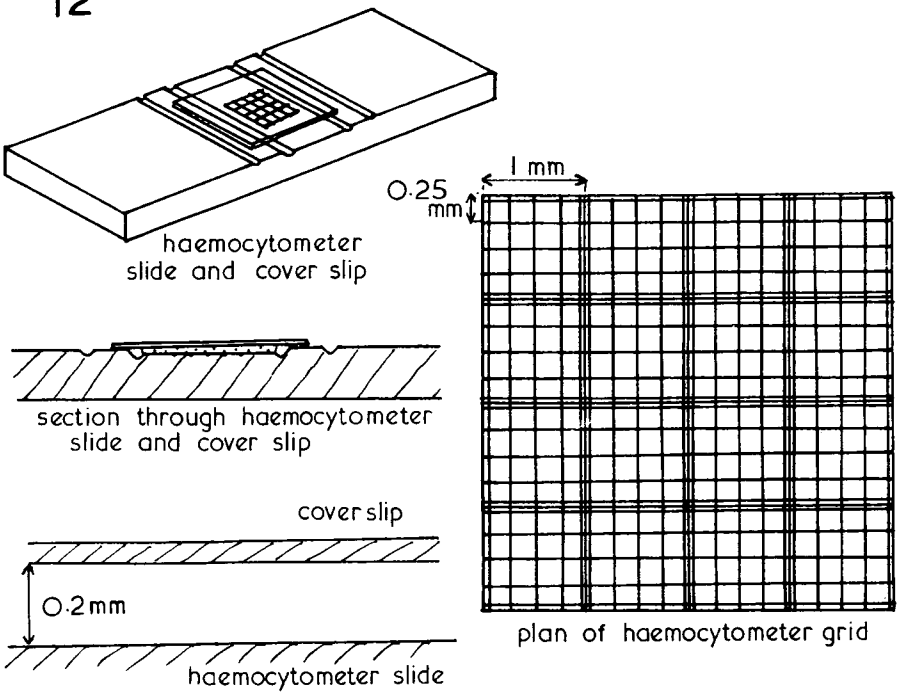
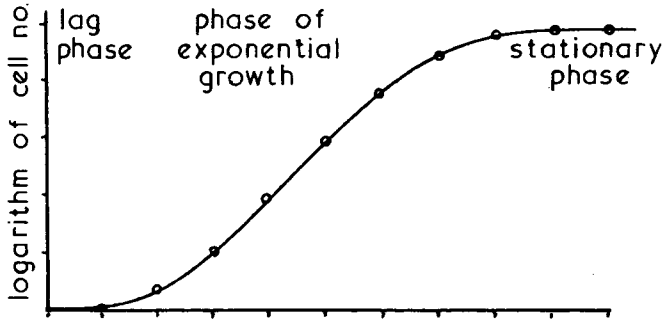


10. Expressing results

Qualitative results (eg much growth, little, or none) can be put into the form of tables, with suitable signs to represent the amount of growth (eg ++, +, −). Quantitative results are best expressed as graphs, with time in days or weeks plotted against the logarithms of cell numbers (Figure 13).



13 idealised growth curve)

11. Culture media for various purposes

Different media may be employed, depending on what use is to be made of the culture, eg simple maintenance, determination of maximum growth rate, induction of sexuality, or some other purpose. What are termed chemically defined media are made up with precise quantities of chemical ingredients, while others rely upon "natural" compounds such as soil, yeast extract, seed grains, etc. The make-up of these cannot be controlled as closely as with the defined media, for the ingredients are complex and liable to slight unanalysable differences. When cultures contain other micro-organisms as well as algae, it is important that the medium chosen does not encourage the former at the expense of the desired components. In general, media with a fairly high organic content (0.1% or more) are suitable only for axenic (bacteria-free) cultures.

For freshwater algae

Soil and water media. These have great advantages so long as axenic cultures are not required. Such media, with well-chosen soil, will result in excellent growth of most unicellular and filamentous algae, with the exception of some of the more exacting planktonic forms. The soil provides not only mineral nutrients and trace elements but the latter are chelated naturally, the pH is buffered, and any toxins arising from the algae are made harmless. These beneficial effects are due partly to the absorbing capacity of the soil and partly to growth substances and other compounds produced by bacteria which germinate from spores resistant to the partial sterilization by steaming used in preparing soil and water media.

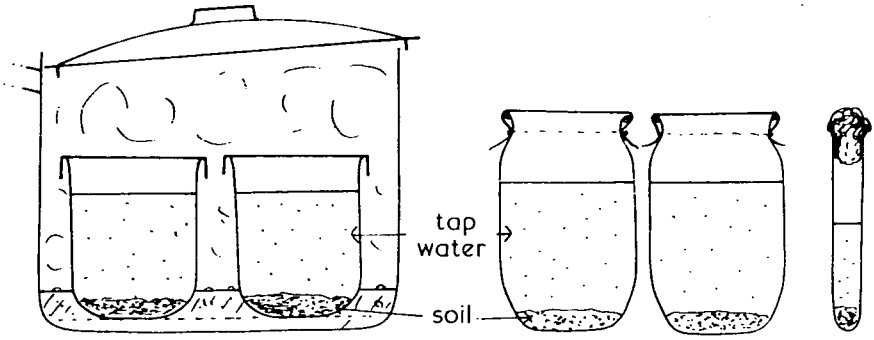
The soil most likely to give good results is that known as a "good garden loam", preferably slightly alkaline, although soil collected from arable fields can be equally good. Whatever the source, the soil should not have been treated recently with either horticultural or agricultural chemicals. Mud from ponds or rivers is seldom satisfactory.

It is important to collect a fair amount of soil in the first place so that, if it proves to be a good one, the remainder can be kept as a stock (air dried) for subsequent experimental work.

At the Culture Centre of Algae and Protozoa, the basic soil and water medium, known as "E", is made up as follows. A layer of about 1 cm of calcareous garden loam is put into a test tube or jar. Tap water is added slowly, the vessel is capped or plugged and the whole is steamed for one hour on each of 2 successive days (Figure 14). This kills any algae or fungi in the soil, but not bacterial spores. It is then allowed to stand for a further day before inoculating.

Variations of the above medium can be made to encourage particular algae. Many eutrophic members of the Chlorophyceae benefit from the addi-

14 soil and water (or "biphasic") cultures



beakers capped with Petri dishes being steamed

jars capped with greaseproof paper and a tube

tion of a small quantity of calcium carbonate (about 3% of the volume of the soil) and a similar amount of ammonium magnesium phosphate is recommended for green euglenoids. Algae from acid habitats may need the addition of sphagnum peat (which could even replace the soil). A little starch inserted below the soil stimulates the growth of saprophytes like *Polytoma* or *Astasia.*

As well as for growth experiments, soil and water media are useful for the long-term maintenance of algae of various kinds. For morphological studies where the presence of bacteria does not matter, soil and water media are usually better than chemically defined ones for retaining the natural appearance of the organisms.

Bold's basal medium (Bischoff & Bold 1963) is a very useful mineral medium for a wide range of algae, including those from more or less eutrophic waters or from soils. It may be supplemented with soil extract and/or vitamins.

Six stock solutions 400 ml in volume are employed, each containing one of the following salts in the concentration listed:

$NaNO_3$	10.0 g
$CaCl_2.2H_2O$	1.0 g
$MgSO_4.7H_2O$	3.0 g
K_2HPO_4	3.0 g
KH_2PO_4	7.0 g
NaCl	1.0 g

To 940 ml of distilled water are added 10 ml of each stock solution and 1.0 ml of each of the 4 stock trace-element solutions prepared as follows:

1. 50 g EDTA and 31 g KOH dissolved in 1 litre H_2O
2. 5 g* $FeSO_4.7H_2O$ dissolved in 1 litre distilled water containing 0.1% by volume of H_2SO_4.

3. 11.5 g* H_3BO_4 dissolved in 1 litre H_2O.
4. 9 g* $ZnSO_4.7H_2O$, 1.5 g* $MnCl_2.4H_2O$, 0.7 g* MoO_3, 1.5 g* $CuSO_4.5H_2O$ and 0.5 g* $Co(NO_3)_2.6H_2O$ all dissolved together in 1 litre H_2O.

* Quantities slightly modified for ease of weighing out.

Chu 10 medium. This well-known medium (Chu 1942) has been used for a wide range of green and blue-green algae and diatoms, particularly those from oligotrophic or less rich eutrophic habitats. The following modification, used by the Windermere Laboratory of the Freshwater Biological Association, has proved very satisfactory.

H_2O	1 litre
$Ca(NO_3)_2.4H_2O$	20.0 mg
KH_2PO_4	6.0 mg*
$MgSO_4.7H_2O$	25.0 mg
Na_2CO_3	20.0 mg
Na_2SiO_3	25.0 mg
Normal HCl	0.25 ml
H_3BO_3	2.5 mg*
$MnCl_2.4H_2O$	1.5 mg*
$(NH_4)_6Mo_7O_{24}.4H_2O$	1.0 mg

Dissolve the following together in a small volume of the water before adding:

EDTA sodium salt	2.0 mg
$FeCl_3$	1.0 mg

Vitamins, if required, are usually dissolved and the mixture sterilized separately by membrane filtration, as heat tends to destroy these compounds. Many strains, however, particularly those of green algae, will grow satisfactorily without added vitamins.

Vitamin B_{12}	0.01 mg
Vitamin B_1	0.001 mg
Biotin	0.001 mg

Two other media recommended for the long-term maintenance of cultures are:

Proteose peptone agar (for axenic cultures)

H_2O	1 litre
Proteose peptone (Difco)	1 g
KNO_3	200 mg
K_2HPO_4	20 mg
$MgSO_4.7H_2O$	20 mg
Agar	10 g

This medium proves satisfactory for many algae, and also quickly reveals the presence of many common contaminants.

Soil extract medium ("E + S") A less rich medium for cultures containing bacteria.

H_2O	900 ml
Soil extract stock solution	100 ml
KNO_3	200 mg
K_2HPO_4	20 mg
$MgSO_4.7H_2O$	20 mg
Agar (optional)	10 g

The soil extract stock solution is made by heating some calcareous garden loam in a steamer with twice its volume of supernatant water (as in Figure 14). It is convenient to make up and sterilize a number of small containers of this extract, each sufficient for one batch of medium. This avoids the risk of contaminating the main stock of extract, in which bacteria would multiply readily.

Media for particular algae under axenic conditions include:

Euglena gracilis medium

H_2O	1 litre
Sodium acetate hydrated	1 g
Beef extract	1 g
Yeast extract	2 g
Bacto tryptone	2 g
$CaCl_2$	10 g
Agar (optional)	10 g

This is also used for other species of *Euglena* and related organisms.

Ochromonas medium

H_2O	1 litre
Liver infusion (dehydrated) Oxoid	1 g
Glucose	1 g
Bacto tryptone	1 g

For *Ochromonas* and other members of the Chrysophyceae.

Polytoma medium

H_2O	1 litre
Sodium acetate hydrated	2 g
Yeast extract	1 g
Bacto tryptone	1 g
Agar (optional)	10 g

For *Polytoma* (a colourless relative of *Chlamydomonas*) and similar apochlorotic algae.

Chlorella medium (Sorokin & Krauss 1958)

H_2O	1 litre
KNO_3	1.25 g
KH_2PO_4	1.25 g
$MgSO_4.7H_2O$	1.0 g
$CaCl_2$	85 mg*
H_3BO_4	115 mg*
$FeSO_4.7H_2O$	50 mg*
$ZnSO_4.7H_2O$	90 mg*
$MnCl_2.4H_2O$	15 mg*
MoO_3	7 mg*
$CuSO_4.5H_2O$	16 mg*
EDTA sodium salt	500 mg

Chlamydomonas reinhardii medium (Sueoka 1960)

H_2O	1 litre
NH_4Cl	50 mg
$MgSO_4.7H_2O$	20 mg
$CaCl_2.2H_2O$	10 mg
K_2HPO_4	720 mg
KH_2PO_4	360 mg
Hutner's trace element solution	1 ml
For colourless mutants, add:	
Sodium acetate hydrated	2 g

Hutner's trace element solution:

H_2O	75 ml
EDTA sodium salt	5 g
$ZnSO_4.7H_2O$	2.2 g
H_3BO_4	1.14 g
$MnCl_2.4H_2O$	510 mg
$FeSO_4.7H_2O$	500 mg
$CoCl_2.6H_2O$	160 mg
$CuSO_4.5H_2O$	160 mg
$(NH_4)_6Mo_7O_{24}.4H_2O$	110 mg

Boil, cool slightly, and bring to pH 6.5–6.8 with KOH (not NaOH). Dilute to 100 ml. The colour should be green, changing to purple on standing.

Blue-green alga medium (medium C of Kratz & Myers 1955)

H_2O	1 litre
$MgSO_4.7H_2O$	250 mg
K_2HPO_4	1 g
$Ca(NO_3)_2.4H_2O$	25 mg

KNO_3	1 mg
Trisodium citrate.$2H_2O$	165 mg
$Fe_2(SO_4)_3.6H_2O$	4 mg
Trace element solution A5	1 ml

Trace element solution A5:

H_2O	100 ml
H_3BO_3	290 mg*
$MnCl_2.4H_2O$	180 mg*
$ZnSO_4.7H_2O$	22 mg*
MoO_3	2 mg*
$CuSO_4.5H_2O$	8 mg*

Several other media for blue-green algae are given by Carr & Whitton (1973)

Erdschreiber medium

Natural or artificial sea water	1 litre
Soil extract stock solution	50 ml
$NaNO_3$	200 mg
$Na_2HPO_4.12H_2O$	30 mg

The sea water is filtered, brought to the boil, cooled, and added to the other ingredients after they have been autoclaved.

Artificial sea water

Natural sea water is more often than not difficult to obtain; moreover, it is variable in quality. At this laboratory, we now use an artificial sea water made from crystals of "Synthetica", produced by Waterlife Research Industries Ltd, 476 Bath Road, Longford, Middlesex, UB7 0ED. This has given admirable results, better in fact than natural sea water, and has the added advantage of being easy to store. It is also cheap.

ASP2 medium (Provasoli et al. 1957)

Certain marine strains which will not grow in Erdschreiber are cultured in a mixture of this and "ASP2", an artificial sea water medium. However, this is very complicated, involves the use of membrane filtration, and is rather beyond the range of this publication. A recipe sheet for it can be obtained on request from this laboratory (please send a stamped addressed envelope).

12. Routine maintenance of algal strains

Growing algae in culture resembles market gardening on a miniature scale, in that each strain has its own requirements and preferences, which have mainly to be discovered by experience. Basic techniques for the routine sub-

culture of strains have already been dealt with, as have suitable media for their growth. The aim is to keep the algae growing, but slowly, so that they remain in a healthy state for as long as possible between successive subcultures, and for this reason subdued light and a cool situation are indicated. However, with some strains, it has been found advantageous to start the cells into active growth after subculture by exposure to increased light and warmth for a few days before transferring the cultures back to their regular situation, and this can best be ascertained by making trials.

The optimum period between successive subcultures varies greatly with the strain, the medium and the conditions. In general, many flagellates in axenic culture need subculturing often, a few even weekly, while non-axenic strains in biphasic culture, especially green and blue-green algae, will often persist for several months in a healthy state. The aim is usually to subculture the organisms when they are at the end of the actively growing (or exponential) phase. Here again, cells of such green algae as *Pediastrum* and *Scenedesmus* may remain viable for months after they have ceased to grow and have fallen to the bottom of the tube forming an orange sludge. Some filamentous green algae grown in soil and water media are best planted when subcultured, using a wire or other suitable implement to push part of the tuft firmly into the soil. If this is not done, they may float as a scum on the surface of the liquid.

With experience, the use of a hand lens will often enable the grower to see whether the algae are multiplying satisfactorily. Quite small flagellates can be observed as moving specks if the tube is examined against the light. However, examination with a microscope at intervals is necessary to make sure that no contaminants such as "weed" algae, fungi or even mites have gained access to the tubes since the last examination. Axenic strains, if not grown upon a nutrient agar such as proteose peptone agar (p. 12) will have to be tested periodically on such a medium for the presence of bacteria, which may remain undetected with normal microscopical examination.

When growing algae on a large scale for physiological or biochemical purposes, it is better to employ several medium-sized culture vessels than one large one, to reduce the risk of losing the lot by contamination.

It is vital that every tube or jar be carefully and adequately labelled. Self-adhesive paper labels should be used bearing the name of the species, the strain designation, the medium, the date, and the cultivator's initials if required. Details of any special treatment can be added.

13. General notes on the isolation of cultures

For many physiological and biochemical purposes, axenic (bacteria-free) cultures are necessary, but their preparation calls for considerable dexterity, experience and patience. There is, however, no reason why the beginner should not attempt the isolation of algae into unialgal but non-axenic culture. It is in any case desirable to produce such cultures before attempting to render them axenic. Moreover, many algae grow more readily in the presence of certain bacteria, and it is perfectly possible to use cultures in this state for a variety of simple growth experiments. Short accounts of methods of isolation are given below.

Care should be taken in the preliminary stages of collecting material, and where applicable enrichment cultures may be prepared as an intermediate stage; both are dealt with below.

In a publication of this scope, the complex subject of isolation can be treated only in a summary fashion, and for more details and other methods such works as those of Pringsheim (1946) and Stein (1973) must be consulted.

14. Collecting algae for isolation

Much excellent advice on the collection of algae is given by Lund (1961). Only a few points can be mentioned here.

Algae collected from the wild should not be overcrowded, should be kept cool, and brought back into the laboratory as soon as possible. Bottles, jars and tubes should always be filled incompletely so as to leave an air space under the cap. If possible, Crustacea and other small animals should not be allowed into the tubes with the algae, as they may consume the latter in the space of a few hours. A tea strainer is useful for filtering off such creatures. Required filamentous forms can be gathered by hand, or forceps can be used. The brown scums of diatoms which form on the surface of mud may be collected with a pipette or spoon.

Upon returning to the laboratory, the samples should be transferred to shallow dishes in a cool light place. After a few hours any mud will have settled, and diatoms may have come to the surface of it. Many flagellates will gather at the meniscus on the side toward the light, and can be collected with a pipette, while a few may swim to the opposite edge. Placing the sample in a small trough or cut-down square bottle diagonal to the light source will often facilitate such collection, as the cells will swim into a corner. Suspended algae may be concentrated in a centrifuge at low speed in tubes

with conical bases. If the speed is too high, they will be damaged and not grow.

If the above jars or dishes are allowed to stand undisturbed for a few days, a succession of species may be observed. However, we have often found that the best procedure for obtaining algae for isolation from a collection is to prepare enrichment cultures, as described in the following section.

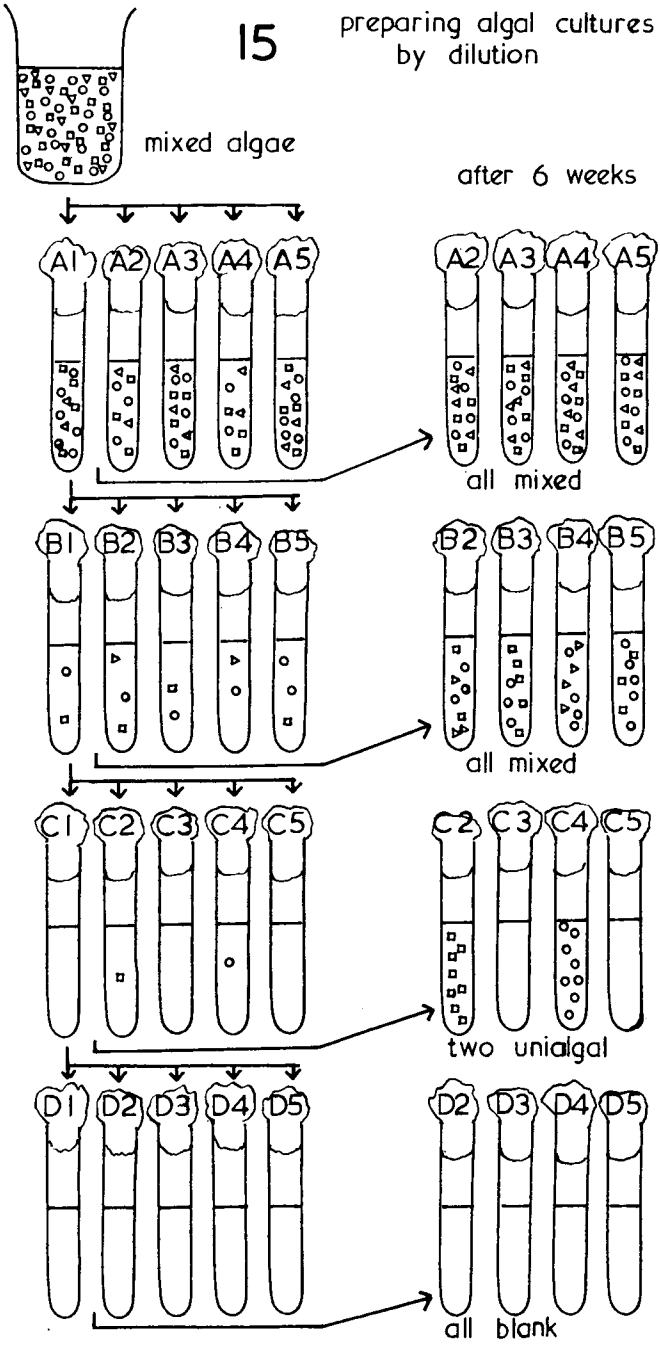
15. Enrichment cultures

These are prepared by putting collected material into culture vessels together with a similar or greater volume of a suitable medium, depending upon the richness of the original sample. The algae are then allowed to multiply under favourable conditions. Studying the succession of forms which appears has, of course, a great interest in itself, but such cultures are invaluable for furnishing suitable material for further isolation, for a variety of reasons. The number of individuals available for isolation is obviously much increased, and these will be in an actively growing state and already becoming adapted to the culture medium and conditions. If the culture is examined day by day, species which were not discernible in the original collection owing to their scarcity will appear for a while before being overtaken by others.

By setting up parallel cultures using a range of media, certain forms will be encouraged by the different conditions, and an idea thus obtained of their requirements under cultivation. For instance, green algae such as *Scenedesmus*, *Pediastrum* and *Chlamydomonas* thrive in a mineral medium, eg Chu 10 (p. 15). If, however, 25% of sea water is added to this medium, the green algae will be discouraged and diatoms multiply. Many flagellates prefer a soil and water medium, while a split pea pushed under the soil before steaming may encourage saprophytic and pale heterotrophic forms. Many more details are given by Pringsheim (1946).

16. Isolating algae by dilution

The dilution method is particularly useful when the starting material is preponderantly unialgal. This condition surprisingly often obtains in the wild, when the contents of a green pool or puddle may consist largely of one or 2 species. The technique can also be used when a "lucky dip" of various species is acceptable. The principle, illustrated in Figure 15, is to dilute a sample containing the wanted species with medium and to use this mixture to set up a batch of, for instance, 5 tubes so that each tube contains a very few cells (group A). Four of these tubes are put to grow, while the contents of the fifth are shared among 5 more tubes (group B), which are topped up with medium to the volume of the first tubes. This second series of tubes is


15
preparing algal cultures by dilution
mixed algae
after 6 weeks
A1
A2
A3
A4
A5
A2
A3
A4
A5
all mixed
B1
B2
B3
B4
B5
B2
B3
B4
B5
all mixed
C1
C2
C3
C4
C5
C2
C3
C4
C5
two unialgal
D1
D2
D3
D4
D5
D2
D3
D4
D5
all blank

similarly treated to the first, the successive subdivisions being carried on as necessary. When the cells in the tubes multiply, which may take 6 weeks or more, the first group of tubes (group A) will probably all be seen to contain algae, while the last and most dilute (in this case, group D), may not contain any. Somewhere between these extremes will lie cultures (group C) in only some of which algae have grown, and it is among these that the unialgal cultures are to be sought, as when 2 or 3 cells are divided between 5 tubes one or more of the latter will be likely to receive one cell only.

In practice, more tubes can be used in each group, say 10. This method is somewhat laborious, and results in much washing up. However, firms such as Sterilin produce sterile disposable dishes with lids, like square Petri dishes, divided by cross walls into 25 compartments each holding a few ml. These make the preparation of dilution cultures much easier.

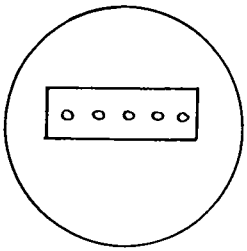
Unfortunately, there is no guarantee that any culture produced by this method is clonal (originating from a single cell), as a tube might receive 2 cells of the same species. However, if clones are needed, any cultures obtained can be further purified. Axenic cultures cannot be produced by this method unless one is very lucky indeed.

Throndsen (in Sournia 1978) gives a more detailed account of the dilution method, particularly as applied to marine plankton organisms.

17. Isolating algae by pipetting

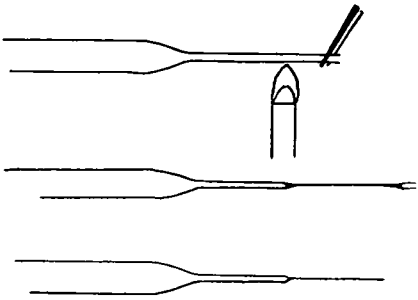
This is a method whereby individual cells are picked up, washed, and inoculated into medium. There are several variants of this technique; a simple one is given here. The process can be carried out using an ordinary microscope with a ×10 or lower-powered objective, an inverted microscope, or for large cells a lower-powered binocular microscope. The method needs a certain amount of skill and practice, and when using an ordinary microscope one has to become accustomed to the inverted image. Aspects are illustrated in Figure 16.

Dry sterile slides stored in Petri dishes or foil are required, together with sterile pipettes as in Figure 1 (provided with a bulb of rubber or PVC), and tubes of medium. Several drops of medium are placed in a row upon one of the slides (placed in another sterile Petri dish for protection from dust). A drop of the alga-containing liquid is placed upon another slide for examination under the microscope and is searched for suitable cells. Before attempting to catch the cells, it is necessary to prepare capillary pipettes by heating the narrow end of a pipette in a small flame (Bunsen burner turned low – use the side of the flame, or a pilot light or spirit lamp) and pulling it out rapidly



sterile slide with drops of medium in Petri dish

16



drawing out a capillary pipette over a small flame

with forceps to a bristle-like thinness. The end of this is then broken off cleanly (catching cells is impossible with a jagged end).

When a cell has been located in the drop under the microscope, and while still watching it, the end of the pipette is dipped into the drop and brought close to the cell, which should then pass into the open end by capillary action. For this operation hold the pipette by its tube only, and do not touch the bulb. Dexterity is required in getting the pipette quickly and accurately to the cell. If there is any delay, liquid entering the pipette sets up currents which sweep away the would-be prize. This and other pitfalls can be avoided only with experience and patience.

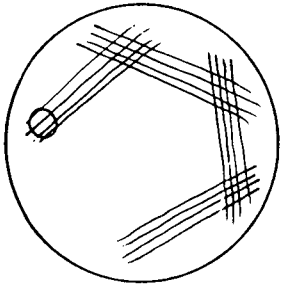
The charged pipette is then removed and emptied (by squeezing the bulb) into the first of the drops of medium on the second slide. The process is repeated if desired until, say, 10 cells have been captured and transferred to the drop. This is then searched and the cells removed one by one and placed in the next empty drop, and so on through several drops, until unwanted individuals of other species (picked up accidentally) have been eliminated.

If it is important to be certain that the resulting culture is a clone, it is advisable to ask one or, preferably, 2 other persons to check that the final drop contains only a single cell. The last stage is to transfer single cells into tubes of medium, to be left to multiply under suitable conditions. The pipette must be sterilized after each cell transfer. When isolating cells into unialgal culture, it is often sufficient to rinse the end in boiling water (in a beaker), drawing in the water and ejecting it again with the bulb. This will kill algal cells adhering to the glass (but not bacterial spores), and as the tip of the pipette is not destroyed it is possible, having found a good one amongst several, to use it until it is accidentally broken; some capillary ends are much better than others for the capture of cells. When preparing axenic cultures, it is usual to draw out a new capillary after each transfer, taking a fresh pipette when necessary. This ensures that bacterial spores are killed.

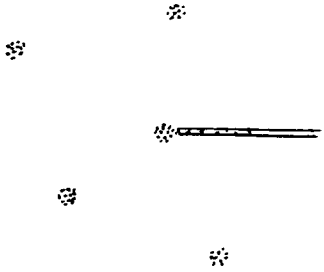
18. Isolating algae by streaking on agar plates (Petri dishes of agar)

This method (called "plating out") is most suitable for members of the green algae which grow well on a solid medium such as Chu 10 agar, and for freshwater diatoms, which often thrive on this medium if 25% of sea water is included.

A Petri dish of the appropriate agar is taken, and a drop of the sample containing algae is placed on the surface of the agar near the edge (Figure 17). A wire loop (Figure 9), sterilized in a flame, is allowed to cool, and with it the drop is smeared into 4 or 5 parallel lines across the surface of the agar near the periphery as shown ("streaking"). Another series of lines is then drawn intersecting the first, and the process is repeated until there are 4 or 5 series of lines. The lid is replaced and the dish left for a culture to grow.



17



"streaking" a drop on an agar plate

picking up cells from a colony

If successful, many small circular colonies of algae will develop, thickly clustered at the site of the original drop and becoming gradually fewer along the successive lines. Among the sparser colonies should be some sufficiently separated from their neighbours for them to be picked up whole or in part and transferred to a tube of medium. Some workers use a sterile loop for this, or a wire with a flattened tip (like a spade), but we have found a capillary pipette, as described in the previous section, to be more effective.

Experience is needed to choose the right time to isolate algae from a streaked plate, for if left too long the whole may become overgrown with unwanted "weed" algae, moulds, bacteria and amoebae. Some of the latter may be facultative pathogens, so it is advisable to sterilize old and overgrown cultures in a pressure cooker or autoclave before washing up.

19. Documentation

When isolating strains of algae, it is essential to keep full records of any retained in culture. These should include the name of the species (if known),

the date, the source of the material, the method of isolation, the medium and any special requirements, together with the name of the isolator.

Note on the meaning of "culture" and "strain"

There is a need to define these terms for they are often used indiscriminately as though they were synonymous.

A culture, as applied to algae, refers to the complex of culture medium, the organism(s) growing in the medium, and the vessel enclosing these. A culture may be mixed (ie containing more than one named organism) or unialgal (one named organism only). Cultures normally (unavoidably) include bacteria, unless special steps are taken to remove or exclude them. Bacteria-free cultures are said to be axenic (Gk, without foreigners).

A subculture is derived from a previously existing one.

A strain is a particular algal population grown in culture and suitably designated. As far as possible its origin is recorded, and the strain will have descended from either one or several cells. Strains are maintained by sub-culturing, sometimes for many years.

If the individuals comprising a particular strain arose from one cell in the first place, by asexual reproduction, this population of descendants is called a clone. The adjective clonal may be applied to cultures as well as strains.

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The books by Pringsheim and Stein are particularly recommended for further reading.

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