A LABORATORY GUIDE TO SOIL MICROBIOLOGY

SOME GENERAL PRINCIPLES AND PRACTICE

J C FRANKLAND, P M LATTER AND J M POSKITT

I   ISOLATION AND MAINTENANCE OF FUNGI AND BACTERIA

II   CULTURE MEDIA

III STERILIZATION

This laboratory guide is an up-date of Merlewood Research and Development Paper No. 43, 1973, and is intended, in particular, for research assistants and students without previous training in soil microbiology. Although it was written with the specific requirements and facilities at Merlewood Research Station in mind, much of the content could be applicable to other laboratories concerned with soil microbiology, especially those with limited resources and only small working collections of cultures. It is not in any way an attempt to produce a comprehensive manual of microbiological methods but rather an introduction to the general principles and procedures that should be adopted when handling and culturing non-pathogenic fungi and bacteria. Techniques for enumerating microorganisms are, therefore, not included. A complete beginner should watch a demonstration of the techniques, subsequently using these notes as reminders and for further explanations.

Two other Merlewood documents are also relevant:

'Safety Guidance Notes' which details special safety precautions that must be adopted by Merlewood workers in the laboratory or field.

'Microbiological Procedures' which gives step-by-step 'recipes' and instructions for particular methods and equipment in current use at Merlewood. This also highlights safety and was drawn up to comply with legal requirements.

ACKNOWLEDGEMENTS

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I   ISOLATION AND MAINTENANCE OF FUNGI AND BACTERIA

'Isolation' is often essential for taxonomic and experimental work on micro-organisms. In the microbiological sense, it is the process of separating a single species of micro-organism from its natural habitat and growing it by itself, without interference from other organisms, on a sterile substratum, i.e. in pure culture. The micro-organism can then be distinguished from other species by its individual characters (even if artefacts of laboratory conditions) and propagated to provide experimental material. Only occasionally is a mixed culture of two species essential for survival or sporulation. Many fungi and bacteria can live for years in culture if competitors are excluded, and if the nutrient medium is renewed periodically. A named collection of such cultures can be invaluable for comparison with freshly isolated and unidentified micro-organims, although some diagnostic characters may be lost and sporulation may cease after long periods in culture.

Methods of isolating micro-organisms from a natural environment, such as soil, litter, air, or water, are numerous. One of the simplest methods of isolating a visible fungus on a leaf, for example, is by picking up its mycelium or fruit body on a sterile needle and transferring it to a nutrient culture medium in a tube or Petri dish. Contaminating organisms, however, may well be carried over and more elaborate procedures are usually needed. Dilution plates and Warcup soil plates, two widely used methods of isolating soil micro-organisms, are described by Johnson and Curl (1972). For references to other isolation methods see Frankland et al. (1990). The basis of all selective isolation techniques is the provision of conditions particularly favourable to the organism concerned and as unfavourable as possible to its competitors. They may involve therefore:

- traps or baits;

- media nutritionally selective for certain species;

- antibiotics or some chemical (eg rose bengal) to kill or inhibit contaminants   or competitors;

- a selective environment, e.g., anaerobic conditions or specific temperatures.

If the first transfer to a culture medium does not result in a pure culture, further cleaning must be carried out as described on p. 20. The final stages of purification are often the most difficult, since two species may be very closely associated, eg some bacterial species on fungal hyphae.
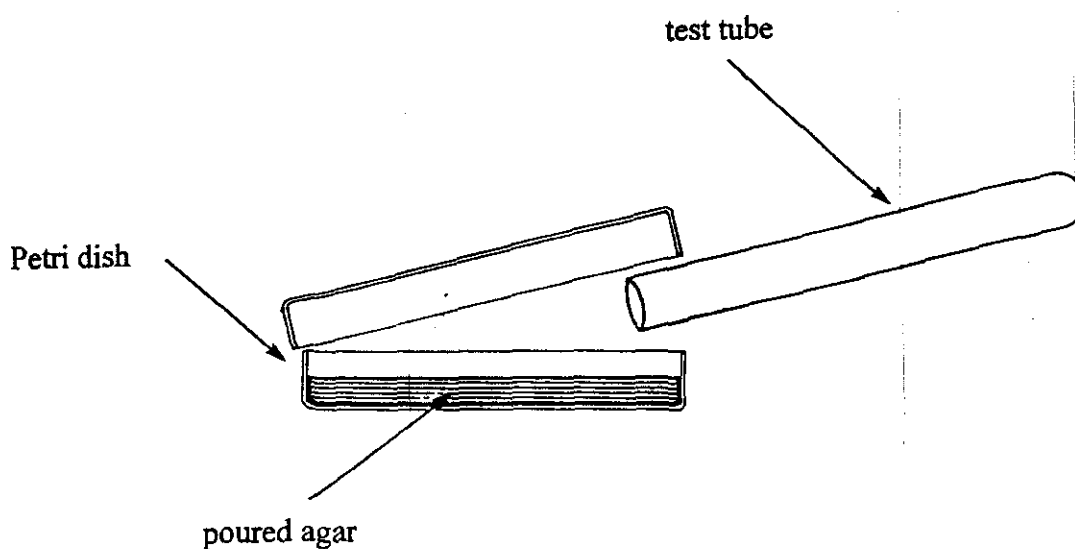
ROUTINE PRECAUTIONS

Throughout all processes of isolating and culturing, re-entry of unwanted organisms must be prevented and the worker protected from infection, and it is good practice to assume that all cultures are potentially pathogenic. The whole environment (air, the worker, the laboratory bench, even a clean laboratory coat) is loaded with bacteria and fungi, most of which can grow on culture media causing contamination. The following routine precautions should therefore be taken.

i. At Merlewood a 'Clean Laboratory' and a Laminar Flow Cabinet is reserved for 'sterile' work, but a Laminar Flow Cabinet is not essential for aseptic technique. Untreated organic materials, such as soil, plants (including litter and roots) and toadstools, are banned from this laboratory (see Mites, p.28). Isolations from such materials should be carried out in a general laboratory.

ii. All instruments and materials that touch the cultures must be sterilized.

iii. The surroundings must be kept scrupulously clean to prevent contamination of sterile objects or pure cultures by dust or aerosols and to protect the worker from accidental infection. Hands should be washed before and after handling cultures; the bench should be swabbed down with disinfectant before and after use, and the room, if it has become heavily infected, should be sprayed (see Section III: Sterilization). Exposure of a clean agar plate for a few minutes will indicate the level of aerial contamination. Dust in crevices, on bench beading and on Bunsen burner fittings is also a source of contamination that can be picked up on the plugs of culture tubes lying on the bench if not guarded against.

iv. In the open laboratory, work close to the source of heat (the updraught of air reduces the risk of micro-organisms settling on the material) in as still an atmosphere as possible, i.e. shut doors and windows, and do not talk or breathe heavily at critical moments! It is possible to work aseptically in quite dirty rooms provided air currents are eliminated as far as possible. In addition, open culture containers for as short a time as possible and protect the apertures and sterile

surfaces from aerial contamination. For example, culture tubes and phials while open should be held with the mouths pointing downwards at an angle of about 30° to the horizontal. Similarly, lids of Petri dishes should be lifted only far enough to allow an instrument or the mouth of another container to be inserted (Fig. 1), and closed plates should always be moved carefully by pressing the lid to the base. When isolations are made from a mixed sample of fungi on a culture plate, fast-growing colonies are sometimes excised while young and non-sporing to prevent them swamping slow-growers. In this case, aerial contamination of the agar surface can be prevented by holding the plate upside down while cutting out the colony.

Figure 1.   Method of pouring agar from a test tube into a Petri dish

The above precautions should be adequate for general culture work, particularly when using test tubes and phials with relatively narrow mouths;

v. For more critical work, or when large surface areas have to be exposed, a Laminar Flow Cabinet should be used. The ENVAIR MK II model at Merlewood provides a sterile downward flow of air that protects the cultures and to some extent the operator from contamination. It can also be used when dispensing sterile media into Petri dishes, etc, but always work away from the mouth of open vessels, as contamination may occur when contaminated clothing or instruments pass over them in the downward flow of air. The operating instructions should be consulted before use.

vi. Disposal of cultures: all discarded living cultures should be killed by autoclaving at $121°C^1$ for at least 15 min (see Section III: Sterilization). This avoids contamination of the air with spores and infection of the worker. Organisms handled at Merlewood are rarely pathogenic, but it is not advisable to inhale heavy doses of any spores or to come into direct contact with living cultures of organisms en masse.

Autoclavable bags that can be supported in a metal bucket are a convenient means of autoclaving cultures in disposable plastic Petri dishes. Local procedures for their final disposal with other microbiological waste are detailed in the Station's safety documents (see Safety Guidance Notes 5.6 Safe disposal of microbiological waste). Dirty glassware encrusted with microbial remains can be soaked in a detergent, such as phosphate-free Decon 90 (Sterling Industrial), or a dilute solution of sodium hypochlorite, after autoclaving and before washing up.

The rule that cultures are never destroyed without consulting the owner and that discards are always labelled as such avoids the occurrence of serious and irreversible mistakes!

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[1] A pressure of 15 lbs in^{-2} used to be quoted, but as there is not an exact equivalent in SI units it is usual now to give the temperature only.

INOCULATION

Tools

Nichrome wire, a darning needle or steel-plated pin mounted in a metal handle with chuck[1], is used as an inoculating tool for transferring inocula to and from media, ideally long enough to avoid contact of the chuck with the culture. To inoculate fungi, nichrome wire can be flattened at the tip to a knife edge for cutting into agar, or the end may be straight or hooked for scraping the colony. For bacteria or liquid inocula, nichrome wire[2] bent into a loop (internal diameter 2-4 mm) or a pipette is used. A loop can also be used to make streak plates of fungi. Nichrome wire has the advantage that it cools rapidly after flame sterilization. Darning needles or steel-plated pins are more rigid and therefore easier to manipulate, but new ones should be heated before use to remove the metallic plating that may flake onto the culture. Fine insect pins[3] fused into a glass-rod handle or, if they have a head, inserted into a chuck handle are recommended for very fine manouevres such as the isolation of single fungal spores from agar media. Disposable, pre-sterilized tools, including loops, are marketed but are more expensive.

General procedure for transfer of inocula between tubes

The procedure for transferring a micro-organism from one tube of solid medium to another illustrates how contamination can be avoided. The inoculating needle, including any chuck, is dipped in alcohol, the alcohol flamed off and the tip heated to redness in a Bunsen burner. Omit the alcohol treatment if there is a danger of spattering potential pathogens. Care should be taken not to burn the inoculum; the heat of the needle can be tested by touching a clear area of agar before touching the organism. Two inoculating instruments may be used alternately, one cooling in a holder, while the other one is in use. During cooling, plugs or caps of tubes (or other containers) are removed, care being taken to touch only the tops, and the mouths of the tubes are flamed.

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[1] Chuck handles: length (20 cm) Fisons Scientific Eqipment; (15 cm) Philip Harris

[2] Nichrome wire or loops: (24 s.w.g.; 0.5 mm diam); Astell Scientific

[3] Fine pins: (38 x 0.40 mm); Asta stainless steel insect pins; Philip Harris.

To transfer fungi from one solid medium to another, a small fragment (the inoculum) is taken from the edge of the colony on the tip of the needle and inserted into the new medium. Sporulating mycelium is preferable, since purely vegetative sectors of a colony often persist indefinitely in producing only vegetative growth. Inocula must be placed in close contact with solid media to prevent desiccation.

To obtain an inoculum of bacteria and yeasts, it is sufficient to touch just the edge of a well-separated colony without scraping the surface; in this case, the smaller the inoculum carried over, the better is the result. This inoculum is then streaked on the surface of the agar (Fig. 2).

After transfer of the inocula, the mouths of the tubes are flamed again, the plugs or caps are replaced, and the needle is immediately flamed to redness to prevent any contamination of the laboratory with any spores or mycelim which may remain on the needle.

test tube slope culture
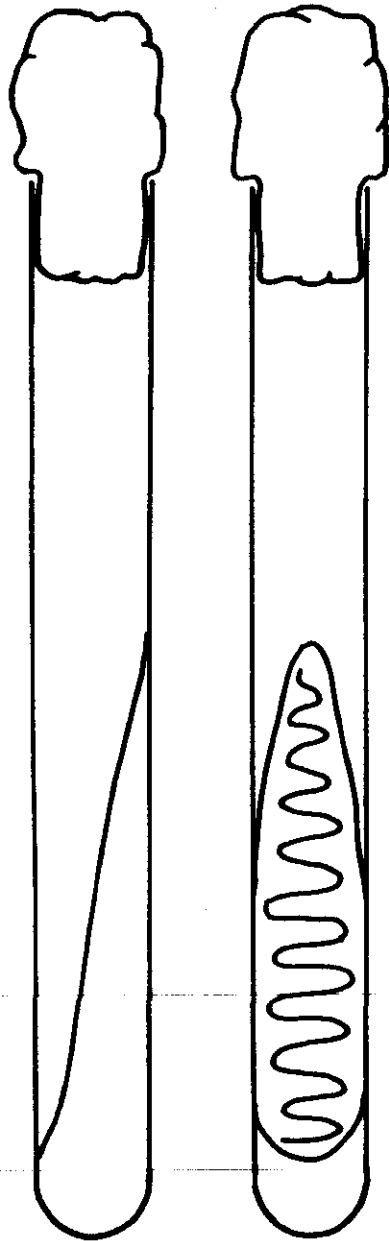
Petri dish culture

method (i)

start

finish

second plate
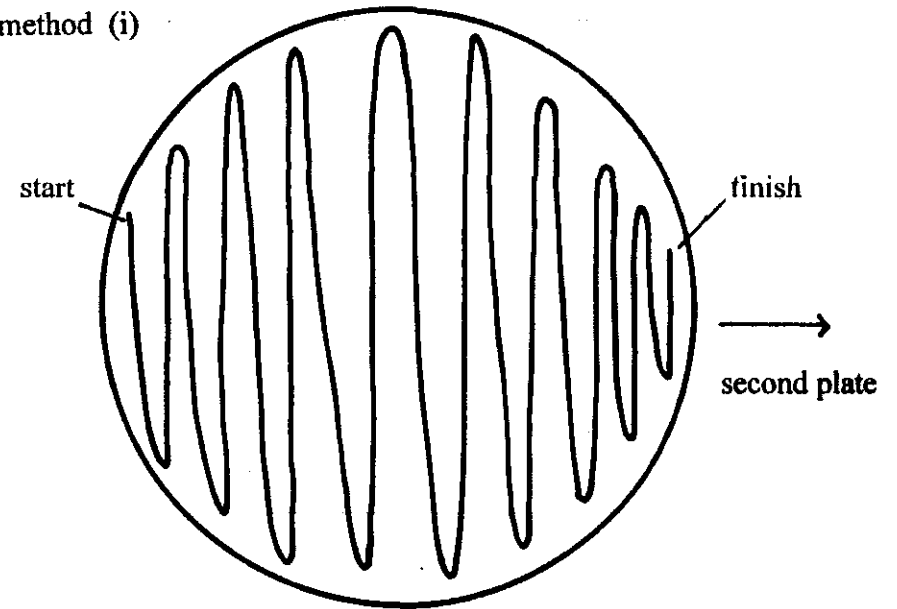
method (ii)

(numbers indicate sequence)

2

1

3

5

4
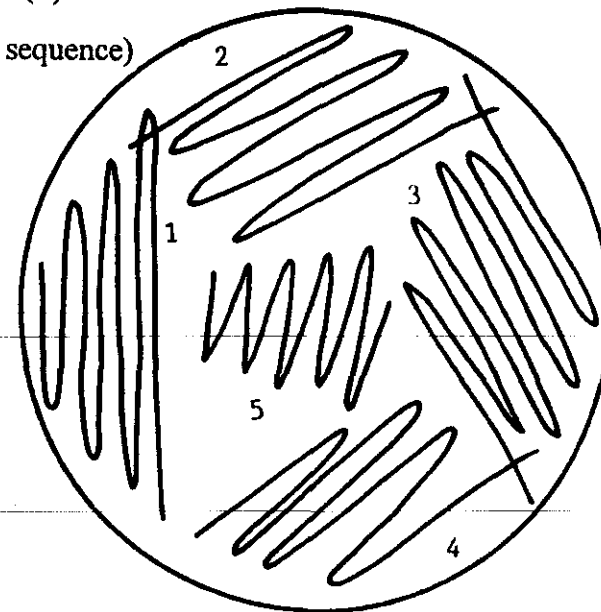
Figure 2. Methods of streaking micro-organisms on solid media

TYPES OF CULTURE

Plate cultures

Plate cultures are required for many morphological and experimental studies but dry up too quickly for long-term storage. A thin layer of molten agar or similar medium is poured into non-vented Petri dishes (Fig. 1), and is allowed to set before, or after, inoculation. The medium, usually 10-15 ml per dish, can be poured from individual tubes or phials, or in larger amounts from conical flasks. An automatic dispenser, e.g. an Agarmatic (New Brunswick Scientific (UK) Ltd) can be used for preparing large batches of dishes. If free water is a problem, allow the plates to dry for two days before inoculation. When growth is well established, desiccation of the agar can be delayed for 2-3 months by sealing the dishes with Parafilm (Northern Media).

Slope cultures

Slope cultures are usually prepared in plugged, rimless, Pyrex test tubes (150 x 16 mm) or screw-capped bottles or phials (see p.68). Test tubes are preferable for cultures under frequent investigation since the organisms can be examined for fruiting structures, mites, etc. under a microscope through the glass. In addition, rims increase the risk of contamination, make packing in baskets difficult, and crack frequently when flamed. Non-absorbent cotton wool is the most satisfactory material for plugging test tubes (see p.65). Foam plugs are preferred by some workers, but they soon lose their shape allowing entry of mites.

Agar medium is poured into the tubes (7-9 ml) or phials (10 ml), sterilized, and then allowed to set in a sloping position by supporting the plugged or capped ends of the containers on a clean rod about 1 cm in diameter. The 'slope' or 'slant' provides a relatively large agar surface in a narrow container for the inoculated organism. In the case of fungi, this enables the various stages of colony development to be observed. The sloped medium should extend up the tube to within about 2-3 cm of the plug and in a 28 ml phial to within about 2 cm of the screw cap (3 cm if a 'short slope' is required, see p.16). The caps of phials should be left slightly loose until cool.

Care should be taken to avoid medium or water on the cotton wool plugs; if wetting does occur, the tube should be discarded, otherwise the plug will shrink on drying, and any agar on it may be overgrown by the culture itself. After cooling, new agar slopes contain condensed water, so they should be handled and stored in the vertical position and allowed to dry to prevent the liquid flowing over the slopes and onto the plugs.

To inoculate a new tube slope with inoculum from another tube, hold the culture tube and the new slope between thumb and first finger, and between first finger and second finger respectively of the left hand, with the plugged ends inclined downwards. In general, the fungal inoculum should be taken from the apex of the colony unless the culture is drying out or appears to be unhealthy in that region. Fungal growth is usually more typical and the slope covered more quickly from a single 'spot' inoculation at the centre of the new slope than from a 'streak' inoculum. Care should be taken to prevent the inoculum touching the hot glass during these operations. The procedure for inoculating a phial slope is similar, but to avoid contamination the interior of the cap must face downwards while the phial is open.

Cultures should always be labelled individually (usually with the culture number, date of inoculation and culture medium) as soon as the inoculation has been completed. Tube labels should be attached just above the apex of the agar slope but below the level of the plug, so that the whole colony can be examined; labels nearer the mouth of the tube are liable to burn during flaming. Wax pencil labelling is satisfactory for temporary cultures if they are kept apart in racks.

Slope cultures are described as 'dry' or 'wet' according to whether the culture is left exposed to air in its container or is flooded with sterile oil or water.

'Dry' slopes are used if the organism has to be examined frequently, dry slope cultues in test tubes are more convenient than those in phials. They are easier to handle when the culture has to be renewed (i.e. subcultured or 'subbed'), contamination during transfers or microscope slide preparation is rare, and the colony can be examined through the glass with a x10 hand lens or under a low-power microscope. Phial cultures do, however, take

up less space and are more sturdy for sending by post.

Dry slope cultures should be packed loosely and stand upright in wire baskets or tube racks, in clean, dry and airy conditions, preferably in the light if they are fungal. Many fungi are known to grow better in daylight (see Smith and Onions 1983). Growth is often restricted if the slopes are stored horizontally, this could be an effect of the carbon dioxide gradient (see Onions *et al.* 1981, p. 273-274). Normal room temperatures are usually satisfactory, but for rapid development the cultures are often incubated at 25°C, the optimum temperature for growth of many mesophilic fungi. The optimum for some basidiomycetes is somewhat lower (21-22°C), and 25-30°C for most bacteria. Strict psychrophiles and thermophiles require respectively lower and higher temperatures.

Short plastic covers over wire baskets of dry slope cultures kept on an open bench are an effective means of protecting the plugs from dust without restricting circulation of air through the sides of the basket (Fig. 5). The covers and surroundings should be swabbed periodically with industrial methylated spirits.

'Wet' slopes, i.e. oiled slopes in phials, are particularly recommended for long-term storage of cultures, but one author (PML) has also had success with oiled and plugged tube cultures. Oil restricts desiccation without complete cessation of respiration. Routine subculturing is therefore reduced from intervals of 3-6 months to 1-2 years or many more, but inocula from oiled slopes always carry over some oil, so that a second transfer must be made to obtain growth of normal appearance.

The slopes are prepared and inoculated in a similar manner to dry tube slopes. If phials with aluminium screw caps ('Universal Containers'; Northern Media) are used, the rubber liners should be removed as they dissolve in oil, restrict aeration and can be toxic. Growth of fungi is better in these wide mouthed bottles than in the narrow mouthed type sometimes used by bacteriologists (see p.68). The phials are slanted rather more steeply (about 30° to the horizontal) than for a dry slope, the shorter slope ensuring complete cover of the sloped agar with oil.

Medicinal liquid paraffin of British Pharmacopoeia quality (specific gravity 0.83-0.89 g), obtainable from chemists is used for oiling. It should be sterilized twice by autoclaving, at 121°C for 15 min, in individual doses, since air, displaced by the oil, carries with it airborne spores, which can adhere to the mouth of the dispenser and contaminate the next culture. Some batches of oil are cloudy with water vapour after autoclaving; usually, this can be removed by incubation in an oven at 30°C for two or more days.

The sterile oil is added to the culture when good growth and, if possible, sporulation have occurred. The surface level of the oil should be about 1 cm above the tip of the slope, and further oil should be added, during storage, before the top of the culture is exposed, or desiccation will occur very rapidly, mycelial strands sometimes acting as wicks. Onions (1971) states that the depth of 1 cm is fairly critical, because the oxygen transmission by layers of mineral oil in excess of 1 cm becomes less favourable.

The oiled phial cultures should be stored at room temperature in clean conditions at low atmospheric humidity. Baird & Tatlock's wooden drawer units used at Merlewood have been particularly satisfactory as containers; they keep out dust, but allow air to enter, and the wood absorbs water vapour given off by the cultures. Lower storage temperatures are not always satisfactory; at 4°C some fungi are killed, and at 8°C contamination may occur from growth of fungi in condensation on the bottles and under the caps (Anon. 1960), but 7°C has been found to be a suitable temperature for bacterial cultures.

Oiled cultures should always be prepared in duplicate, because they are more susceptible to contamination during preparation than dry tube cultures. The lids can be numbered, but labels with full details should be stuck at the base of the bottle, on the reverse side of the slope so that the colony is not obscured.

Sterile water may be used instead of oil as described by Boeswinkel (1976); Ellis (1979); Smith & Onions (1994) and Jones et al. (1991). This simple and inexpensive method of preserving cultures has not, however, stood the test of time to the same extent, and the cultures have to be subcultured more frequently.

Deep frozen slope cultures (Carmichael 1962; Smith & Onions 1983) may also be stored in a deep freeze (-17 to 24°C) for 4-5 yr. The cultures should not be thawed and refrozen, so they should be subcultured in the frozen state, using a stronger implement than a needle, otherwise a fresh slope culture must be prepared. This method has not been successful with basidiomycetes at Merlewood.

More elaborate and expensive methods of preserving agar cultures such as freeze drying and use of liquid nitrogen are only appropriate for large culture collections (see Smith & Onions 1983).

Liquid cultures

Liquid cultures can be set up in conical flasks, medical flats (medicine bottles), or similar containers. A thin layer of liquid medium is placed in each container; the depth should not exceed 1 cm for aerobic organisms. After sterilization, the medium is inoculated with a small piece of culture, or with a suspension of bacteria, fungal spores or homogenized mycelium. Medical flats are incubated in a horizontal position to increase the area of growth. More uniform growth of cultures throughout the liquid is often obtained by use of shake cultures. This is a means of improving aeration of liquid cultures and, with many fungi, results in growth in the form of pellets.

Damp chambers

This method (Keyworth 1951) is used only for fungi. The increased humidity of a damp chamber culture induces many species to fruit on plant material, so that they can be identified *in situ* or isolated more readily. Many fungi which never grow on agar media can be obtained by this technique.

The base of a glass Petri dish is covered with a standard filter paper (9 cm diameter) and the inner rim lined with a strip of Carlson Ford filter paper (type XE5; 100% cellulose; Carlson Filtration Ltd). this thick and highly absorbent paper obtainable in sheets retains water for several weeks at room temperature, so that frequent watering of the chambers is unnecessary. A complete base lining of Carlson Ford paper, however, would make the substratum too sodden, reduce fungal growth and encourage bacteria. The chamber is autoclaved dry in tins or in thick newspaper to absorb moisture, and the fresh material to be examined, such as a leaf or root, is then placed in the centre, taking the usual precautions to prevent entry of contaminants which might grow on the filter paper. The chambers are watered from a sterile dispenser by wetting the paper rim, so that water diffuses inwards to the centre of the dish. Free water should not come into contact with the specimen. Incubation is usually carried out at room temperatures in the light. Examinations should be made within 2 days, but the plates can be kept for months to allow fruit bodies of basidiomycetes to develop.

CLEANING TECHNIQUES

Fungi

Repeated transfers of an inoculum are made from the edge of a colony to fresh medium, usually in a Petri dish, until all the contaminant has been left behind. Use of tap water agar, addition of an antibiotic and/or inoculation onto the base of the Petri dish before pouring the agar is often helpful. The method is successful only if the organisms grow out at different rates from the inoculum. Broad spectrum antibiotics include the fungicides novobiocin (10 mg l^{-1}; sodium salt, BDH) and benomyl (5 ppm; Benlate; Boots) both of which can be autoclaved. To obtain 5 ppm of benomyl, dissolve 0.1 g Benlate in 100 ml sterile distilled water and add 5 ml of this solution to 1 litre of medium. Detailed instructions for use of the bacterial inhibitors penicillin, streptomycin and crystomycin (a mixture of antibiotics; Glaxo) in culture media are given by Onions *et al.* (1981, p.376). However, use of antibiotics all of which can damage the organism physiologically or genetically should be avoided whenever possible. If the fungus is sporing, a streaked inoculum may separate out single spores from which clean colonies will develop.

Hyphal tip isolation. A single, clean hyphal tip in a Petri dish colony is located under the microscope. The objective is then replaced by a sterilized dummy, consisting of a metal tube with a cutting edge, which is lowered to cut out the hyphal tip on a block of agar. The block is then removed with a needle to fresh sterile medium.

Raper's (1937) ring method (see Onions *et al.* 1981) for fast-growing fungi contaminated with bacteria is advocated by several text books, but we have rarely been successful with this technique.

The dilution method for single spore cultures is described by Onions *et al.* (1981).

Bacteria

The aim is to disperse the organisms as much as possible and to obtain single colonies.

Pour plate method. Separated colonies are produced when using the soil dilution plate method, but a quicker procedure is often satisfactory. For this an inoculum from a culture, or a loopful of bacterial suspension, is transferred to a tube of molten agar medium, this tube is shaken by rubbing it between the palms of the hand, the loop re-sterilized, and a loopful of the inoculated medium taken to another tube of agar, which is also shaken. The agar from both tubes is then poured into Petri dishes. Well-separated colonies should result in the second dish at least.

Streak-plate method. An inoculum from a culture or a suspension is streaked successively on the surface of one or two plates of well-dried, solidified sterile agar, using an inoculating loop. There are various ways in which this can be done, as shown in Fig. 2. In method (i) the loop is flamed between each plate, the inoculum then being taken from the end of the streak on the previous plate. In method (ii) the loop should be flamed between each streak, unless contamination is very slight.

MAINTENANCE OF CULTURES, INCLUDING TYPE AND VIRILITY

The aim should always be to preserve the species in a culture collection true to type, with the same characters (when transferred to a particular medium) as when they were first isolated and described. General storage conditions for 'dry' and 'wet' slopes have been given on p.15-16.

The frequency at which subculturing should be carried out depends on the particular organism and on the storage conditions, particularly temperature. Cultures should be transferred before the culture has 'staled' and the agar has shrunk far from the glass, and never left to dry out completely. Some fungi that do not spore freely will not survive for six months, even in a refrigerator, without subculturing. This may be due to 'staling' of the culture by toxic waste products. Spores of some fungi remain viable for years, while others, e.g. certain common Mucorales, have a viability of less than six months. In general, 'dry' slope cultures of soil fungi kept at 18-20°C should be subcultured within 4-6 months and 'wet' slopes within two years. Although the period between subculturing of cultures can be extended by storage in a cold room at 4-7°C or even in a refrigerator, increased humidity and condensation increases the incidence of fungal and bacterial contamination and mites.

Useful lists showing the longevity of certain genera under various conditions of storage are given by Smith & Onions (1994).

Media

Cultures for identification are often grown on relatively rich media, e.g. potato dextrose agar for most fungi, and Czapek-Dox agar for penicillia. These produce a particularly characteristic type of growth and colouring. Comparison of the gross morphology of large numbers of cultures is carried out most easily on such a medium, although weaker media may be necessary for development of particular features, e.g. fruit bodies of fungi. For long-term storage of fungi, a natural and less sugary medium should be used such as weak potato carrot agar, but a change of diet should be given from time to time to preserve full vigour. On sugary media, many bacteria and fungi gradually decline and cease to spore, although there are exceptions requiring high sugar concentrations for survival. Potato

carrot agar is a good 'starvation' medium for inducing sporulation of many fungi, but wood decomposers and dematiaceous fungi may spore readily on cornmeal or oatmeal agar. Most soil bacteria grow well and store satisfactorily on tryptone-soya agar.

Light

If a change of medium fails, an increase of light will sometimes reverse a decline of fungi. Many species are stimulated by light; some require regular exposure to diffuse daylight, others require bright sunlight.

Temperature, Aeration and Humidity

Micro-organisms are limited to particular ranges of temperature, outside which they will not grow; these vary with both different species and strains. Optimum growth is obtained within a more narrow range (see p....). Incubators are used to maintain uniform conditions, at the required temperature during growth, or for examining growth at different temperatures.

Oxygen is required by all organisms and is normally obtained from the air. Aerobic cultures are sufficiently well aerated in Petri dishes and in containers with cotton wool plugs (if they are not too tight). Anaerobes are inhibited by gaseous oxygen and obtain it in a combined form. Anaerobic incubators are available for growth in various gas mixtures; for small numbers of cultures, anaerobic jars can be used.

The moisture content of laboratory air is normally satisfactory for cultures, but it can be increased by placing a dish of water in an incubator, or by using a controlled-humidity oven. Booth (1971, p. 43) discusses the use of graded solutions of salts, glycerol or sucrose to achieve specific humidities in incubators.

Genetic change

Some organisms undergo genetic changes and a sector of the colony develops with several characters differing from the original. Abnormal conditions are often responsible. The

new form should be separated before it swamps the 'parent'. Despite careful culturing, however, some species decline after long periods in culture. Fungi, eg *Chaetomium* and *Fusarium*, often produce large quantities of floccose mycelium, differing in colour from the original; other fungi may become entirely conidial losing all vegetative mycelium. In any collection, loss of virility of a small number of cultures is inevitable without detailed knowledge of the nutritional requirements.

GENERAL TECHNIQUES FOR INDUCING SPORULATION

Fungi

Change of medium. Sporulation will often occur if a fungus is transferred to a weak, shallow, 'starvation' medium, e.g. potato extract, potato-carrot or tap water agar, on which vegetative growth is reduced. *Mortierella* species will generally spore prolifically when transferred from agar to tap water. A small portion of the agar culture with well developed aerial mycelium is floated on sterile water in a watch glass until sporing mycelium has grown over the surface of the water. Good results have not been obtained with Merlewood tap water, which is chlorinated, therefore water from other sources should be used. *Alternaria* species often spore on the thin layer of agar obtained when a slope is rotated rapidly just before setting.

Light. Most leaf-and stem-fungi require the stimulation of light for sporulation, and short exposure to sunlight or near ultra-violet rays (300-380 nm) often stimulates production of spores or fruit bodies of other fungi, although heavier doses may kill mycelium and spores. Several fungi, e.g. *Trichoderma*, will often spore in a few days if they are placed on a sunlit bench. If this fails, the fungus (3-4 days old) should be exposed for 2 weeks or more to light of short wavelength in a 'black light' (near UV; wavelength 300-380 nm) apparatus (Fig. 3; see also Smith & Onions 1994; Smith 1991). Plate cultures should develop in an incubator before exposure to alternating 12 h periods of UV light and darkness. Although disposable plastic Petri dishes are recommended by Smith (1991, p. 158), we have had more success with glass plates in which desiccation is less of a problem. Weak media are recommended for these irradiated cultures. Dematiaceous hyphomycetes, Coelomycetes and ascomycetes are particularly responsive to this treatment.
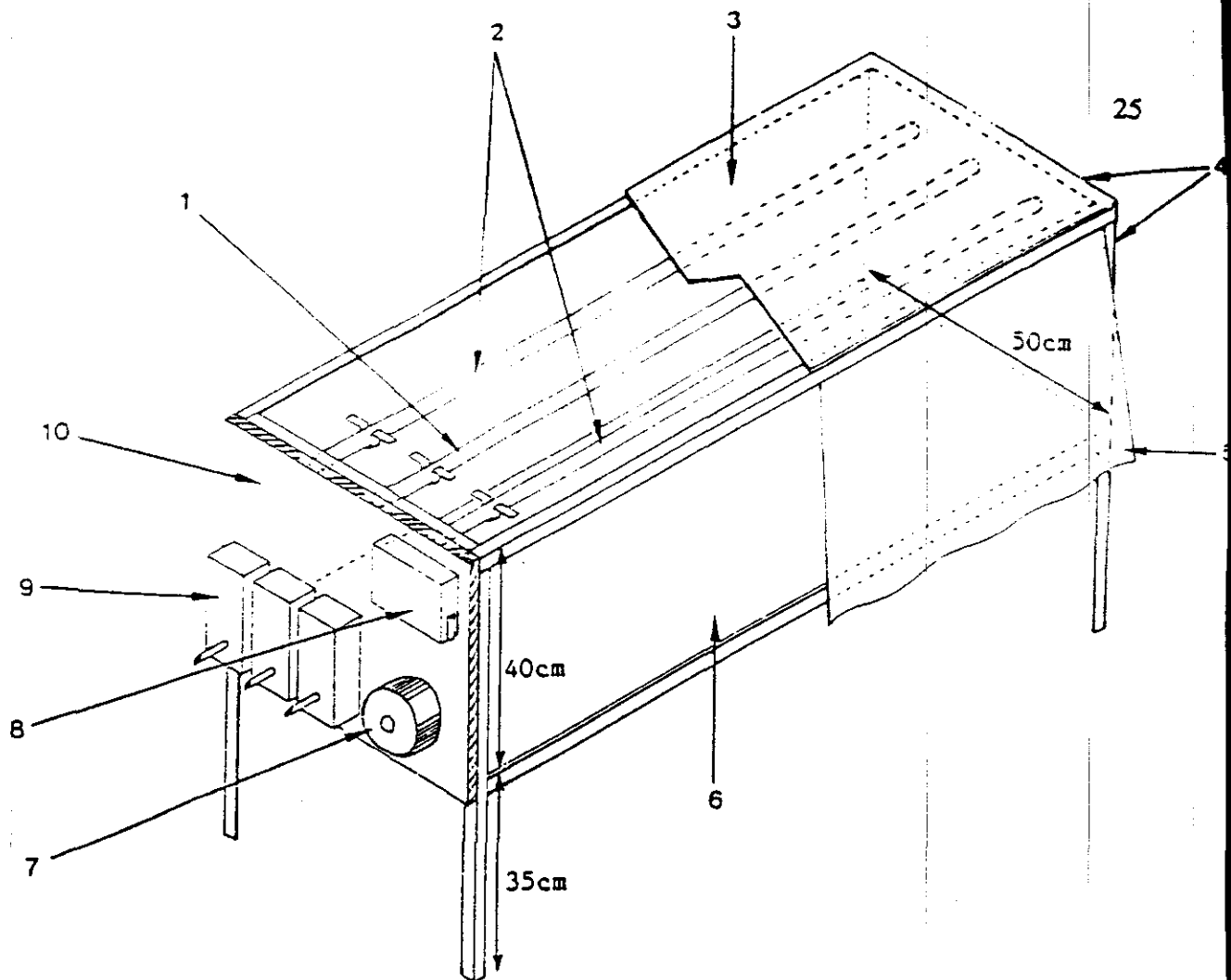
Figure 3.   'Black light' apparatus

Model used at Merlewood from specifications, pers. comm., former Commonwealth
Mycological Institute, Kew.

1.   One 120cm 'black light' (Phillips TL/40W/08)

2.   Two 120cm 'natural', cool-white fluorescent tubes (Phillips TL/40W/08)
       The tubes are supported by Terry clips 12.5cm apart and 35cm above the platform

3.   Hardboard roof under which lights are mounted

4.   Dexion frame 75cm high

5.   Curtain

6.   Hardboard platform for cultures

7.   24h dial, synchronous time-switch to give 12h dark / 12h light cycle

8.   Main switch

9.   Choke and starters (one per tube)

10.   19 mm plywood board to hold control gear

Change of temperature and treatment with red-heat. Sudden changes of temperature, e.g. from cold-room temperatures to 25°C in an incubator, will sometimes induce sporulation, but the method is not usually as successful as a change of medium or lighting. Stroking a culture with a red-hot needle, often has, however, spectacular results. This simple technique is particularly successful with some *Trichoderma* species which produce their green conidial heads along the singed furrow.

Sporulation on slide cultures. When identifying fungi, it is often essential to see how the spores are borne. The spores of many fungi are all too readily detached from the mycelium when transferred from a culture to a liquid mount. Various methods have therefore been devised to induce fungi to spore on a microscope preparation, so that they can be examined *in situ* under a microscope without disturbance. The slide culture technique (Riddell 1950) is one of the most useful of these.

Agar medium is poured into a Petri dish to a depth of about 2 mm (14 ml per dish). Graph paper is placed under the dish, and a 1 cm square of the set agar is cut out with a sterile scalpel and transferred to the centre of a slide, previously dipped in alcohol, flamed and cooled. The slide is then placed on a tile wiped with alcohol and protected by the lid of a Petri dish. Each vertical side of the block is inoculated centrally with a minimum amount of the fungus, and then a large No. 0 sterile cover slip (22 x 26 mm) is placed over it. The cultures are supported on two glass rods in a Petri dish lined with filter paper, soaked in a 20% solution of glycerine; only the dish itself need be sterile. The glycerine keeps the agar moist but not too wet as would water, and the culture dishes do not usually require rewetting if kept at room temperature for 7-14 days. Meanwhile, the slide cultures can be lifted out for examination under a microscope. Alternatively, the squares can be cut from an agar plate already inoculated with the fungus and mounted as before, Many fungi will grow out over the slide and cover slip and sporulate freely. At an appropriate stage of growth, two preparations are made, one from the slide and one from the cover slip. The cover slip is carefully removed from the agar block and a drop of 95% alcohol added to wet and kill the fungus growing over it. When most of the alcohol has evaporated, the mountant is added (lacto-glycerol or lactophenol with or without a fungal stain: see p.34) and the cover slip is lowered on to a slide. The agar

block is then carefully removed from the slide and discarded so that a slide preparation of the fungus can be made in a similar manner to that on the cover slip. The preparations are sealed as described on p.33) but care should be taken not to cover the fungus, which will be attached close to the edge of the mount.

A simpler alternative method has been tried with reasonable success. Two cover slips are placed in a Petri dish before sterilization. Agar is carefully poured into the dish, so that the cover slips remain in place on the base of the dish. Before inoculation, a disk of agar is removed from the area above the cover slips, using a flamed and cooled cork borer slightly smaller than the diameter of the cover slip. The plates are inoculated and the area of the cover slips observed until a thin film of growth occurs with good development of sporing structures. The cover slips are then removed, quickly flamed on the top surface to remove condensation and mounted as usual. The use of two cover slips allows for breakage when mounting. A recommended variation of this method, in which the cover slip is inserted under the set agar is described by Onions et al. (1981, p.303).

Bacteria

Sporulation of bacteria, i.e. species of Bacillus or Clostridium, is enhanced in media containing trace elements. Soil extract, which also supplies trace elements, has a similar effect. The inclusion of sugar in a medium inhibits sporulation.

MITES

All fungal cultures should be protected from mites (Fig. 4). A laboratory may escape an invasion for years, and then, almost overnight, a culture collection can be lost. Simple preventive measures, described below, are therefore well worthwhile. Most fungal-feeding mites are barely visible to the naked eye and are not usually noticed until damage has been done. Lids of Petri dishes, even when sealed with tape, and cotton wool plugs do not keep them out, although plugs are better than the various metal/plastic closures on the market. Paraffin-waxed plugs were once in vogue, but mites can tunnel through the wax. They crawl from one culture to another carrying contaminants, particularly spores of *Cephalosporium* which can be almost impossible to eliminate from other fungi. New laboratories with undried-out plaster, and cupboards and incubators, in which a relatively high humidity can build up, are particularly vulnerable. In a mass outbreak, a whole building can be infected from a single source in about a week, and mites can be seen with a hand lens even on 'clean' glassware and polished benches. They gather on finger prints, which can form a nutrient supply for the fungi brought by the mites, each print supporting a minute, grazed colony.
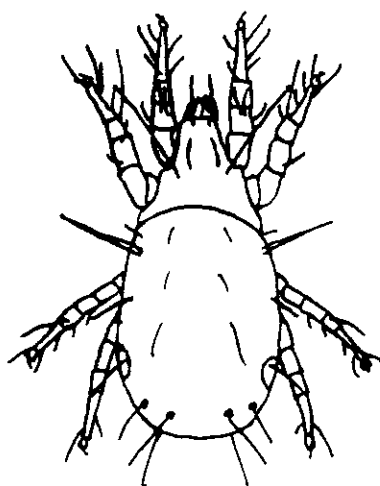
Figure 4. A typical mite : actual size 0.5mm

Their not uncommon occurrence in cameras may be due to the presence of fungi on oil accumulating on viewfinders from contact with eyelashes (New Scientist, Letters, 14 December 1991, p.57). Many modern university laboratories are contaminated, probably because of the difficulty of separating different types of project.

Prevention

i. Constant vigilance.

ii. General cleanliness. Mites and their eggs are carried on hands, shoes, clothing, soil and flies. They are also blown about in dust. It is difficult to exclude all sources of infection, but books, files and packing materials should be kept off the culture bench. Cupboards and benches should be wiped down regularly, giving particular attention to cracks and crevices.

iii. Strict exclusion of all plants and organic matter (moss, litter, wood shavings, house plants, toadstools, soil, chocolate, cheese, etc) from a laboratory where cultures are kept. Deep enamel or plastic trays should be used and washed immediately after use if examination of such materials is necessary in nearby rooms. Mite outbreaks spread readily even from one end of a building to another.

iv. Storage of cultures in dry, airy conditions at room temperature. It is better to keep 'dry' slope cultures on a laboratory bench or free-standing stack than shut away in a cupboard. Mites move and breed slowly at low temperatures, but dampness is a greater risk than high temperatures. Duplicate sets of cultures kept in different locations are an extra safeguard.

v. Vaseline traps. Movement of mites between Petri dishes and culture baskets is impeded by smearing tiles and shallow trays with Vaseline (Fig. 5). The baskets can be stood on corks above these trays. 'Air attack' and transfer of mites from

hands and clothing are, however, still possible.

vi.   Use of repellants. Yellow, unrefined tractor vaporizing oil (TVO), which contains

an impurity repellent to mites without ill effects on fungi, was once widely used to

protect cultures, but because of supply difficulties and a possible health hazard it is

now replaced at Merlewood by Vaseline as described above. Dried herbarium

specimens can be protected with a few crystals of paradichlorobenzene or

naphthalene, but these repellants can cause sterility and mutations in living

cultures. Again, these chemicals must be treated as health hazards.
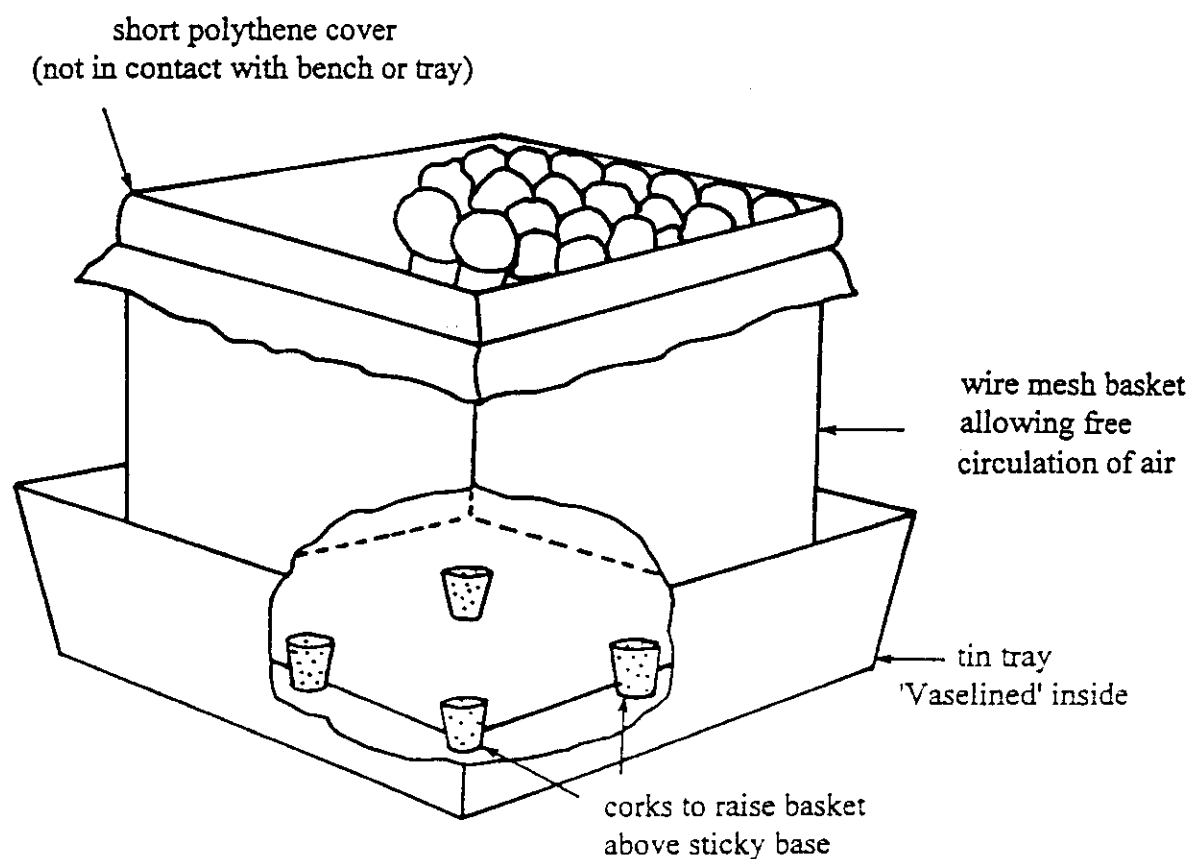
Figure 5.   Method of protecting tube cultures from dust and mites

vii. Separation of culture baskets from one another, particularly those containing penicillia, the odour of which appears to be especially attractive to mites, will slow down an invasion.

viii. Quarantining of suspect cultures is very important. Cultures received from other laboratories should be kept apart from the main collection for some weeks, and new isolates taken directly from fresh plant and fungal material should not be mixed with other cultures until it is certain that animals and eggs have not been transferred with the inoculum.

Signs of attack

i. A characteristic earthy smell, if many cultures are infected.

ii. Mouldy plugs or labels.

iii. Cultures that appear to have been grazed, or which are growing over the glass and plug from spores carried by the mites. The culture may just look 'untidy' with churned up agar.

iv. Minute colonies growing in lines. This often indicates that spores have been dropped in the 'foot-steps' of an animal.

v. Fungal contaminants in the culture. The cause of these should always be investigated.

vi. Mites with their faecal pellets and eggs often congregate at the base of the culture tube between the agar and the glass. The adults and eggs are usually visible with a x10 hand lens.

Remedies

All cultures should be examined through the glass under a low power microscope. Clean fungi should be separated, and infected cultures destroyed immediately by autoclaving, or quarantined and subcultured repeatedly. Re-examination over a long period is necessary to allow for hatching of eggs. Cupboards should not be re-used, because further outbreaks from eggs in crevices sometimes occur over a period of many years. Infected cultures can be fumigated in an air-tight box or tin with paradichlorobenzene crystals, but there will be a risk of adverse effects on the fungi. Various acaricides with relatively low toxicity to fungi have also been recommended by the International Mycological Institute, including Kelthane (Murphy Chemical Co,) and Acteltic (ICI Agrochemicals). These are used to wash down cupboards and benches when an outbreak of mites has occurred. Drops of Kelthane may also be added to the base of the plug if attempts are to be made to save an infected culture. Safety precautions must be taken when using these hazardous chemicals. Refrigeration of equipment (not cultures!) for 24 hours can also be effective in killing mites.

When mite control is a continuing problem, the method of protecting slope cultures by sealing the tube above the plug with cigarette paper (sold in packets for rolling cigarettes) using copper sulphate-gelatin glue is simple and very effective (Snyder and Hansen, 1946; Smith & Onions, 1994).

MICROSCOPE SLIDE MOUNTS

However carefully living cultures are maintained, some will deteriorate, cease to sporulate or die. Slide mounts are therefore usually prepared as soon as possible after isolation.

Slides and cover slips for mounts should be spotlessley clean and grease-free. Some slides can be conveniently stored in a jar of alcohol to remove grease and wiped on a clean linen cloth just before use.

Fungi

Methods of observing fungi in the living state have been reviewed by Booth (1971a) and Frankland *et al.* (1990). Booth includes slide chambers for time-lapse photography.

For non-living preparations, lactophenol (Onions *et al.* 1981, p. 312) has been the standard mycological mountant for many years and measurements in taxonomic works used to assume use of this medium or water. Lactophenol is also a good clearing agent, but lacto-glycerol (lactic acid, 25 ml; glycerol, 50 ml; distilled water, 25 ml) is now used at Merlewood whenever possible to avoid the use of toxic phenol. These liquid mountants do not harden and must be sealed to hold the cover slip in place. Two coats of Glyceel (British Drug Houses Ltd) or of clear, unpigmented nail varnish (thinned if necessary with a little acetone), neither of which reacts with lactophenol, are used as sealants, using a ringing table for round cover slips. Many seals for lactophenol mounts react eventually (perhaps after many years) with the mountant, so that the specimen is completely obscured by a mass of crystals.

Glyceel and the varnish have been recommended by the International Mycological Institute because they (particularly the latter) have stood the test of time, but futher coats are required for long storage periods. Glyceel is the most convenient sealant for general use, see also Onions *et al.* (1981, p. 317). Euparal (GBI (Labs) Ltd) is recommended as a permanent mountant for Jones and Mollison agar films and similar bulky mounts.

Mounted specimens can be examined unstained (by either bright field or phase contrast microscopy) or stained. Water-soluble aniline blue (= cotton blue or soluble blue) in phenol is a general purpose stain for fungi (5% aqueous phenol, 15 ml; 6% aqueous (W.S.) aniline blue, 1 ml; glacial acetic acid, 4 ml or 0.05 g of stain in 100 ml lactophenol). It is particularly recommended for soil and litter preparations such as Jones and Mollison agar films in which the hyphae need to be distinguised against a background of organic and mineral material. Filter 1 h after preparation, stain for 1 h, rinse in water and/or dehydrate in 95% alcohol (Parkinson *et al.* 1971, p. 21 and 62). **N.B.** The staining is a slow process and may continue during storage, sometimes leading to overstaining.

Trypan blue (0.05% in 100 ml lactophenol; Onions *et al.* 1981, p. 316) results in more even staining of the walls and contents of well-teased out fungal specimens than aniline blue/cotton blue, but according to Dade quoted by Gurr (1965, p. 240) it may fade after 2-3 years. Some authors recommend a more concentrated stain (0.1-0.5% trypan blue in 45% acetic acid or lactophenol), but this can lead to over-staining. If the background is too heavily stained, the stain can be drawn off and the specimen remounted in lactophenol. See Gurr (1965), Watling (1981) and Frankland *et al.* (1990) for further recommendations.

Bacteria

Living bacteria can be observed using phase-contrast microscopy. To observe motility, use young, 24h cultures. Place an inoculating loop into a liquid culture or condensation water at the bottom of a slope culture, and touch the centre of a 16 mm square cover slip to produce a small drop of the culture. Then add a small drop of water to each corner of the cover slip and invert it over a cavity slide. The cover slip will adhere to the slide and the hanging drop should be suspended over the centre of the well. A ring of Vaseline on a flat slide may be used to form a similar chamber. If properly prepared and sealed, the drop is protected from evaporation and air currents, thus avoiding any external influence on the movements or drifting of the organisms within the drop. Reduce the microscope illumination as much as possible to avoid loss of motility from the effect of heat.

For permanent mounts a smear preparation is made on a slide which should be grease-free

(dipping in alcohol and flaming will remove light grease deposits, but wiping with a clean linen cloth can be even more effective). Pass the microscope slide through a Bunsen flame three times. Place a drop of distilled water on the slide, and take a small inoculum from the culture to mix with the drop of water. Spread the drop in a thin even film over the slide and allow to dry in air (a smear easily seen with the naked eye is too thick!). Again pass the slide, film side up, rapidly through a Bunsen flame to 'fix' the smear. Just enough heat should be applied, so that the slide can be tolerated on the back of the hand.

After cooling, the slide can be stained with Gram stain, or other stains as appropriate. The recipe for the Gram stain varies and should be checked with the worker concerned. The preparations can be observed directly under oil immersion or mounted in Euparal.

See general text books for other mountants, seals or stains used in bacteriology.

DRIED HERBARIUM CULTURES OF FUNGI

Dried cultures of ascomycetes and Sphaeropsidales with fruit bodies are useful for reference, because the spores can be re-examined after the culture has been soaked and teased out. The following method (Anon. 1960) is recommended.

Tap water agar (1.5% is poured onto the smooth side of 4.5" (11.4 cm) squares of hardboard. The culture to be dried down is removed from the Petri dish, test tube or McCartney phial and placed on the melted agar; slope cultures must be flattened first by slicing off some agar. Old, dry tube cultures can be loosened by heating a little water in the bottom of the tube. The cultures are protected and allowed to dry. After 2-5 days they are ready to loosen with a razor blade, peel off and trim. They are stored in envelopes but must be protected from mites.

Procedures for drying, rehydration and examination of the fruit bodies of macrofungi are described by Largent et al. (1977) and Watling (1981). To obtain cultures from dried herbarium caps and gills, place a portion in a cavity slide suspended in a damp chamber. Leave overnight at 24°C in darkness, then plate a suspension on potato dextrose agar containing novobiocin (see p.20).

Freeze-drying of fungal cultures is discussed in detail by Smith and Onions (1983). Freeze-dried (-60°C) mycelium has been prepared at Merlewood for immunological research using an Edwards Super Modulyo freeze dryer (see Microbiological Procedures) on deep frozen material. The bottled samples can be stored in polythene bags with silica gel, in a cold room, until needed.

II. CULTURE MEDIA

Culture media are used as a nutritive source, on which to isolate and grow micro-organisms under artificial and controlled conditions, after their removal from a natural habitat.

A good medium should:

* simulate the natural environment;

* permit satisfactory growth;

* be reproducible; for experimental purposes, the exact composition must be accurately known.

In practice these three points cannot be completely satisfied in any one medium and a compromise is made according to the purpose of the experiment. It is, for instance, virtually impossible to reproduce exactly the natural conditions under which micro-organisms flourish. Also, to obtain satisfactory growth and readily recognised characters a far richer source of pure nutrients is normally used. However, micro-organisms show a considerable degree of adaptability and suitable artificial media can generally be found. It seems highly unlikely that any one medium, containing all the necessary substances, will be found for the culturing of all organisms, so many different media are used, each more or less specific for a particular purpose or organism. Thus, general media are used to culture as many organisms as possible, and selective media to culture only one type or group of organisms.

The basic needs of all organisms include water, 'food' (nutrients), and a suitable physical environment. Nutrient substances necessary for growth and reproduction include carbon (C), nitrogen (N), hydrogen (H), oxygen (O), sulphur (S), phosphorus (P), calcium (Ca), sodium (Na) and various metal salts and vitamins in low concentrations. The proportions of these elements needed by different organisms, or by the same organism, for different physiological processes are not the same. Also, some organisms obtain compounds of

carbon or nitrogen, or the latter in its elemental form, from the air, and for these microbes either carbon or nitrogen is excluded from the medium. Others require carbon or nitrogen in various forms, from simple inorganic to complex organic compounds. Autotrophic organisms are those which grow in the absence of organic carbon, while heterotrophs require an external supply.

The organic and inorganic constituents of media are mixed in the proportions believed to satisfy these various requirements.

Media are usually made up as liquids or as a solid gel with agar or gelatin; the solid condition aids manipulation and observation of the cultures. Solid media, found to be successful for bulk growth of mycorrhizal fungi, can also be prepared by mixing Perlite or Vermiculite (inert, volcanic substances with high water-holding capacity; obtainable from garden shops) with peat and adding a nutrient solution. The ratio of the components can be varied to give the required pH and moisture content. Semi-solid media are sometimes used e.g. for anaerobes.

THE CONSTITUENTS OF CULTURE MEDIA

Water

Tap water contains many accessory compounds and varies in composition from one locality to another, and it is often chlorinated to inhibit growth of harmful bacteria with the result that media made with it would be subject to variation in constitution and might be bacteriostatic. For most scientific purposes, tap-water is therefore purified by distillation or de-ionization processes. Water treated by one of these methods should be used in preference to untreated tap water, unless otherwise specified; tap water is used in some cases to provide trace elements in culture media.

Nutrients

Organic carbon and nitrogen are supplied as fresh, or treated, natural products. Extracts of potatoes, carrots, soil etc., make good, easily prepared media. They are usually heated to extract the nutrients and may be supplemented with other constituents.

Sugars and other carbohydrate constituents are added as sources of organic carbon, or in order to test the ability of an organism to degrade them. Common sugars used are glucose (dextrose) and sucrose. Starch, cellulose, pectin, and glycerol are polysaccharides frequently used.

Meat extracts, e.g. Lablemco, supply nitrogen and other nutrients. They stimulate activity and accelerate growth of micro-organisms, but have not proved particularly useful in soil microbiology.

Peptones are meat digests and their most important function in culture media is to provide an available source of nitrogen. Forms of peptone used include tryptone and casein hydrolysate.

Yeast extract is an excellent stimulator of growth. It is a complete food and also a rich source of the B vitamins and is used to supply these factors in culture media.

Inorganic nutrients are supplied as mineral salts, particularly the chlorides, phosphates, and sulphates amongst the acid radicals, and calcium, potassium and sodium amongst the bases. Nitrogen is supplied in the inorganic form as nitrates, nitrites, or as ammonium salts. Carbonates are sometimes added to check development of acidity.

Trace elements, vitamins and other growth factors, are also needed for the successful cultivation of micro-organisms. The failure of an organism to grow on a certain medium can be due to the absence of one or more of these essential growth substances. They may be added as trace element or vitamin mixtures, but in most complex media sufficient quantities are present as contaminants of the other chemicals used, particularly those of natural origin. Soil extract solution fulfills similar requirements.

Constituents to adjust the physical state of media

pH of culture media is important because organisms live only between certain limits of acidity and alkalinity. To adjust the pH to the required level, dilute solutions of potassium hydroxide or hydrochloric acid are added, (see Microbiological Procedures). The pH of all media should be known. In general, bacteria prefer neutral, and fungi an acid pH.

Buffers are substances which increase a solution's resistance to pH changes. Organic compounds or phosphates may be added as buffers, but in complex media the ingredients used provide sufficient buffering capacity.

Osmotic pressure of a medium must be in equilibrium with the cell contents of the organism, or it will burst owing to unequal pressures. Sodium chloride is often added to adjust the osmotic pressure to the correct level. Standard recipes for media should provide suitable osmotic pressure so that adjustment is not needed.

Indicators of physical or biochemical state

Acid/alkaline indicators, and other chemicals, that change colour under particular conditions are used in media to demonstrate biochemical changes, or to indicate a physical state, eg, anaerobiosis. The materials used are normally non-nutritive.

Solidifying agents

Agar is used to solidify media. It is a substance extracted from certain seaweeds. Although of a carbohydrate nature, it is attacked by only a very few organisms and is regarded as an inert constituent of media. It is a hydrophilic colloid with unusual melting and setting properties. It dissolves in water and becomes liquid at 98°C, and then remains liquid until cooled to approximately 40-45°C, when it solidifies. The modern refined agars give satisfactory gels at a concentration of 1.2 - 1.5% and the medium is sufficiently clear for most purposes to make filtration unnecessary. Semi-solid media can be prepared with agar down to a concentration of 0.05%.

The dried powder (more readily-handled granules are also available from Merck) dissolves in water when heated at 100°C for 1 hour. Agar media should not be adjusted to a pH lower than 5.8 prior to sterilization, or the agar will be hydrolyzed and will not set on cooling. For a lower pH value the agar can be adjusted after sterilization by adding sterile acid, or a higher concentration of agar can be used. Agar media must be cooled to 45-50°C, before adding micro-organisms or heat-sensitive constituents. The concentration of agar used in solid media results in a correct moisture content. It is important to remember that agar loses its gelling strength if repeatedly autoclaved or melted (see Penn, 1991).

Organisms are sometimes grown on other solid substrata, e.g. gelatin, silica gel (an inorganic gel; see Smith and Onions, 1994), potato or carrot slices, and on plant material in damp chambers (p ...).

For more critical work, as in molecular biology, *agarose*, the refined carbohydrate component of agar, is used.

Gelatin is soluble in boiling water, and solidifies on cooling to form a transparent gel. A firm gel is formed at 15% concentration, but it is not used now as a substitute for agar in the preparation of solid media because it is attacked and decomposed by many organisms, and it melts at temperatures normally used for incubation (i.e., above 20°C). It is sometimes added to media to test the ability of organisms to attack it and cause liquefaction.

TYPES OF CULTURE MEDIA

Classified on composition

Non-synthetic or natural culture media include natural ingredients of variable chemical composition, eg, vegetable or meat mixtures and digests. Many of these are now obtainable from manufacturers in a dehydrated form and have a relatively uniform composition. Nevertheless, it is impossible to obtain identical preparations of the same natural medium from different manufacturers or from different batches of the ingredients. Thus, it has become standard practice at Merlewood to use Difco malt extract in preference to the Oxoid product in the preparation of antigenic mycelium, because the former tends to produce less precipitate in liquid culture.

Synthetic or defined media are of known chemical composition. They may be composed entirely of inorganic salts, or be a mixture of inorganic salts and organic compounds. The exact chemical composition of all the ingredients is known so that two batches of the same medium can be duplicated to a high degree of accuracy. Synthetic media are also used when it is necessary to ascertain the effect of an organism on a certain compound.

Mixed culture media are often prepared containing synthetic and natural compounds.

Classified on function

General recipes are used for growing as many types of micro-organism as possible on one medium. They are sometimes known as broad spectrum media. Examples are: tryptone soya agar for bacteria, and Czapek-Dox agar (often with added yeast extract) and malt extract agar for fungi. Note that Mucorales will not grow on Czapek-Dox because they cannot utilise sucrose, whereas *Fusarium* grows well on potato sucrose agar (Smith & Onions, 1994).

Selective media are used to isolate particular types of bacteria or fungi. Their composition is such that only the required organisms develop well, so that they can be selected and

isolated.

Biochemical test media are used to test the different biochemical activities of micro-organisms. Many types of micro-organism may grow on these media, but the composition is devised so that changes caused by particular enzymes can be easily detected, e.g. liquefaction of gelatin, and production of acid from sugars.

Ionically-balanced solutions, such as Ringer's solution and physiological saline, are of the correct density to keep organisms alive but contain no nutrients for growth. These solutions are used when high dilutions or washed cell suspensions are prepared for microbial counts and experimental work. Under these conditons, the natural materials which would normally maintain correct osmotic pressure become too dilute.

MEDIA PREPARATION

See 'Microbiological Procedures' for instructions on use of equipment such as balances, dispensers and sterilizers.

Useful manuals and references for media recipes include: McLean & Cook (1958), Oxoid (1979), Booth (1971b), Parkinson et al. (1971), Johnson & Curl (1972), Gerhardt et al. (1981), Hawksworth et al. (1983), Smith & Onions (1994), Difco (1984), Gams et al. (1987). It is convenient to keep recipes used frequently on a card-index file, particularly since they are often obtained from published papers.

Manufactured media in dehydrated form, obtainable from Difco, Oxoid and other firms are increasingly used for convenience, particularly when only small quantities of media are required, but freshly prepared media are often preferable for some species of fungi or bacteria. Smith & Onions, (1994) infact stated that experience at the International Mycological Institute has shown that cultures grow more satisfactorily on freshly prepared media especially vegetable decoctions.

All media should be made up quickly and sterilized, if possible, on the same day to avoid microbial contamination. If sterilization has to be delayed, media should not be left in the laboratory, or at a warm temperature, but should be removed to a refrigerator or cold room. Reheating or prolonged storage affects the chemical nature of culture media, so they should be prepared in the quantity required for current work.

Pyrex or other heat-resistant glassware is normally used for storage of solid and liquid media. Medical flats may be used for liquid media, but solid media take too long to melt in flats, and the containers often crack. New glassware should be washed thoroughly before use, as it often contains removable salts which would alter the composition of the medium. The containers may be plugged or capped. See also p. 65-67 . Perlite/peat media can be prepared in wide-necked, conical flasks or 1 litre Kilner jars. Care must be taken to avoid over filling these vessels as the medium swells when autoclaved. The lids of the jars should be left undone when autoclaving and clipped down as soon as they are cool enough to handle.

Schott (250 ml) or Duran (100 - 1000 ml) bottles, both obtainable from Fisons, are convenient for sterilizing, shaking and pouring small quantities of media or water. They are sterilizable up to 140°C, and the caps and drip-resistant, plastic pouring rings are completely interchangeable. The caps should be loose during autoclaving.

When preparing a medium (especially when precipitation is a problem), dissolve all the ingredients, other than agar, in separate amounts of water using a glass rod or magnetic stirrer and gentle heat as appropriate. For general media, it is often satisfactory to mix all the ingredients together. The recipe usually states whether any antibiotic or inhibitor should be added before or after sterilization. Agar should be mixed with at least half the total amount of water before adding it to the other solutions. Some water should always have been reserved for rinsing out the various containers at this stage. The bulked liquid should be mixed thoroughly in a large flask (no more than half full) and the pH determined and adjusted if necessary. Dispense as required and autoclave.

Sterilization by autoclaving

Before sterilization in an autoclave, aliquots of the molten medium are transferred to appropriate containers, such as tubes or smaller flasks. These should not be more than 2/3 full to allow for boiling or frothing. The aliquots should be selected as the correct quantities for slope cultures, or in amounts that will avoid repeated opening of the containers in use. Care should be taken to prevent spillage of the medium on the rim of the containers, otherwise contamination may occur at a later stage. If any insoluble ingredients (e.g. $CaCO_3$) are included in the recipe, the medium should be kept well mixed during preparation and dispensing. When pouring plates from a flask of medium, cool to about 55°C to reduce condensation (see Penn, 1991).

Sterilization should be carried out according to the recipe. Under-treatment might not ensure sterility whereas over-treatment might change the physical or chemical nature of the medium. Screw-caps should be loosened to avoid explosion when autoclaving. Leave

the media in the autoclave to cool at the end of the sterilization cycle, if possible, to avoid unsterile air being sucked in during cooling. Screw tops should then be tightened. Any sterile ingredients needed to complete the medium or to adjust the pH are usually added immediately before use. Antibiotics can be added to the cooled agar using a syringe filter unit (Millipore or similar).

Automatic sterilizing and dispensing

A bench-top agar sterilizer with variable volume dispensing pump (see p.14) is recommended as a speedier means of preparing large batches of media for Petri dishes or test tubes. The dissolved media can be sterilized for a given length of time, and then held at a pre-set temperature ready for dispensing in a laminar-flow cabinet. The dispenser pump can also be used on its own to dispense media or other liquids from a bulk container into smaller vessels.

Sterile culture media should be stored in a cold room (2-4°C) if not required immediately, and all media containing the growth inhibitor rose bengal, including inoculated plates, should be kept in the dark, because the dye becomes ineffective when exposed to light.

Any containers emptied of nutrient solutions during media preparation should be rinsed in hot water immediately. Organisms from the air can grow quickly in any residual film of medium.

LABELLING

Good labelling is important. Sessions paper labels for culture tubes and phials are highly recommended; adhesion is good, but they peel off easily when the containers are dry. All such labels should be removed before autoclaving as they are much more difficult to remove once wetted. Slide labels (Gallenkamp) can be removed by soaking in detergent. Paper labels should be marked with an H or hard HB lead pencil to prevent smudging if they get damp.

Both plastic Petri dishes and glassware can be marked with a permanent black Pentel pen as the ink is autoclave-proof. The markings can be removed with acetone from dry glassware or with a pan scrubber during washing up. Chinagraph (Royal Sovereign) wax pencils are a useful temporary alternative particularly when the labelling has to be removed for photography. Containers to be stored in the deep freeze should have paper labels marked with pencil, because Pentel pen marks smudge when frozen and then thawed.

III. STERILIZATION

'To sterilize' means to kill, or completely remove, all forms of life. For experimental and culture work on micro-organisms, sterile media and equipment are essential, and subsequent contamination by live organisms on dust in the air, or on other surfaces, must be prevented. When complete sterilization is not feasible, methods which involve the partial killing or inhibition of microbial growth are used. Death may, or may not, involve disintegration of the organisms.

Organisms differ in their resistance to heat and other sterilizing agents, and any sterilizing procedure must allow for the most resistant forms.

The amount of treatment required will be affected by the following:

- Concentration and age of the cells. A more concentrated suspension will need longer or more drastic treatment;

- The size of the container is important in any heat or other physical treatment. The treatment must reach the whole of the material for the required time;

- Organic matter of any form and particularly fatty materials and mucilage protect the cells against sterilization. This is one reason why soil is difficult to sterilize.

'To disinfect' means to partially kill, or only inhibit, micro-organisms, usually certain undesirable forms, such as human pathogens or food spoilage organisms.

Various methods are employed to kill or remove organisms, according to the nature of the article to be sterilized and the use to which it will be put. Some of the classic methods are described in detail by Gerhardt et al. (1981) and Russell et al. (1991). Each method has its own particular use with well-defined limitations. Two main processes are involved:

- Killing of organisms by some form of heat, dry or moist, or by other physical methods including radiation and use of chemicals;

- Mechanical removal by filtration.

HEAT

Of all methods of sterilization, heat and particularly moist steam under pressure is the most simple and reliable method and therefore the most frequently used. Death by dry heat is primarily an oxidation process, whereas moist heat causes coagulation of cell proteins. The vessels used should be loosely packed to allow good circulation of hot air or steam. For this reason newspaper is better than aluminium foil for wrapping articles to be sterilized in an autoclave; it also has the advantage that it absorbs moisture.

Dry heat

The flame of a Bunsen burner will kill organisms almost instantly and is used for many purposes. The Spencer type of Bunsen burner (Fig. 6) is best, since this gives a larger and hotter flame than others. The ring should be adjusted to give a strong blue flame. A hooded safety Kampf or Bactiburner (Fisons) is used when spattering of infectious material from an inoculating tool must be reduced. Modern electric burners are also available.

As described under Inoculation (p .11), inoculating needles and other instruments are heated to red heat in the Bunsen flame and allowed to cool before use. Care must be taken to flame all parts which may contact the sterile container in use. The mouths of culture tubes are also flamed, after opening, to kill organisms that might have settled on the rim during storage. Similarly, flaming is used when joining up any apparatus, e.g. for filtration. The capillary tips of glass pipettes may also be quickly flamed before use.

Needles, scalpels and other instruments may be sterilized by dipping them in industrial alcohol and then burning off the spirit. This process can be repeated several times. Since the alcohol burns in direct contact with the organisms, a lower heat is necessary. This method is therefore better than direct flaming for articles which may be damaged by red heat, eg, glass rods, forceps, watch glasses, pestles and mortars, but it is less efficient and 'spattering' of live organisms may occur.

wide flange for air input

screw ring to adjust air intake
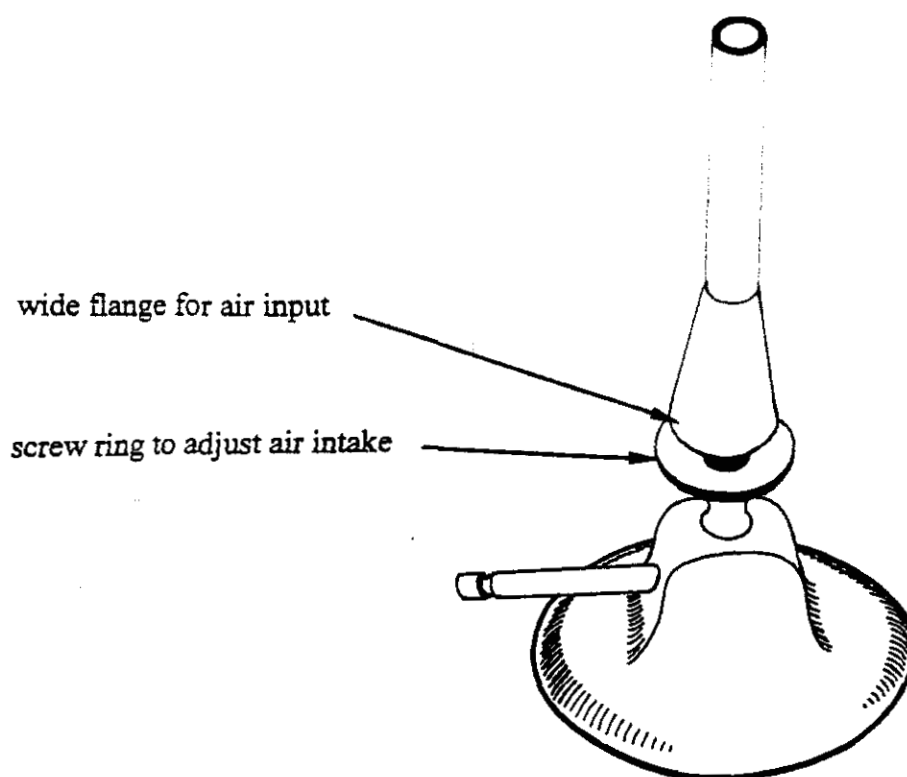
Figure 6.   A Spencer-type Bunsen burner

Hot-air ovens (see use of Ovens in 'Microbial Procedures'), heated by gas or electricity, can be used to sterilize dry glassware. A temperature held at 160°C for one hour should destroy all organisms. Articles for sterilization should be wrapped in aluminium foil, placed in special metal canisters, or in any container that, with its cap, will withstand heat. Newspaper wrapping must <u>not</u> be used in ovens! For powders or other solid materials, the greatest surface areas possible should be exposed and containers should not hold more than 30-60 g of the solid.

Moist heat

Moist heat, or steam, is used to sterilize media and any apparatus that may be damaged by dry heat.

Boiling water kills most vegetative organisms but does not kill bacterial spores. A temperature of 80-100°C for ten minutes kills all vegetative organisms and most fungal spores and is referred to as *pasteurization*. Flowing steam and steam under pressure are far more effective.

Steam under pressure (see Using the Autoclave in 'Microbiological Procedures') is the usual method. When water is boiled under pressures above atmospheric, temperatures higher than 100°C are reached; this moist heat is a very efficient sterilizing agent and can be used for many purposes. The temperature usually employed is 121°C for 15-20 min. Exposure to this temperature kills (with few exceptions) all forms of organisms, including spores. Sufficient time must be allowed for the replacement of all air by steam (in non-automatic autoclaves), otherwise the correct temperature will not be reached. At the end of the boiling time, the steam cools and water will condense on the articles; all plugged containers must therefore be covered with some absorbent paper to prevent wetting of the plugs. Completely sealed containers must not be used because they may explode.

Since the temperature used is lower than that used for dry heat sterilization, the method can be used to sterilize anything that will withstand heating to at least 115°C, such as,

i. Plugged glass containers

ii. Screw-top containers with rubber liners (remove red rubber liners if they are to be used for cultures, and all liners when using oil)

iii. Discarded cultures

iv. Media, with the exception of some sugary media, or media containing vitamins, which would be decomposed by heat. Sugary media can be sterilized under pressure as concentrated solutions at pH 7.0 at 115°C for 30-35 min. Any such variations in temperature or holding time should be indicated on the medium recipe card.

v. Soil placed in thin layers in flat containers such as glass Petri dishes.

Note that polypropylene, polycarbonate and some other plastics can be autoclaved but not polyethylene or polystyrene.

Intermittent steaming at 100°C (Koch sterilization) is occasionally used for heat sensitive materials. The material being sterilized by this method must be capable of supporting germination of spores and quick microbial growth. It is placed in an autoclave at a low setting (100°C at zero pressure) or in a steamer (no longer available at Merlewood) for 20-60 min, on each of three successive days. One exposure kills only the vegetative organisms but the spores then germinate, being in a favourable medium, and are destroyed in the vegetative state during subsequent heatings. The method can be used for sterilizing media containing sugars or gelatin which are decomposed by higher temperatures, or for sterilizing soil. It is not always effective against very resistant spores and may be more harmful to ingredients than a short autoclaving process under pressure. Usually, therefore, the latter is preferred.

IRRADIATION

This method is often less harmful to the material being sterilized than heat, but complete sterilization is not always achieved. High-energy ionizing radiations such as X-rays and gamma-rays are lethal to living cells and have good penetrating power. They affect the metabolism of the cell, but some of the enzyme funtions may continue after the cell ceases to be viable.

For a comparison of the effects of certain sterilization treatments, including radiation, on leaf litter see Howard & Frankland (1972).

Ultra-violet light

Light of a certain wavelength within the ultra-violet region (2×10^{-7} - 4×10^{-7} m) is lethal when it makes direct contact with living organisms. Plate glass or water will absorb ultra-violet light, and because the waves have low penetrating power the reverse side of any object will not be affected. These facts limit its use in many situations. The optimum wavelength for maximum killing is 2.6×10^{-7} m and even *Bacillus* spores can be killed in two hours by a 15 W lamp at 2.5 cm distance when continuously stirred, if only a thin layer of suspension is exposed.

An ultra-violet lamp is sometimes used for partial sterilization of air in rooms and hoods, or for equipment, but it will be ineffective in crevices and corners. See safety precautions in 'Codes of Practice'.

X-rays

X-rays are electromagnetic and of shorter wavelength than light (5×10^{-9} - 6×10^{-12} m). They have been used to kill soil animals and their eggs in the partial sterilization of plant litter. A total of 0.02×10^{4} Gy in three separate doses can be sufficient for this if the position of the samples is changed between doses to facilitate penetration .

Gamma-rays

Gamma-rays, of very high penetrating power, resemble X-rays but are of shorter wavelength (10^{-10} - 10^{-13} m) and are emitted from an element at only one or two fixed wavelengths. They are even more effective than X-rays, and gamma-radiation is now frequently used to sterilize such materials as soils and plant tissues, which are difficult to sterilize by other methods without considerable structural and chemical damage. Packages of plant matrial and soil, etc. can be irradiated at a commercial plant (e.g. Isotron PLC, Bradford). A total dosage of 2.5 x 10^4 Gy (2.5 Mrad) from a ^{60}Co source has been found to be satisfactory for 2-3 g samples of air dry soil and plant litter, resulting in minimal physical and chemical change. The samples can be irradiated in plugged flasks or sealed polythene bags packed in cardboard boxes, and then stored unopened until required. A period of at least two weeks should elapse for free-enzyme activity to cease before use. The brown discolouration of glassware caused by the irradiation can be reversed by heating the containers in an oven for some hours.

Microwaves

Microwaves of relatively long wavelength (30 cm - 1 mm) may also be used for sterilizing small samples of plant material and soil. Microwave ovens provide a cheaper alternative to commercial gamma-irradiation (see 'Microbiological Procedures' for details). However, to date, microwave sterilization has not been tested as thoroughly for efficiency and side effects as gamma-irradiation. Moreover, strong side effects on soil properties, such as lowering of water-holding capacity have been reported (Huhta et al., 1989). For further discussion of this method see Ferriss (1984); Speir et al. (1986); Gibson et al. (1988) and Russell et al. (1991).

CHEMICALS

Many strong chemicals as opposed to 'disinfectants' will effect sterilization but are not normally suitable for general laboratory use.

Disinfectants

Disinfectants are used in the laboratory to prevent infection and to maintain general cleanliness, e.g. for wiping benches or spraying air. When air is sprayed, the liquid droplets also bring down dust, greatly reducing this source of contamination, but cultures should always be removed from the area being sprayed.

The following terms are used for disinfectants:

* *bactericides* or *fungicides* (suffix -cide) kill vegetative organisms but not all spores. With efficient agents, a 99-100% kill is achieved and they are then satisfactory for many cleansing purposes. Proprietary products with the suffix -cide do not all, however, reach this standard;

* *bacteriostats* or *fungistats* (suffix - stat) are substances that only prevent or inhibit growth, without killing all the organisms.

The same substance may show either property at different concentrations.

Powerful disinfectants include Savlon, containing chlorhexidine and cetrimide, and Chloros, an hypochlorite; both are ICI products (see p... for local supplier of these liquids). They can be used to kill organisms accidentally spilt or for immersing slides of suspected pathogens. The concentrated solutions should be diluted according to the manufacturer's instructions for use as bactericides and fungicides. The neat liquids are toxic chemicals, hypochlorites being strong oxidizing and bleaching agents, so it is important to follow the safety procedures when handling them. In addition, hypochlorite disinfectants should not be allowed to come in contact with any phenolic substances, since chlorine gas is produced on mixing.

Surface disinfectants such as Buraton (Stirling Industrial), alcohol (70-95%) or thymol (0.1% in 1% alcohol; see recipes file) can be used for wiping benches before and after use. Spray bottles of Buraton are useful for decontaminating incubators (S. Dawson, pers. comm.). Alcohol (95%) is also used as a dip for instruments before flaming, but the dip should be renewed regularly as the alcohol becomes more dilute on exposure, and a build up of resistant spores is a possibility.

Thymol, which contains phenolic substances, may also be used in more concentrated form (2% in 95% alcohol) as a spray in enclosures for more complete cleansing, but a room should not be entered for 30 min afterwards as the vapour is harmful. To destroy fungal spores and mites a more powerful fumigant such as formaldehyde (Smith 1969) can be used, and may be necessary in rooms that have become heavily contaminated. Sodium or potassium permanganate is placed on an iron tray on the floor and formalin poured on to wet the heap. Gaseous formaldehyde is then evolved. All windows and ventilators should be closed during the operation and the room quickly evacuated for at least 24 h (preferably over a weekend).

Propylene oxide gas has been used at Merlewood to sterilize soils and similar materials. It is an inflammable and highly toxic substance but completely evaporates from the material after treatment. For an assessment of its use see Howard & Frankland (1972). More powerful sterilants must be used for soil in bulk quantities.

Surface sterilization

Less powerful agents are required for sterilizing the surface of delicate plant parts and fungal tissue before isolating hyphae from within the samples. Washing in several changes of sterile water on a shaker may be sufficient to remove surface spores and debris, otherwise a dilute chemical solution, e.g. mercuric chloride, sodium hypochlorite or potassium permanganate (Booth 1971a, p. 5) can be effective. A 10% aqueous solution of sodium hypochlorite is recommended for surface sterilization of fungal rhizomorphs and fruit body stipes. The material is dipped in the solution for a few seconds and then rinsed in sterile water. The hypochlorite solution quickly loses strength so should be freshly prepared. Hydrogen peroxide has been used successfully for surface sterilization of thick-

coated, tree seeds before plating on agar. Details of the method are given in 'Microbiological Procedures'.

FILTRATION

The passage of gases or liquids through sterile filters removes organisms from them. Solutions of heat-sensitive compounds, and media for critical experiments, are often sterilized by this process.

Filtration is not the mere mechanical removal of particles of a certain size by slightly smaller pores; it also depends on physico-chemical factors affecting adsorption. For example, an earthenware filter consists mainly of magnesium and calcium silicates, and according to the electric charge on a particle so it will be adsorbed or be able to pass through. The maximum pore size of any bacterial filter is always greater than the size of the particles it can remove.

Liquids

In the filtration of liquids, acidity promotes adsorption, also the microbial content, the presence of protein or particulate material, the temperature, pH, and viscosity of the solution will all affect the duration and effectiveness of sterilization. The method can only be used for liquids in which the ingredients are in complete solution. Single ingredients are usually sterilized by filtration in a concentrated solution, and then added to the rest of the medium which has been sterilized by the usual methods. Since any soluble products of metabolism will remain, the growth of organisms during preparation of the medium must be avoided.

Membrane filters were developed as a method of counting bacteria in liquids, but are also used to sterilize solutions by filtration. Special filter holders are available. To sterilize the membranes, they are placed between sheets of filter paper in a Petri dish and autoclaved. They are destroyed after use, so that no cleaning is involved. Millipore, Oxoid, or Sartorius membrane filters are commonly used, but a wide range of porosities can be obtained from other firms.

Sintered glass filters are available in five porosities, the finest of which (no. 5) will remove bacteria from liquids.

A number of other filters mentioned in the literature are seldom used now in scientific work, e.g. candle filters, or asbestos Seitz filters. The latter are liable to yield alkaline compounds or traces of iron, have undesirable adsorption properties, and often shed fibres into the medium.

As fluids do not readily pass through filters by gravity, it is necessary to use a positive or negative pressure. Negative pressure (suction) from a vacuum pump is the most convenient method of filtration (Fig. 7), the fluid being drawn through the filter into a previously sterilized container, usually a Buchner flask (a conical flask of thick glass with a side-arm). An air filter (p...) is used to sterilize incoming air, and all outlets or side-arms of flasks are plugged with cotton wool during sterilization by autoclaving.

The smallest negative pressure that produces satisfactory filtration should be used, starting with a small pressure and gradually increasing as filtration proceeds. The duration of filtration should not be prolonged, or small motile and flexible organisms may pass through the filter. A high negative pressure should always be avoided, as small particles would be rapidly forced into the pores and clog the filter. For general purposes, a negative pressure of 100-200 mm of mercury is usually sufficient.

A disadvantage of the negative pressure method is that the filtered fluid has to be transferred to another container and contamination may occur. If positive pressure from a compressor is used, the fluid can then be filtered directly into any type of container required.

A sterility check should always be done on the filtered material, and solutions known to be badly contaminated should be filtered twice, using a fresh, sterile filter for the second filtering.

Air

Dry, non-absorbent cotton wool is a most effective air filter and for that reason is used to plug test tubes (p.65), pipettes and other open apparatus, or is packed into a flask or open cylinder to remove organisms from flowing air. Absorbent cotton wool would hold moisture after exposure to steam, which would allow passage of organisms and subsequent growth of bacteria and moulds. The wool should be of best quality, i.e. long fibred and free from short, broken fibres and dust.

Special air filters that can be fitted to air lines or rooms for microbiological purposes are available, although laminar flow cabinets have reduced the need for them as separate items. High-efficiency Particulate Air (HEPA) Filter systems can have a retention efficiency of 99.97% for airborne particles 0.3 μm or more in diameter. Filtration efficiency is reduced by increased air velocity, neutralization of electrostatic charges on the particles or relative humidities over 95%. 'Pre-filters' in the system, which remove the larger particles and some of the smaller ones, are changed several times before the final filter has to be replaced. A less sophisticated, portable Air Filter System (NSA, Model No. 1200A) with a 'tri-media' filter is used at Merlewood in still air to reduce aerial contamination in the laboratories after various operations such as sub-culturing have taken place. The manufacturers claim it is 95% efficient in filtering out spores and other aerial particles >5 μm.

suction vacuum pump
(tap or electric)

screw taps to hold
or release pressure

vacuum guage

liquid
trap

these parts should
be sterilized

orifice for positive
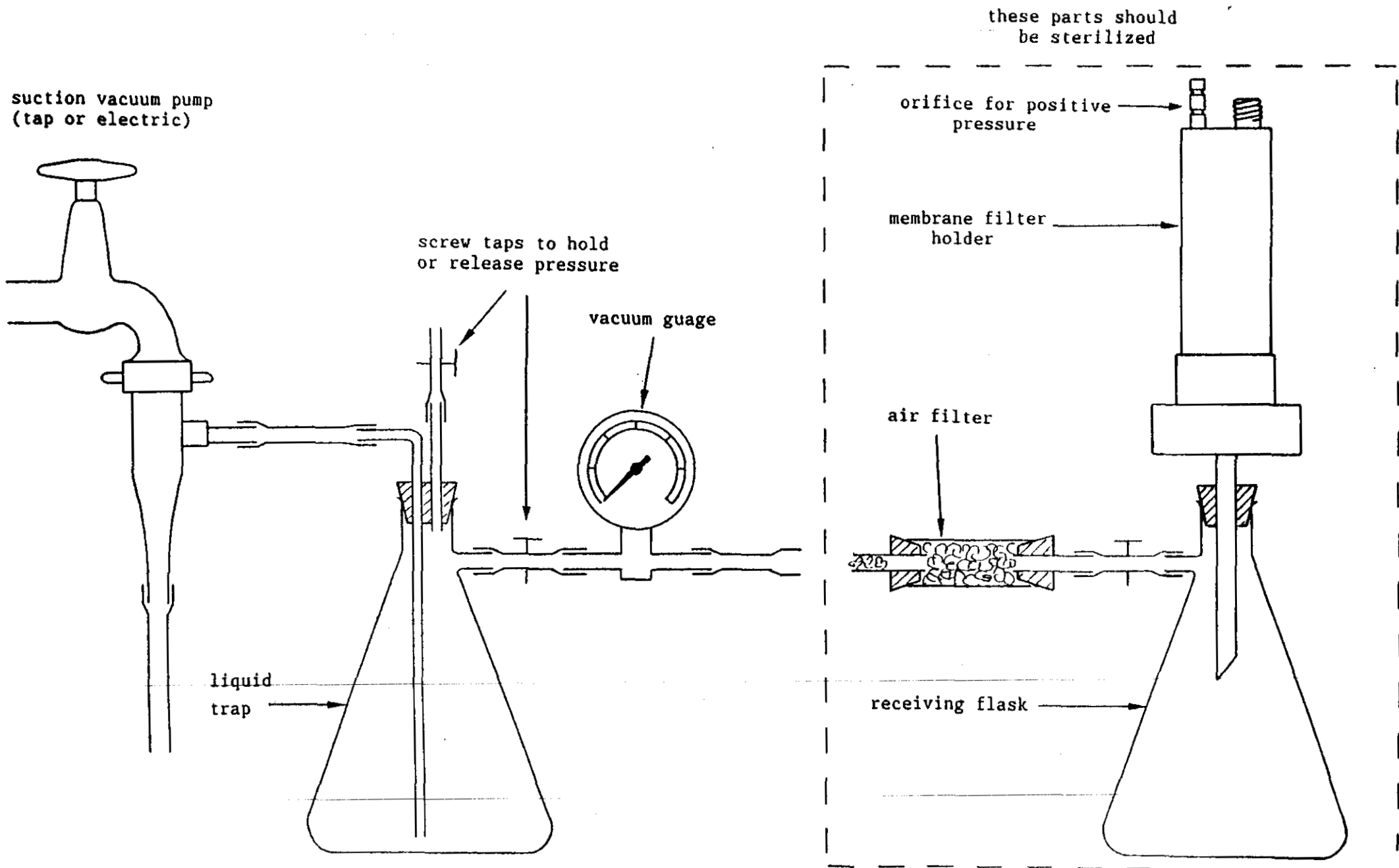pressure

membrane filter
holder

air filter

receiving flask

Figure 7   Membrane filtration assembly

RESTRICTION OF MICROBIAL GROWTH BY LOW TEMPERATURES OR DESICCATION

Unwanted microbial growth can also be arrested by freezing or drying. A proportion of organisms will die when frozen, the proportion depending on the species and the suspending medium. In general, bacterial spores are little affected by low temperatures. Low temperature nearly always arrests the growth of micro-organisms and some form of cold storage is essential for storage of culture media and of certain field samples prior to examination.

Some cells also die when dried, but again desiccation has little effect on many spores. However, few micro-organisms grow in a dry environment, so that drying alone is a useful and well-known method of preventing spoilage of materials.

EQUIPMENT FOR STERILE CULTURE WORK

Petri dishes

Petri dishes or 'plates' are chambers invented by the wife of Petri, a microbiologist, for the growth of micro-organisms in a culture medium. They consist of a shallow dish with a loose lid which allows access of air (as required by fungi and many bacteria) but which keeps out contamination for long periods, if handled carefully in clean surroundings, owing to the tortuous route of the air passage.

Pre-sterilized, disposable plastic Petri dishes are now the type most in use, and may be obtained in polythene packs of 20 in boxes of 500 (Northern Media or Sterilin Ltd). The standard dish of clear plastic is circular, 10 cm x 1.5 cm deep, but various sizes, colours and shapes, including compartmented plates, are available. Non-vented dishes should be used except in experiments in gas jars as when requiring anaerobic conditions, for which single, double and triple vented dishes are available, small nibs raising the lid slightly from the base.

Although largely superseded by plastic, glass Petri dishes can also be used. These can be sterilized in an autoclave wrapped in thick newspaper but more efficiently in a hot-air oven (at 160°C for 1 h <u>from the time of reaching this temperature</u>) packed in metal canisters made for the purpose or in square 1 kg biscuit tins that hold 24 dishes. The canisters have holes in the lids which must be aligned during autoclaving to allow access of steam. The lid is turned to close the canister after treatment. Remaining dishes should not be regarded as sterile once the containers have been opened.

Growth of fungi sometimes appears to be more healthy and sporulation to occur more frequently in glass dishes than plastic; this may be due to the presence of trace elements in the glass. Griffin (1981) gives an interesting illustration of the effect of such trace element contamination from different makes of glass in which *Aspergillus niger* had been grown (Table 1).

Table 1.   The effect of glassware on the zinc requirement of *Aspergillus niger* (after Griffin, 1981; source: Steinberg, 1919).

	Dry weight of mycelium (mg)	
	Control	Zn added
Glassware	without added Zn	(10 mg l^{-1})
Jena	950	987
Kavalier Bohemian	301	943
Pyrex	287	957

Whether plastic or glass, best quality, precision-made Petri dishes with flat bases should be used whenever an even depth of culture medium is an important requirement.

Test tubes, flasks, plugs and caps

The interior of open test tubes, flasks and medical flats, etc, should be carefully protected from contamination by organisms in unsterile air during the preparation of media. They should be kept covered, therefore, until plugged or capped.

Cotton wool plugs for test tubes should be about 5 cm (2") long, with 2 cm (0.75") protruding from the tube, and a tight fit with plenty of 'body', so that they retain their shape when pushed in and out repeatedly. This prevents dust from working down the sides into the tube. Small pointed plugs even if they have a good-sized top are easily dislodged when the tubes are handled (Fig. 8). Moreover, in a mite attack (see p. 28), the tubes with the shorter plugs are usually the first to be contaminated, as mites and spores have a shorter distance to travel before they drop onto the culture. It should be possible to hold the tube by the plug without supporting the tube, but they should not be so tight that they restrict aeration.

top heavy plug
easily displaced

too short insertion

gap allows entry
of contaminants
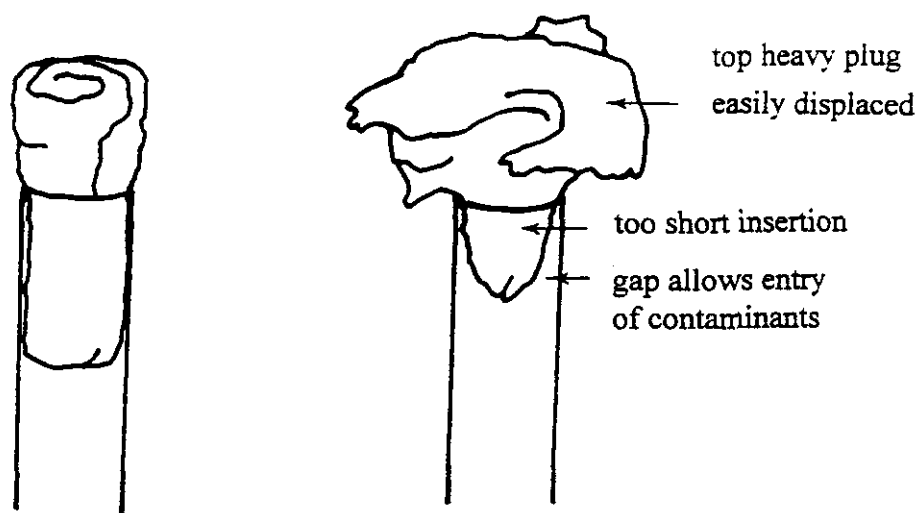
Figure 8.   Good and poor cotton wool plugs

There are two common methods of making cotton wool plugs. They have separate
devotees, but the end result is what matters.

For the first, tear off a piece of cotton wool about 10 cm (4") square, fold two opposite edges into the centre, and roll up across the folds and push the plug into the tube to the level finally required. The shape will be retained after sterilization, so that the plug can always be returned to the same position.

For the second, tear off a rectangular piece of wool, fold in the edges to make a piece 6 cm (2.5") long and of a width twice the diameter of the tube, fold again lengthwise, lay the wool across the mouth of the tube and push its centre into the tube by means of a glass or metal rod.

The size of plug required for larger containers such as flasks can be found by trial and error.

Foam plastic plugs ready cut to shape (see p. 14) are inserted into tubes most easily by using straight, flat-sided forceps.

Metal or polypropylene caps of various types can also be used for short-term tube cultures. They act in a similar way to a Petri dish in excluding contamination, so are not effective closures against mites. They also have the fault that they are too easily dislodged in use and must be carefully matched to the size of the individual tubes if they are to fit properly.

If using aluminium screw caps on McCartney phials (*without oil*), the rubber liners should be black as some red rubber releases toxic substances (see also p. 15 and 54). Polypropylene caps are useful for certain purposes, but they may loosen during storage.

Rubbers stoppers are not recommended.

Culture bottles

Useful sizes of glass bottles include: the bijou bottle (7 ml - 0.25 fluid oz[1]), small McCartney (14 ml; ½ oz) and large phials (28 ml; 1 oz; 'Universal Container') with aluminium screw caps; the latter which are the usual choice for fungal cultures have wider necks than the others; medical flats 110 ml (holding 50-100 ml medium), 560 ml (holding 250-500 ml); Schott and Duran bottles for culture media (see p. 46).

When tubes, flasks and bottles have to be stored for some time, the tops should be loosely covered against dust with foil, polythene sheeting or paper, but cotton wool plugs covered too tightly may hold moisture resulting in contamination.

Pipettes and syringes

Pipetting should not be done by mouth in a microbiological laboratory. A suction bulb or a safety pipette should always be used instead.

Pipettes that require sterilization in an autoclave or oven should be plugged with non-absorbent cotton wool. A small, pointed instrument or wire can be used to do this, the plugged top then being passed through a flame to remove protruding cotton wool. Plugged pipettes can be autoclaved in large glass tubes with cotton wool stoppers, or sterilized by dry heat in metal canisters. A plug of cotton wool pushed to the base of the container will prevent breakage of the pipette tips. They can also be wrapped individually or in small groups in paper for autoclaving or in foil for ovening. If sterilized in an oven, the plugs will char but the pipettes remain dry. Only metal canisters that have an outlet should be used for steam sterilization.
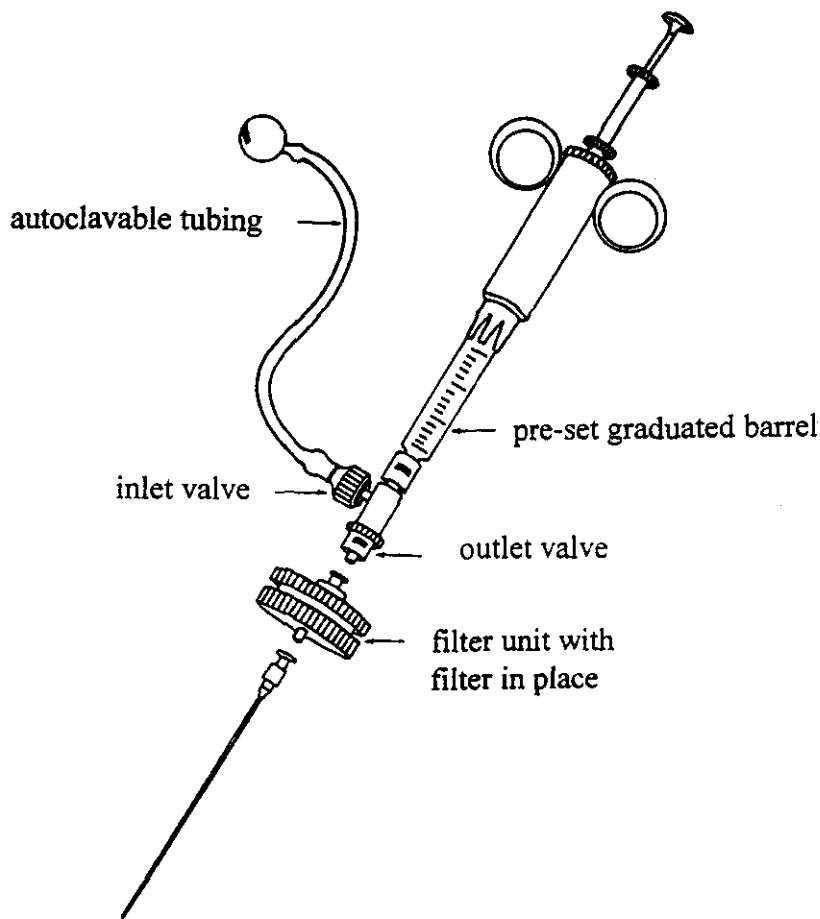
Automatic pipettes, such as the Gilson precision air-displacement, Eppendorf and Volac pipettors (Northern Media), with disposable, sterile tips are available in various sizes. Liquid is pipetted into the plastic tip but not into the body of the pipette, and the tip can be discarded and replaced. This operation should be carried out in a Laminar Flow Cabinet. These pipettes are excellent for repeated use as in dilution counting. Completely

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[1]   'The fluid oz unit is still quoted in catalogues; 1 fluid oz = 28.31 ml

autoclavable pipettes are also available.

Syringes with a filter attachment are obtainable in various sizes with autoclavable parts of glass, metal, and sturdy plastic tubing for aseptic work. The type currently in use at Merlewood (Fig. 9) has a two-way valve and can be fitted with a Millipore or similar filter. Liquid is drawn into the syringe through a tube and dispensed in fixed amounts through the filter into a sterile medium. The syringe and tubing can be autoclaved, wrapped loosely in foil or newspaper. It can then be used in a Laminar Flow Cabinet for adding small quantities of antibiotics, and other components that cannot be autoclaved, to a sterile medium.

autoclavable tubing

pre-set graduated barrel

inlet valve

outlet valve

filter unit with
filter in place

Figure 9.   Membrane filter syringe with Luer fittings

LIST OF SUPPLIERS (1995)

Agar Scientific Ltd, 66a Cambridge Road, Stansted, Essex, CM24 8DA. Supplier of Aniline Blue W.S. (water soluble) and Euparal.

Aldrich Chemical Co Ltd, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4JL. Supplier of Aniline Blue (= Methyl Blue; Acid Blue), FDA, FITC, Rose Bengal, Trypan Blue.

Astell Scientific Equipment, 172 Brownhill Road, London, SE6 2DL.

Baird & Tatlock (London) Ltd, Millbuck Industrial Estate, Moston Road, Sandbach, Cheshire, CW11 9YA.

British Drug Houses Chemicals Ltd, Broom Road, Poole, Dorset, BN12 4NN. Supplier of Glyceel.

Carlson Filtration Ltd, Butts Mill, Barnoldswick, Lancashire, BB8 5HP. Contact: Philip Brown.

Difco Laboratories, P O Box 14B, Central Avenue, West Moseley, Surrey, KT8 OSE.

Edwards High Vacuum International, Manor Road, Crawley, West Sussex, RH10 2LW.

Envair (UK) Ltd, York Avenue, Haslingden, Rossendale, Lancashie, BB4 4HX.

Fisons Scientific Equipment, Bishop Meadow Road, Loughborough, Leicestershire, LE11 ORG. Supplier of Aniline Blue W.S., Euparal, Rose Bengal.

Gallenkamp, Bleton Road West, Loughborough, Leicestershire, LE11 OTR. (A subsidiary of Fisons).

GBI (Labs) Ltd, Heaton Mills, Heaton Street, Denton, Lancashire.

Gibco Bio-cult, 3 Washington Road, Paisley, PA3 4EP. Supplier of Trypan Blue.

Glaxo Chemicals Ltd, Cobden Street, Montrose, DD10 8EB.

Philip Harris Ltd, Lynn Lane, Shenstone, Lichfield, Staffordshire, WS14 OEE.

Hygiene Cleaning Supplies, Middlegate, White Lund Industrial Estate, Morecambe, Lancashire, LA3 3BN. Local supplier of Savlon and Chloros.

Imperial Chemical Industries Ltd, P O Box 14, The Heath, Runcorn, Cheshire, WA7 4QJ.

ICI Agrochemicals UK Sales, Woolmead House West, Bear Lane, Farnham, Surrey, GU9 7UB.

Isotron plc, Euroway Trading Estate, Bradford.

Merck (BDH Laboratory Supplies), Merck House, Poole, Dorset, BH15 1TD.

Millipore (UK) Ltd, The Boulevard, Blackmoor Lane, Watford, Hertfordshire, WD1 8YW.

Murphy Chemical Co, Wheathampstead, St Albans, Hertfordshire, AL4 8QY.

New Brunswick Scientific (UK) Ltd, 6 Colonial Way, Watford, Hertfordshire, WD2 4PT.

Northern Media, Sainsbury Way, Hersle, North Humberside, HU13 9NX.

NSA Air Filter Systems, NSA House, 39 Queen Street, Maidenhead, Berkshire, SL6 1NB.

Oxoid Ltd, Wade Road, Basingstoke, Hants, RG24 OPW.

Phase Separations, Deeside Industrial Park, Deeside, Clwyd, CH5 2NU. Supplier of Aniline Blue W.S. and Trypan Blue.

Royal Sovereign Pencils (Chinagraph). Local supplier: Rainbow Office Supplies, 48 Branthwaite Brow, Kendal, Cumbria, LA9 4TN.

Sartorius Ltd, Longmead Business Centre, Blenheim Road, Epsom, Surrey, KT19 9QN.

William Sessions Ltd, The Ebor Press, York, YO3 9HS.

Sigma Chemical Company Ltd, Fancy Road, Poole, Dorset, BH17 7NH. Supplier of Aniline Blue (= Methylene Blue), FDA, FITC, Rose Bengal, Trypan Blue.

Sterilin Ltd, Sterilin House, Clockhouse Lane, Feltham, Middlesex, TW14 8QS.

Sterling Industrial, Onslow Street, Guildford, Surrey, GU1 4YS.

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